IDENTIFICATION AND CONTROL OF THE GEOGRAPHIC ORIGIN OF PLANT MATERIALS: INVESTIGATION OF AMBIENT INFLUENCES AND ENVIRONMENTAL SELECTION

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PUBLISHED IN: Frontiers in Sustainable Food Systems, Frontiers in Nutrition and Frontiers in Forests and Global Change

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-929-2
DOI 10.3389/978-2-88976-929-2

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Citation: Horacek, M., Hölzl, S., Magdas, D. A., Ondreickova, K., Wunderlin, D., eds. (2022). Identification and Control of the Geographic Origin of Plant Materials: Investigation of Ambient Influences and Environmental Selection. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-929-2

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frontiers | Frontiers in Sustainable Food Systems

TYPE Editorial
PUBLISHED 01 August 2022
DOI 10.3389/fsufs.2022.985249

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Crop Biology and Sustainability, a section of the journal Frontiers in Sustainable Food Systems

RECEIVED 03 July 2022
ACCEPTED 11 July 2022
PUBLISHED 01 August 2022

CITATION
Horacek M, Magdas DA, Ondreickova K, Hölzl S and Wunderlin DA (2022) Editorial: Identification and control of the geographic origin of plant materials: Investigation of ambient influences and environmental selection. *Front. Sustain. Food Syst.* 6:985249. doi: 10.3389/fsufs.2022.985249

Editorial: Identification and control of the geographic origin of plant materials: Investigation of ambient influences and environmental selection

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KEYWORDS

provenance, terroir, geographic indication, geographic origin, traceability, environmental conditions

Editorial on the Research Topic
Identification and control of the geographic origin of plant materials: Investigation of ambient influences and environmental selection

Control of the claimed geographic origin of plant commodities like food and timber has become an important task, as the origin of these commodities is relevant with respect to several issues. We can consider, for example, the implications for foods that carry a recognized Geographical Indication/ Protected Designation of Origin (GI/PDO). GI/PDO foods often have significantly higher prices than the same food, growing or produced in non-specific areas (e.g., sparkling wine from Champagne (France) as opposed to sparkling wine from a non-specific area; fruits, vegetables, and spices from certain regions, e.g., Saffron La Mancha (Spain), Blue Mountain Coffee (Jamaica), etc.).

Another issue related to the geographic origin is food traceability, which refers to the need of determining the geographical origin of food, and it way "from farm to fork". Food traceability helps to locate the origin of contaminated, adulterated, and/or faked food, when it has been found in markets and needs to be traced back to its (geographic) origin. Food traceability is also important to verify commodities/ products from a given region, which trade/importation have been banned for some issue related to health, pollution, etc., or to fight customs fraught.

Besides the topic of food traceability, the control of provenance of wood also is an important issue, in particular with respect to the protection of national parks and primeval forests, fighting illegal logging, and all of its accompanying consequences. Control of timber origin is not limited to developing (tropical) countries but carried out globally. Furthermore, wood is used in many ways since ancient times (from construction materials to tools, paper, veneer, instruments and even jewelry), thus there are a multitude of interests to investigate its geographic origin. The method of choice for the identification and verification of geographic origin covers a wide range of research: isotopic composition, multielemental profiles, genetic variation, and many others generically named "fingerprinting". Generally, there are two main approaches used to identify the geographical origin of food/ wood:

Environmental influences (including geology)

Most of the methods applied investigate differences in the plant composition, from major compounds to trace elements, resulting from the influence of the respective ambient environment. These influences are archived in the plant materials e.g., variations in element concentrations due to differences in soil and bedrock, irrigation water, fertilizers, and emissions (either natural or anthropogenic), among many other influences. As a consequence of these differences, the geographical origin of plants can be determined using the above mentioned fingerprinting methods.

Variations in isotope ratios can result from a multitude of influencing factors (e.g., the influence of climate and weather on H- and O- isotopes in the water, but also agricultural practice, with or without synthetic fertilizers, etc.). Namely, the influence of the geographical area, including soil, irrigation water, natural emissions (volcano, etc.), in addition to anthropic factors, like use of fertilizers, emissions from vehicles, industries and residential plots can produce characteristic isotopic (H, C, N, O, S, Pb and Sr isotopes, among others) and elemental fingerprints.

Research of isotope ratios of non-conventional elements has been started to be also applied for investigations on the geographic origin of plants. However, the processes influencing the isotope ratios of these elements are extremely diverse, and in most cases not yet sufficiently investigated.

Variations in concentrations of organic compounds in plant material are usually caused by a plant's reaction to ambient influences and are thus also environmentally driven. The analytical techniques as NMR, GC-EA-IRMS and LC-EA-IRMS are producing an increasing amount of information on isotopic changes in organic molecules, which, added to the isotopic and multielemental fingerprint, should produce a more accurate evaluation of the geographical origin in plants and plant-based materials/food. Infrared-related and NMR screening techniques are also widely applied in both targeted and untargeted metabolomics studies. The relevant advantage of these techniques consists in the high number of compounds that are simultaneously determined in a single run, (potentially) enabling the discrimination of the geographical origin of a commodity by using suitable chemometric methods.

Genetic/molecular relatedness of individuals

Molecular analysis related to geographic origin base on the assumption that geographically nearer individuals are usually more closely related to each other than distant individuals. Investigated are either the plant or animal of interest itself, or its associated microbiome that are the microorganisms associated to a plant or animal. These microorganisms can be of endogenous or exogenous origin. Among the endogenous microorganisms, endosymbiotic bacteria and fungi in plant roots are very suitable. Soil is most often used from exogenous sources, or specifically rhizosphere, which represents a very close connection between the soil and the roots of plants. In the rhizosphere, a very high metabolic activity takes place, and the microbial diversity is several times higher than in the surrounding soil. Methods based on the molecular fingerprint of associated microorganisms are therefore used to trace the geographical origin of plant material. With the development of more sophisticated next-generation sequencing techniques, the capture of individual microorganisms and their subsequent differentiation between two or more geographical locations is at a better level than a few years ago.

Special issue contributions

Most of the articles published in this volume (eight of nine) belong to the first group and report differences between different geographic origins resulting from the respective environmental conditions. The article by Bhagat et al., describes a feasibility study to utilize the plant microbiome for discrimination of geographic origin.

Two articles are dealing with the control of geographic origin of tropical timber: The paper related to stable isotope ratio analysis of timber to protect two forest concessions in Gabon by Watkinson, Rees, Gwenael, et al., establishes an approach for the origin assessment of forest products and timber from two distinct sites. The samples were analyzed for their isotope patterns. The results of this pilot study indicate that the ^{34}S ratio to have a significant discrimination potential for the origin of the timber samples from Gabon.

The study by Watkinson, Rees, Hofem, et al., on the stable isotope ratio of timber from two islands of the Solomon Islands is regarded as a baseline for future isotope studies on

timber of this geographic origin. As the Solomon Islands are currently deforested at an alarming pace, measures for the control of geographic origin are urgently required. However, further studies on this topic are still necessary, as certain features, e.g., species-related differences in sulfur isotopes require scientific explanations.

Three articles report on the provenance, or provenance-related (influence of storage) issues, of food (saffron and cucumbers), and identification and differentiation of dietary supplements from algae: The paper by Kejžar et al., describe a survey of 18 commercial algae products collected on the Slovenian market. Their characterization was performed based on the elemental profile, isotope content, and antioxidant potential. The sample differentiation was performed through chemometrics [Principal Component Analysis (PCA)], but did not result in a complete discrimination of different micro-algae types or different geographic origins, thus additional data are required for this purpose. The article by Bhagat et al., presents a feasibility-study successfully differentiating the geographic origin of three saffron (to be precise: the microbiome of the crocus corms of crocus sativus, saffron are the filaments of the crocus flower) samples by analysis of the microbiome associated with these samples, thus using molecular (instead of environmental) markers for the investigation of geographic origin. Even though only a very low number of samples were investigated, the results are promising and request further studies of this approach. The article by Horacek and Papesch, deals with potential changes in the isotopic composition of vegetable foods (cucumber) due to storage of the goods. They report significant changes, even though during saleable conditions of the goods the variations remained insignificant. Although these results probably cannot directly be translated to other plant food commodities, this study documents that storage of (plant) food under non-ideal conditions might lead to changes in the isotopic pattern, which needs to be considered when evaluating the authenticity of product claims.

Four articles investigate the provenance of wine: Leder et al. and Griboff et al. apply a combination of isotope ratios and element concentrations for the discrimination and control of geographic origin of wine. Both studies identify $\delta^{18}O$, in addition to the elemental concentration of Sr, Mg and Na as geographic origin-relevant parameters. but disagree on the relevance of others, e.g., $\delta^{13}C$, which was relevant in the study about Croatian, but irrelevant in the study comparing Argentine and Austrian wines. Griboff et al., also identify many elements as markers of geographic origin, while Leder et al., report that the elemental composition is mainly influenced by oenological practice. This matter needs further investigation, even though the particular bedrock geology of Argentina should explain, at least partially, the observed differences in element concentrations. In Horacek et al., a comparison of $\delta^{13}C$ and $\delta^{18}O$ of wine samples of two vintages from Central and Southeastern Europe and Argentina has been published. They show a quite good differentiation among the European samples, but a rather incomplete one of the Argentinean wine samples. These data show that wine samples of distant geographic origins still might possess similar $\delta^{13}C$ and $\delta^{18}O$ values, requiring additional data to construct a reliable fingerprint to differentiate wines from diverse areas (e.g., the multielemental concentrations presented by Griboff et al.).

Muñoz et al., report on the differences of fruit yield and phenolic profiles of wines, produced from two clone types, from different geographic locations within the Mendoza area in Argentina. They found notable differences related to altitude between these investigated two clone types, including differences in the phenolic profiles. The results indicate that fruit yield and the phenolic composition of wines are influenced by the environment, in addition to the plant material and their interaction, resulting in higher concentration of some specific phenolic compounds at lower temperatures and higher altitude.

Author contributions

MH wrote the manuscript. DM, KO, SH, and DW contributed parts of the manuscript and revised an earlier version. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Nutrition

ORIGINAL RESEARCH
published: 05 February 2021
doi: 10.3389/fnut.2020.618503

Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach

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OPEN ACCESS

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Specialty section:
This article was submitted to Food Chemistry, a section of the journal Frontiers in Nutrition

Received: 17 October 2020
Accepted: 29 December 2020
Published: 05 February 2021

Citation:
Kejžar J, Jagodic Hudobivnik M, Nečemer M, Ogrinc N, Masten Rutar J and Poklar Ulrih N (2021) Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach. Front. Nutr. 7:618503. doi: 10.3389/fnut.2020.618503

Dietary supplements based on algae, known for their nutritional value and bioactive properties, are popular products among consumers today. While commercial algal products are regarded safe by numerous studies, information about the production and origin of such products is scarce. In addition, dietary supplements are not as strictly regulated as food and medicinal drugs. We characterized different algal products (kelps: Laminariales, *Spirulina* spp., *Chlorella* spp., and *Aphanizomenon flos-aquae*), obtained on Slovenian market, based on their elemental composition (X-ray fluorescence, inductively coupled plasma–mass spectrometry), antioxidative potential [DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, total phenolic content], and stable isotope values [carbon (C), nitrogen (N), and sulfur (S); elemental analyzer isotope ratio mass spectrometry (EA-IRMS) method]. Antioxidative potential is consistent among products of the same type, with *A. flos-aquae* samples having 4.4 times higher antioxidative potential compared to *Chlorella* spp. and 2.7 times higher compared to *Spirulina* spp. Levels of toxic trace elements (arsenic, cadmium, mercury, and lead) are below the maximum allowed values and as such do not pose risk to consumers' health. Samples of *Spirulina* spp. have relatively high δ^{15}N (7.4 ‰ ± 4.4‰) values, which indicate use of organic nitrogen sources in certain samples. Likewise, different elemental composition and isotopic ratios of stable elements (C, N, and S) for the samples with *Spirulina* spp. or *Chlorella* spp. are the consequence of using different nutrient sources and algae-growing techniques. Statistical analysis (principal component analysis) has confirmed that all tested *A. flos-aquae* samples originate from the same source, supposedly Klamath Lake (Oregon, USA). Hawaiian *Spirulina pacifica* can also be differentiated from all the other samples because of its characteristically high metal content (iron, manganese, zinc, cobalt, nickel, vanadium). *Chlorella* spp. and *Spirulina* spp. require further analyses with larger number of samples, as differentiation is not possible based on results of this study.

Keywords: algae, *Spirulina, Chlorella, Aphanizomenon flos-aquae*, antioxidative potential, stable isotopes, elemental composition, toxic elements

INTRODUCTION

There are numerous algae-based dietary supplements available on the market, which indicates their widespread use among consumers. Dietary supplements are not subject to strict regulations like drugs and imported food. Therefore, continuous evaluation of efficacy, safety, and origin is required to guarantee quality of dietary supplements. Comparison between different products is also complicated because of addition of unknown compounds, which is a common practice among manufacturers. Microalgae (unicellular eukaryotes and cyanobacteria) are interesting organisms to cultivate because of their ability to synthesize bioactive compounds and accumulate minerals and high nutritional value. They are able to grow in modified mediums, including wastewater, which additionally improves economic viability of cultivating microalgae (1). Currently, most producers of microalgae-based commercial products are located in Asia or Australia and show an impressive growth. Production share of food/feed microalgae products owned by European companies is estimated to be approximately 5% of the global market (2).

Microalgae-based products (*Spirulina* spp.—*Arthrospira* spp., *Chlorella* spp., and *Aphanizomenon flos-aquae* or AFA) have the highest market share among "algal" dietary supplements. *Spirulina* spp. products, in tablet or powder form, are mostly consumed because of their nutritional profile: protein (60–70%), carbohydrates (14–19%), fat (8%), dietary fibers (3%), vitamins (<1%), and phytochemicals (3, 4). Algae products are also regarded as a significant source of major elements, such as iron (Fe), calcium (Ca), phosphorus (P), potassium (K), sodium (Na), and magnesium (Mg), and trace elements, including manganese (Mn), zinc (Zn), copper (Cu), selenium (Se), and chromium (Cr). Recommended daily amount of aforementioned algae can provide substantial amount of these minerals and even fulfill recommended dietary allowance for iron intake (4, 5). Studies on content of toxic elements and cyanotoxins are scarce (6). Studies (7–10) done on safety of microalgae do not necessarily reflect safety of algal products, as commercial cultivation practice is unknown and not subject to strict regulations. Results acquired from laboratory grown algae are potentially misleading as different growing conditions significantly impact the content of certain elements and synthesized metabolites in algae (1). Determining efficacy and safety of algal products therefore requires analysis and comparison of individual samples from different manufacturers.

Manufacturers provide little to no information regarding the origin and manufacturing practices of their algae-based dietary supplements. Nutrient composition and toxic compounds differ, depending on the location of sample production. Reasons are various environmental conditions and agrotechnical measures. Thus, to ensure quality and safety of the products used in daily nutrition, determination of product's origin is of great importance (11). Variable environmental conditions that influence microalgal growth can consequently affect the stable isotopic composition of carbon, nitrogen, and sulfur (C, N, and S). These parameters along with elemental composition could be used to verify the quality and origin of microalgal products.

The aim of our study is to differentiate commercially available algal products on Slovenian market by characterizing them based on antioxidative potential, elemental composition and stable isotope composition of C, N, and S, as they can reflect different growing conditions, sources of nutrients and origin. To our knowledge, such an approach has not yet been used in any previous study of algae-based supplementary products.

MATERIALS AND METHODS

Sample Collection

In the present study, 18 samples were obtained from several physical stores and web stores in Slovenia (2018). Dietary supplements were selected based on several types of algae [kelps: Laminariales ($n = 2$), *Spirulina* spp. ($n = 7$), *Chlorella* spp. ($n = 5$), and AFA ($n = 4$)] with different types of production—conventional and organic. The samples were intended for sale on Slovenian market and have declaration in Slovene language (**Table 1**).

Sample Preparation

Samples in tablet form were ground to fine powder, and samples in capsules were opened. All samples were subsequently stored in powdered form in plastic containers with screw caps. During analysis, all samples were stored at room temperature and kept away from direct sunlight, following the manufacturers' storage guidelines. Sample preparation step was repeated for each individual analysis.

Sample Extract Preparation for Determination of Total Phenolic Compounds and Antioxidative Potential

Five hundred milligrams of fine powder sample was added to 10 mL 80% methanol solution in a centrifuge tube. After vortex mixing it for 5 min, it was incubated in ultrasound bath for 30 min at 40°C. Following incubation, samples were centrifuged for 20 min at 9,400 rcf (at 20°C) and filtrated through filters with 0.32-μm pore width into centrifuge tubes. Ten milliliters of 80% methanol solution was added to resulting sample sediment, and the whole procedure was repeated for each sample. We added 80% methanol solution until 20-mL volume was reached. Sample extracts were prepared in duplicate. Extract of each duplicate sample was stored in four vials, containing 5 mL extract each (total 20 mL per sample duplicate) at −20°C.

Total Phenolic Content

The total phenolic content (TPC) of algal methanolic extracts was determined by Folin–Ciocalteu method (12). Twenty milliliters of Folin–Ciocalteu reagent solution was prepared by mixing MilliQ water (resistivity of 18.2 MΩ*cm (at 25°C) and total organic C value <5 ppb) in ratio 1:10. Mixture in screw cap tube was prepared by adding 0.2 mL Folin–Ciocalteu solution, 0.2 mL sufficiently diluted methanolic sample extract, 1 mL Na carbonate solution (mass concentration of 75 g/L), and 2 mL MilliQ water. After thorough vortex mixing, the mixture was incubated for 2 h in the dark at room temperature, followed by 5-min centrifugation at 2,000 RPM.

TABLE 1 | List of algae-based dietary supplement samples obtained on Slovenian market with the information on purity, origin, and suggested daily use.

Algae	Sample	Purity	Origin	Declared growing practice
Laminaria digitata and *Ascophyllum nodosum*	S1	Additives	Not specified	Conventional
Macrocystis pyrifera	S5	Additives	Not specified	Conventional
Aphanizomenon flos-aquae	S2	Additives	Klamath Lake	Conventional
A. flos-aquae	S10	Pure	Klamath Lake	Organic
A. flos-aquae	S11	Pure	Klamath Lake	Conventional
A. flos-aquae	S12	Additives	Klamath Lake	Conventional
Chlorella pyrenoidosa	S3	Pure	Not specified	Conventional
Chlorella sp.	S4	Pure	China	Conventional
Chlorella sp.	S7	Additives	Not specified	Conventional
Chlorella vulgaris	S8	Pure	Outside of EU	Organic
Chlorella sp.	S9	Pure	Outside of EU	Organic
Spirulina platensis	S6	Pure	Not specified	Conventional
Spirulina pacifica	S13	Additives	Hawaii	Conventional
Spirulina sp.	S14	Pure	Outside of EU	Organic
S. platensis	S15	Pure	Outside of EU	Organic
S. platensis	S16	Pure	Taiwan	Organic
S. pacifica	S17	Additives	Hawaii	Conventional
Spirulina maxima	S18	Additives	Italy	Conventional

Spectrophotometer was calibrated using blind sample. The sample was prepared in the same way as other samples, except that 0.2 mL of 80% methanol was added instead of 0.2-mL sample extract. All measurements were performed at wavelength of 750 nm. Sample dilution ratio was determined for each individual sample by test runs using the same procedure. Kelp samples' total phenolic compounds content was too low to be detected by our method (even after the manipulation of sample dilution ratio in final mixture). Calibration curve for TPC analysis was prepared with gallic acid in triplicate with concentrations 5, 10, 15, and 20 mg/mL. Gallic acid solutions were prepared according to the same protocol as samples, in 80% methanol solution. Results were expressed as mg gallic acid equivalent (GAE)/g solid sample mass.

DPPH Assay

The free radical–scavenging activity of algal extracts were measured by the decrease of absorbance of methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (13). A stock methanolic solution of DPPH (0.0837 μM) was prepared by mixing 3.3 mg DPPH in 100 mL of pure methanol. Absorbance of stock DPPH solution was ∼1.1 at 517 nm. Final mixture was prepared by mixing 0.5 mL of sufficiently diluted sample extract and 2.5 mL of methanolic DPPH solution. Blank samples were prepared with 0.5 mL diluted sample extract and 2.5 mL pure methanol. Control sample was prepared with 0.5 mL pure methanol and 2.5 mL methanolic DPPH solution. Absorbance was measured after 30-min incubation period at room temperature in the dark.

Sample dilution ratio was determined for each individual sample by test runs using the same procedure. Antioxidative potential of kelp samples was below detection of our method and could not be determined. On the contrary, AFA samples required dilution in ratio 1:5. Calibration curve for DPPH analysis was prepared with gallic acid in triplicate with concentrations 6, 4.5, 3, 1.5 and 0.75 μg/mL. Like samples, gallic acid was prepared in 80% methanol solution.

Elemental Composition Determination Using X-Ray Fluorescence

X-ray fluorescence (XRF) analysis was performed at Jožef Stefan Institute, Ljubljana. Powdery samples were pressed into tablets. Quality assurance for element analysis was performed using standard reference materials 1573A National Institute of Standards and Technology (NIST) tomato leaves and 1547 NIST peach leaves, acquired from the NIST (Gaithersburg, MD, USA). XRF was used to determine the following elements: bromine (Br), calcium (Ca), chlorine (Cl), iron (Fe), iodine (I), potassium (K), manganese (Mn), phosphorus (P), rubidium (Rb), sulfur (S), silicon (Si), strontium (Sr), titanium (Ti), and zinc (Zn).

Sample Preparation for Inductively Coupled Plasma–Mass Spectrometry

Samples were digested using an UltraWAVE digestion system (Millestone, Italy); 0.05–0.1 g of sample was weighted directly into Teflon vial, followed by addition of 2 mL of 65% HNO_3 (Suprapur, Merck, Germany). Digestion temperature program was as followed: from room temperature to 240°C in 20 min, held on 240°C for 15 min, and then cooled to 40°C (approximately 1 h). Maximum pressure was set at 100 bar. Loading gas (N_2) was set at 25 bar and room temperature.

Digested samples were transferred into plastic vials and filled to 10-mL mark with MilliQ water. Samples were additionally diluted with 5% HNO_3 in 1:5 ratio. Because of visible residuals, the samples were filtered through 0.45-μm hydrophilic syringe filters (Millipor Millex-HV, Merck, Germany). Quality assurance for element analysis was performed using standard

reference material BCR-414 (trace elements in plankton) with known elemental composition. Reagents' blanks were prepared according to the same protocol as samples.

Elemental Composition Determination Using Inductively Coupled Plasma–Mass Spectrometry

Inductively coupled plasma–mass spectrometry (ICP-MS) analysis was performed on an Agilent 8800 triple quadrupole instrument (Agilent Technologies, California, USA). Calibration curve for mercury (Hg) content determination was prepared using NIST 3133 Hg standard solution in the following concentrations: 0, 0.1, 0.5, 1, and 5 ng/mL. Elements [V, Mn, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Pb and Fe] were determined using MULTI XVI (Merck, Germany) multielement standard solution for ICP-MS. Calibration curve was prepared in 5% HNO_3 by using the following concentrations of MULTI XVI: 0, 0.1, 0.5, 1, 5, 10, 50, 100, and 250 ng/mL.

Each sample was analyzed in dilutions (with 5% HNO_3) to 10 and 100 mL. Samples S1, S4, S7, and S10 were prepared in duplicate (two parallels for 10-mL dilution, two parallels for 100-mL dilution). ICP-MS was used for determination of following trace elements: As, Cd, Co, Cu, Hg, Mn, Mo, Ni, Pb, Se, Sr, V, and Zn.

Stable Isotope Ratio Analysis of Light Elements Using EA-IRMS

Stable isotope ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ were expressed as δ values in ‰ according to the following equation (14):

$$\delta^{i}_{j}E = \frac{{}^{i}_{j}R_{sample} - {}^{i}_{j}R_{ref}}{{}^{i}_{j}R_{ref}} \quad (1)$$

where E represents element (C, N, S), R is isotope ratio between heavier "*i*" and lighter "*j*" isotopes ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in the "sample" and reference material ("ref"). Values for C were expressed relative to V-PDB (Vienna-Pee Dee Belemnite) standard, N values relative to AIR, and S values relative to V-CDT (Vienna Cañon Diablo Troilite) standard.

Stable isotope ratios of light elements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in algae samples were simultaneously determined by isotope ratio mass spectrometry with preparation system for solid samples IsoPrime 100–Vario PYRO Cube (OH/CNS Pyrolyser/Elemental Analyzer, Elementar Analysensysteme GmbH, Germany). Four milligrams of sample and 4 mg of tungsten oxide (WO_3) were weighted directly into tin capsules, sealed, and placed into the automatic sampler of the elemental analyzer. Each sample was measured in triplicate, and the average value was considered. Quality assurance for stable isotope ratio analysis was performed using the following reference materials: USGS-43: $\delta^{13}C = -21.28 \pm 0.10‰$, $\delta^{15}N = +8.44 \pm 0.10‰$, $\delta^{34}S = +10.46 \pm 0.22‰$; B2155 Protein Sercon: $\delta^{13}C = -26.98 \pm 0.13‰$, $\delta^{15}N = +5.94 \pm 0.08‰$, $\delta^{34}S = +6.32‰ \pm 0.8‰$; Casein Protein CRP: $\delta^{13}C = -20.34 \pm 0.09‰$, $\delta^{15}N = +5.62 \pm 0.19‰$, $\delta^{34}S = +4.18‰ \pm 0.74‰$. Measurement precision value was ±0.2‰ for $\delta^{13}C$, and ± 0.3‰ for $\delta^{15}N$ and $\delta^{34}S$.

Statistical Analysis

Statistical calculations were carried out using the XL-STAT software package (Addinsoft, Long Island, NY, USA, 2019). First, basic statistics were applied to the data. Because most of the data were not normally distributed (Shapiro-Wilk test, $p < 0.05$), the nonparametric Mann–Whitney *U* test was used for comparison of element content between different microalgae products. In all analyses, $p < 0.05$ was considered as statistically significant.

Further, principal component analysis (PCA) was applied. PCA is an unsupervised pattern recognition multivariate statistical tool able to analyze numerical dataset structured in an M observations/N variables table and recognize underlying patterns in the dataset. The results of such analysis are displayed as biplots, which are simultaneous representations of variables and observations in the space of selected two PCA axes (e.g., PC1/PC2). The biplots enable visualization and increase interpretability of relation and trends among observations and variables on a two-dimensional map and identify similarities and differences among observations, association with variables, and also impact and role of a particular variable in discrimination and clustering of observations.

PCA was used to enable identification of characteristic parameters that are able to discriminate samples based on antioxidative potential, stable isotope composition of light elements and elemental composition. Samples of kelps (Laminariales) were excluded from PCA, because of relatively low content of algae in dietary supplement products and significant physiological differences compared to microalgae.

RESULTS AND DISCUSSION

Total Phenolic Content and Antioxidative Potential

Average values of TPC of different dietary supplements based on algae, expressed as mg GAE/g solid sample, were 12.3 ± 1.2 for samples of *Chlorella* spp., 23.2 ± 6.4 for *Spirulina* spp., 96.4 ± 7.5 for AFA, and 1.6 ± 1.6 for kelp (**Figure 1**). AFA samples contained the highest amounts of TPC, followed by *Spirulina* spp. and *Chlorella* spp. For comparison, Al-Dhabi and Valan Arasu (15) determined TPC values of 2.4–24.4 mg GAE/g sample for *Spirulina* spp. Kelp samples had negligible TPC content, presumably due to the low algae content in the sampled product itself (13–14% according to the declaration). It should be noted that kelp samples were not homogenous (brown algal parts tabletized with filler); therefore, the measurements might be incorrect.

Average antioxidative potential values, determined by the DPPH method, for different types of algae-based dietary supplement samples (expressed as mg GAE/g solid sample) were 1.33 ± 0.08 for *Chlorella* spp., 2.17 ± 0.43 for *Spirulina* spp., and 5.90 ± 0.71 for AFA (**Figure 1**). Antioxidative potential of kelp was significantly lower in comparison to other algae samples and as such could not be measured by using our method.

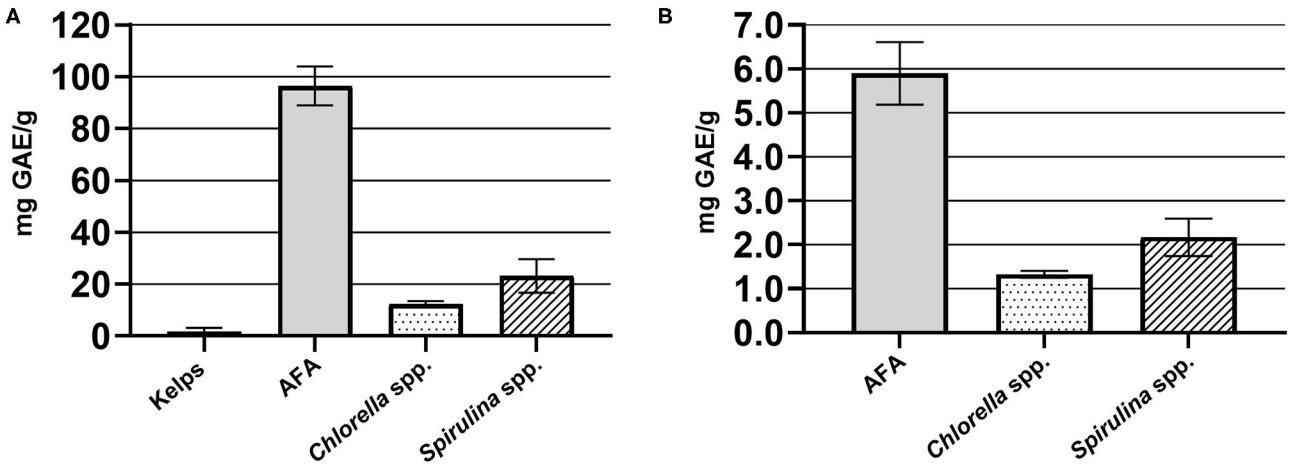

FIGURE 1 | (A) Average total phenolic compounds (Folin–Ciocalteu method); **(B)** average antioxidative potential values (DPPH method) in different types of algae-based dietary supplement samples, expressed as mg gallic acid equivalent (GAE)/g solid sample.

AFA samples showed the highest measured antioxidative potential, which was 4.4 times the value of *Chlorella* spp. samples and 2.7 times the value of *Spirulina* spp. samples. There were no significant differences among similar products from different manufacturers regarding antioxidative potential. The latter is evident from the relatively small deviations in measured values among sample groups with the same algae type (**Figure 1**). Consequently, antioxidative potential allows discrimination of samples between different algae types. It should be noted that presented results of antioxidative potential do not assess the efficacy in relation to health benefits of algal products, as our research goal is mainly characterization of different product types.

Stable Isotope Composition of Light Elements

δ^{13}C value in algae is reflected by their C source (16). Average δ^{13}C value of *Spirulina* spp. samples was −23.0‰ ± 4.0‰. Unusually high δ^{13}C value of −17.4‰ of sample S18 could be explained by declared addition of corn maltodextrin, which has characteristic δ^{13}C value of C4 plants (from −15‰ to −12‰) (17). *Chlorella* spp. had similar δ^{13}C values with an average value of −27.5‰ ± 5.7‰, with sample S3 having the lowest value of −37.1‰ (**Table 2**). The low δ^{13}C value determined in *Chlorella* spp. could be related to the growing conditions in a closed system. Closed systems are closed bioreactors with higher control over growing conditions (pH and temperature), higher photosynthetic efficiency, lower water evaporation rate, and lower CO_2 loss to the atmosphere (18). It was found that the δ^{13}C value in a closed system exhibits lower δ^{13}C value of CO_2. Because of relatively small deviation among samples of *Spirulina* spp. and *Chlorella* spp., we assume they utilize similar sources of HCO_3^- and CO_2 during cultivation, presumably ones that have shown to be the most efficient from manufacturer's point of view.

Spirulina spp. samples had an average δ^{15}N value of 7.4‰ ± 4.4‰, indicating organic source of N in samples with high δ^{15}N values (>6‰), possibly due to wastewater use (19). This also applies to kelp samples. Higher δ^{15}N values also indicate usage of modified mediums for algae cultivation, as the latter can significantly improve economic viability of the project. One sample (S6) of *Spirulina platensis* differed from other samples with δ^{15}N value of 1.2‰, indicating inorganic source of N or molecular N (air) fixation. Differences in δ^{15}N values between samples can also be explained by other factors, such as (i) different climate, which is hard to control in open bioreactors, and (ii) recycling of growth medium (1). Samples of *Chlorella*

TABLE 2 | δ^{13}C, δ^{15}N and δ^{34}S values in algal dietary supplement samples.

Algae	Sample	$\delta^{13}C_{V-PDB}$ (‰)	$\delta^{15}N_{AIR}$ (‰)	$\delta^{34}S_{V-CDT}$ (‰)
Kelp	S1	−25.5	8.8	15.5
	S5	−22.1	10.3	16.8
Aphanizomenon flos-aquae	S2	−16.8	1.8	4.9
	S10[a]	−15.4	−0.5	5.1
	S11	−15.8	0.4	5.3
	S12	−14.1	−0.6	5.3
Chlorella spp.	S3	−37.1	−0.7	1.7
	S4	−25.9	6.6	−0.9
	S7	−25.7	3.4	−3.0
	S8[a]	−22.0	−3.4	1.3
	S9[a]	−27.0	11.8	−0.9
Spirulina spp.	S6	−27.7	1.2	−0.2
	S13	−25.8	10.8	8.8
	S14[a]	−26.1	8.8	11.3
	S15[a]	−22.1	7.6	−0.6
	S16[a]	−21.8	6.2	11.5
	S17	−24.4	13.8	7.8
	S18	−17.4	13.3	11.0

[a] *Declared as organic.*

spp. generally showed lower δ^{15}N values (3.5 ± 6.0‰) compared to *Spirulina* spp. samples. This is probably due to *Chlorella* manufacturers using less optimized and modified growing methods compared to *Spirulina* manufacturing. *Spirulina* is able to grow in saline environments (8), which is exploited to prevent contamination by other microorganisms when growing in "low-quality" media. As original media use mostly inorganic source of N (1), we can assume that most samples were grown using modified media. Lack of information from manufacturers makes interpretation of results rather difficult, as we do not have any insight into geographical factors that may affect fractionation.

AFA samples had similar stable isotope composition of C and N, which indicates that samples originate from the same source (all AFA products are declared to originate from Klamath Lake, OR). Small deviations in stable isotope composition of C and N in sample S2 were probably due to presence of additives in the product. Relatively high values of δ^{13}C (−15.5 ± 1.1‰) in AFA samples might indicate photosynthetic fixation of CO_2 from air as their primary source of C. Values of δ^{15}N were around zero (0.3 ± 1.1‰), indicating fixation of molecular N from air.

The δ^{34}S values in algae-based dietary supplement samples ranged from −3.0 to 16.8‰, with the lowest values observed in *Chlorella* spp. and the highest in kelps (brown algae). Variability in δ^{34}S values among the samples of same algae species (with the exception of AFA) was on average higher for C and N. This variability can be explained by different origins of samples or differences in organic load during growing conditions. However, the information regarding the distribution of the δ^{34}S values of aquatic resources and organisms is scarce. There are three potential sources of S in algae, depending on the proximity to the ocean, geology, and redox chemistry. For example, the δ^{34}S of marine sulfate and vegetation near the ocean are approximately +20‰ but decrease to +6‰ over 100 km (20, 21). In the Hawaiian Islands, δ^{34}S values of sulfates from volcanic ash and basalt-derived soils ranging from 6.3 to 15.4‰ (22) have been reported and are also in agreement with our data.

Elemental Analysis

The results of the elemental composition in microalgae supplements are presented in **Supplementary Table 1**. No statistically significant difference between different types of algae supplements was observed for Si, V, Mn, Ti, Co, Ni, Rb, Cu, Se, Mn, Fe, and Hg. A significantly higher content of Ca was observed for *Spirulina* spp. products, whereas *Chlorella* spp. displayed the highest P level. This observation agrees with the study performed by Rzymski et al. (7). AFA products exhibited high Ca and Mo concentrations that differ statistically significantly from other products. The highest concentrations of Sr, Br, and Cl were determined in kelps samples and differ significantly from other products mainly from *Chlorella* spp. Zn levels did not differ between *Spirulina* spp. and *Chlorella* spp., but they are significantly higher than those observed in AFA and kelps.

Iron Content

Average Fe content (mg Fe/g solid sample) in samples of *Chlorella* spp. was 1.00 ± 0.52, *Spirulina* spp. 1.36 ± 1.33, AFA 0.44 ± 0.05, and kelps 0.13 ± 0.01 (**Figure 2**). High deviation among *Spirulina* spp. samples is due to higher Fe content in Hawaiian *Spirulina pacifica* samples (3.29 ± 0.27). Iron content in *Spirulina* reflects the Fe content in the medium used for cultivation (23).

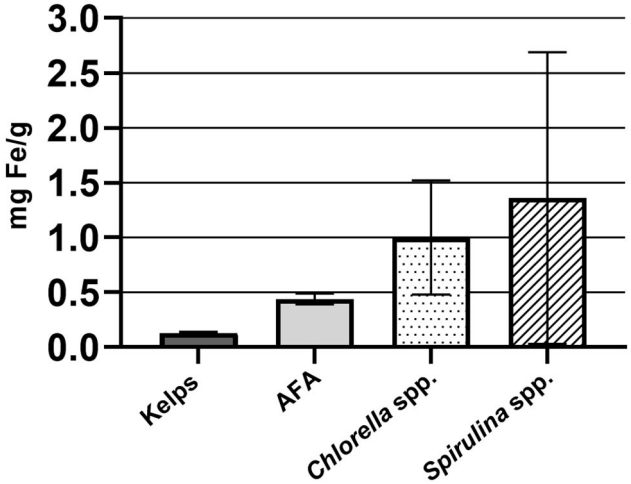

FIGURE 2 | Average iron content in algal dietary supplement samples, expressed as mg iron/g solid sample.

Iodine Content

Iodine content in kelp samples was 183 mg/kg solid sample for S1 and 221 mg/kg for S5, with 3% and 11% deviation from values declared on the product. Variability of I content in kelp-based supplements is therefore lower compared to edible seaweed (24). Other algae samples had I content below the limit of detection of XRF method.

Toxic Elements Content in Algal Dietary Supplements

Statistically significant difference in As concentration was found between *Chlorella* spp. and *Spirulina* spp. samples and AFA and kelp-based algae. Average total arsenic (As) content of samples was 0.26 ± 0.17 mg/kg for *Chlorella* spp. and 0.73 ± 0.96 mg/kg for *Spirulina* spp. samples (**Figure 3**). AFA and kelp-based samples had higher total As content, which was between 3.5 and 6.5 mg/kg solid sample. At the time of writing, European Commission (25) has not set upper tolerable level for As content in dietary supplements. It should be noted that our analysis determined only total As. For health risk assessment, determination of As species is required as the inorganic form of As is more toxic compared to the organic form.

Cd content was below maximum allowed value for dietary supplements of 1.0 mg/kg, set by European Commission (25) (**Figure 4**). Kelp sample (S5) had notable Cd content of 0.55 mg/kg solid sample and as such differs significantly from other samples. Other samples had Cd content between 0.011 and 0.064

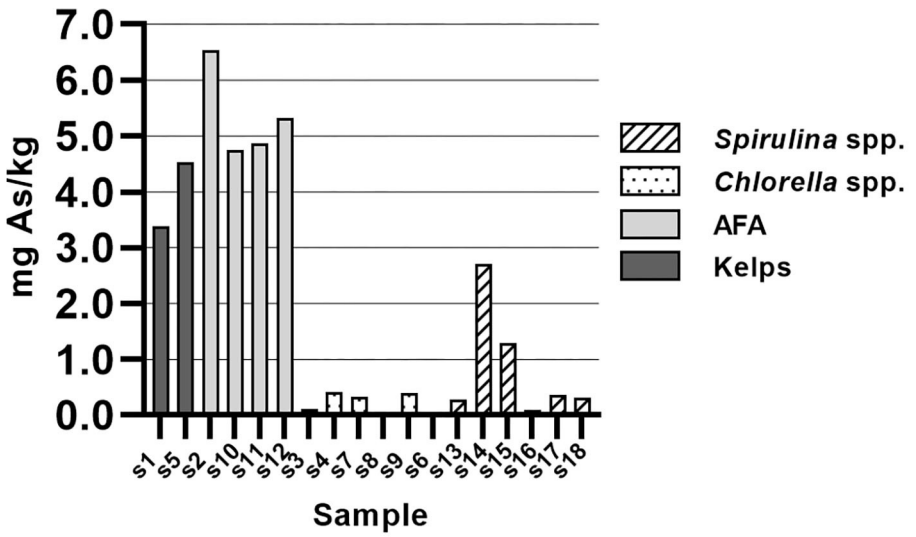

FIGURE 3 | Arsenic content in samples of algal dietary supplements, expressed as mg total arsenic/kg solid sample.

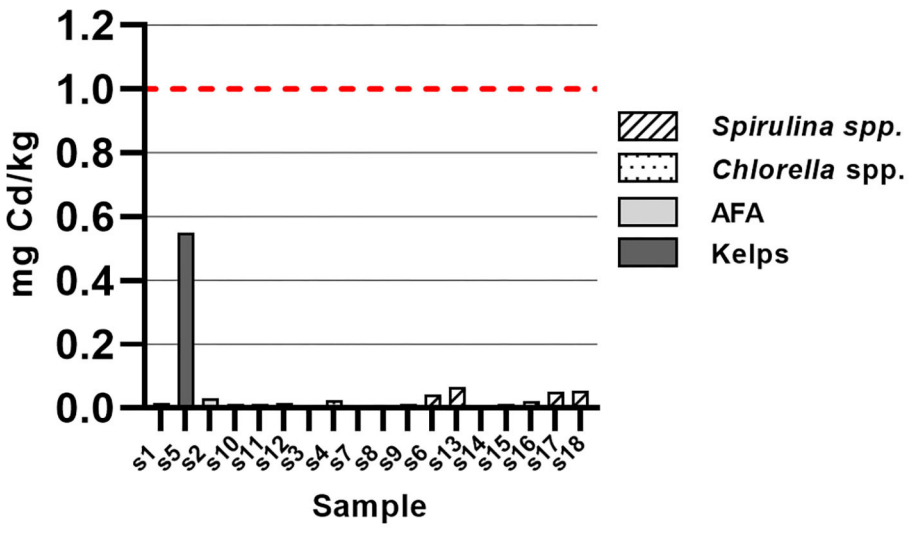

FIGURE 4 | Cadmium content in samples of algal dietary supplements, expressed as mg total cadmium/kg solid sample.

mg/kg. With respect to Cd content, none of the samples pose risk to consumers' health.

Samples of kelps (S1) and AFA (S2) exceeded maximum allowed value of Hg in dietary supplements by factor of 3.5 and 4.4, respectively (**Figure 5**). Maximum allowed value of Hg in dietary supplements is set at 0.1 mg/kg by European Commission (25). Hg content of other samples was below maximum allowed value. One sample of *Chlorella* spp. (S8) had Hg content below LOD. Despite the exceeded values of Hg in two samples, it should be noted that maximum Hg content for fish is set much higher (compared to dietary supplements) at 1.0 mg/kg fish muscle (25). By ingesting manufacturer's recommended daily dose of sample (4.02 g) with highest Hg content, we would ingest 1.76 μg of Hg. In contrast, eating 100 g of fish flesh with Hg content at limit (1.0 mg/kg) would equate to ingesting 0.1 mg Hg, which is 57 times higher than daily dose of sample (S2) with the highest Hg content.

Pb content was below maximum allowed value (3.0 mg/kg) (25) in all samples of algal dietary supplements (**Figure 6**). Kelps sample (S1) had Pb content below LOD. Pb content of samples was 0.35 ± 0.22, 0.23 ± 0.19, and 0.02 ± 0.00 mg/kg for *Spirulina* spp., *Chlorella* spp., and AFA, respectively, where *Spirulina* spp. differ significantly from AFA samples. In contrast with our

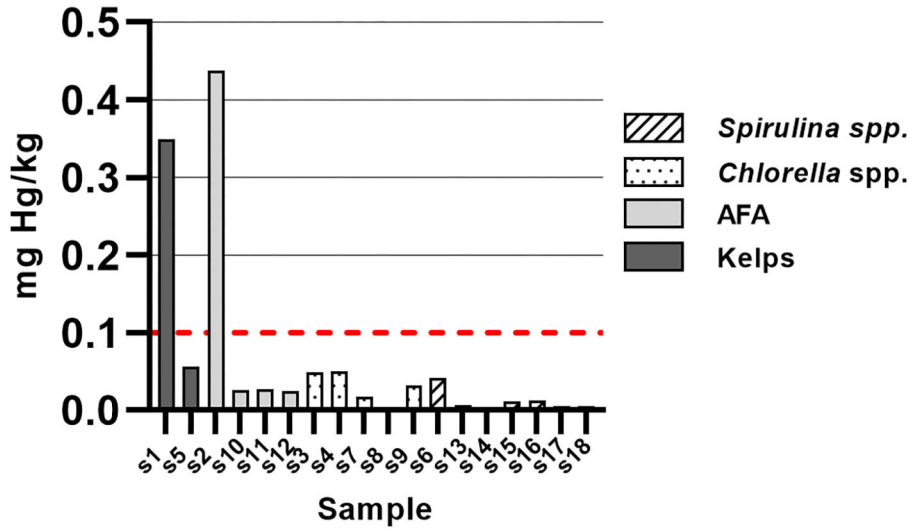

FIGURE 5 | Mercury content in samples of algal dietary supplements, expressed as mg total mercury/kg solid sample.

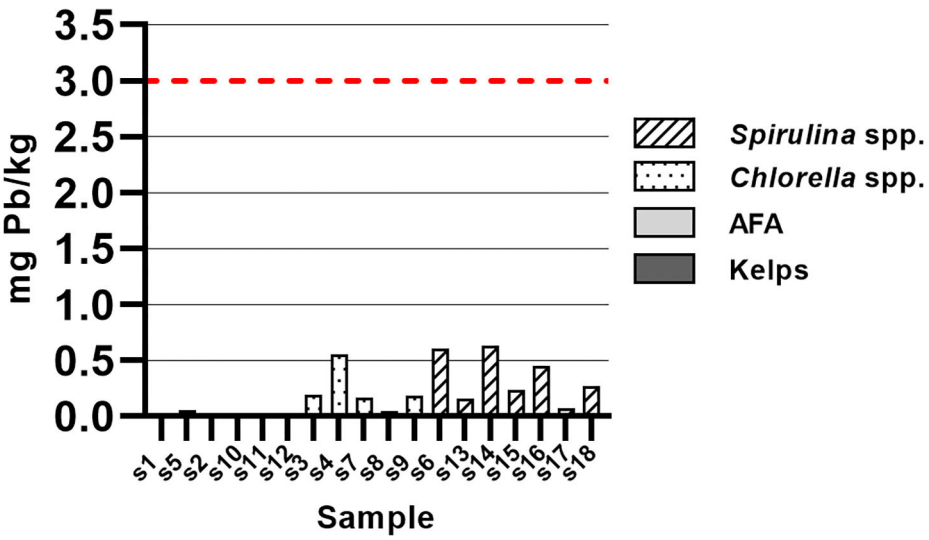

FIGURE 6 | Lead content in samples of algal dietary supplements, expressed as mg total Pb/kg solid sample.

results, Rzymski et al. (7) report of average Pb content of 2.6 ± 1.3 mg/kg for *Chlorella* spp. samples and 2.6 ± 1.9 mg/kg for *Spirulina* spp. samples, where 40% of *Chlorella* spp. and 30% of *Spirulina* spp. samples exceeded maximum allowed value.

Toxic elements such as Hg, Cd, Pb, and As were detected only in trace amounts and as such do not pose risk to consumers' health, which is in agreement with other studies (7, 10). It should be noted that algal products also require determination of algal toxins to fully evaluate their safety (6).

PCA of Microalgae Samples

AFA-based products are distinguishable from other samples (**Figure 7**). They have characteristically high antioxidative potential (TPC and DPPH); high Mo, Ca, and Sr content; low P content; relatively low δ^{15}N values; and high δ^{13}C values. Sample S2 slightly deviates from AFA group, possibly due to additives (all other AFA samples are declared as pure). Based on our PCA, we can claim that AFA samples originate from the same source, supposedly Klamath Lake, OR. Interestingly,

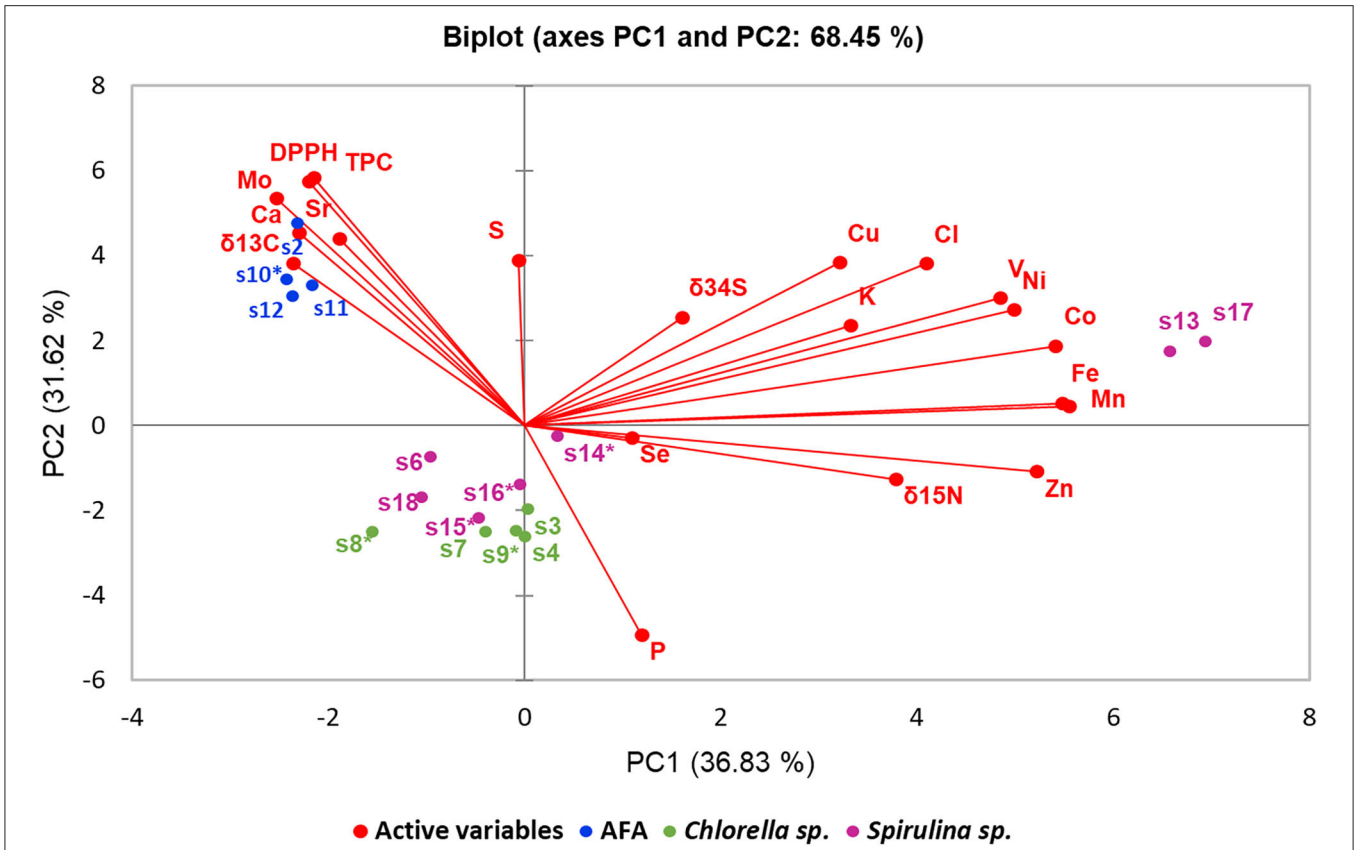

FIGURE 7 | Principal component analysis of algal dietary supplements analysis results (antioxidative potential, elemental, and stable isotope composition data). The biplot shows data of samples as dots (scores) and analysis results (loadings) as vectors. Samples marked with * are declared as organic.

sample S10 is declared as organic, whereas other samples have no such declaration. Such labeling discrepancy is unexpected, considering all our AFA samples are advertised to originate from Klamath Lake.

Samples of *Chlorella* spp. and *Spirulina* spp. cannot be reliably distinguished using PCA (with exception of Hawaiian *S. pacifica*) because of lack of characteristic parameters of respective microalgae (**Figure 7**). Organically grown *Spirulina* spp. and *Chlorella* spp. also do not exhibit any characteristic parameters, including δ^{15}N values, where high values usually indicate assimilation of organic N originating from wastewater. Two *S. pacifica.* samples (S13 and S17), originating from Hawaii, are well separated from other samples based on Fe, Mn, Zn, Co, Ni, V, K, Cl, Cu, and δ^{15}N values and δ^{34}S values, whereas the sample from Italy (S18) cannot be distinguished from other *Spirulina* spp. samples originating from non-EU countries.

Hawaiian *S. pacifica* samples S13 and S17 (**Figure 7**) significantly differ from other analyzed samples. That is largely due to significantly higher content of elements such as Co, Mn, Fe, Ni, V, and Zn compared to other samples, which is shown by their respective loadings (**Figure 7**).

By combining results from stable isotope composition, antioxidative potential, and elemental composition, we can reliably discriminate *S. pacifica* and AFA from our samples. Discrimination between *Chlorella* spp. and *Spirulina* spp. is not possible based on our results because of insufficient number of samples and scarce information provided by the manufacturers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NO, NP, and JK: Conceptualization. JK, NP, NO, and JM: Methodology. MJ, JK, and MN: Validation. JK, MN, and MJ: Formal analysis. JK, MJ, JM, and MN: Investigation. NP, NO, and MN: Resources. JK: Writing—original draft preparation and visualization. JK, MJ, NO,

NP, and MN: Writing—review and editing. NP and NO: Supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by Slovenian Research Agency (P4-0121, P1-0143 and J4-1773: Lactic acid fermentation for enrichment of microalgae biomass with new nutrients).

ACKNOWLEDGMENTS

We would also like to acknowledge the technical support provided by Stojan Žigon and Doris Potočnik for stable isotope analysis. We would also like to thank our colleague Miha Bahun for providing help in the laboratory.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.618503/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 04 March 2021
doi: 10.3389/fnut.2021.625613

Geographical Discrimination of Croatian Wines by Stable Isotope Ratios and Multielemental Composition Analysis

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The $\delta^{18}O$ and $\delta^{13}C$ (analyzed by isotope ratio mass spectrometry, IRMS) and concentration of 22 selected elements (analyzed by inductively coupled plasma—optical emission spectrometry, ICP-OES) in 190 Croatian microvinified and commercial wine samples from continental and coastal winegrowing areas and from three viticultural zones (B, CI, and CII) were measured to investigate whether multivariate statistical methods could provide the fingerprint for geographical origin determination. The highest power for discrimination of wines produced in Croatian winegrowing areas was achieved by general discriminant analysis (GDA) showing correct classification of 97.9% of all investigated samples, 100.0% of microvinified samples and 84.8% of commercial samples in the cross-validation matrix. The most significant markers for discrimination of coastal and continental areas found by GDA were $\delta^{18}O$ and Co, followed by K, Rb, Sn, Li, and $\delta^{13}C$ in descending order. GDA showed higher levels of correctly classified samples from three viticultural zones in Croatia if only microvinified samples were employed in the analysis (94.9%) than for all samples together (86.3%) or for commercial samples (66.1%) in the cross-validation matrix. The discrimination of viticultural zones B, CI, and CII in Croatia was achieved by $\delta^{18}O$, Co, Rb, Li, K, and Sn. The results obtained showed that the relationships between the isotopic ratios and concentrations of different considered elements combined with appropriate statistical model represent a powerful tool in discrimination of wines produced in different Croatian winegrowing areas.

Keywords: Croatian wines fingerprint, elements, geographical origin, inductively coupled plasma optical emission spectroscopy, isotope ratio mass spectrometry, stable isotopes

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Food Chemistry, a section of the journal Frontiers in Nutrition

Received: *03 November 2020*
Accepted: *07 January 2021*
Published: *04 March 2021*

Citation:
Leder R, Petric IV, Jusup J and Banović M (2021) Geographical Discrimination of Croatian Wines by Stable Isotope Ratios and Multielemental Composition Analysis. Front. Nutr. 8:625613. doi: 10.3389/fnut.2021.625613

INTRODUCTION

The adulteration of food and beverages is a growing global problem. Consumer awareness of the food safety importance has been steadily increasing in recent years as well as activities that include adulteration of food products for economic gain (1, 2). Following these trends, analytical methods for determination of the authenticity of food products, including wine, are also constantly being developed and upgraded accordingly (3, 4). Appropriate chemometric analysis of the data provided by those methods are needed and proposed for wine (5–7) and other food types, i.e., honey (8),

cheese (9), and meat (10). Authenticity and commercial value of wine are often associated with geographical origin, and some countries or regions are known for producing high commercial value wines (3). Wine is a product that is often adulterated by the addition of sugar and/or water, as well as through intentional mislabeling of origin for economic gain (6, 11, 12). Hence, the use of analytical methods to verify the declared composition and origin is of high-interest both for wine producers and consumers (13, 14). This is also increasingly recognized in Croatia (15–19), where viticulture and winemaking represent a significant economic activity, especially through the growing tourism industry (20).

The relationship between the isotope data of wine and physical variables related to the climate and geography of the production area is a very interesting topic, as is evident in many published papers in the last 20 years (5, 11, 21–28).

Just like the stable isotopes, mineral elements are also considered to be good indicators of geographical origin of wine since they are neither metabolized nor modified during the wine production (29). Distinction of wine region through trace element composition is due to their close connection with their transfer from rock to soil and from soil to grape. The multi-element composition of wine is strongly influenced by the solubility of inorganic compounds in the soil and, in principle, the multielement composition of wine reflects the soil geochemistry of the grape growing region (30). Recent research has been conducted to determine the geographic origin based on the composition of the elements assuming that the chemical composition of the wine reflects the soil composition (31), in which case their determination enables the establishment of a "fingerprint" for each element and creates the possibility of establishing a link between wine and their geographical origin (26). The potential of multielement "fingerprint" techniques to identify the geographical origin of wine was established in many investigations in different countries: Portugal (32, 33), Italy (34, 35), Slovakia (36), Croatia (37, 38), Spain (39–41), Romania (42), Cyprus (7, 14), Slovenia (4), Serbia (43), Macedonia (44), Ukraine (45), Turkey (46), Argentina (47), South Africa (31, 48, 49), and California (50), USA.

Numerous researchers applied combined isotopic and multielement methods to determine the geographical origin of wine. One of the oldest such studies was carried out on French wines from the Bordeaux region (51). The characterization of Swiss vineyards using isotope data in combination with trace elements and classical parameters has also demonstrated the possibilities of multidimensional statistical data processing (52). IRMS, ICP-OES, and NMR analysis of authentic wines that are part of a Cypriot bank of authentic wines as well as analysis of Cypriot commercial wines have been carried out and the observed variations in isotopes and elements were compared with grape varieties, environmental conditions, and geographical origin (7, 14). The possibilities of isotope and multielement techniques as "fingerprints" have been explored in regional differentiation of Romanian wines for 2 years of harvest and various autochthonous and introduced varieties (53). The differentiation of wine samples from the border areas of Austria, Czech Republic, Slovakia (and from Serbia) was investigated by applying different techniques (e.g., IRMS, NMR, ICP-MS, ICP-OES, EPR, HPLC, UV-VIS, etc.) showing promising possibilities for provenance studies (54).

Research to determine the geographical origin of wine has not only been conducted in Europe. Articles published by Argentine (55), Brazilian (56), Chinese (57), and American (58) authors are also available in the scientific literature. The most important conclusion of these studies is that the combined application of isotopic and multi-element methods with multivariate statistical methods will provide a promising statistical model for the classification of wines with regards to their geographical origin.

There are few published studies on determining the geographical origin of Croatian wines with regard to isotopic data (24), elemental profiles (37, 38, 59), or some other aspects of wine quality, i.e., polyphenolic composition (17). Present work is the first study to combine isotopic and multielemental methods for discrimination of Croatian wines according to their geographical origin.

The geographic position of Croatia is a meeting point of a continental climate in the eastern and central parts of the country, and the Mediterranean climate in the southern, coastal areas. It is divided into three viticulture climate zones (B, CI, and CII; **Figure 1**) according to the Winkler (60) division system and into four winegrowing regions or geographical indications (61), which include 16 protected denominations of origin (PDO) registered at database for EU geographical indications eAmbrosia (62).

Temperature based bioclimatic Winkler index (WI) uses a growing degree base of 10°C (growing degree-days, GDD) and correlate viticulture with the climate through five viticulture regions (60). Accordingly, zone B (1391-1670 GDD or WI Region II) corresponds to the wine region of Croatian Uplands. The zone CI (1671-1940 GDD or WI Region III) appertains to the wine region of Slavonia and Croatian Danube. Zone CII (1941-2220 GDD or WI Region IV)

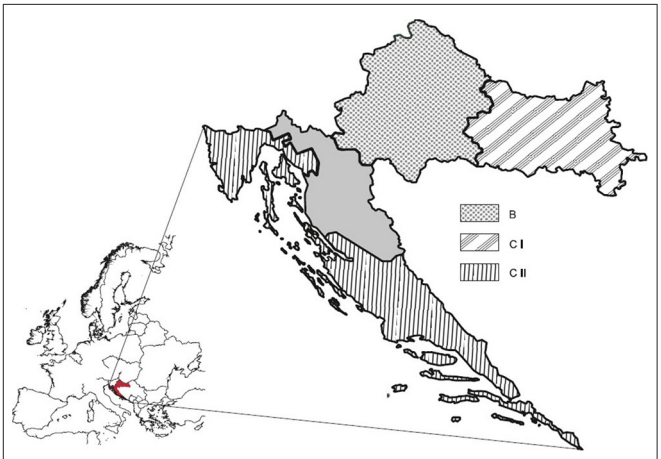

FIGURE 1 | Croatian winegrowing zones according to EU Regulation (63). Continental Croatia = zone B (Croatian Uplands) + zone CI (Slavonia and Croatian Danube). Coastal Croatia = zone CII = Croatian Istria and Kvarner + Dalmatia. Gray area, no winegrowing region.

includes two wine regions: Croatian Istria and Kvarner, and Dalmatia.

Croatia joined the EU in 2013 and consequently participates in the EU Wine Isotopic Databank in order to comply with EU legislation (64, 65). Following these requirements, we produced and analyzed microvinified wines that are a part of the Croatian national and EU Wine Databank bank as well as commercial wines from Croatian wine producing regions. $\delta^{18}O$ of wine water and $\delta^{13}C$ of wine ethanol were determined by Isotope Ratio Mass Spectrometry (IRMS) and concentrations of Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Pb, Rb, Sn, Sr, V, and Zn by Inductively Coupled Plasma—Optical Emission Spectrometry (ICP-OES). The aim of this study was to evaluate the obtained isotopic and multielement data by appropriate statistical methods in order to identify suitable geographical origin markers of Croatian wines and to obtain a chemometric tool for discrimination of wines produced in different Croatian winegrowing areas and zones.

MATERIALS AND METHODS

Wine Samples

One-hundred and ninety wines of two vintages (2015 and 2016) were analyzed. In total, 78 samples were a part of the Croatian bank of authentic wines produced by microvinification in accordance to the EU legislation (65), and 112 wines were conventionally produced and obtained by the Croatian Agency for Agriculture and Food, Center for Viticulture, Enology, and Edible Oils Analysis after the procedure of placing the wine on the Croatian market. Both indigenous and international white and red vine varieties were represented among the samples. The majority of these wines were monovarietal and evaluated as top quality after physicochemical analysis and sensory evaluation. The following parameters were determined: relative density, alcoholic strength, total dry extract, reducing sugars, pH, total acidity, volatile acidity, ash, free, and total sulfur dioxide. Obtained results were in accordance to the specifications of declared PDO registered at database for EU geographical indications eAmbrosia (62). The limits of tested parameters for Croatian top quality PDO wines listed in specifications are: alcoholic strength $\geq$ 8.5% (v/v) for zone B wines, and $\geq$9.0 % (v/v) for the wines from CI and CII zones; reducing sugar-free extract $\geq$ 18, 19, and 20 g L^{-1} (for white, rose, and red wine, respectively); total acidity (as tartaric acid) $>$ 3.5 g L^{-1}; volatile acidity (as acetic acid) $\leq$ 1.1 g L^{-1} for white and $\leq$1.2 g L^{-1} for red wines; ash content $\geq$ 1.5, 1.6, and 1.8 g L^{-1} (for white, rose, and red wines, respectively). With regard to the aspect of food safety, it is important to emphasize that the concentration of SO_2 in all samples did not exceed the limits: 150 mg L^{-1} for red and 200 mg L^{-1} for rose and white wines with the residual sugar content $\leq$ 5 g L^{-1}; and 200 mg L^{-1} for red and 250 mg L^{-1} for rose and white wines with the residual sugar content $>$ 5 g L^{-1}. Details about the origin of wines according to the winegrowing areas, zones, regions, type of production, harvest, and varieties are given in **Table 1**.

Sample Preparation for $\delta^{13}C$ Measurement

Determination of alcoholic strength of the wine samples was performed by electronic density meter coupled with near infrared spectrometer (DMA 4500 and Alcolyzer, Patent Anton Paar® (66); Anton Paar, Austria). Wine samples with a volume of 200 mL were distillated at ADCS—Automated Distillation Control System (Eurofins, Nantes, France), operated by the ADSC V1.1.9.0 software. Karl Fischer DL31 volumetric titrator (Mettler Toledo, Greifensee, Swizterland) operated by LabX light titration v2.6 software (Mettler Toledo, Greifensee, Swizterland) was used for the determination of the distillate water content (% w/w) in all obtained distillates in order to calculate the alcoholic strength (% w/w) and yield of each performed distillation. Eurokarl Windows v.1.0.0.0 software (Eurofins, Nantes, France) was used for transfer of the obtained alcoholic strength data to the ADSC V1.1.9.0 software (Eurofins, Nantes, France). Reagents used for the Karl-Fischer titration were Titrant 5, Solvent and 1% water standard for the standardization procedure of the solvent were obtained from Merck (Darmstadt, Germany). The requirements for the distillation procedure are described in the OIV method (OIV-MA-AS311-05:R2011) (67).

Sample Preparation for ICP-OES Measurements

Residue of wine after obtaining the ethanol for $\delta^{13}C$ measurement by ADCS distillation was used as described by Miloš et al. (68). The residue was returned to its initial volume and diluted 1:1 by 2% (v/v) HNO_3.

Sample Preparation for $\delta^{18}O$ Measurement

No sample preparation was required.

ICP-OES Measurements

The determination of 22 elements was conducted by 2000 Dual View Optima ICP-OES (Perkin Elmer, Shelton, Connecticut, USA) equipped with a Meinhard spray chamber (Meinhard, Golden, Colorado, USA), nebulizer, and peristaltic sample delivery system. The instrument was controlled by the ICP WinLab 1.35 Perkin Elmer software. The flow conditions for plasma gas, auxiliary gas, and nebulizer gas were 15.0 L min^{-1}, 0.2 L min^{-1}, and 0.8 L min^{-1}, respectively. Radio frequency generator power was set at 1,300 W. Samples were analyzed by calibration curve method including the internal standard. Operating conditions of the used method were previously published (69).

The 60 % (v/v) ultrapure HNO_3 (Merck, Darmstadt, Germany) was used diluted to 2% (v/v) by ultrapure water (18 MΩ cm^{-1} resistivity, Simplicity, Millipore, Molsheim, France) and used as blank, to prepare appropriate stock and calibration solutions and to dilute the samples. A 1 g L^{-1} ICP grade standard solution of yttrium (Perkin Elmer, Waltham, Massachusetts, USA) was used as an internal standard at the concentration of 100 µg L^{-1}. Multi-element standards were prepared in-house by mixing of certified, traceable, ICP grade single element standards: 1 g L^{-1} of B and Cr (Acros Organics, New Jersey, USA), As, Ca, Cd, Mg, Mo, Na, Pb, Zn, and 10 g/L of K (Perkin Elmer, Waltham, Massachusetts, SAD), 1 g L^{-1} of Al, Ba, Co, Cu, Fe,

TABLE 1 | Geographical areas, wine-growing zones, and regions, type of production (microvinified—A, commercial—C), harvest (2015 and 2016), and varieties (indigenous varieties are marked with an asterisk) of the investigated wine samples (Σn = total number of samples).

Area	**Continental Croatia = (zone B + zone CI)**		**Coastal Croatia = zone CII**		***Σn***
n (Area)	120		70		**190**
n (A)	42		36		**78**
n (C)	78		34		**112**
n (2015)	53		38		**91**
n (2016)	67		32		**99**
Zone	**B**	**CI**	**CII**		
Region	**Croatian Uplands**	**Slavonia and Croatian Danube**	**Croatian Istria and Kvarner**	**Dalmatia**	
n (Region)	78	42	29	41	**190**
n (A)	25	17	13	23	**78**
n (C)	53	25	16	18	**112**
n (2015)	31	22	16	22	**91**
n (2016)	47	20	13	19	**99**
White wine varieties (*n*)		Chardonnay (9), Gewürztraminer (3), Grüner Sylvaner (5), Kraljevina* (1), Moscato Giallo (1), Müller Thurgau (1), Pinot Blanc (4), Pinot Gris (5), Riesling Italico (47), Rhein Riesling (7), Sauvignon Blanc (8), Škrlet* (2), mixture of white varieties (1).	Bogdanuša* (1), Cetinka* (1), Chardonnay (6), Malvazija istarska* (8), Maraština* (1), Moscato Giallo (1), Pošip bijeli* (6), Vugava* (1), Žlahtina* (1).		**120**
Red wine varieties (*n*)		Blaufränkisch (5), Cabernet Sauvignon (8), Merlot (9), Pinot Noir (1), Zweigelt (2), mixture of red varieties (1).	Babić* (1), Cabernet Franc (1), Cabernet Sauvignon (2), Merlot (14) Plavac mali crni* (23), Plavina* (1), mixture of red varieties (2).		**70**

Li, Mn, Rb, Sn, Sr, and V (Reagecon, Shannon, County Clare, Ireland). To eliminate potential contamination, all glassware, and polypropylene storage bottles were rinsed by HNO_3 (2% v/v), and three times by ultrapure water, and allowed to dry before use.

Calibration was performed for each element at appropriate level (**Table 2**) and limits of detection and quantification were calculated as well as the recovery and measurement uncertainty (70, 71). The control charts of the standard reference material were used through the study period to ensure the quality of measurement results.

IRMS Measurements

IRMS measurements were performed by IRMS Delta V Plus (Thermo Fischer Scientific, Bremen, Germany) coupled to Gasbench and Elemental Analyzer FlashEA 1112 Series, for $\delta^{18}O$ and $\delta^{13}C$ measurements, respectively. Instruments were controlled by the Isodat 3.0 software (Thermo Fischer Scientific, Bremen, Germany). The isotopic ratios of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ are expressed in the delta notation, $\delta^{13}C$ and $\delta^{18}O$, respectively, as part per thousand (‰). Determination of stable isotope ratio of $\delta^{18}O$ in wine water was performed as described in the OIV method (OIV-MA-AS2-12:R2009) (67) after equilibration with helium and CO_2 mixture at 24 ± 1°C for 24 h. The samples were analyzed against in house reference material calibrated by the certified reference materials VSLAP2 and VSMOW2 obtained at International Atomic Agency, Vienna, Austria. Determination of stable isotope ratio of $\delta^{13}C$ in obtained ethanol was performed as described in the OIV method (OIV-MA-AS312-06:R2001) (67). The samples were measured against the certified reference material BCR-656 (Institute for Reference Materials and Measurements, Geel, Belgium). Chemicals used for filling the combustion reactor for conversion the sample ethanol in carbon dioxide were copper (II) oxide, silver cobaltous/cobaltic oxide, and chromic (III) oxide (Thermo Fischer Scientific, Bremen, Germany). The quality of measurement results was validated by control charts of appropriate reference materials, which were recorded during the study and confirmed by participating to the interlaboratory comparisons organized by Eurofins (Nantes Cedex, France). Satisfactory quality of isotope measurement results was confirmed by obtained z-scores ($-2.00 \leq z \leq 2.00$). Both methods for isotopic measurements are accredited in accordance to HRN EN ISO/IEC 17025:2017 (72), which confirms laboratory ability to perform valid and comparable stable isotope results.

Statistical Analysis

Results of isotopic and elemental analyses were uploaded to the software package Statistica 10.0 (Statsoft, Tulsa, Oklahoma, USA) and evaluated by descriptive statistical analysis (average

TABLE 2 | Ranges of calibration, limits of detection (LOD), and limits of quantification (LOQ) expressed as concentration in matrix, recovery (%), and expanded measurement uncertainty (%) for all elements.

Element (γ)	Calibration range (γ)	LOD (γ)	LOQ (γ)	Recovery (%)	Measurement uncertainty U (%)
Al (mg L^{-1})	0.1–2.0	0.0004	0.0015	93	5
As (μg L^{-1})	15–300	9	28	97	11
B (mg L^{-1})	0.25–5.0	0.001	0.002	94	12
Ba (mg L^{-1})	0.05–1.00	0.00001	0.00005	99	5
Ca (mg L^{-1})	5.0–100.0	0.004	0.014	101	15
Cd (μg L^{-1})	1–20	0.3	0.9	101	4
Co (μg L^{-1})	0.5–10.0	0.4	1.2	106	11
Cr (μg L^{-1})	0.5–10.0	0.3	1.2	106	16
Cu (mg L^{-1})	0.05–1.00	0.0004	0.0013	98	5
Fe (mg L^{-1})	0.5–10.0	0.003	0.009	101	7
K (mg L^{-1})	100–2,000	0.05	0.16	106	8
Li (μg L^{-1})	1–20	0.004	0.014	93	7
Mg (mg L^{-1})	5–100	0.002	0.005	99	7
Mn (mg L^{-1})	0.25–5.00	0.00004	0.00014	101	6
Mo (μg L^{-1})	0.5–10	0.1	0.2	107	33
Na (mg L^{-1})	1.0–20.0	0.00	0.01	98	7
Pb (μg L^{-1})	15–300	5	16	101	5
Rb (mg L^{-1})	0.25–5.00	0.0003	0.0009	96	9
Sn (μg L^{-1})	0.05–1.00	0.004	0.012	99	5
Sr (mg L^{-1})	0.05–1.00	0.000004	0.000014	100	7
V (μg L^{-1})	5–100	0.3	1.1	107	8
Zn (mg L^{-1})	0.25–5.00	0.0005	0.0018	101	7

values and standard deviations) and General Linear Model—Analysis of Variance (GLM-ANOVA) followed by *post-hoc* Tukey test and multivariate analysis methods. For statistical processing, elements with the values below the LOD were set to LOD/2 (73). Multivariate analysis was performed by principal component analysis (PCA) using the Unscrambler® software package, version 11.0 (CAMO AS, Norway) and general discriminant analysis (GDA) using the Statistica software package 10.0 (Statsoft, Tulsa, Oklahoma, USA). For visual presentation of results MS Excel® [Microsoft Office Professional Plus 2019, Microsoft Excel 2019 MSO (16.0.10354.20022)] was used.

RESULTS

All elements (Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Pb, Rb, Sn, Sr, V, and Zn) were analyzed by ICP-OES in appropriate linear calibration ranges (μg L^{-1} or mg L^{-1}), which are presented together with limits of detection (LOD), limits of quantification (LOQ), recovery (%), and expanded measurement uncertainty (%) in **Table 2**. The achieved recovery was between 93% (for Al and Li) and 107% (for Mo and V). Assessment of expanded measurement uncertainty (with the coverage factor of $k = 2$, which gives a 95% confidence level for normal distribution) showed the highest expanded uncertainty for Mo (33%) and the lowest expanded uncertainty for Cd (4%).

GLM-ANOVA showed the significant interaction effect of the harvest year ($F = 10.535$; $p < 0.001$), type of production ($F = 15.553$; $p < 0.001$), and viticulture zone ($F = 9.274$; $p < 0.001$) on tested measurands (stable isotopes and elements). The effect of the type of production was significant for the harvest ($F = 4.843$; $p < 0.001$) and viticulture zones ($F = 2.133$; $p < 0.001$), and also the mutual interaction of these three effects was significant ($F = 1.709$; $p < 0.005$) indicating that these attributes were useful in characterizing the differences among the measured values in wines. The significance between the effects of harvest year and viticulture zones was not found.

The results of stable isotopes ($\delta^{18}O$ and $\delta^{13}C$) and 22 elements measurements in a set of 190 Croatian wine samples are given in **Table 3** according to the area of production (continental and coastal) and viticulture zones (B, CI, and CII) in Croatia, together with the GLM-ANOVA and *post-hoc* Tukey test results. The measurands with important significance found by GLM-ANOVA ($p < 0.05$) for the type of production (microvinified vs. commercial), harvest year (2015 vs. 2016), area (continental vs. coastal), viticulture zones B vs. CI, B vs. CII, and CI vs. CII are marked by numbers from 1 to 6, respectively. The average values of measured stable isotopes $\delta^{18}O$ and $\delta^{13}C$ were 1.37 ± 2.56‰ SMOW and −27.57 ± 1.47‰ V-PDB, respectively. The ICP-OES analyses results showed that Croatian wines contain elements that may contribute to the daily dietary intake of essential metals (i.e., copper, iron, and zinc) but can also have potentially toxic effects if metal concentrations are not kept under allowable limits. Analyzed wines contain also elements that have no nutritional value but are known to be potentially toxic, like

TABLE 3 | Summary of results (average values with standard deviations) for all measurands and samples according to the area of production (continental and coastal Croatia) and winegrowing zones (B, CI, and CII) in Croatia.

Measurand (Unit)	GLM-ANOVA ($p < 0.05$)[a]	All samples ($n = 190$)	Continental Croatia = Zone B + Zone CI ($n = 120$)	Coastal Croatia = Zone CII ($n = 70$)	Zone B ($n = 78$)	Zone CI ($n = 42$)
$\delta^{18}O$ (‰ SMOW)	1, 2, 3, 4, 5, 6	1.37 ± 2.56	−0.22 ± 1.47	4.09 ± 1.52	−0.61 ± 1.44	0.51 ± 1.23
$\delta^{13}C$ (‰ V-PDB)	2, 3, 5, 6	−27.57 ± 1.47	−28.31 ± 1.01	−26.29 ± 1.24	−28.38 ± 0.99	−28.20 ± 1.06
Al (mg L^{-1})	1, 3, 5	0.59 ± 0.52	0.51 ± 0.42	0.74 ± 0.64	0.48 ± 0.39	0.56 ± 0.48
As (μg L^{-1})		7.5 ± 5.4	7.2 ± 5.1	8.2 ± 5.9	7.2 ± 5.0	7.2 ± 5.2
B (mg L^{-1})	1, 3, 5, 6	2.98 ± 1.14	2.62 ± 1.03	3.60 ± 1.07	2.76 ± 0.97	2.36 ± 1.10
Ba (mg L^{-1})	6	0.11 ± 0.05	0.11 ± 0.05	0.10 ± 0.04	0.11 ± 0.05	0.12 ± 0.04
Ca (mg L^{-1})	1, 2, 3, 5	85.0 ± 22.7	89.3 ± 21.2	77.5 ± 23.6	92.1 ± 20.9	84.1 ± 20.9
Cd (μg L^{-1})	2	0.7 ± 1.0	0.7 ± 1.0	0.8 ± 0.8	0.7 ± 1.2	0.5 ± 0.7
Co (μg L^{-1})	2	5.9 ± 4.4	5.5 ± 3.9	6.6 ± 5.1	5.8 ± 4.4	4.9 ± 2.6
Cr (μg L^{-1})	2	19.0 ± 17.0	17.4 ± 12.0	21.8 ± 23.1	17.4 ± 11.8	17.4 ± 12.6
Cu (mg L^{-1})		0.18 ± 0.14	0.17 ± 0.15	0.18 ± 0.12	0.18 ± 0.17	0.16 ± 0.09
Fe (mg L^{-1})	1	1.91 ± 1.39	1.86 ± 1.43	20.1 ± 1.34	1.78 ± 1.32	2.00 ± 1.61
K (mg L^{-1})	3, 5, 6	788 ± 226	730 ± 180	889 ± 260	730 ± 179	730 ± 185
Li (μg L^{-1})	3, 6	4.6 ± 3.2	5.2 ± 3.5	3.6 ± 2.3	4.7 ± 3.3	6.0 ± 3.6
Mg (mg L^{-1})	1, 4, 5	81.3 ± 17.9	79.8 ± 16.6	83.9 ± 19.8	76.8 ± 15.9	85.4 ± 16.5
Mn (mg L^{-1})	4, 6	0.96 ± 0.52	1.01 ± 0.58	0.87 ± 0.39	0.92 ± 0.63	1.19 ± 0.45
Mo (μg L^{-1})	2	4.3 ± 2.1	4.4 ± 2.2	4.1 ± 2.0	4.7 ± 2.3	4.0 ± 2.0
Na (mg L^{-1})	1, 3, 5, 6	14.3 ± 18.1	10.6 ± 7.0	20.8 ± 27.3	10.7 ± 7.9	10.2 ± 5.0
Pb (μg L^{-1})	1, 2, 3	30.2 ± 18.7	28.0 ± 17.2	33.8 ± 20.5	29.1 ± 16.5	26.1 ± 18.6
Rb (mg L^{-1})	3, 5, 6	1.08 ± 0.41	0.99 ± 0.42	1.22 ± 0.35	1.05 ± 0.43	0.88 ± 0.37
Sn (μg L^{-1})	2, 3, 5, 6	55.0 ± 30.3	48.4 ± 31.1	66.3 ± 25.3	46.8 ± 29.2	51.6 ± 34.6
Sr (mg L^{-1})	1	0.46 ± 0.21	0.46 ± 0.17	0.46 ± 0.28	0.46 ± 0.18	0.45 ± 0.15
V (μg L^{-1})	1	83.6 ± 16.8	82.4 ± 15.8	85.6 ± 18.3	80.0 ± 15.1	86.7 ± 16.2
Zn (mg L^{-1})		0.69 ± 0.37	0.67 ± 0.32	0.73 ± 0.44	0.69 ± 0.34	0.64 ± 0.27

[a]*Measurands with $p < 0.05$ for: (1)—type of production; (2)—harvest year; (3)—continental and coastal area; (4)—zones B and CI; (5)—zones B and CII; (6)—zones CI and CII.*

arsenic, cadmium, and lead (74, 75). The results showed that the lowest concentration of all samples had the micro-elements Cd (0.7±1 μg L^{-1}), Mo (4±2 μg L^{-1}), Li (5±3 μg L^{-1}), Co (6±4 μg L^{-1}), and As (8±5 μg L^{-1}). The highest concentrations had the macro-elements K (788±226 mg L^{-1}), Ca (85±23 mg L^{-1}), Mg (81±18 mg L^{-1}), and Na (14±18 mg L^{-1}). The determined concentrations of As, B, Cd, Cu, and Pb that are related to the safety of wines were within the acceptable limits established by the OIV—International Organization of Vine and Wine (76). Maximum permitted concentration (80 mg L^{-1}) prescribed by the OIV was exceeded only for Na in three samples. The obtained results suggest that moderate consumption of Croatian wines may contribute to the daily dietary intake of essential minerals and trace elements without the danger of exceeding admissible daily dose or causing a toxic effect according to dietary reference values for nutrients of European Food Safety Authority (EFSA) (77).

A study of the data structure by PCA was carried out to aid in interpretation of the obtained data and to establish whether the wines from different wine producing geographical areas and viticulture zones constitute distinctive, well-defined groups. PCA was performed for all wines and variables (isotopes and elements) to determine whether different geographical regions of origin (areas and zones) had influenced the isotopes and elements profile. In this context, 2 isotopic ratios and 22 elements posed as the investigated variables, while wines posed as the cases under investigation.

PCA performed for entire dataset of microvinified and commercial wines ($n = 190$) is explaining only 65% of variability by first seven factors. The first two factors (PC1 and PC2) represent 29.2% of the initial data variability and 40% with the third factor (PC3). The remaining 25% of significant variability is hidden in the remaining four factors (PC4–PC7). Total variability of the first seven factors and eigenvectors of correlation matrix for all samples obtained by PCA is shown in **Table 4**.

The PCA model for entire data set was validated by segmented cross validation (random method, 20 segments, and 9 samples per segment) and significance of the variables (p-value) was estimated by a t-test. Most of the measurands were found to be significant ($p < 0.05$) for the differentiation of the geographical origin according to the obtained p-values (**Table 4**). Only Cu and Zn showed no significance in either of PC1–PC7. Because the calibration set (the raw data set, $n = 190$) explained only 65%, and the validation set correctly explained only 39% of total variability for first seven PCs (**Table 4**), it was concluded that overall uncertainty of the model is fairly high. Hence, the

TABLE 4 | Eigenvectors (EV) of correlation matrix for all samples ($n = 190$) obtained by PCA for first seven factors (PC1–PC7 with eigenvalues of correlation matrix > 1), total variability % for calibration set (TV), total variability % obtained by cross validation (CV), and significance of the variables (highlighted are the $p < 0.05$).

	PC1		PC2		PC3		PC4		PC5		PC6		PC7	
TV (%)	15,64		13,52		10,87		7,43		6,41		5,82		5,15	
CV (%)	7,90		10,19		7,61		3,59		1,62		3,09		4,63	
Measurand	**EV**	***p***	**EV**	***p***	**EV**	***p***	**EV**	***p***	**EV**	***p***	**EV**	***p***	**EV**	***p***
$^{18}O/^{16}O$	0.08	0.03	0.04	0.06	−0.48	0.00	−0.15	0.00	0.20	0.00	0.09	0.23	−0.18	0.06
$^{13}C/^{12}C$	0.08	0.04	0.08	0.01	−0.46	0.00	0.04	0.44	0.27	0.00	0.06	0.46	−0.12	0.22
Al	0.28	0.00	−0.09	0.01	−0.09	0.02	−0.40	0.00	−0.14	0.11	0.07	0.44	0.26	0.08
As	0.08	0.09	−0.01	0.71	−0.08	0.02	−0.18	0.02	−0.26	0.00	−0.26	0.02	0.58	0.00
B	0.04	0.03	0.23	0.00	−0.29	0.00	0.10	0.00	−0.31	0.00	−0.31	0.00	−0.12	0.05
Ba	0.23	0.00	0.02	0.54	0.26	0.00	−0.15	0.06	0.28	0.00	−0.33	0.00	−0.17	0.04
Ca	0.11	0.01	0.24	0.00	0.27	0.00	0.24	0.00	−0.28	0.00	0.02	0.82	0.07	0.38
Cd	0.11	0.00	−0.24	0.00	−0.15	0.00	0.48	0.00	0.09	0.06	−0.04	0.42	0.18	0.01
Co	0.36	0.00	−0.29	0.00	−0.02	0.62	0.08	0.12	−0.05	0.21	−0.07	0.32	−0.12	0.26
Cr	0.22	0.00	−0.17	0.00	−0.10	0.29	0.02	0.82	−0.21	0.01	0.05	0.68	−0.35	0.06
Cu	0.01	0.92	0.00	0.98	0.03	0.65	0.07	0.64	−0.25	0.31	0.19	0.25	−0.36	0.40
Fe	0.13	0.05	−0.25	0.00	−0.07	0.30	−0.17	0.17	0.21	0.12	−0.03	0.86	0.18	0.49
K	0.11	0.01	0.16	0.00	−0.23	0.00	0.12	0.09	−0.07	0.21	−0.49	0.00	0.02	0.80
Li	0.22	0.00	−0.11	0.00	0.28	0.00	−0.20	0.03	0.08	0.42	−0.07	0.67	−0.15	0.23
Mg	0.28	0.00	0.34	0.00	0.03	0.13	0.19	0.00	0.24	0.00	0.09	0.04	0.11	0.16
Mn	0.28	0.00	0.01	0.49	0.25	0.00	−0.09	0.15	0.23	0.02	−0.23	0.08	−0.19	0.07
Mo	0.24	0.00	−0.21	0.00	0.06	0.15	0.33	0.00	−0.26	0.00	0.06	0.23	−0.07	0.45
Na	0.29	0.03	−0.02	0.59	−0.11	0.04	−0.27	0.12	−0.20	0.30	0.28	0.22	−0.02	0.88
Pb	0.24	0.00	−0.34	0.00	−0.18	0.00	0.17	0.00	0.08	0.11	0.03	0.64	0.14	0.17
Rb	0.03	0.53	0.18	0.00	−0.14	0.01	−0.19	0.04	−0.19	0.10	−0.36	0.01	−0.22	0.14
Sn	0.11	0.01	0.36	0.00	−0.10	0.02	−0.08	0.14	0.10	0.19	0.30	0.00	0.06	0.52
Sr	0.30	0.00	0.19	0.00	0.09	0.18	−0.01	0.92	−0.15	0.14	0.10	0.27	0.12	0.23
V	0.30	0.00	0.34	0.00	0.04	0.04	0.23	0.00	0.20	0.00	0.06	0.16	0.09	0.19
Zn	0.16	0.08	0.11	0.05	0.01	0.86	−0.11	0.47	−0.22	0.36	0.22	0.32	−0.03	0.92

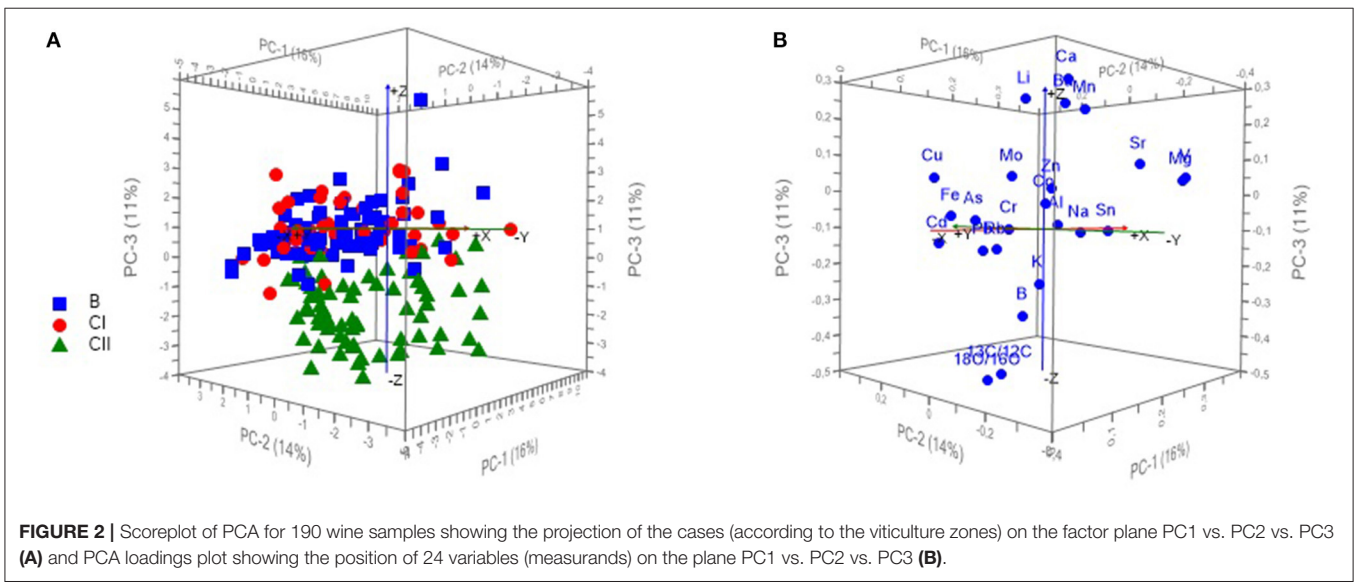

FIGURE 2 | Scoreplot of PCA for 190 wine samples showing the projection of the cases (according to the viticulture zones) on the factor plane PC1 vs. PC2 vs. PC3 **(A)** and PCA loadings plot showing the position of 24 variables (measurands) on the plane PC1 vs. PC2 vs. PC3 **(B)**.

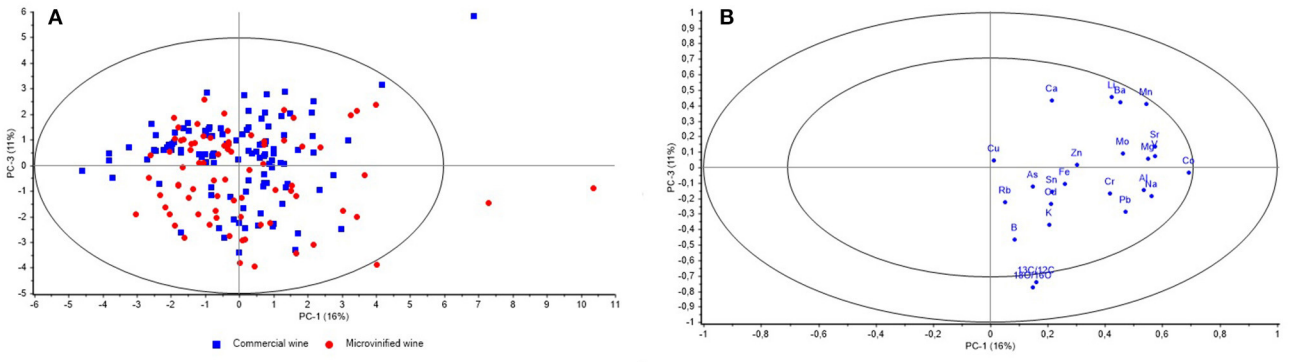

FIGURE 3 | Scoreplot of PCA for 78 samples of microvinified wines and 112 samples of commercial wines showing the projection of the cases (according to the type of production) on the factor plane PC1 vs. PC3 **(A)** and PCA correlation loadings plot showing the position of 24 variables (measurands) on the plane PC1 vs. PC3 **(B)**.

additional statistical tool of multivariate analysis (GDA) needed to be applied.

Scoreplot of PCA for 190 wine samples is showing the projection of the cases (according to the viticulture zones) on the factor planes PC1 vs. PC2 vs. PC3 (**Figure 2A**), where wines from the continental part of Croatia (zones B and CI) are positioned mostly on the positive side of PC3 while the wines from coastal Croatia (zone CII) remained on the negative side of the PC3. Positioning of the variables on the factor planes PC1 vs. PC2 vs. PC3 can be observed at **Figure 2B** indicating the strongest influence of $\delta^{18}O$ and $\delta^{13}C$ on the grouping of the samples from coastal Croatia.

Since microvinified samples are part of the Croatian national isotope database of authentic wines, PCA was also carried out to visualize the effect of the type of production on the positioning of the samples on the factor planes in order to establish whether the microvinified wines can be used as a representative set for the authenticity evaluation of declared geographical origin of commercial wines by used set of variables (stable isotopes and elements). In addition to the entire data set, the PCA was also performed for microvinified and commercial wines separately. Eigenvalues of correlation matrix (>1) showed that 76% of total variability is explained by the first eight factors for the microvinified, and 71% for commercial data set (**Supplementary Table 1**).

The scoreplot of PCA for microvinified wines and commercial wines (**Figure 3A**) is showing the projection of the cases according to the type of production on the factor plane PC1 vs. PC3 and with the 95% confidence interval. Rather uniform distribution of microvinified and commercial samples in the PC1 and PC3 planes can be observed, indicating the same effect of the measured values (stable isotopes and elements) influencing the distribution of the samples, both microvinified and commercial. **Figure 3B** is showing PCA correlation loadings plot with the position of 24 variables (measurands) in the plane PC1 vs. PC3 and the Hotelling's T^2 ellipse representing 50 and 100% of modeled variance (r^2 = 0.5/1). The highest effect on the variability explained by

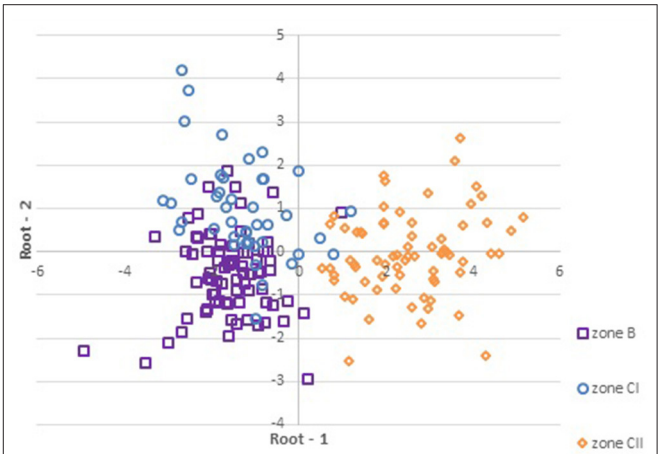

FIGURE 4 | Projection of the scores (GDA) of the samples (n = 190) depending on zoning system in the plane defined by the two standardized canonical discriminant function coefficients (Root 1 and Root 2).

PC3 have variables $\delta^{18}O$ and $\delta^{13}C$ positioned between the two ellipses.

GDA analysis was performed to choose the variable with the most significant contribution to the discrimination between continental and coastal winegrowing areas of Croatia and then for the viticulture zones B, CI, and CII. The reduced number of variables was used based on the significance obtained by PCA (Cu and Zn were omitted). Also, the model was validated through cross-validation using the set of microvinified wines as the model, and the set of commercial wines as unknown samples.

Figure 4 depicts the projection of the cases (zones) on the Root 1 vs. Root 2 where wines from the continental part of Croatia (zones B and CI) are positioned mostly on the negative side of root 1 while the wines from coastal Croatia (zone CII) remained on the positive side.

Multivariate test of significance (Wilks test, $p \leq 0.05$; **Supplementary Table 2**) showed that most significant for geographical areas discrimination (coastal and continental) are

TABLE 5 | Classification matrix obtained by GDA showing the percentage of correctly predicted classifications (%) vs. the observed classifications for continental and coastal winegrowing areas and zones B, CI, and CII.

Winegrowing areas and zones	Complete data set (all wines) ($n = 190$)	Authentic wines ($n = 78$)	Crossvalidation data set (commercial wines) ($n = 112$)
Continental Croatia	97.5	100.0	87.2
Coastal Croatia	98.6	100.0	79.4
Total	**97.9**	**100.0**	**84.8**
Zone CII	100.0	100.0	79.4
Zone B	91.0	96.0	67.9
Zone CI	54.8	82.4	44.0
Total	**86.3**	**94.9**	**66.1**

$\delta^{18}O$ and Co, followed by K, Rb, Sn, Li, and $\delta^{13}C$, and for discrimination of samples according to the zones most significant are $\delta^{18}O$ and Co, followed by Rb, Li, K, and Sn, in descending order. Classification matrix obtained by GDA, showing the percentage of correctly predicted classifications (%) vs. the observed classifications for continental and coastal winegrowing areas and zones B, CI, and CII, is shown in **Table 5**. In the entire dataset ($n = 190$) the GDA classification matrix correctly classified 97.9% of the samples in regards to the winegrowing areas, while for the microvinified and commercial wines, correct classification was achieved for 100.0 and 84.8% of the samples, respectively.

Regarding the three viticulture zones, 86.3% of correct classification was achieved for the entire dataset, while for the microvinified and commercial wines, correct classification was achieved for 94.9 and 66.1% of the samples, respectively.

Regarding the differentiation between two zones (B and CI) of the continental part of Croatia, which can also be observed at the **Figure 4** to some extent, GDA showed correct classification for 67.9% of the samples from zone B and for only 44.0% of the samples from zone CI. Correct classification was obtained for 79.4% of the CII zone samples.

DISCUSSION

The isotopic and multielement composition of the analyzed wines and statistical methods were used as chemical descriptors in order to establish criteria for wine classification and differentiation according to geographical origin.

The measurands with important significance found by GLM-ANOVA ($p < 0.05$) and marked from 1 to 6 in **Table 3** are herein discussed in more detail. *Post-hoc* test (Tukey test) was conducted to evaluate the significance of the influence of the type of sample production, vintages, and winegrowing areas (continental and coastal Croatia), and viticulture climate zones (B, CI, and CII) on the measurands.

GLM-ANOVA of obtained results for all samples in regards to the type of production (**Table 3**, measurands denoted by 1) showed statistically significant differences between microvinified and commercial samples for $\delta^{18}O$, Al, B, Ca, Fe, Mg, Na, Pb, Sr, and V, which could be caused by oenological practices employed for production of commercial samples but lacking at the microvinification process, and by the differences in the size of actual samples (25 kg of grapes for microvinified vines). The $\delta^{18}O$ values differences could also imply the mislabelling of the commercial samples in regard to the geographical origin or vintage, or possibility of water addition. Nevertheless, intensive rainfall during grape harvest also will be reflected in the isotope ratios values (13). To establish the possibility of fraudulent activities more elaborate investigation of isotopic ratios should be employed (23).

Regarding the harvest year (2015 and 2016), there were significant differences for $\delta^{18}O$, $\delta^{13}C$, Ca, Cd, Co, Cr, Mo, Pb, and Sn (**Table 3**, measurands denoted by 2), showing the contribution of the seasonal meteorological conditions influencing their uptake (5, 78). $\delta^{18}O$ of water values were more positive (in average for 1‰) in the 2015 than in the 2016 harvest. This can be explained by the higher rainfall in September during the 2016 harvest (average 152 mm) (79) compared to the 2015 harvest (average 88 mm) (80). Similar influence of rainfall on $\delta^{18}O$ of wine water was observed by previous research (13, 56). Variations of $\delta^{13}C$ can also be the result of plant growth conditions, which can significantly modify 13C isotope values (81), in particular, the use of CO_2 from photorespiration by the plant that reacts to water deficit by closing the stomata (82).

Regardless of the studied vintage year or the type of production, the GLM-ANOVA of isotopic ratios and multielement content enabled the discrimination of the two studied winegrowing areas (continental and coastal Croatia) and three winegrowing zones (B, CI, and CII). The statistically significant discrimination of the continental and coastal winegrowing areas was achieved for the following measurands: $\delta^{18}O$, $\delta^{13}C$, Al, B, Ca, K, Li, Na, Pb, Rb, and Sn (**Table 3**, measurands denoted by 3). These measurands were also identified as the key explanatory factors in various combinations for geographical origin determination by other researches, i.e., for Spanish (40, 41), Italian (34, 35), Romanian (53, 83, 84), Cypriot (7), USA (85), Brazilian (56), or Chinese (57) wines.

Wine samples from continental vineyards presented significantly lower average values of $\delta^{18}O$ than those from coastal vineyards (−0.2 and 4.1‰ SMOW, respectively). These differences between geographical areas can be explained by the specific climatic conditions of each individual area, such as temperature, humidity, as well as meteorological conditions. The mean values of $\delta^{18}O$ found in this research are consistent to those obtained for Croatian wines of vintages 1999–2001 (24). Obtained $\delta^{18}O$ values are also in accordance with the wines from different European regions (22). The range of $\delta^{13}C$ values of wines from the two investigated geographical areas is variating from −26.3‰ V-PDB in coastal part of Croatia to −28.3‰ V-PDB in continental area. The mean values of $\delta^{13}C$ found in the present work are similar to those obtained by previous research (22, 24).

Samples from the coastal Croatian vineyards had significantly higher content of Al, B, K, Na, Pb, Rb, and Sn than the continental vineyards. The values of Na were almost double in coastal (21 mg L^{-1}) than in continental areas (11 mg L^{-1}) due to the proximity of the Adriatic Sea. This influence of the sea on the elevated Na content was observed by other investigations (7, 86, 87). As opposed to this, the continental vineyards were characterized by higher levels of Ca (89 mg L^{-1}) and Li (5 μg L^{-1}) than in coastal vineyards (76 mg L^{-1} of Ca and 4 μg L^{-1} of Li).

Statistically significant discrimination between two continental winegrowing zones B and CI is achieved only by the $\delta^{18}O$, Mg, and Mn (**Table 3**, measurands denoted by 4). As expected with regards to geographical and climatic conditions, average values of $\delta^{18}O$ of wine water from the eastern continental part of Croatia (zone CI) were higher than those of the wines from vineyards in the western continental region of Croatia (zone B), 0.51 and −0.61‰ SMOW, respectively. Both elements, the Mg and Mn, had higher content in zone CI (85 and 77 mg L^{-1}, respectively) than in zone B (1.2 and 0.9 mg L^{-1}, respectively).

In regards to the differentiation of the coastal zone CII vs. continental zone B, significant were the same measurands (**Table 3**, measurands denoted by 5) as for entire costal vs. continental area, with the exception of Pb and the addition of Mg, which was able to discriminate between zones CII and B (84 and 77 mg L^{-1}, respectively).

$\delta^{18}O$, $\delta^{13}C$, B, Ba, K, Li, Mn, Na, Rb, and Sn (**Table 3**, measurands denoted by 6) enabled the differentiation of the coastal zone CII vs. continental zone CI. Average values of element B were significantly higher in coastal zone CII (3.6 mg L^{-1}) than in continental zone CI (2.4 mg L^{-1} respectively). Ba was found to be significant only in discrimination of zones CI and CII, but it also enabled the geographical origin differentiation in the research of Croatian (37), Italian (35), Romanian (42), and South African (31) wines.

Compared to the PCA results for the entire data set, which explains 65% of the variability, the set of microvinified samples has a higher percentage of explained variability (75%) and better presents the geographic origin than the whole data set (**Supplementary Table 1**). This difference can be explained by the fact that microvinified samples do not have the influence of elements from the production process, i.e., Al, B, Cu, K, Fe, Mn (88–91). In these samples, the distinction of geographical origin is achieved only by endogenous measurands that reached the wine naturally, i.e., stable isotopes (5) or elements Mg, Sr (57, 88) and/or as natural contaminant such as Na (91). Even commercial samples evaluated separately by PCA have explained more variability (71%) than the whole set (**Supplementary Table 1**). This can also be explained by the influence of a technological process that is more or less similar in all commercial samples. Hence, it can be concluded that the combination of samples of different types of production leads to less explained overall variability.

As seen at **Figure 2A**, the 3D representation of the samples obtained from PCA using the raw data matrix (190 samples and 24 measurands) and the first three components indicates a satisfactory separation of samples according to the geographical area, although the first three components explained only 40% of the total variation. The samples from continental Croatia (zone CII) are well-distinguished from the samples from coastal Croatia (zones B and CI). The differentiation of continental zones B and CI by PCA method was not achieved.

It is shown that $\delta^{18}O$ and $\delta^{13}C$ (**Figure 2B**) have the strongest influence on separation of the CII zone from B and CI zones in the plane PC1 vs. PC2 vs. PC3 (**Figure 2A**). The significance of this influence is also visible at the **Figure 3B**, which is showing Hotelling's T^2 ellipse representing 50 and 100% of modeled variance ($r^2 = 0.5/1$). The highest effect on the variability explained by PC3 have variables $\delta^{18}O$ and $\delta^{13}C$ positioned between the two ellipses.

Overlapping of microvinified and commercial samples in the PC1 vs. PC3 planes (**Figure 3A**) is showing similar impact of the measured values on observed variability. This is an indication that the microvinified wines can be used as a representative set for the authenticity evaluation of declared geographical origin of commercial wines by used set of variables (stable isotopes and elements). However, the positioning of three microvinified samples from CII zone and one commercial sample from zone B outside of the 95% confidence interval can be noticed. This could be the result of specific microclimatic and pedologic characteristics of individual vineyards locations and it can be supported by the research of Croatian winegrowing regions (92) where it was found that both Western and Eastern continental Croatian regions, ranging from 1,323.9 to 1,652.5 GDD for the observed climatologic period (1988–2017) belong to the Winkler Regions I and II (zone A and B). In regards to Coastal Croatia in the same period, values ranged from 1,496.5 to 2,483.5 GDD, which is Winkler Region II to V (zones B, CI, CII, and CIII). The reason for outlying of the commercial sample from the zone B should be explored in more detail, considering all relevant meteorological and winegrowing parameters such as precipitation, harvest date, grape variety, and to use a representative number of reference samples (23).

GDA was found to be the most distinguishing chemometric tool for discrimination of Croatian wines according to the area of geographical origin. As seen at **Table 5**, the highest power for discrimination of wines produced in coastal and continental Croatia showed GDA by correct classification 100.0% of microvinified samples, 97.9% of all investigated samples, and 84.8% of commercial samples in the cross-validation matrix.

GDA showed somewhat weaker separation (**Figure 4**) of the zones B and CI in comparison to the excellent discrimination of continental and coastal areas. This can be explained by incompliance between official borders of the zones (**Figure 1**) (63) and actual situation presented by previous research (92), which established that within the zone B exist a smaller area corresponding to the Winkler Region I (zone A) and that Slavonia and Croatian Danube fall into Region II, which is zone B and not CI as stated by the current EU division system.

This can explain the deviation of some samples outside of the designated zones in particular if taking into consideration that the most dominant marker of the geographical origin identified by this research is the $\delta^{18}O$, which is also strongly influenced by the climate (5, 22).

The analysis of bioclimatic indices in Croatian winegrowing regions (92) would enable more accurate interpretation of isotopic and multielement data found in this research as the tools for Croatian wine geographical origin determination. Furthermore, current administrative division of the zones established by the EU legislation (63) is defining the limits and conditions for certain oenological practices (enrichment limits/increase in the natural alcoholic strength) where climatic conditions have made it necessary in certain winegrowing zones. Consequently, a question arises of interpreting the isotopic data from EU wine data bank in regards to chaptalization, requiring a larger number of representative samples and expert interpretation. The shortcomings of Croatian vineyards zoning are also suggested by projections of further warming and drying of the climate in Croatia (93), making the existing viticulture zoning even less adequate.

This study verified that stable isotopes of oxygen and carbon have proven to be most valuable indicators of discrimination of wines from Croatian winegrowing areas and zones and especially in the combination with the multielemental composition analysis, which was conducted here for the first time for Croatian wines.

Results suggest that the proposed methodology is a powerful tool and it could add extra value to local Croatian wines by emphasizing the wine authenticity importance, especially in the light of the growing tourism industry and increasing awareness of winemaking significance as economic activity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RL and MB: conceptualization. RL and IVP: methodology and writing-original draft preparation. JJ and RL: formal analysis and investigation. MB: writing-review and editing and supervision. All authors: contributed to the article and approved the submitted version.

FUNDING

This research was supported by the Center for Viticulture, Enology and Edible Oils Analysis, Croatian Agency for Agriculture and Food, Zagreb, Croatia.

ACKNOWLEDGMENTS

The authors would like to thank to the wine producers for the support of this study by donation of grapes for microvinification.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.625613/full#supplementary-material

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Conflict of Interest: JJ was employed by Department of Physico-Chemical Testing, Center for Viticulture, Enology and Edible Oils Analysis, Croatian Agency Agriculture and Food, Zagreb, Croatia, during the research and as she performed the ICP-OES analyses. After the experimental part of the research was finished, she changed her job and her current employment is at company Primevigilance d.o.o., Zagreb, Croatia.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
published: 31 May 2021
doi: 10.3389/fsufs.2021.638941

Isotope Analysis (^{13}C, ^{18}O) of Wine From Central and Eastern Europe and Argentina, 2008 and 2009 Vintages: Differentiation of Origin, Environmental Indications, and Variations Within Countries

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In this study, we compare the stable isotope composition of oxygen and carbon of wines from four Central and Southeastern European countries and from Argentina to study the similarities and differences in the isotope signatures and, thus, the potential of differentiation of the various wine-growing countries. We observe similar trends for wines from Austria, Slovenia, and Romania with respect to the vintages 2008 and 2009, which are absent in the Montenegrin and Argentinean samples. It is speculated that the weather develops similarly for Austria, Slovenia, and Romania, as these countries are positioned at a similar latitude and not too far away from each other (general central and eastern European weather situation), whereas Montenegro is not influenced by the latter being situated farther south and dominantly influenced by the Adriatic Sea. Investigations on further vintages are needed to test this assumption.

Keywords: oxygen isotope, carbon isotope, water, alcohol, authenticity, geographic origin

OPEN ACCESS

Edited by:
Matteo Balderacchi, Independent Researcher, Piacenza, Italy

Reviewed by:
Constantinos Georgiou, Agricultural University of Athens, Greece
Matteo Perini, Fondazione Edmund Mach, Italy

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Specialty section:
This article was submitted to Crop Biology and Sustainability, a section of the journal Frontiers in Sustainable Food Systems

Received: *07 December 2020*
Accepted: *24 March 2021*
Published: *31 May 2021*

Citation:
Horacek M, Ogrinc N, Magdas DA, Wunderlin D, Sucur S, Maras V, Misurovic A, Eder R, Čuš F, Wyhlidal S and Papesch W (2021) Isotope Analysis (^{13}C, ^{18}O) of Wine From Central and Eastern Europe and Argentina, 2008 and 2009 Vintages: Differentiation of Origin, Environmental Indications, and Variations Within Countries. Front. Sustain. Food Syst. 5:638941. doi: 10.3389/fsufs.2021.638941

INTRODUCTION

In the last century, several wine scandals occurred in the 70's and 80's in Europe, a few of them even had lethal consequences (https://www.spiegel.de/wirtschaft/tod-in-italien-a-7490f6a9-0002-0001-0000-000013519771?context=issue). To tackle this situation seriously affecting the consumers' confidence, rights, and health, in 1990, the EU commission passed a regulation and installed the EU-wine database. For this database, every wine-producing EU-country has to collect a certain number of grape samples in the vineyards every year, transform the grapes into wine by micro-vinification, and measure the isotope pattern of these samples by isotope ratio mass spectrometry (IRMS) and site-specific natural isotope fractionation nuclear magnet resonance (SNIF-NMR). EU amended and replaced the

earlier ones. Initially, the isotope investigations were solely intended for wine authenticity control, to identify any illegal addition of water or sugar (chaptalization and sweetening).

As the isotope ratio of most biogenic materials and especially plant tissue is influenced by their environmental conditions, the control of declared geographic origin can also be carried out by these analyses. As wine from certain wine-growing regions and also vineries receive far higher prices (e.g., sparkling wine from Champagne/France) than wine from other areas or wine producers, there is also a need to control the geographic origin. The investigation of the geographic origin of wine by analyzing the isotope pattern is founded on the idea that every region and locality has unique environmental conditions. Stable isotope measurements for the control of declared food origin investigate the isotope ratio of elements influenced by, e.g., weather and water availability, distance from the sea, altitude, soil, and natural and anthropogenic emissions (e.g., Rossmann, 2001; Camin et al., 2007, 2010; Horacek and Min, 2010; Horacek et al., 2010, 2015; Schellenberg et al., 2010). The isotope ratio in precipitation is a result of the climate and geographic position (Bowen and Revenaugh, 2003). Water vapor evaporating from a water surface is isotopically depleted with respect to the water from which it emanates (Dansgaard, 1964). This isotopic fractionation is temperature-dependent, with a strong fractionation at low temperatures and minor fractionation at elevated temperatures (temperature effect). Water vapor migrating in clouds over a continent becomes isotopically successively more and more depleted in ^{18}O and ^{2}H (continental effect), as the heavy oxygen and hydrogen isotopes preferentially enter the liquid phase (rain, snow) and are, in this way, removed from the clouds (Gat and Gonfiantini, 1981).

For wine, the weather is a very important influencing factor, not only with respect to quality and quantity, but also regarding the stable isotope pattern. Martin and Martin (2003) identified that $\delta^{13}C$ is positively correlated with the mean temperature and negatively correlated with the amount of precipitation during the sugar accumulation and grape ripening period in wines. In addition, they showed that the wine water $\delta^{18}O$ value is positively correlated with the temperature and negatively with the amount of precipitation, but also additional influences by other climatic parameters. Furthermore, in Californian wines, a good correlation was found for $\delta^{18}O$ and the daily relative humidity in the 3 weeks prior to harvesting, for crop evapotranspiration in September, and for the average maximum daily temperatures from July (Ingraham and Caldwell, 1999). Other studies demonstrate the influence of relative humidity of a period of 30 days prior to harvest and the $\delta^{18}O$ of atmospheric humidity on the wine water $\delta^{18}O$ (Hermann and Voerkelius, 2008). For Italian wines, a statistical evaluation of the relevant influencing parameters (including geographical and climatic information) on the isotope values has been carried out by Camin et al. (2015); for Austrian wines, this has been preliminary done by Heinrich et al. (2016). A study on the differentiation of the geographic origin of wine from a border region applying a diverse combination of physico-chemical methods, to investigate strengths and limitations of these methods, and the potential of their combination has been carried out by Horacek et al. (2019a,b).

FIGURE 1 | Map indicating the countries of origin for the investigated wine samples.

As the weather conditions play such a fundamental role for the isotope pattern of wine, the year of harvest is important since the weather varies from year to year (e.g., Christoph et al., 2003, 2015; Magdas et al., 2012; Philipp et al., 2018). The investigation of wine harvested in 2008 and 2009 is of relevance for wine control, as (I) wine is a commodity that is not only consumed as young wine, but also as aged wine, with the (suitable) aged wine becoming more esteemed and, thus, expensive, and (II) the environmental conditions do vary from year to year, but similarities between certain years are usually found (e.g., cold-humid vs. hot-dry weather) and, thus, past vintages show ranges of variations between years, relevant for control of wine without vintage information.

In the present study, wine samples from Austria, Slovenia, Romania, Montenegro, and Argentina (**Figure 1**) have been collected, processed, and analyzed for their carbon and oxygen isotope values. The isotope results of samples are compared with respect to vintages and with respect to the investigated samples from the other countries. The aim of this work was to investigate if any pattern exists in both vintages and the potential of discrimination of the samples from other origins. Furthermore, comparison of European wines with samples from Argentina shows whether there is a distinctive correlation of distance and isotope patterns of the wine samples. We hypothesize that we can differentiate the wine samples by their isotope patterns due to their geographic origin. The further away the geographic origin, the better the discrimination for identical vintages.

Wine Regions

Wine samples of the years 2008 and 2009 from Austria, Slovenia, Romania, Montenegro, and Argentina have been collected (**Table 1**) and analyzed. As some of these samples (from Austria and Slovenia) are the official ones for the EU wine database, which were harvested in selected vineyards by governmental collectors and vinified applying a standardized protocol, an assignment to a certain area, locality, or winery was not done to retain data confidentiality. The Romanian samples come from only two of the wine producing regions, Oltenia and Muntenia, both located in the very south of Romania (Magdas et al., 2012) and, therefore, do not represent the isotopic variation of the entire Romania, which is significantly larger (see Magdas et al., 2012). The Montenegrin wine samples come from "13. Jul Plantaže," the biggest winery in Montenegro, which produces around 50% of the entire Montenegrin wine production. Argentinean wine samples come from the Mendoza area, except for one sample from the Neuqen region.

SAMPLES AND METHODS

The wine samples from Austria, Romania, and Slovenia were collected, processed, and analyzed according to the EU regulation EC No. 555/2008 and the Compendium of the OIV (OIV-MA-AS312-06, 2001; OIV-MA-AS2-12, 2009; International Organisation of Vine and Wine (OIV), 2014). For the other countries (Argentina and Montenegro), commercially produced bottled wines were collected and analyzed. All samples were distilled using an automated distillation control system using cadiot distillation columns by Eurofins/Nantes, France. Distilled samples have a yield better than 90%. Carbon isotope ratio was analyzed of the distillated ethanol, oxygen isotope ratio of the wine water in bulk wine samples. The samples from Austria and Montenegro were analyzed at the Austrian Institute of Technology GmbH Tulln stable isotope laboratory. Argentinean wine samples were measured at the BLT Wieselburg stable isotope facility. Slovenian samples were processed at the Agricultural Institute of Slovenia and analyzed at the Jožef Stefan Institute, Ljubljana and the Romanian samples at the National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca. Instrumentation details and descriptions can be found in **Supplementary Material 1**.

The results are expressed in the conventional δ-notation in ‰ with respect to the V-SMOW (Vienna-Standard Mean Ocean Water) and with respect to the V-PDB (Vienna-PeeDee Belemnite) standards for oxygen and carbon, respectively, which are as follows:

$$\delta X‰ = ((Rsample/Rstandard) - 1)x\ 1000 \quad (1)$$

where X is ^{13}C or ^{18}O and R is the ratio of $^{13}C/^{12}C$, or $^{18}O/^{16}O$, respectively. The enlarged reproducibility of measurements of $\delta^{13}C$ and $\delta^{18}O$ were better than ±0.3 and ±0.5‰, respectively, for all laboratories. For quality control and comparability of the results, identical or comparable certified standards and reference materials were analyzed together with the wine samples. Among the measured standards are V-SMOW ($\delta^{18}O = 0.0‰$) and SLAP ($\delta^{18}O = -55.5‰$) (both provided by the International Atomic Energy Agency (IAEA); and BCR 660 ($\delta^{13}C = -26.72‰$) and BCR 656 ($\delta^{13}C = -26.91‰$) [both produced by the Institute of Reference Materials and Measurements (IRMM)].

Statistical Evaluation

The statistical evaluation has been carried out using classifications by the linear discriminant analysis (LDA). The chemometric processing of experimental data was made using the SPSS Statistics 24 (IBM, USA). The algorithm behind this method is based on finding a linear combination among the analyzed variables, which separates the predefined classes of samples. By these combinations, a model is obtained, which is validated by the "leave-one-out" cross validation method. This method removes each sample from the sample-set and reclassifies it as an unknown. Outlier test was performed using the Grubbs test by the Graphpad Software (graphpad.com; USA) and orthogonal partial least squares discriminant analysis (OPLS-DA) by the SIMCA (https://www.sartorius.com/en/products/process-analytical-technology/data-analytics-software/mvda-software/simca; Germany).

RESULTS

A short summary is given in **Table 1** and graphically shown in **Figures 2**, **3A,B**. Austrian wine samples from 2008 have $\delta^{13}C$ values ranging from −25.8 to −29.7‰, and from 2009, from −25.3 to −30.0‰. The $\delta^{18}O$ values of the Austrian samples are between −3.0 and +1.8‰ for 2008 and between −2.6 and

TABLE 1 | Number (*n*) of wine samples per year and country, average $\delta^{13}C$ and $\delta^{18}O$ values, and standard deviation for 2008 and 2009.

	n	*n*	$\delta^{13}C$		$\delta^{18}O$		$\delta^{13}C$		$\delta^{18}O$	
Country	**2008**	**2009**	**Av $\delta^{13}C$ 2008**	**STDEV**	**Av $\delta^{18}O$ 2008**	**STDEV**	**Av $\delta^{13}C$ 2009**	**STDEV**	**Av $\delta^{18}O$ 2009**	**STDEV**
Austria	50	50	−28.0	±0.8	−1.3	±1.1	−28.2	±0.9	0.3	±1.3
Slovenia	20	22	−28.4	±0.8	1.4	±2.1	−28.0	±1.0	2.0	±2.4
Romania	9	14	−25.7	±0.8	3.5	±0.8	−27.0	±1.0	4.0	±1.7
Montenegro	3	3	−27.4	±0.7	8.0	±0.2	−27.8	±0.2	8.1	±0.2
Argentina	6	5	−27.2	±0.5	2.8	±2.1	−27.2	±0.3	2.0	±0.8

+3.1‰ for 2009 (**Figures 2**, **3A,B**). Average values for 2008 are −28.0 ± 0.8‰ and −1.3 ± 1.1‰ and for 2009, −28.2 ± 0.9 ‰ and 0.3 ± 1.2‰ for ^{13}C and ^{18}O, respectively. The Austrian samples from the same year form a cluster with a slightly positive correlation of the investigated parameters. The cluster of the year 2008 shows an about 1.5‰ lower value than the cluster from 2009, but almost no difference in the $\delta^{13}C$ averages. The presented data are the first published combined C- and O-isotope results of wine from Austria.

The Slovenian samples range in $\delta^{13}C$ from −29.5 to −27.1‰ for the year 2008 and between −29.9 and −26.6‰ for the year 2009. Oxygen isotope values vary between −3.0 and +6.7‰ for 2008 and −2.0 and +5.3‰ for 2009 (**Figures 2**, **3A,B**). The 2008 and 2009 average values for $\delta^{13}C$ are −28.4 ± 0.8‰ and −28.0 ± 1.0‰, respectively. For $\delta^{18}O$, the average values are 1.4 ± 2.1 and 2.0 ± 2.4‰ for 2008 and 2009, respectively. The Slovenian sample results of the same year are falling into two clusters, one cluster hosting the "enriched" isotope samples and the other one, the "depleted" samples. The two investigated parameters are clearly positively correlated and separated from each other by a 1.5–2‰ gap in the oxygen isotope values. In addition, for the Slovenian samples, the vintage 2008 have lower $\delta^{18}O$ values compared with 2009, and also, the $\delta^{13}C$ is lower. The presented values are in good agreement with the data (wine samples from 1996–1998) published by Ogrinc et al. (2001). In the mentioned publication, the two clusters described above have also been identified ("enriched" cluster: coastal area, "depleted" cluster: Sava and Drava areas), however, for the investigated years, these clusters overlap. This fact might be due to the annual weather variations, but also, the larger sample set investigated by Ogrinc et al. (2001) might have influenced the outcome to some extent. For the vintages 2008 and 2009, it seems to be a complete separation as two separate clusters are clearly present, however, this is a speculation as the exact geographic origin of the wine samples is not revealed.

The Romanian samples vary in $\delta^{13}C$ from −27.2 to −24.4 and −28.7 to −24.8‰ for the years 2008 and 2009, respectively. In $\delta^{18}O$, the Romanian samples range between 1.4 and 4.2 and 1.3 and 6.7‰ for the years 2008 and 2009, respectively (**Figures 2**, **3A,B**). Average $\delta^{13}C$ values are −25.7 ± 0.8 and −27.0 ± 1.0‰ for 2008 and 2009, and average $\delta^{18}O$ values are 3.5 ± 0.8 and 4.0 ± 1.7‰ for 2008 and 2009, respectively. In addition, for the Romanian samples, a significant difference in the average values is present for the 2008 and 2009 clusters. The data fit very well with the published data by Magdas et al. (2012).

The wine samples from Montenegro have $\delta^{13}C$ values between −28.1 and −26.8‰ and −28.0 and −27.5‰ for the years 2008 and 2009, respectively. The $\delta^{18}O$ values are within 7.4 and 8.7 and 7.5 and 8.7‰ for the years 2008 and 2009, respectively (**Figures 2**, **3A,B**). The sample average $\delta^{18}O$ is 8.0 and 8.1‰ and the sample $\delta^{13}C$ are −27.4 ± 0.7 and −27.8 ± 0.2‰ for the years 2008 and 2009, respectively. The values cluster at more or less the same place for the two investigated years. The Montenegrin samples show a very homogenous pattern. These are, to our knowledge, the first published data for the Montenegrin wine. They are in agreement with the isotope results of the wine from the coastal area of Croatia (Christoph et al., 2003).

The Argentinean samples range in $\delta^{13}C$ from −28.1 to −26.7‰ and −27.6 to −26.8‰ for the years 2008 and 2009, respectively. In $\delta^{18}O$, the values are between 0.8 and 6.5 and 1.0 and 3.2‰ for 2008 and 2009, respectively (**Figures 2**, **3A,B**). Average $\delta^{18}O$ values are 2.8 ± 2.1 and 2.0 ± 0.8‰ and average $\delta^{13}C$ values are 27.2 ± 0.5 and 27.2 ± 0.3‰ for 2008 and 2009, respectively. The samples from both years cluster more or less at the same range (between −27.5 and −26.5‰ for carbon and 0.8 and 3.2‰ for oxygen), with the exception of one sample from 2008 possessing an entirely different $\delta^{18}O$ value coming from a different wine-growing area in Argentina. Grubbs outlier test confirmed the value as an outlier (Z: 2.51775358743, Significant outlier. $P < 0.05$, Critical value of Z: 2.35472945013). If this sample is ignored, the average values are almost identical for both years. Nevertheless, the small sample set has to be considered with respect to the completeness, as obviously, an increase in the samples might significantly influence the result. The outlier sample was produced in another wine-growing region (Neuqen) of Argentina than the other samples, which came from Mendoza, the largest Argentinean wine production area. If we disregard that sample, the isotope results are more or less identical for both years. The isotope values are well in agreement with the literature data (Di Paola-Naranjo et al., 2012, Christoph et al., 2015). The single "outlier" datum is a sample from the southernmost Argentinean wine-growing region (Neuqen area) possessing a significantly different environmental conditions than the other Argentinean wine producing areas.

Statistical evaluation by LDA shows differing results for the vintages 2008 and 2009. Whereas for 2008, an average correct classification of around 80% (84.1% initial classification, 79.5% cross-validation) is achieved for the samples from the five investigated countries (**Figure 4**), the correct classification

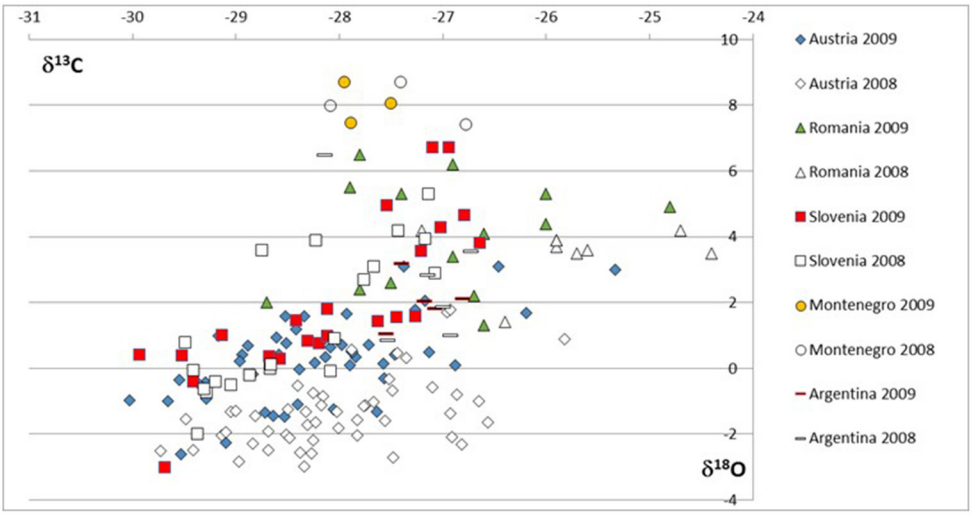

FIGURE 2 | $\delta^{13}C$ vs. $\delta^{18}O$ of the investigated wine samples (from Austria, Slovenia, Romania, Montenegro, and Argentina) from 2008 and 2009.

decreases to around 55% (56.4% initial classification, 54.3% cross-validation) in 2009 (**Figure 5**). Taking into account only the three bigger sample sets (with sample numbers of nine and above for each vintage) from Austria, Romania, and Slovenia increases the results to almost 90% (89.9% initial classification, 88.6% cross-validation) for 2008 (**Figure 6**) but to only above 60% (62.8% initial classification, 62.8% cross-validation) for 2009 (**Figure 7**). Combining both vintages of the investigated samples from all five countries result in ca. sixty-five percentage correct classifications (67.0% initial classification, 65.4% cross-validation; **Figure 8**). For details, see **Supplementary Material 3**. OPLS-DA demonstrated a significance in the differentiation between the samples of the respective countries [$P < 0.0001$ for all countries including both vintages (**Supplementary Material 2**)] and also a significance in the differentiation of both vintages ($p < 0.00348$), due to the differences in the oxygen isotope values. Significantly lower $\delta^{18}O$ values were observed in 2008 compared to 2009.

DISCUSSION

Austria

Despite the rather small size of the entire wine-growing area in Austria, the wine isotope values are very heterogeneous as the wine-producing regions in Austria are situated north, east, and south of the Eastern Alps, which have a strong influence on the regional weather. Nevertheless, the general weather trend (as a main influencing factor for the C- and O-isotope ratios) influences the vintages and resulted in significantly higher $\delta^{18}O$ values for the year 2009 than 2008, although there is some overlap. Still, it is to be expected that most Austrian wines of these years can be assigned to the respective year (most likely due to the oxygen isotope value) as demonstrated by the statistical evaluation, where almost 70% of the vintages were correctly classified (**Supplementary Material 3**). The carbon isotope values, on the other hand, are very similar and do only show very moderate differences, despite the variation in $\delta^{18}O$. The most likely explanation is a larger amount of precipitation in September 2008 (or generally within the last 4 weeks prior to harvest; Christoph et al., 2003) resulting in lower $\delta^{18}O$ values for the 2008 vintage.

Slovenia

The results of the Slovenian wines fall clearly into two clusters, as described above. Many of the 2009 vintage samples have higher $\delta^{18}O$ values than the 2008 vintage, although the shift toward higher values in 2009 is lower in its magnitude than for the Austrian samples of the same vintage. This indicates that the weather condition responsible for the variation of the oxygen isotopes in Austrian wines also influenced the Slovenian vintage (**Figures 2**, **3A,B**), but to a slightly lower extent than the Austrian samples (see **Table 1**). However, as the trend lines of the two vintages are very similar in Slovenia, it seems that in addition to the variation in $\delta^{18}O$, some positively correlated trend also occurs in the $\delta^{13}C$ values of the Slovenian wine samples, possibly indicating a slightly higher draft stress condition, resulting in higher $\delta^{13}C$ ratios for the 2009 vintage as well. By initial classification and cross-validation, ca. eighty percentage of the Slovenian samples were correctly assigned to the respective vintages (**Supplementary Material 3**).

Romania

In addition, the $\delta^{18}O$ values of the Romanian samples show the influence of the general weather condition in 2008 and 2009 with lower values in 2008 and higher in 2009, with a similar shift between the 2 years as for the Slovenian wine samples. However, in 2009, the samples show a larger spread of values, indicating a differentiation in the regional weather conditions in Romania.

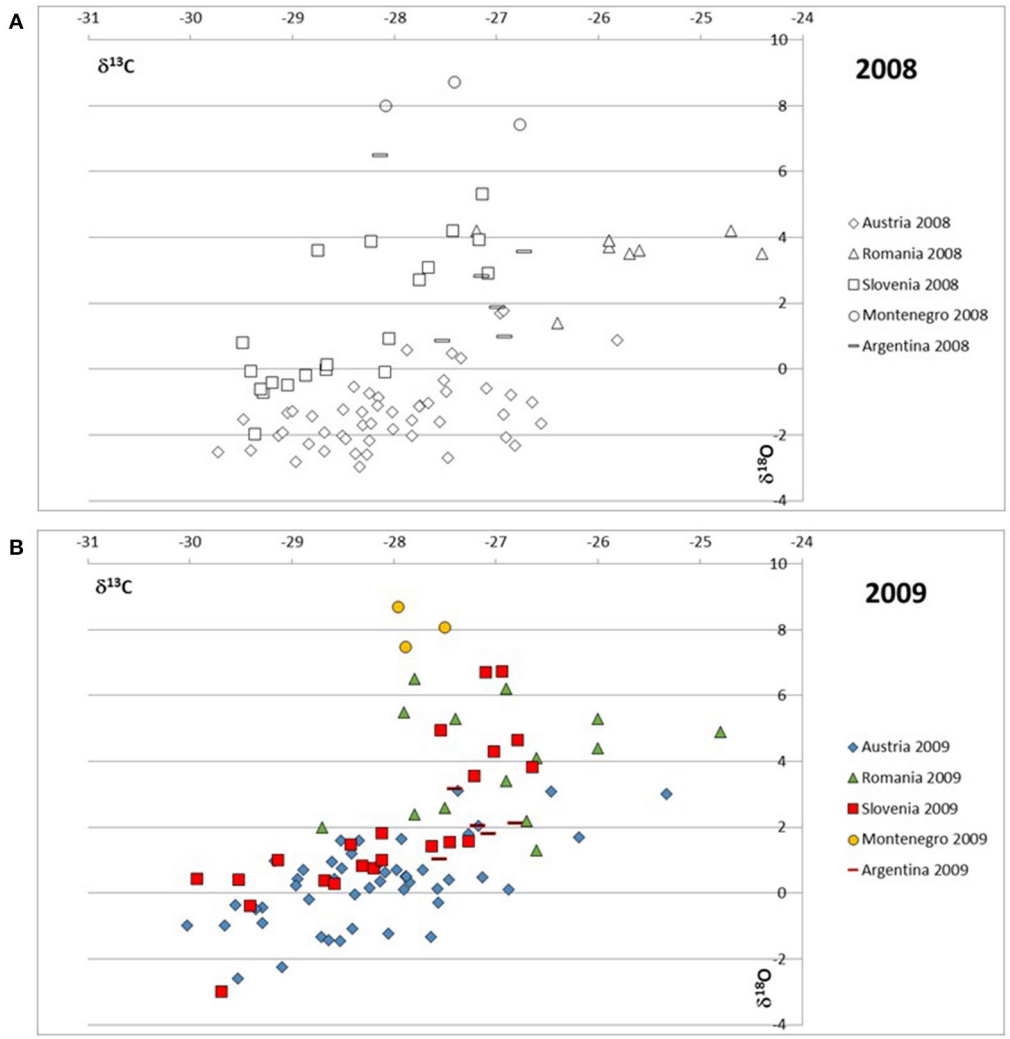

FIGURE 3 | (A) $\delta^{13}C$ and $\delta^{18}O$ values of vintage 2008. For this year, an almost complete discrimination is achieved for the samples of the investigated countries (except for Argentina). **(B)** $\delta^{13}C$ and $\delta^{18}O$ values of vintage 2009. In 2009, there is a trend to more extreme values (high and low) for the samples from Austria, Slovenia, and Romania with respect to 2008. Due to this broadening, the point clouds overlap.

This assumption is also supported by the $\delta^{13}C$ values that have a smaller range in 2008 than 2009.

Montenegro

The Montenegrin wine samples show very similar values for both years, indicating stable and homogenous conditions during the investigated years and suggest that Montenegro was not influenced by the general weather conditions of central and eastern Europe. This lead to having identical trends in the wine isotopes in the three countries discussed above, or that such weather conditions did not significantly influence the vine and wine isotope pattern due to stable agricultural conditions as water supply and isotopic composition of water available to the vines. It has to be taken into account that the samples are just from one producer and, thus, do not reflect the variation among the produced Montenegrin wines. On the other hand, however, this producer accounts for about 50% of the Montenegrin wine production and the samples demonstrate homogenous conditions resulting in homogenous isotope values for the investigated vintages.

Argentina

The Argentinean wine samples generally show similar values for both investigated vintages, with one outlier coming from a separate wine area (Neuqen). The $\delta^{18}O$ values are low if we take into account the warm and arid climates of most wine-growing regions in the western Central and Northern provinces of Argentina. This can be explained by the irrigation with river water from high mountains, which might lead to a significant lowering of the oxygen isotope values (Gómez-Alonso and García-Romero, 2010), as the river water coming from the High Andes mountain range has low isotope values, and the general

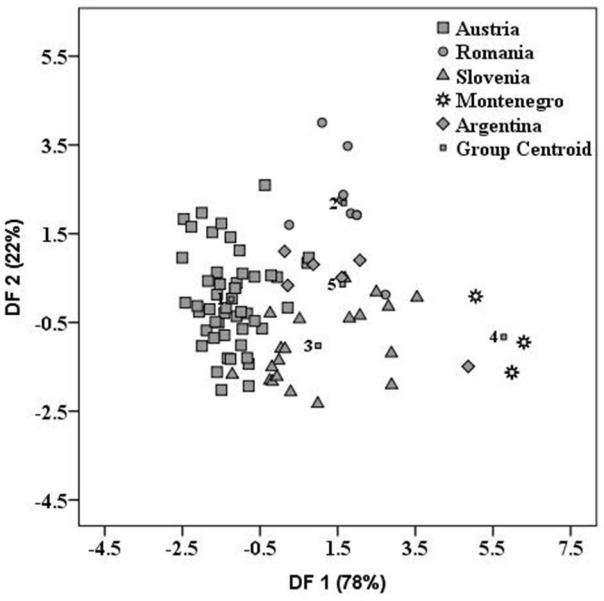

FIGURE 4 | LDA evaluation of the investigated samples from Austria, Slovenia, Romania, Montenegro, and Argentina for 2008.

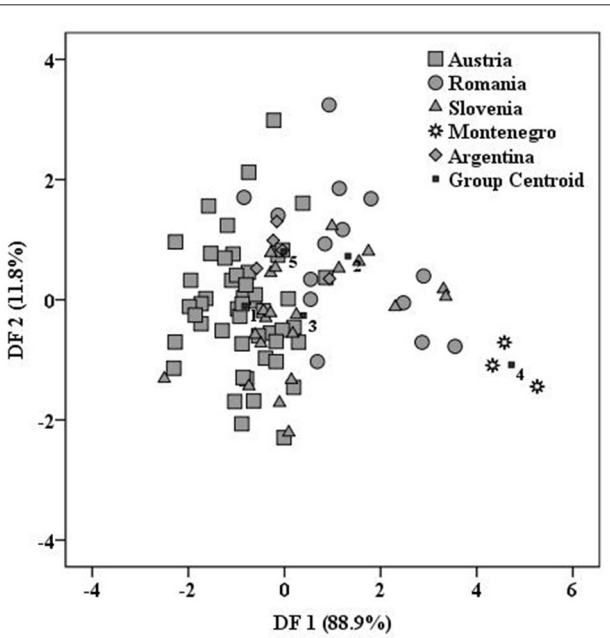

FIGURE 5 | LDA evaluation of the investigated samples from Austria, Slovenia, Romania, Montenegro, and Argentina for 2009.

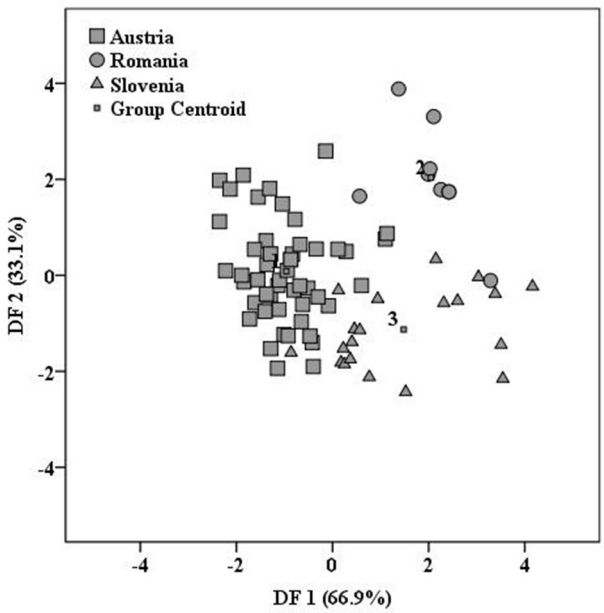

FIGURE 6 | LDA evaluation of the investigated samples from Austria, Slovenia, and Romania for 2008.

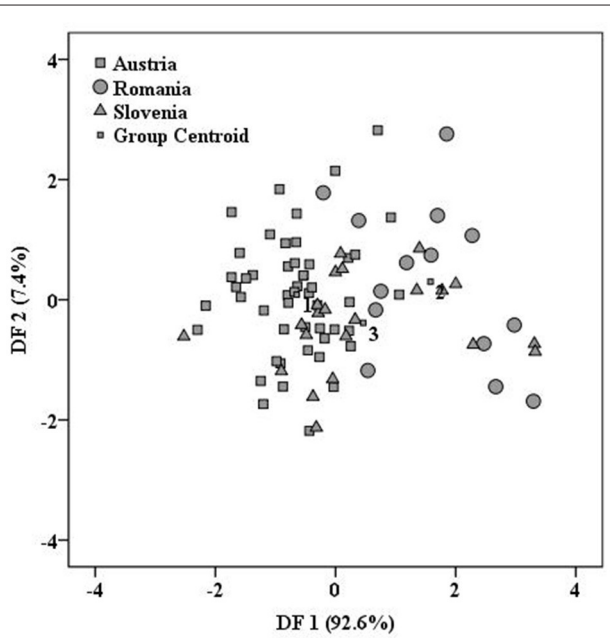

FIGURE 7 | LDA evaluation of the investigated samples from Austria, Slovenia, and Romania for 2009.

climate situation in Central and Northern Argentina. The sample from the Neuqen area exhibiting a significantly higher $\delta^{18}O$ value indicates (but this needs to be verified with more samples of different vintages) the use of local water in this area enriched in oxygen isotopes.

Differentiation of Geographic Origin

Regarding the differentiation of geographic origin for the 2008 vintage, the investigated samples from the different regions only slightly overlap (**Figures 2**, **3A,B**). This indicates that, given certain environmental and weather conditions, there is a high

potential to differentiate geographic origin by measuring $\delta^{13}C$ and $\delta^{18}O$ with respect to the wine from Austria, Slovenia, Romania, Montenegro, and Argentina, even if no further indication of regional origin or exact locality is known. This is quite remarkable, as southern Austrian and northeastern Slovenian wine-growing areas are adjacent and a significant overlap of the data should be expected.

This overlap, though, occurs for the Austrian and Slovenian (supposedly the Drava and Sava cluster) samples of the 2009 vintage, enabling an incomplete differentiation of this vintage from these two areas only (**Figures 3A,B**). Furthermore, the year 2009 seems to have caused stronger regional differences within Austria, Slovenia and Romania, as the samples of these countries show a larger spread of values with respect to 2008. Still, a differentiation of geographical origin can be achieved, if additional information of origin (region, locality, winery) will be given for the investigated samples.

Due to its southern and coastal position, Montenegrin wine always (with respect to the investigated vintages and samples) can be differentiated from wine of the other countries investigated, but literature data indicates an overlap with wine from adjacent regions in Croatia (Christoph et al., 2003).

Argentinean wine samples exhibit quite homogenous values, but the "outlier sample" from a separate wine-growing area indicates that a much larger variations in the Argentinean wine isotope values can be expected, if a more comprehensive study (e.g. Griboff et al. 2021[1]) includes samples from every wine-growing area of this large country.

The Argentinean samples overlap with the samples of the other countries investigated, except for the samples from Montenegro. This demonstrates that despite a large distance between different origins, wine samples from these areas can still sometimes have similar isotope patterns. However, the more parameters are investigated, the less likely is such a similarity (Di Paola-Naranjo et al., 2012). Identified trends and influences need to be verified by further vintages, to unequivocally classify them as constantly existing besides the annual weather variations.

As the general pattern of lower $\delta^{18}O$-values in 2008 and higher in 2009 occurs for the Austrian, Slovenian, and Romanian samples, we assume the existence and influence of a "general central and eastern European weather situation for the investigated relevant period resulting in these features. The Montenegrin samples do not show any influence of this phenomenon, potentially indicating that it is absent in Southern Montenegro. However, it has to be taken into account that the Montenegrin samples only represent one winery (although one of the biggest in Europe) and might not be representative for all of Montenegro.

CONCLUSIONS

The two investigated vintages from Austria, Slovenia, and Romania show significant congruent variations in the isotope pattern with lower $\delta^{18}O$ values in the 2008 vintage (**Figures 2**, **3A**). This indicates the existence and influence of a "general central and eastern European weather situation." Dominantly, the oxygen isotope value is more relevant for geographic differentiation. The Romanian samples also show a significantly larger variation in $\delta^{13}C$ in 2009 indicating stronger variable specific climatic conditions in the different wine-growing areas in that vintage. Montenegrin wine samples have similar values for both investigated years and are, thus, not influenced by the proposed weather situation. The Montenegrin wine can easily be distinguished by its enriched isotope values. The Argentinean wine samples significantly overlap with other investigated samples, evidencing the possibility of similar patterns despite the large distances between the compared regions. The investigation of further parameters as $(D/H)_I$ and $(D/H)_{II}$ by SNIF-NMR, Sr-isotopes, and trace element pattern can reduce these similarities. For 2008, the investigated samples from the different Central and Southern European countries show almost no overlap and can, thus, be nicely discriminated. For 2009, the samples have a larger overlap. The isotope variations between 2008 and 2009 are significant and enable identification of vintages in most cases for the investigated wines from Austria and Romania.

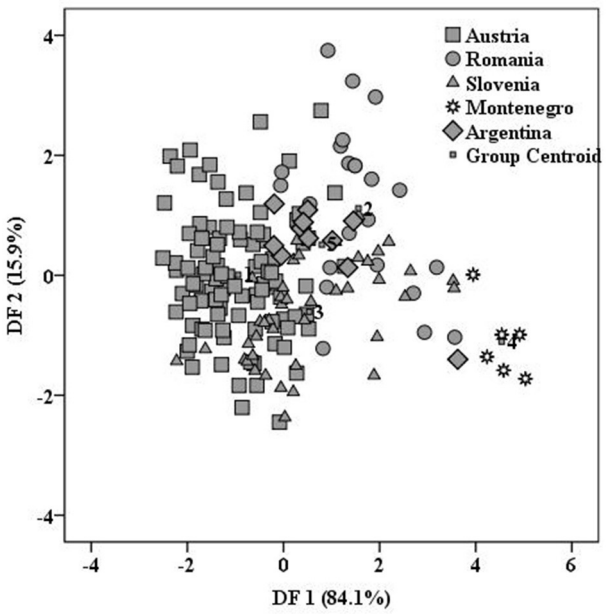

FIGURE 8 | LDA evaluation of the investigated samples from Austria, Slovenia, Romania, Montenegro, and Argentina for both investigated vintages 2008 and 2009.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

[1]Griboff, J., Horacek, M., Wunderlin, D. A., and Monferrán, M. V. (2021). Differentiation between Argentine and Austrian wines based on isotopic and multi-elemental composition (In review).

AUTHOR CONTRIBUTIONS

MH designed the project, analyzed the Austrian, Montenegrin, and Argentinian wine samples, and wrote the manuscript. NO analyzed the Slovenian samples, performed statistical evaluation, and contributed to the manuscript. DM analyzed the Romanian samples, performed the statistical evaluation, and contributed to the manuscript. DW provided the Argentinean wine samples and contributed to the manuscript. SS, VM, and AM provided the Montenegrin wine samples and contributed to the manuscript. FČ organized vinification of Slovenian grape samples and wine distillation. RE organized vinification of Austrian grape samples. SW and WP analyzed Austrian wine samples. All authors contributed to the article and approved the submitted version.

FUNDING

AM and MH benefited from a bilateral project (Austria-Montenegro by ÖAD, grant number: ME 05/2011), NO and MH benefited from a bilateral project (Austria –Slovenia by ÖAD, grant number: SI 11/2011), DW and MH benefited from a bilateral project (Austria-Argentina by ÖAD, grant number: AR 12/2013), and DM and MH benefited from a bilateral project (Austria—Romania by ÖAD, grant number: RO 07/2009).

ACKNOWLEDGMENTS

The processing of samples and isotope measurements have been carried out at the AIT by Joachim Heindler and at the HBLFA Wieselburg by Elisabeth Riegler, which are thankfully acknowledged. The constructive review of two reviewers and skillful handling by the editor are acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.638941/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
published: 19 July 2021
doi: 10.3389/fsufs.2021.688393

Microbiome Fingerprint as Biomarker for Geographical Origin and Heredity in *Crocus sativus*: A Feasibility Study

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OPEN ACCESS

Edited by:
Everlon Cid Rigobelo, São Paulo State University, Brazil

Reviewed by:
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Specialty section:
This article was submitted to Crop Biology and Sustainability, a section of the journal Frontiers in Sustainable Food Systems

Received: *30 March 2021*
Accepted: *14 June 2021*
Published: *19 July 2021*

Citation:
Bhagat N, Sharma S, Ambardar S, Raj S, Trakroo D, Horacek M, Zouagui R, Sbabou L and Vakhlu J (2021) Microbiome Fingerprint as Biomarker for Geographical Origin and Heredity in Crocus sativus: A Feasibility Study. Front. Sustain. Food Syst. 5:688393. doi: 10.3389/fsufs.2021.688393

Host–microbiome interactions are specific and not random, making them defining entities for the host. The hypothesis proposed by various researchers earlier, that both plants and animals harbor specific inheritable core microbiome, is being augmented in the present study. Additionally, a case for using microbial fingerprint as a biomarker, not only for plant identification but also as a geographical indicator, has been investigated, taking *Crocus sativus*, saffron, as a study material. *Crocus sativus*, a monogenetic herb, on account of its male sterility and vegetative propagation, is reported to lack genome based molecular markers. Cormosphere microbiome (microbiome associated with corm) has been compared across three geographical locations, in two continents, to identify the core and unique microbiome, during the vegetative phase of its growth. Microbiome analysis done at phylum and genus level, using next generation sequencing technology, revealed that cormosphere at three locations harbored common phyla. At genus level, 24 genera were found common to all three geographical locations, indicating them to be part of the core microbiome of saffron. However, there were some bacterial genera unique to Kashmir, Kishtwar, and Morocco that can be used to develop microbial markers/geographical indicators for saffron grown in these regions. This is a preliminary study, indicating that the location specific bacterial community can be used to develop microbial barcodes but needs further augmentation with high coverage data from other saffron growing geographical regions.

Keywords: barcodes, microbiome, microbial fingerprint, biomarker, cormosphere, saffron

INTRODUCTION

Molecular markers are important tools for plant genome analysis, crop improvement, and development of barcodes for authentic plant identification (Mishra et al., 2016). The development of molecular markers relies on genetic variation in the plants; however, plants lacking genetic variation due to asexual reproduction harbor unidentified or poor resolution molecular markers. *Crocus sativus* is one amongst such plants that lack genetic variations. *Crocus sativus*, commonly known as saffron, is an autumn-flowering

perennial sterile triploid plant bearing eight chromosomes per set, i.e., $2n = 3x = 24$ (Goldblatt et al., 2006; Ahmad et al., 2021). Due to male sterility, it does not produce viable seeds and propagates vegetatively, thereby lacking any variations at the genomic level. In order to propagate vegetatively, clusters of corms, underground bulb-like, starch-storing organs are dug up, divided, and replanted in the soil (Golmohammadi, 2014; Moshtaghi, 2020). The stigmas of saffron flowers are plucked and dried, to be used as a seasoning and coloring agent in food (Mzabri et al., 2017; Jafari et al., 2020). It is the world's highest priced spice with medicinal and aromatic properties, so referred to as 'golden condiment' (Monika and Neha, 2014; Pandita, 2021; Su et al., 2021). This exotic spice is cultivated worldwide in many countries, among them Iran, Spain, India and Morocco are the major producers (Mykhailenko et al., 2020; Gupta et al., 2021). Saffron is reported to be a monomorphic plant, having no variations at a genomic level across different saffron accessions across the world, as depicted by various molecular markers like RAPD, AFLP, and SSR (Rubio-Moraga et al., 2009; Busconi et al., 2015). However, there are variations in the phenotypic and biochemical characteristics such as the percentage of various metabolites (crocin, picocrocin, and saffranal) reported from different geographical locations (Othman et al., 2020). However, there are no authentic molecular markers that can identify these variations till date (Alavi-Kia et al., 2008; Keify and Beiki, 2012; Mir et al., 2021).

In the last three decades, microbes are established to have a pronounced effect on the biotic or abiotic environments they inhabit (Gupta et al., 2021). No eukaryote lives as an individual but is a meta-organism or holobiont (Guerrero et al., 2013; Vandenkoornhuyse et al., 2015). As established in humans, plants have their own microbiota and studies have shown that the rhizosphere (hotspot for microbial activities) has tremendous microbial density associated with almost 10^{11} microbial cells/gram of soil, represented by >30,000 prokaryotic species (Berendsen et al., 2012; Chaparro et al., 2014; Ofek-Lalzar et al., 2014). The sum total of the genomes of microbes associated with any environmental niche is referred to as "microbiome", the term coined by Joshua Lederberg (Lederberg and McCray, 2001).

The rhizosphere of many plants has been reported to contain specific microbiomes that are essential for plant survival and are referred to as the plant's second genome (Berendsen et al., 2012; Ofek-Lalzar et al., 2014). The microbiome and its dynamics have been extensively studied in the rhizosphere of *Arabidopsis*, *Zea mays*, *Triticum aestivum*, *Oryza sativa*, *Vitis vinifera*, *Crocus sativus*, etc. (Ambardar and Vakhlu, 2013; Ambardar et al., 2014, 2016; Mendes and Raaijmakers, 2015; Kandel et al., 2017). Studies also suggest that though the microbiome associated with any plant is organ specific, it may show temporal variation, primarily during developmental stages, geographical location, and cultivation practices (Peiffer et al., 2013; Chaparro et al., 2014; Edwards et al., 2015; Lin et al., 2020).

Despite temporal and spatial variations, the core microbiome is specific for each plant. Core microbiome may be defined as a congregation of micro-organisms within a host's microbiome that remains unaltered across different growth stages, niches, and geographical locations. Core inheritable microbiome, present in all the organisms, is hypothesized to be inherited as a core genome (Walters et al., 2018). The composition of core microbiome is reported to depend on the host plant, and this hypothesis was tested in *Arabidopsis* by Lundberg et al. (2012) wherein specific microbial communities common to tested plant varieties were established. The presence of core microbiome has been established in rice (Eyre et al., 2019), sponges (Schmitt et al., 2012), corals (Ainsworth et al., 2015; Hernandez-Agreda et al., 2016, 2017), wheat (Kuzniar et al., 2020), human gut (Qin et al., 2010), human hands (Fierer et al., 2008), and sand beaches (Newton et al., 2013).

The present study was initiated to discover the core microbiome of saffron cormosphere, along with unique genera specific to the saffron cultivated at a particular location. Unique genera can be used to develop molecular markers/geographical indicators. In the present study, core microbiome of saffron cormosphere comprises 24 genera that remain unaltered in three locations across two continents, i.e., Africa and Asia. In addition, unique microbes associated with the corm at each location have also been identified and cataloged, which can be used as molecular markers/geographical indicators.

MATERIALS AND METHODS

Sample Collection

Plant samples were collected from three geographical locations, two in Jammu and Kashmir, India, i.e., Kishtwar (33°19′12.00″ N 75°46′12.00″ E) and Kashmir (34.02°″ N 74.930″ E), and one from Morocco (Taliouine, 30°31′58.00″ N 7°55′32.00″ W) (**Figure 1**). Plant samples were collected from Kishtwar during three consecutive years (2011, 2012, and 2013; coded as C11, C12, and C13, respectively), whereas from Kashmir in 2013 and Morocco in 2016. In all the cases, sampling was done during the vegetative phase of saffron life cycle. The sample collection was done as per the protocol developed by Ambardar et al. (2014). The temperature and humidity of the sites at the time of sample collection have been tabulated in **Table 1**.

In a separate experiment, corm samples and saffron field soil from Kishtwar region [referred to as Kishtwar-L 17 (L for lab)] were collected during the dormant phase of life cycle (August, 2017) and stored at room temperature (20–25°C) till further use. Corms were sown in the soil (Kishtwar soil) at the start of vegetative phase (October, 2017) and were grown for 2 months. The corms were grown in pots that were incubated in plant growth chamber with day temperature (25 ± 5°C), night temperature (10 ± 5°C), and 70% relative humidity and watered after every 5 days with autoclaved distilled water for a period of 2 months. Cormosphere samples were collected after 2 months in vegetative phase.

Metagenomic DNA Extraction

The loose soil adhered to corms was removed by shaking corms vigorously. Corm sheath was peeled off from the corms under sterile conditions (inside laminar air flow) and was

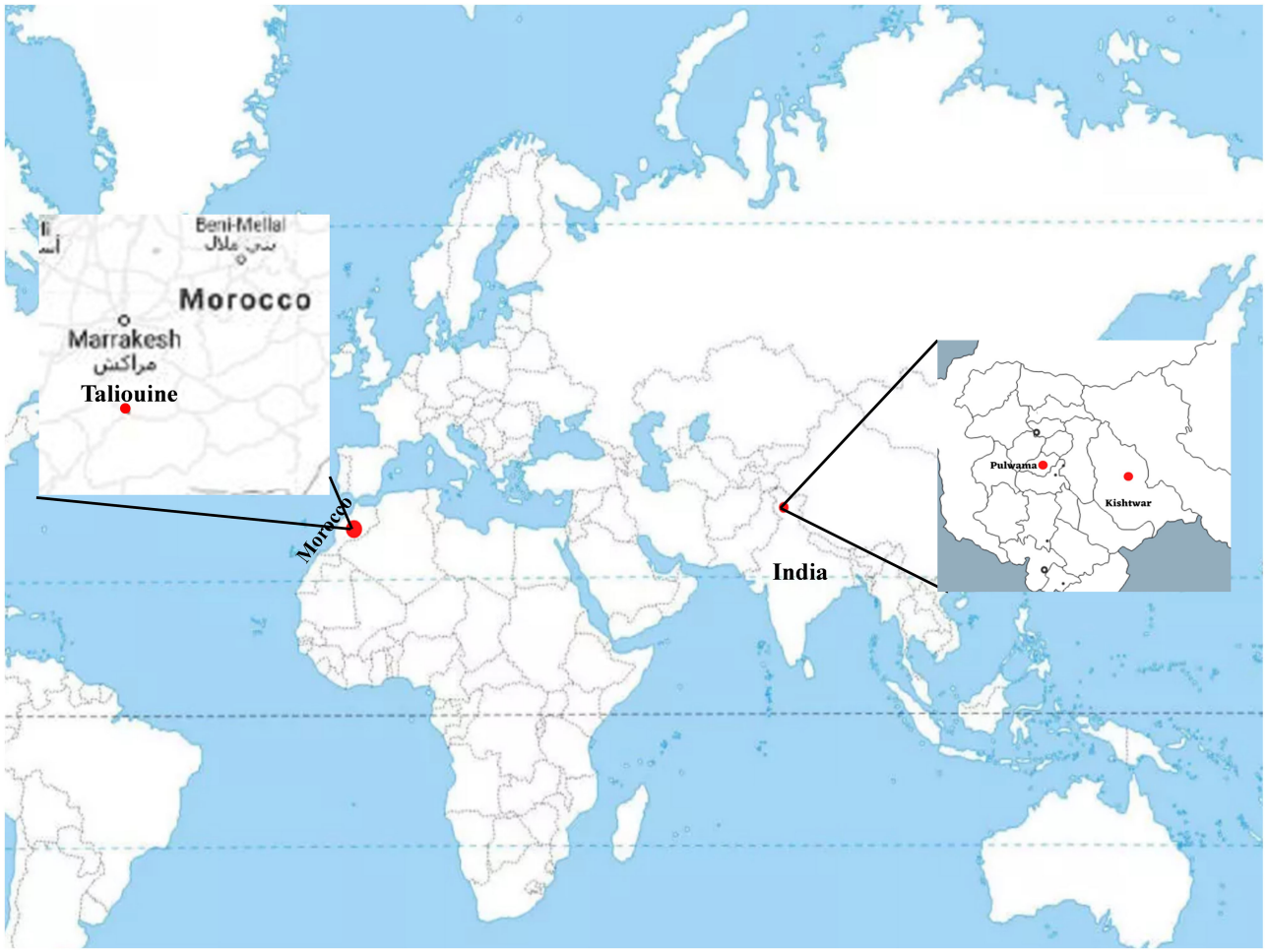

FIGURE 1 | Geographical location of sample collection sites on world map. Kishtwar (33°19′12.00" N 75°46′12.00″ E), Kashmir (34°1′30″ N 74°.5′80″ E), and Morocco (Taliouine, 30°31′58.00″ N 7°55′32.00″ W).

TABLE 1 | The temperature and humidity of the three geographical regions: Kishtwar (during different years), Kishtwar-L 17, Kashmir, and Morocco.

S. No	Conditions		Kishtwar			Kishtwar-L 17	Kashmir	Morocco
			2011	2012	2013	2017	2013	2016
1	Temperature (°C)	High	14	12	14	25 ± 5	14	24
		Low	−4	−3	−4	10 ± 5	−4	12
		Average	3	4	4		4	
2	Humidity		76%	80%	74%	70%	74%	62%
3.	Yield (kg/hectare)		0.36	0.22	0.45	–	–	–

TABLE 2 | Mapping percentage of reads to different domains in all six samples [Kishtwar 2011 (C11), Kishtwar 2012 (C12), Kishtwar 2013 (C13), Kishtwar-L 17, Kashmir (2013), and Morocco (2016)].

Domain	C11 (%)	C12 (%)	C13 (%)	Kishtwar-L 17 (%)	Kashmir (%)	Morocco (%)
Bacteria	96.3	98.78	98.31	90.12	85.29	95.28
Eukaryota	3.47	1.06	1.51	9.74	14.14	4.53
Archaea	0.13	0.12	0.09	0.06	0.18	0.09
Viruses	0.02	0.02	0.02	0.03	0.29	0.06
Unclassified sequences	0.08	0.02	0.05	0.05	0.1	0.04

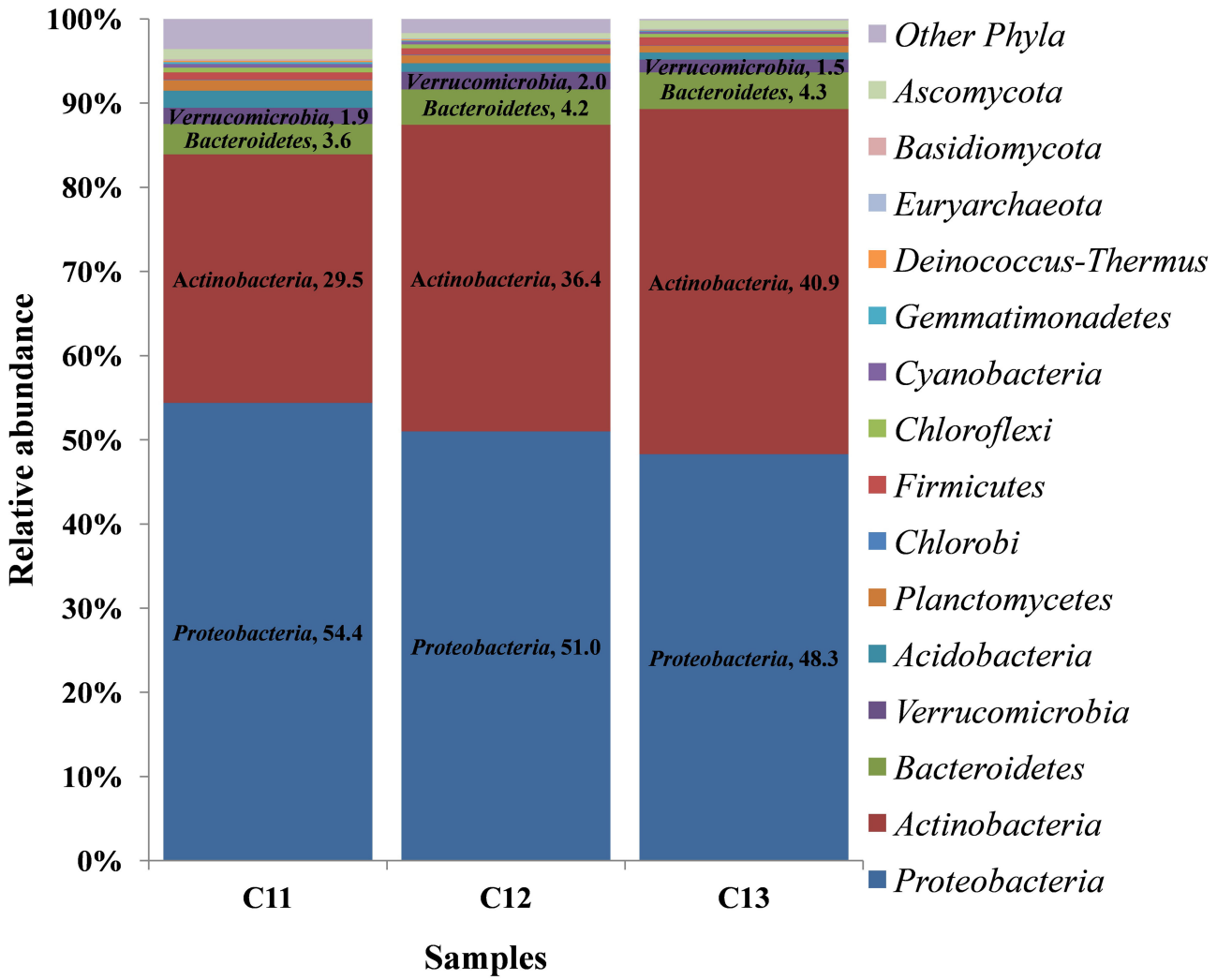

FIGURE 2 | Relative abundance of different phyla in the three consecutive years of Kishtwar (2011, 2012, and 2013). The bar graph depicts 15 phyla (12 bacterial, 2 fungal, 1 archaeal) cataloged from the cormosphere of saffron grown in Kishtwar.

used for metagenomic DNA extraction. Metagenomic DNA was isolated using three different standardized protocols: Zhou et al. (1996), Wechter et al. (2003), and Pang et al. (2008) to capture maximum microbial diversity. The metagenomic extraction was done in triplicates for each sample with all the three protocols. These triplicate metagenomic DNA extracts were subsequently pooled to get maximum possible diversity for each sample. The quality of metagenomic DNA was accessed on 0.8% agarose gel and quantified using Invitrogen Qubit® 2.0 Fluorometer (Thermofischer, Foster City, CA, USA).

Whole-Genome Shotgun Sequencing and Bioinformatic Analysis

The library preparation of isolated metagenomic DNA from all the samples was done using *Truseq Nano* DNA Library preparation kit (Catalog No. 20015964, Illumina, CA, USA) as per the instructions of manufacturer. To ensure maximum yield, a high-fidelity amplification step was performed using sparQ HiFi PCR Master mix (Quanta bio, QIAGEN, Beverly Inc. Catalog No. 95192-050). The quality of amplified libraries was analyzed on Bioanalyzer 2100 (Agilent Technologies, catalog number: G2939BA) and was further sequenced using Illumina NextSeq 500 platform with 2 × 150 base paired-end configuration from Xcleries labs limited, Ahmadabad, Gujarat, India. The raw reads were quality checked using FastQC tool kit (Brown et al., 2017). The low quality reads having phred score less than 30 were filtered and trimmed using software Trimmomatic (Bolger et al., 2014). The *de novo* assembly of high quality paired end reads for all samples was done using CLC Genomics Workbench 6.0 (CLC bio, Aarhus, Denmark) with the following parameters: minimum contig length: 200, automatic word size, perform scaffolding, mismatch cost: 2, insertion cost: 3, deletion cost: 3, length fraction: 0.5, similarity fraction: 0.8. The assembled contigs were then uploaded on MG-RAST (Metagenomic Rapid Annotation using Subsystem Technology) server version 4.0.3

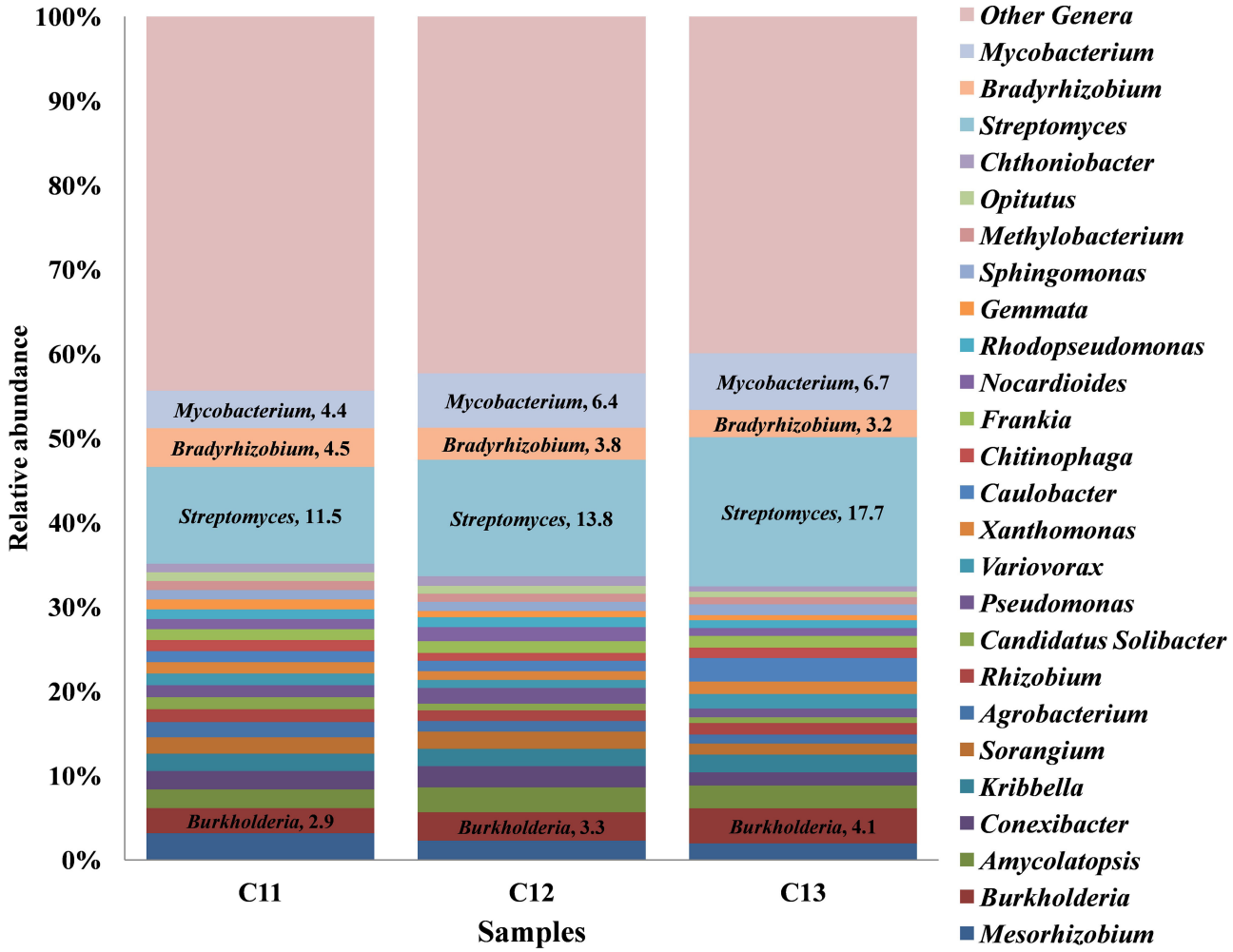

FIGURE 3 | Relative abundance of top 25 genera cataloged from Kishtwar cormosphere in the three consecutive years (2011, 2012, and 2013). The bar graph represents 42 genera common in all the three Kishtwar samples out of 52 genera cataloged in this study.

with default parameters for analyzing taxonomic hit distribution and calculation of Shannon-alpha diversity values (Meyer et al., 2008).

Taxonomic classification up to phylum and genus level was represented in the form of heat maps and principal component analysis (PCA) plots using ClustVis tool (Metsalu and Vilo, 2015). Rarefaction curve was generated using PAST software version 4.03 (Hammer et al., 2001). Circos plot was generated using the tool Circos Version 0.63-9 (Krzywinski et al., 2009).

RESULTS

Whole metagenome sequencing data of the six samples (Kishtwar 2011, 2012, 2013; Kishtwar-L 17, Kashmir 2013; Morocco 2016), collected from three locations during the vegetative period of growth, ranged between 5.3 million reads and 40 million reads. Reads mapped to bacteria and eukaryota ranged from 85.29 to 98.78% and 1 to 14.2% in all the samples, respectively (**Table 2**). The dynamics of the microbiome of Kishtwar for three consecutive years (2011, 2012, and 2013) were studied, and the average of the 3 years was taken as reference. Subsequently, the Kishtwar average was compared to the microbiome of saffron cormosphere of Kishtwar-L 17, Kashmir (2013), and Morocco (2016) to discover core and location specific unique microbiome.

Structure and Inheritability of Kishtwar Cormosphere Microbiome for the Three Consecutive Years

At the phylum level, 15 phyla (12 bacterial, 2 fungal, 1 archaeal) were cataloged from the cormosphere of saffron grown in Kishtwar during three consecutive years (2011, 2012, and 2013). The three most dominant phyla were *Proteobacteria* followed by *Actinobacteria* and *Bacteroidetes.* The relative abundance of *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* varied among the samples, and it was observed that the percentage of *Proteobacteria* reduced gradually from 2011 to 2013 (54.4 to

TABLE 3 | Core microbiome of Kishtwar analyzed in 3 years (42 genera) in C11, C12, and C13 with their relative abundance (%).

Genera	C11 (%)	C12 (%)	C13 (%)	Phyla
Streptomyces	11.50	13.80	17.7	*Actinobacteria*
Bradyrhizobium	4.58	3.81	3.22	*Proteobacteria*
Mycobacterium	4.46	6.45	6.71	*Actinobacteria*
Mesorhizobium	3.15	2.28	1.96	*Proteobacteria*
Burkholderia	2.99	3.35	4.14	*Proteobacteria*
Amycolatopsis	2.20	2.95	2.69	*Actinobacteria*
Conexibacter	2.17	2.52	1.57	*Actinobacteria*
Kribbella	2.06	2.03	2.09	*Actinobacteria*
Sorangium	1.92	2.05	1.31	*Proteobacteria*
Agrobacterium	1.80	1.27	1.06	*Proteobacteria*
Rhizobium	1.52	1.25	1.37	*Proteobacteria*
Candidatus Solibacter	1.43	0.79	0.66	*Acidobacteria*
Pseudomonas	1.40	1.83	1.05	*Proteobacteria*
Variovorax	1.39	0.96	1.71	*Proteobacteria*
Xanthomonas	1.35	1.03	1.48	*Proteobacteria*
Caulobacter	1.31	1.24	2.78	*Proteobacteria*
Chitinophaga	1.30	0.91	1.23	*Bacteroidetes*
Frankia	1.27	1.39	1.38	*Actinobacteria*
Nocardioides	1.20	1.67	0.92	*Actinobacteria*
Gemmata	1.15	0.74	0.60	*Planctomycetes*
Sphingomonas	1.12	1.09	1.29	*Proteobacteria*
Methylobacterium	1.05	0.95	0.84	*Proteobacteria*
Opitutus	1.03	0.92	0.66	*Verrucomicrobia*
Chthoniobacter	1.01	1.14	0.61	*Verrucomicrobia*
Micromonospora	0.89	1.04	0.79	*Actinobacteria*
Phenylobacterium	0.75	0.67	0.85	*Proteobacteria*
Actinosynnema	0.62	0.87	0.97	*Actinobacteria*
Arthrobacter	0.59	0.75	0.92	*Actinobacteria*
Rhodococcus	0.56	0.63	0.78	*Actinobacteria*
Erythrobacter	0.52	0.54	0.66	*Proteobacteria*
Novosphingobium	0.52	0.58	0.76	*Proteobacteria*
Sinorhizobium	0.52	0.51	0.45	*Proteobacteria*
Starkeya	0.50	0.49	0.54	*Proteobacteria*
Sphingobium	0.50	0.41	0.59	*Proteobacteria*
Sphingopyxis	0.49	0.49	0.68	*Proteobacteria*
Saccharopolyspora	0.48	0.37	0.48	*Actinobacteria*
Hyphomicrobium	0.48	0.84	0.38	*Proteobacteria*
Streptosporangium	0.46	0.51	0.50	*Actinobacteria*
Dyadobacter	0.45	0.72	0.84	*Bacteroidetes*
Pedobacter	0.41	0.48	0.58	*Bacteroidetes*
Verrucomicrobium	0.41	0.75	0.68	*Verrucomicrobia*
Rhodopseudomonas	1.16	1.17	0.91	*Proteobacteria*

48.3%). On the other hand, *Actinobacteria* and *Bacteroidetes* increased from 29.5 to 40.9% and 3.6 to 4.36%, respectively (**Figure 2**).

At the genus level, a total 52 genera were cataloged from three Kishtwar samples. Surprisingly, *Streptomyces* (C11 = 11.5%, C12 = 13.8%, and C13 = 17.7%) belonging to phylum *Actinobacteria* was the most abundant followed by *Mycobacterium*, *Bradyrhizobium*, and *Burkholderia* all belonging to *Proteobacteria* (**Figure 3**). The abundance of three genera *Streptomyces*, *Burkholderia*, and *Mycobacterium* increased from C11 to C13, whereas *Bradyrhizobium* decreased from 4.58% to 3.22%. Out of total 52 genera cataloged, 42 genera were found common in C11, C12, and C13, which correspond to 81% of total genera. The relative abundance of the 10 genera that were not common (19%) was found to be less than 1% of each genus.

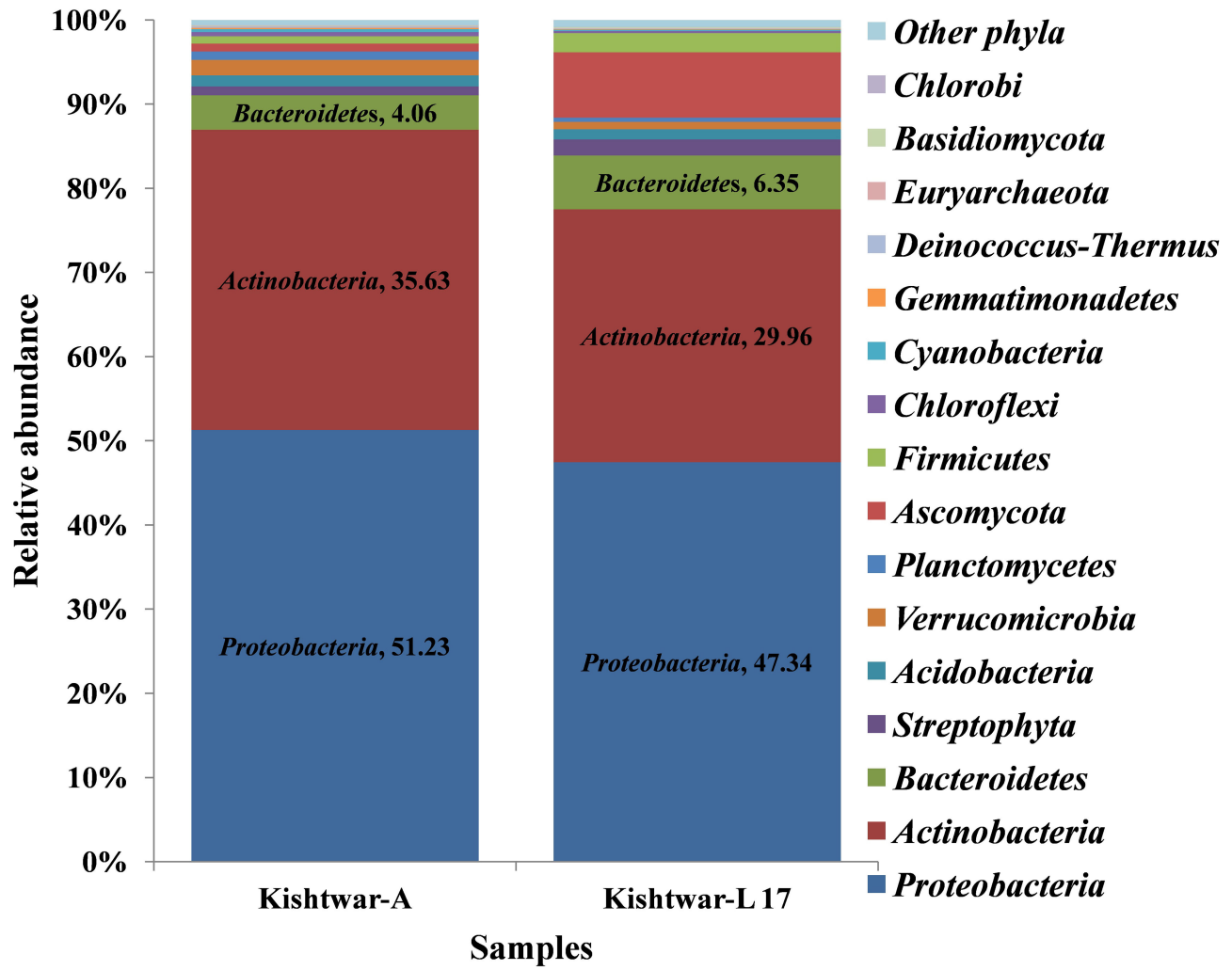

FIGURE 4 | Abundance of different phyla in Kishtwar-A and Kishtwar-L 17. The bar graph depicts 15 phyla (12 bacterial, 2 fungal, 1 archaeal) cataloged from the cormosphere of saffron grown in Kishtwar fields and one laboratory grown sample.

Core Bacterial Microbiome of C11, C12, C13

Core bacteriome of the saffron plant grown in Kishtwar comprised of 42 genera that belongs to five different phyla and corresponds to 81% of the total microbiome (52 genera). Based on diversity/relative representation, 50% (21 genera) belonged to *Proteobacteria*, 31% (13 genera) to *Actinobacteria*, 7.2% (3 genera) to *Bacteriodetes*, 7.2% (3 genera) to *Verrumicrobia*, and 2.3% (1 genus) to phylum *Planctomycetes* in the core microbiome of Kishtwar. However, on the basis of abundance, 10 most abundant genera were *Streptomyces* followed by *Mycobacterium*, *Bradyrhizobium*, *Burkholderia*, *Mesorhizobium*, *Conexibacter*, *Sorangium*, *Agrobacterium*, *Rhizobium*, and *Pseudomonas*. The relative abundance of core genera has been tabulated in **Table 3**.

In addition to 42 common genera, 10 genera were randomly present in one or two of the three samples. Two genera (*Stenotrophomonas* and *Anaeromyxobacter*) belonging to phylum *Proteobacteria* were present only in C11. Three genera such as *Bacillus* (*Firmicutes*), *Mucilaginibacte* (*Bacteriodetes*), and *Brevundimonas* (*Proteobacteria*) were present only in C13. The Shannon α-diversity of C11, C12, and C13 was found to be 443, 395, and 359, respectively, thereby indicating decrease in microbial diversity from 2011 to 2013. However, decrease in microbial diversity may be due to the depletion of few rare genera having less abundance in consecutive years. To conclude, it was observed that 81% of genera were common in the three consecutive years but their relative abundance varied.

Comparative Stability and Inheritability of the Corm Microbiome in Kishtwar Average and Kishtwar-L 17

The Kishtwar microbiome (Kishtwar average of C11, C12, and C13, hereafter referred to as Kishtwar-A) was taken as reference microbiome for comparing the diversity and abundance of microbes in Kishtwar-L 17 (plants grown under

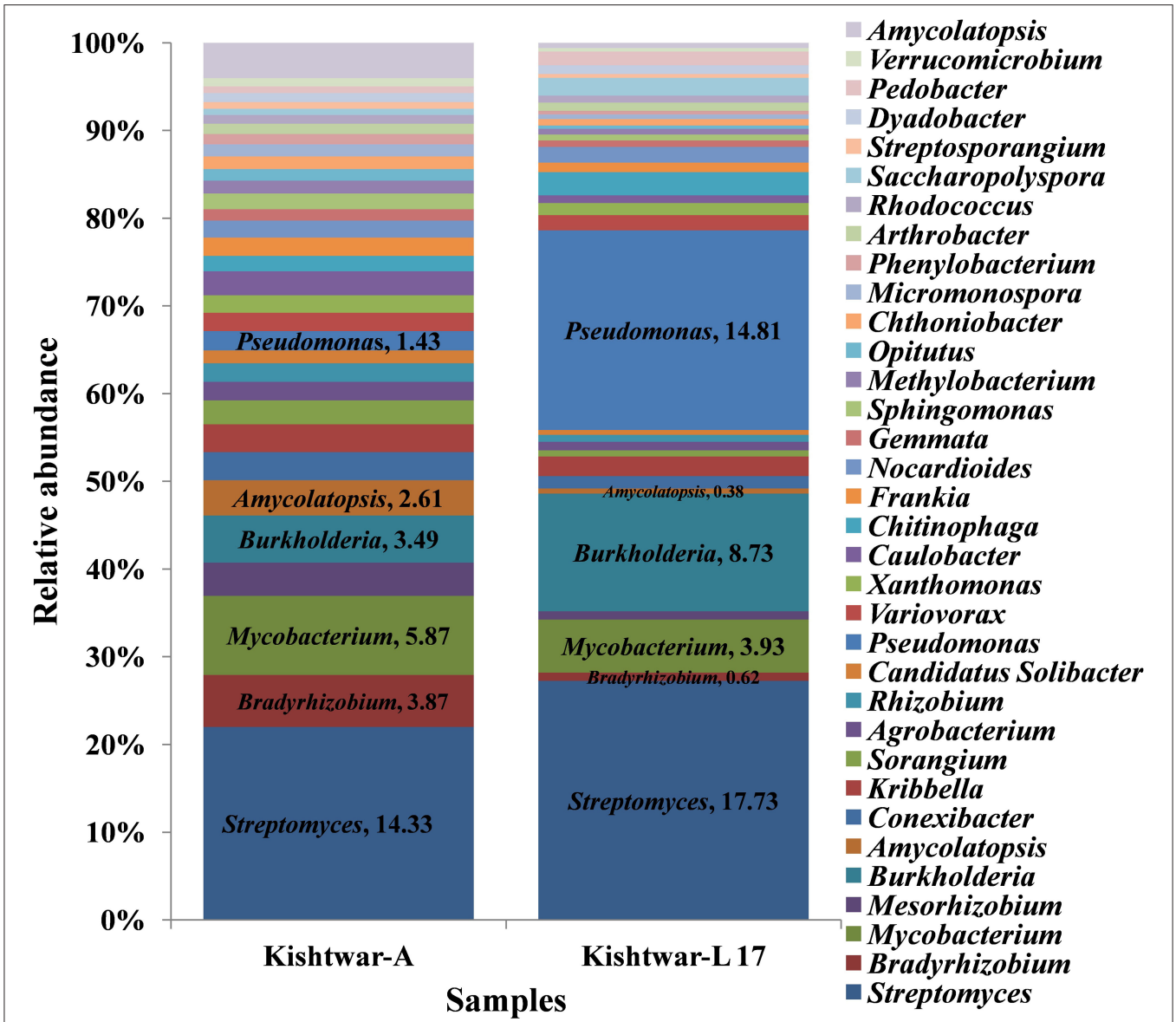

FIGURE 5 | Relative abundance of common 34 genera cataloged from Kishtwar-A and Kishtwar-L 17.

controlled laboratory conditions) again to confirm stability and inheritability of the corm microbiome. All 15 phyla present in Kishtwar with 3 year average, referred to as Kishtwar-A, were present in Kashmir-L 17. The three major dominant phyla were the same, i.e., *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, but their relative abundance varied between Kishtwar-L 17 and Kishtwar-A. *Proteobacteria* and *Actinobacteria* were less abundant, whereas *Bacteroidetes* were more abundant in Kishtwar-L 17 (**Figure 4**).

At the genus level, 34 genera were found to be common in Kishtwar-A and Kishtwar-L 17 that constituted the core of Kishtwar region. Similar to Kishtwar-A, the most abundant genera in Kishtwar-L 17 was *Streptomyces* (17.73%), but the pattern and abundance of other genera varied; *Pseudomonas* (14.81%) and *Burkholderia* (8.73%) had higher abundance, and *Mycobacterim* (3.93%) and *Bradyrhizobium* (0.6%) were less abundant compared to Kishtwar-A (**Figure 5**). The alpha diversity index (Shannon indices) was more in Kishtwar-A (399) compared to Kishtwar-L 17 (354). This is further complemented by the rarefaction curve wherein C11 showed the highest species richness and least by Kishtwar-L 17 (**Figure 6**). In addition, there were six genera unique to Kishtwar-L 17, namely, *Achromobacter*, *Enterobacter*, *Serratia*, *Nocardia*, *Terriglobus*, and *Sphingobacterium*, whereas eight genera (*Rhodopseudomonas*, *Hyphomicrobium*, *Sphingopyxis*, *Novosphingobium*, *Sphingobium*, *Erythrobacter*, *Starkeya*, and *Actinosynnema*), present in Kishtwar-A, were completely absent in Kishtwar-L 17.

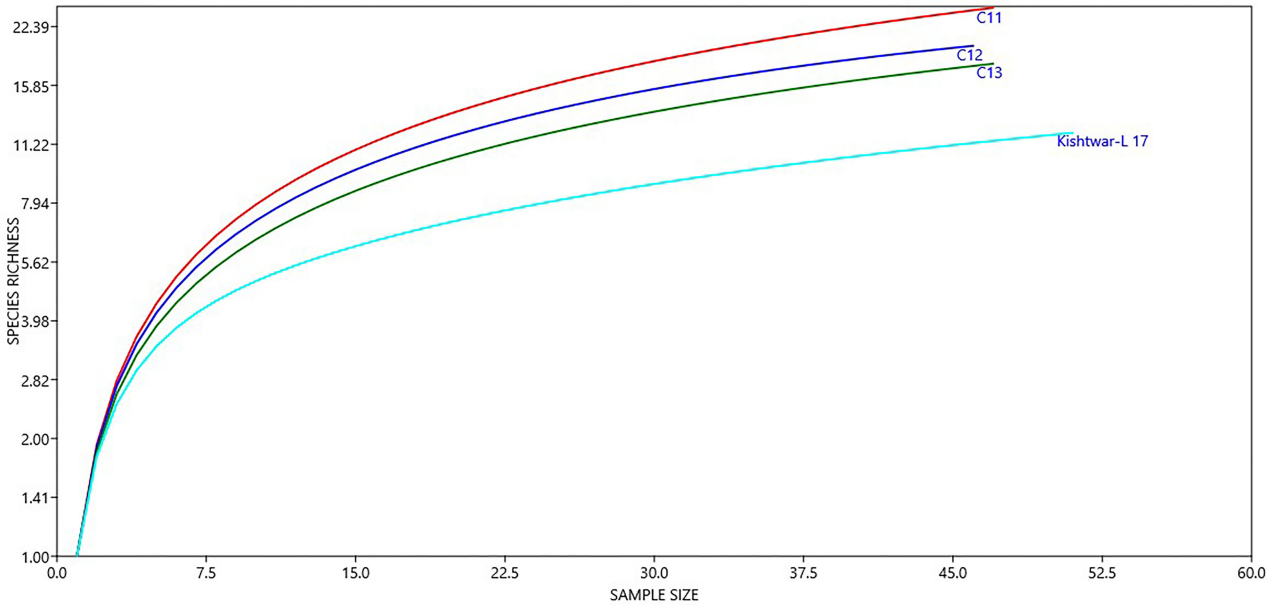

FIGURE 6 | Diversity of different genera in Kishtwar samples (C11, C12, C13, and Kishtwar-L 17). Presence of higher number of species in C11 indicates that it is more enriched than C12, C13, and Kishtwar-L 17.

Diversity and Structure of Cormosphere Microbiome of Kashmir and Morocco in Comparison to Reference Kishtwar-A Cormosphere Microbiome

At the phylum level, 15 phyla (12 bacterial, 2 fungal, 1 archaeal) were cataloged from the cormosphere of saffron corms grown in Kashmir and Morocco. The three dominant bacterial phyla among the samples were *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. *Proteobacteria* was most dominant (Kashmir = 48.46%, Morocco = 55.06%) followed by *Bacteroidetes* (Kashmir = 12.93% and Morocco = 16.92%) and *Actinobacteria* (Kashmir = 10.98% and Morocco = 16.34%) *Proteobacteria* (51.23%), followed by *Actinobacteria* (35.64%), *Bacteroidetes* (4.06%), and *Verrucomicrobia* (1.85%). Though the number of phyla was same in Kishtwar-A, Kashmir, and Morocco, heat maps hierarchical clustering clustered Kashmir and Morocco in one clad and Kishtwar-A in different clad. This represented that the co-relation distance and average linkage of Kishtwar-A were different from Kashmir and Morocco (**Figure 7**). Relative abundance of different phyla of Kishtwar-A, Kashmir, and Morocco is represented in **Figure 8**.

At the genus level, abundance dynamics of bacterial genera varied among samples (**Figure 9**). Kashmir was dominated by *Pseudomonas* (12.78%), *Streptomyces* (3.33%), and *Chitinophaga* (2.65%). Morocco was dominated by *Pseudomonas* (8.66%), *Caulobacter* (5.46%), and *Pedobacter* (4.68%) compared with Kishtwar-A, which was dominated by *Streptomyces* (14.33%), *Mycobacterim* (5.87%), and *Bradyrhizobium* (3.87).

Comparative Analysis of Core Bacteriome of Three Different Geographical Locations

Of the total 73 genera cataloged, 24 genera were found common in all the three geographical locations, viz., Kishtwar, Kashmir, and Morocco, corresponding to 32.8% similarity in microbial community across geographical regions in Asian and African sub-continent. Core microbiome of saffron across different geographical location comprised of 24 genera; out of 24 common genera, 13 genera (54.2%) belonged to phylum *Proteobacteria*, 5 genera (20.8) to *Actinobacteria*, 3 genera (12.5%) to *Bacteriodetes*, and 3 genera (12.5%) to *Verrumicrobia* (**Figure 10**). The core genera with their relative abundance have been tabulated in **Table 4**.

Notable differences were observed in the cormosphere microbiome composition of Kashmir, Kishtwar, and Morocco as depicted by principal component analysis (PCA) that clusters Kishtwar (C11, C12, and C13) samples in close proximity and Kashmir and Morocco samples that clustered away in different coordinate, thereby indicating differences in diversity/abundance pattern (**Figure 11**). In addition, alpha diversity indices like Shannon indices were highest in case of Kashmir (507) as compared to Morocco (415) and Kishtwar-A (399). This was further complemented by rarefaction curves wherein Kashmir samples were more enriched as depicted by the higher number of species in Kashmir, in comparison to Kishtwar-A and Morocco (**Figure 12**).

In addition to the core microbiome, five bacterial genera were unique to Kashmir (*Marivirga*, *Microscilla*, *Planctomyces*, *Plesiocystis*, *Candidatus koribacter*), seven genera were unique to Morocco (*Rahnella*, *Bdellovibrio*, *Methylovorus*,

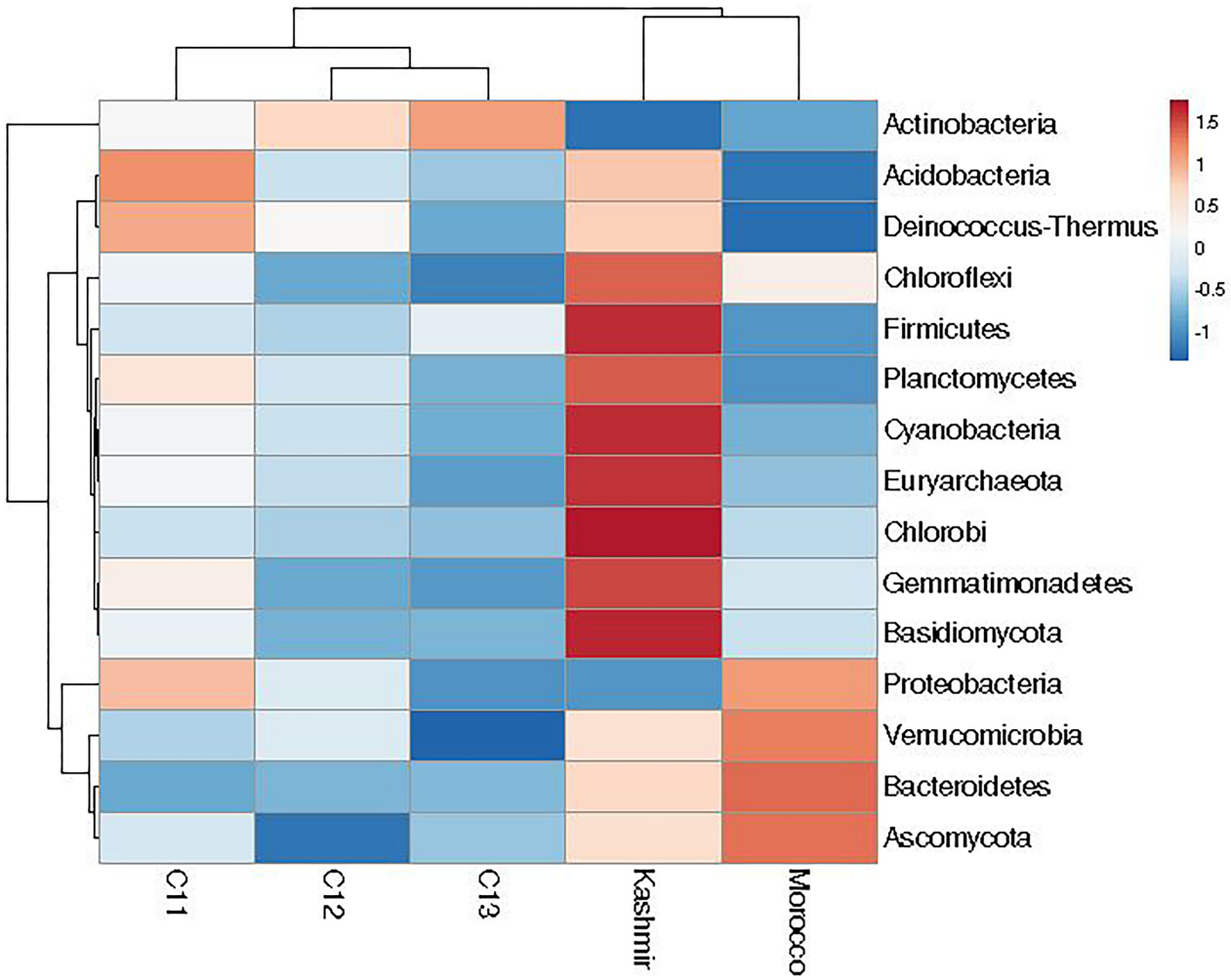

FIGURE 7 | Relative abundance of 15 phyla in the saffron cormosphere from all the three geographical locations (Kashmir, Kishtwar, and Morocco).

Chryseobacterium, and *Algoriphagus*), and the five unique genera identified specific to Kishtwar were *Rhodococcus*, *Kribella*, *Streptosporangium*, *Saccharopolyspora*, and *Amycolatopsis* (**Figure 13**).

To conclude, Kishtwar consecutive 3 year samples showed 81% similar genera across the years. The genera that were absent in one or other Kishtwar samples had less than 1% representation in total reads and may be attributed to sequencing bias due to the low relative amount present in whole metagenomic data. This needs to be confirmed by repeated sampling and sequencing across various geographical locations. However, 32.8% of the genera were common in all three locations that can be designated as core microbiome of saffron cormosphere. Bacterial genera that were present in one geographical location and completely absent in the other two locations were observed, thereby representing the specific interactions of these bacterial genera to each geographical location. Unique genera to each place can be developed into biological markers as geographical indicators.

DATA AVAILABILITY STATEMENT

The sequencing data has been submitted to NCBI under the accession number PRJNA705068.

DISCUSSION

The present study was initiated to investigate the possibility of using microbial fingerprints as molecular marker and/or geographical indicator, particularly in monogenetic crops. Microbial biomarkers have been reported to be used for the determination of biogenic chemical concentration (e.g., phospholipid fatty acids; Lupwayi et al., 2017), evaluation of microbial activity (e.g., photosynthetic activity; Corcoll et al., 2011), and capacity (e.g., adaptation; Pesce et al., 2016). Species specific biomarkers are reported to detect the toxicity in the environment caused by a pathogen bacteria or toxic *Cyanobacteria* (Bonnineau et al., 2012; Guasch et al., 2017). In

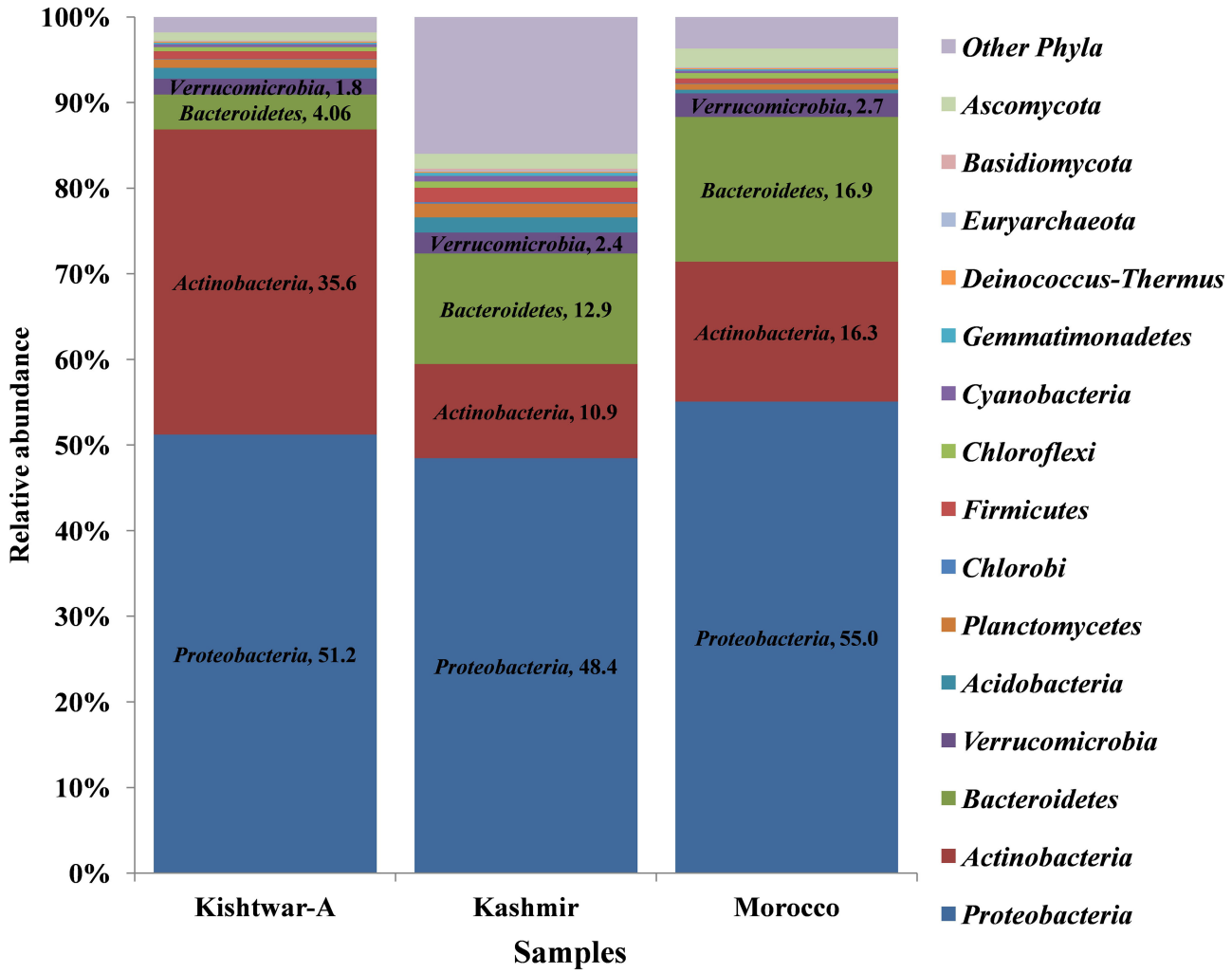

FIGURE 8 | Representation of relative abundance of different phyla in all the three geographical locations (Kishtwar-A, Kashmir, and Morocco). Fifteen major phyla (12 bacterial, 2 fungal, 1 archaeal) were cataloged from the cormosphere of saffron grown in three geographical locations. *Proteobacteria* was dominant in all the three locations followed by *Actinobacteria* and *Bacteroidetes* in Kishtwar-A and *Bacteroidetes* and *Actinobacteria* in both Kashmir and Morocco.

plants also, microbiome is reported to have significant variation based on different geographical locations, such as reported in the case of vineyards, Amazonian hardwood, and fragrant orchid. The researchers co-relate the variations in the plant microbiome to various factors such as location/genetics/abiotic and biotic factors (Coller et al., 2019; Skaltsas et al., 2019; Lin et al., 2020). However, reports are emerging on the core microbiome that remains unaltered at various developmental stages of the plants or plants from different locations/niches (Chen et al., 2018; Toju et al., 2018; Lin et al., 2020). The significance of molecular markers/barcodes in selecting/identifying different varieties needs no elaboration.

However, some plants are reported to lack high resolution molecular markers on account of sterility. *Crocus sativus*, saffron, is reported to lack genetic variation on account of vegetative propagation through corms, as it is male sterile plant (Nemati et al., 2019). The present study was initiated to discover if microbiome based markers can be used as geographical indicators in saffron grown at different geographical locations, i.e., Kashmir and Kishtwar in India and Taliouine in Morocco, also to identify core microbiome in saffron across different locations, if any. The microbiome associated with corm, grown at these three locations, was isolated and analyzed at the vegetative stage to keep the development stage as a constant factor.

The bacteriome and mycobiome of *Crocus sativus* have been reported earlier by our group, but that study was specific to Kashmir for 1 year only (Ambardar et al., 2014). In the present study, microbiome analysis of cormosphere was initiated in saffron grown in Kishtwar during three consecutive years to check the inheritability of cormosphere microbiome. About 81% of the microbial genera were common in the cormosphere of all 3 years, though their abundance varied (**Figure 2**). The abundance of the genera belonging to phylum *Proteobacteria* decreased from C11 (54.4%) to C13 (48.3%), whereas an increase in the

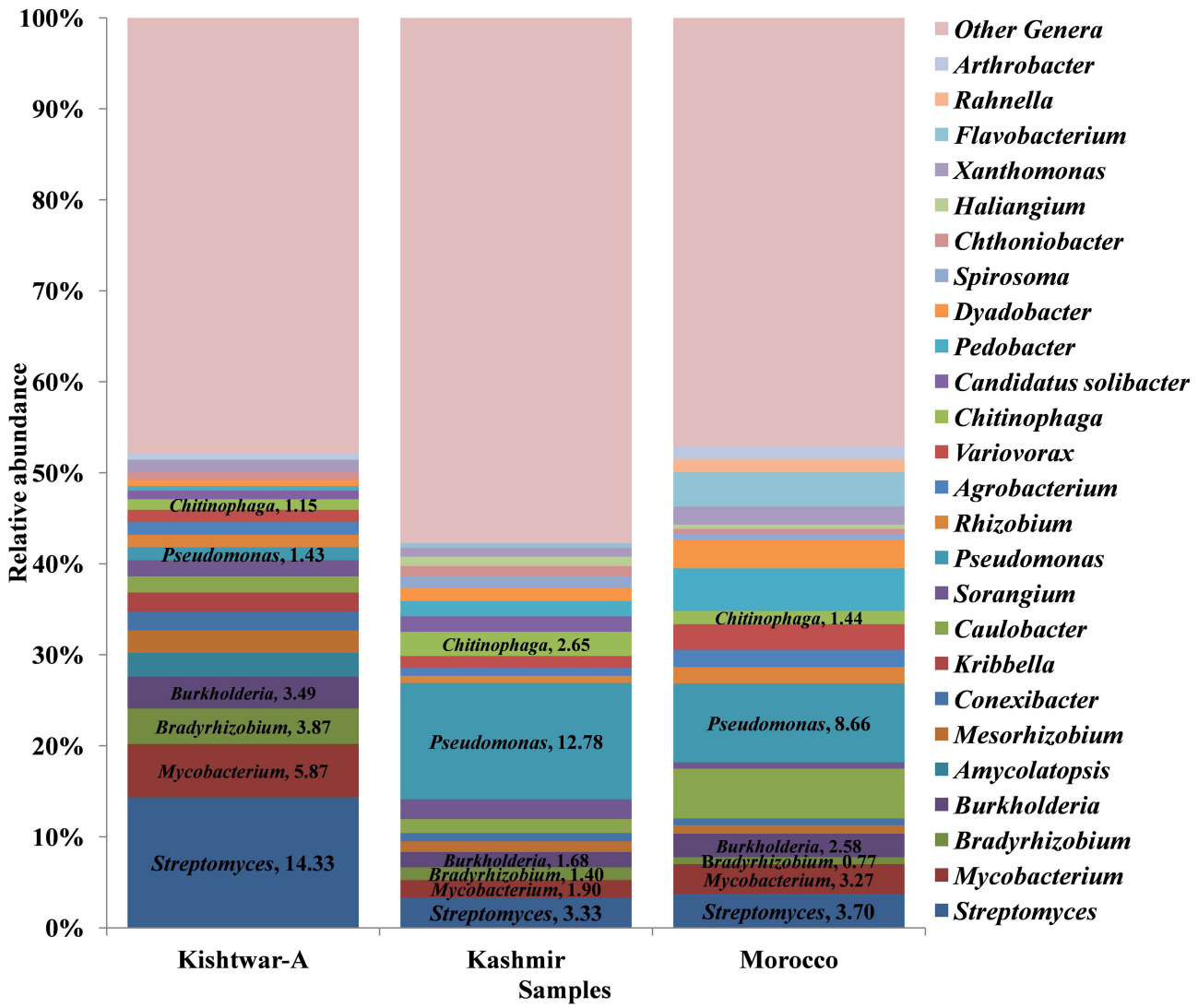

FIGURE 9 | Relative abundance of top 25 genera cataloged from cormosphere of all the three geographical locations (a total of 73 genera were cataloged from three geographical locations: Kishtwar-A, Kashmir, and Morocco). *Streptomyces* was the dominant genera in Kishtwar-A followed by *Mycobacterim*, *Bradyrhizobium*, and *Burkholderia*. *Pseudomonas* was the dominant genera in both Kashmir and Morocco followed by *Streptomyces*, *Chitinophaga*, and *Sorangium* in Kashmir and *Caulobacter*, *Pedobacter*, and *Flavobacterium* in Morocco.

abundance of *Actinobacteria* and *Bacteriodetes* was observed from C11 (Actinobacteria−29.5%, *Bacteriodetes*−3.6%) to C13 (Actinobacteria−40.9%, *Bacteriodetes*−4.36%). Since saffron is a perennial crop, and farming practices were not changed, the climate was more or less the same and yield also did not show any co-relation (**Table 1**); the exact reason for this variation in abundance is not clear. Eighty-one percent of the genera, common to all the 3 years, are being proposed as the core microbiome of saffron cormosphere grown in Kishtwar. Core microbiome have been earlier reported in various plants across different geographical location, climatic gradients, genotypes, and seasons, namely, *Salvia miltiorrhiza* (Chen et al., 2018), angiosperm plants (Fitzpatrick et al., 2018), *Coffea arabica* (Fulthorpe et al., 2020), *Orzya sativa* (Eyre et al., 2019), *Phaseolus vulgaris* (Pérez-Jaramillo et al., 2019), Switchgrass (*Panicum virgatum L.*) and Miscanthus (*Miscanthus x giganteus*) (Grady et al., 2019), and *Panax ginsen,* (Nguyen et al., 2016). On the similar lines, phyllosphere microbiome of Switchgrass was compared for two consecutive years (2016 and 2017) and 60% of leaf communities were found common (Grady et al., 2019), which was comparatively less than the present study (81% of cormosphere community was common in saffron).

In the present study, once the inheritability of the core microbiome in field conditions was established, the same was tested under laboratory conditions wherein the saffron corms and soil were collected from Kishtwar in 2017, and grown in pots in the laboratory. Except for the variation of temperature that was around 25°C, the rest of the parameters were kept close to natural conditions, such as soil and light intensity. Out of 81% genera common during three consecutive years from Kishtwar, only 65%

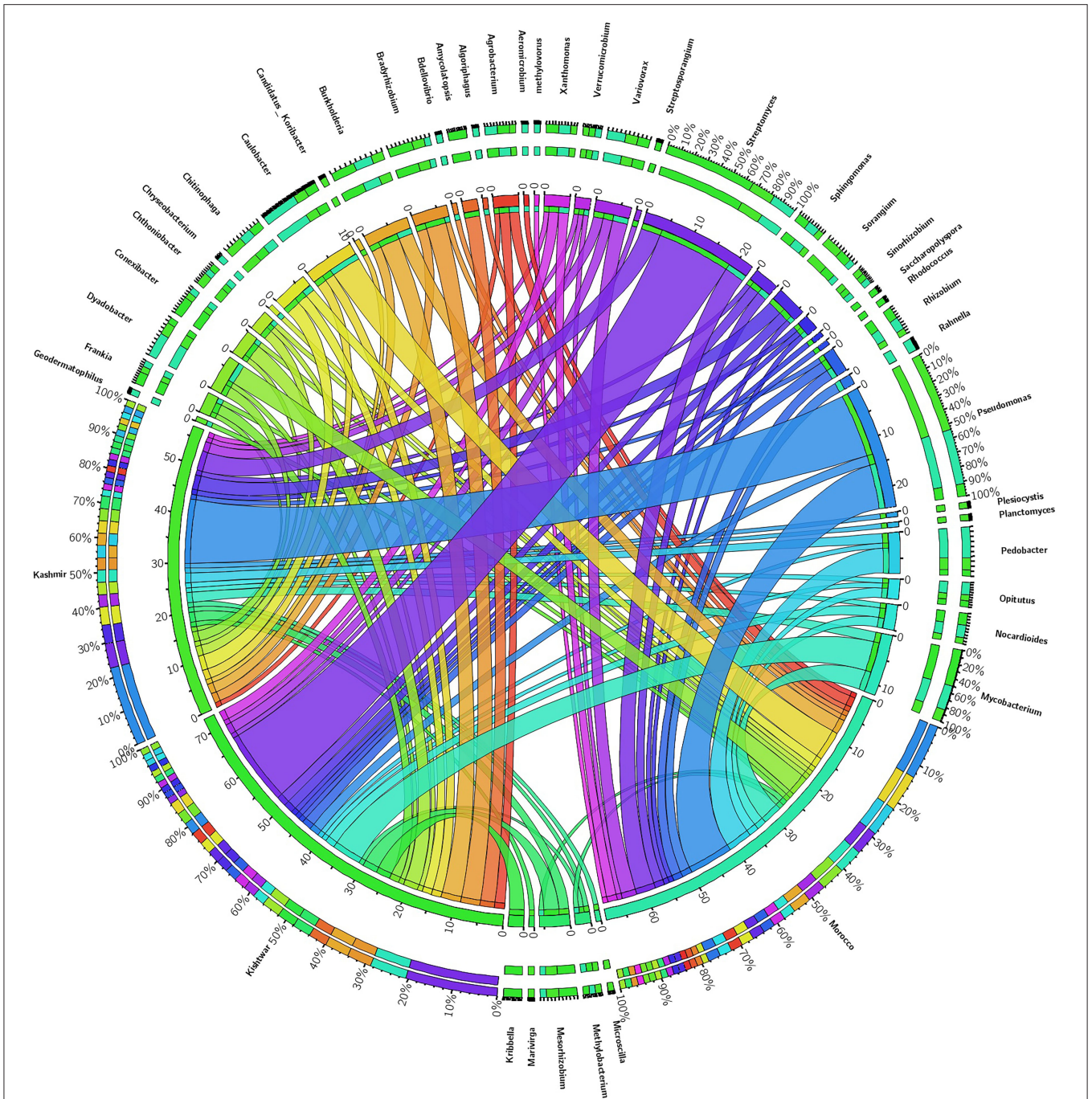

FIGURE 10 | Abundance of core genera and unique genera in three geographical locations. Central line (ribbons) represents the abundance of each genus and connects genera to samples. The outer three rings are the stacked bars representing the relative contribution of particular genera in every sample.

were retained under laboratory conditions. Eight genera were missing under lab conditions, but additional six genera were present and were completely absent under field conditions. The aim of laboratory cultivation was to check if the unique genera present in Kishtwar samples are retained if corms are dug and grown somewhere else. It was observed that the unique genera were retained as the five unique genera present in Kishtwar 3 years (C11, C12, and C13) were also present in these samples. This further establishes that the unique genera are inherited and can be used as geographical indicators or markers. In addition, all the microbes, representing the core microbiome of saffron corm characterized from different locations, were also present in laboratory grown samples (discussed in the following sections).

In total, 15 phyla (12 bacterial, 2 fungal, 1 archaeal) cataloged were common among three different locations. The most dominant phylum was *Proteobacteria* followed by *Actinobacteria*,

TABLE 4 | Core microbiome of three geographical regions (24 genera) (Kishtwar-A, Kashmir, and Morocco) with their relative abundance (%).

Genera	Kishtwar-A	Kashmir	Morocco	Phyla
Streptomyces	14.33	3.33	3.70	*Actinobacteria*
Bradyrhizobium	3.87	1.40	0.77	*Proteobacteria*
Mycobacterium	5.87	1.90	3.27	*Actinobacteria*
Mesorhizobium	2.46	1.15	0.95	*Proteobacteria*
Burkholderia	3.49	1.68	2.58	*Proteobacteria*
Conexibacter	2.09	0.92	0.73	*Actinobacteria*
Sorangium	1.76	2.15	0.69	*Proteobacteria*
Agrobacterium	1.37	0.86	1.88	*Proteobacteria*
Rhizobium	1.38	0.79	1.81	*Proteobacteria*
Pseudomonas	1.43	12.78	8.66	*Proteobacteria*
Variovorax	1.35	1.30	2.82	*Proteobacteria*
Xanthomonas	1.29	0.95	1.98	*Proteobacteria*
Caulobacter	1.78	1.53	5.46	*Proteobacteria*
Chitinophaga	1.15	2.65	1.44	*Bacteroidetes*
Frankia	1.35	0.66	0.61	*Actinobacteria*
Nocardioides	1.26	0.42	1.26	*Actinobacteria*
Sphingomonas	1.16	0.52	1.21	*Proteobacteria*
Methylobacterium	0.95	0.62	0.54	*Proteobacteria*
Opitutus	0.87	0.91	1.30	*Verrucomicrobia*
Chthoniobacter	0.92	1.12	0.50	*Verrucomicrobia*
Sinorhizobium	0.49	0.42	0.71	*Proteobacteria*
Dyadobacter	0.67	1.42	3.18	*Bacteroidetes*
Pedobacter	0.49	1.67	4.68	*Bacteroidetes*
Verrucomicrobium	0.62	0.73	0.78	*Verrucomicrobia*

Bacteroidetes, *Streptophyta*, *Acidobacteria*, and *Verrucomicrobia* (**Figure 8**). *Proteobacteria* have also been reported to be the dominant phylum in the rhizosphere of other plants such as *Gossypium hirsutum*, *Artemisia argyi*, *Ageratum conyzoides*, *Erigeron annuus*, *Bidens biternata*, *Euphorbia hirta*, and *Viola japonica* (Qiao et al., 2017; Lei et al., 2019). *Proteobacteria* are the main microbial contributors to plant growth promoting rhizobacteria (PGPR), reported so far. They are fast growing and play a role in diverse metabolic activities that enhance plant growth (Philippot et al., 2013; Rampelotto et al., 2013; Schillaci et al., 2019; Mukhtar et al., 2020). *Actinobacteria* are the gram positive bacteria that are known to promote plant growth and having biocontrol activity (Viaene et al., 2016). Likewise, *Bacteriodetes* are the pathogen suppressing members and also have a role in plant growth promotion by phosphorus mobilization (Lidbury et al., 2021). This explains the dominance of *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* in the cormosphere, which are among the most dominant phyla in the soil and maybe getting transferred from the soil, just like in rhizosphere (Miyashita, 2015; Fierer, 2017; Compant et al., 2019). To our knowledge, cormosphere microbiome has not been studied in any other plant, except for saffron reported previously by our group (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016; Kour et al., 2018) from Kashmir, India (Chamkhi et al., 2018), and from Taliouine, Morocco. Previous reports by our group have also confirmed the dominance of *Proteobacteria* in Kashmir saffron rhizosphere and cormosphere, even though the method used was cultivation dependent and 16S targeted metagenome sequencing was different than the methods used in the present study (Ambardar and Vakhlu, 2013; Ambardar et al., 2014) (**Supplementary Material 1**).

Core Microbiome of *C. sativus* Across Different Geographical Locations

A total of 73 genera were identified after taxonomical characterization of *C. sativus* cormosphere microbiome from reference samples collected from Kishtwar-A, laboratory grown Kishtwar-L 17, Kashmir, and Morocco. *Streptomyces* was dominant in Kishtwar-A as well in Kishtwar-L 17, whereas *Pseudomonas* was dominant in Kashmir and Morocco. *Pseudomonas* and *Streptomyces* have been reported in the rhizosphere of various plants like *Arabidopsis thaliana*, tomato, rice, and maize including saffron (Ambardar and Vakhlu, 2013; Ambardar et al., 2014; Chen et al., 2018; Kour et al., 2018; Chu et al., 2019; Qessaoui et al., 2019). *Pseudomonas* isolates have been reported to solubilize phosphate, produce siderophores, ammonia, and indole-3-acetic acid, and colonize the roots of tomato plants (Qessaoui et al., 2019) and also have been reported to induce salt tolerance in *Arabidopsis thaliana* (Chu et al., 2019). *Pseudomonas* has also been reported to be dominant in saffron rhizosphere grown in Kashmir, India,

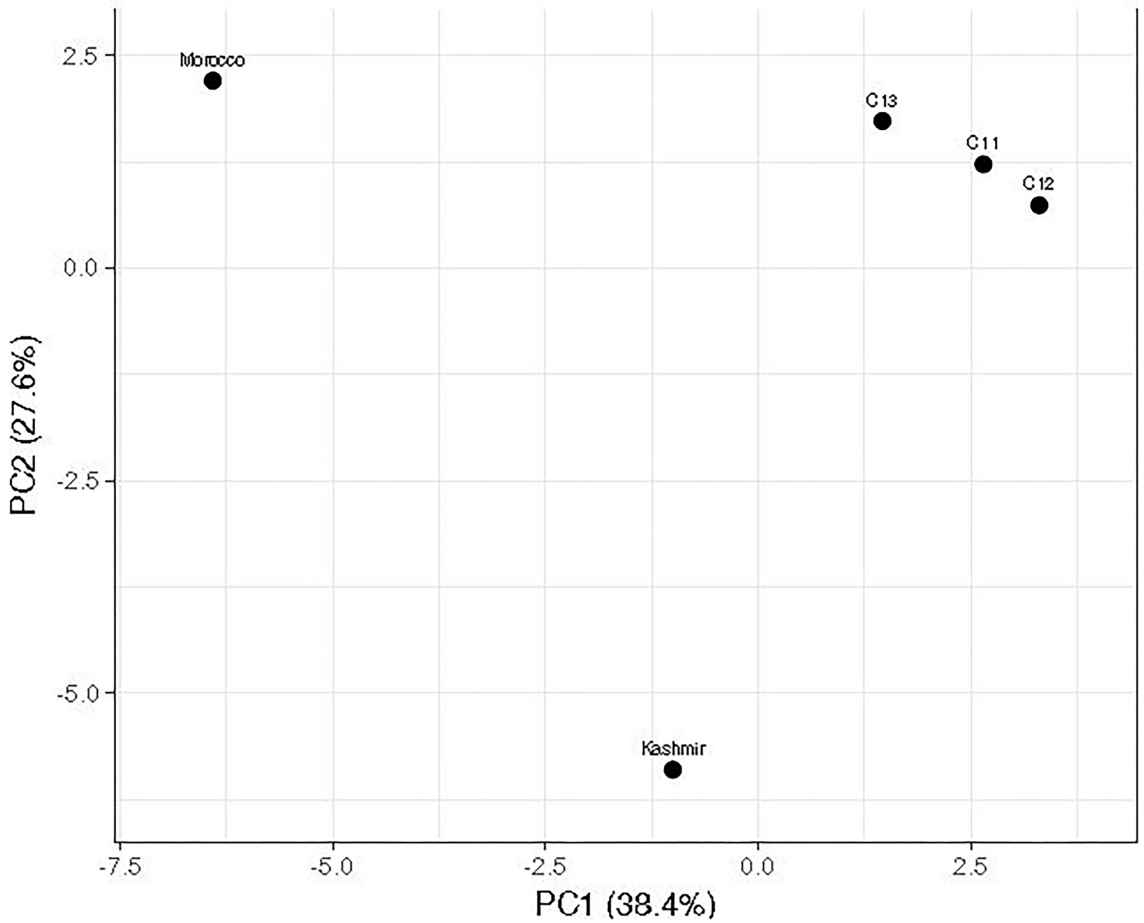

FIGURE 11 | Comparison of microbial diversity of Kishtwar during three consecutive year's sample (C11, C12, and C13) with Kashmir and Morocco. It represents that Kashmir and Morocco are significantly different from the rest of the sample as it does not cluster with Kishtwar sample.

analyzed using both cultivation dependent and cultivation independent method (Ambardar and Vakhlu, 2013; Ambardar et al., 2014), and Morocco, analyzed using cultivation dependent approaches (Chamkhi et al., 2018). *Streptomyces* is the largest genus of *Actinobacteria* phylum comprising 500 species and has been found to promote plant growth (Lehr et al., 2007, 2008; Schrey and Tarkka, 2008; Viaene et al., 2016). It is reported to produce secondary metabolites having antibiotic activities and volatile organic compounds (VOCs) that can exert biocontrol activity (Lucas et al., 2012; Viaene et al., 2016). *Streptomyces* have been previously reported from the saffron rhizosphere also using cultivation independent approaches (Ambardar et al., 2014). In addition, to *Pseudomonas* and *Streptomyces*, 53 have also been reported previously in saffron in Kashmir and Morocco (Ambardar and Vakhlu, 2013; Ambardar et al., 2014, 2016; Chamkhi et al., 2018; Kour et al., 2018) (**Supplementary Material 2**).

Interestingly, we found that 24 genera were common to all, representing 32.8% of collective bacteriome in the four samples (three field and one laboratory sample). These 24 genera are being proposed to represent the core microbiome of cormosphere of *C. sativus* across different geographical locations. Core microbiome across different geographical locations has been earlier reported in various plants such as *Salvia miltiorrhiza* (Chen et al., 2018), *Phaseolus vulgaris* (Pérez-Jaramillo et al., 2019), *Oryza sativa* (Eyre et al., 2019), and *Gymnadenia conopsea* (Lin et al., 2020) and also in Vineyards soil (Coller et al., 2019) and *Dalbergia spruceana* (Skaltsas et al., 2019). Core microbiome of 21 *Salvia miltiorrhiza* seeds represented 54% of whole microbiome cataloged from seven different geographic origins, and *Pantoea*, *Pseudomonas*, and *Sphingomonas* were enriched core taxa (Chen et al., 2018). In the case of *Phaseolus vulgaris* rhizosphere, core microbiome represented 25.9% of total microbiome in native and agricultural soils (Pérez-Jaramillo et al., 2019). Core microbiome of 30 phylogenetically diverse angiosperm plants constitutes 40% of whole microbiome (Fitzpatrick et al., 2018). In rice seeds, the core microbiome was enriched in *Rhizobium*, *Pantoea*, *Sphingomonas*, *Methylobacterium*, *Xanthomonas*, *Paenibacillus*, *Alternaria*, and *Occultifur* that have been also

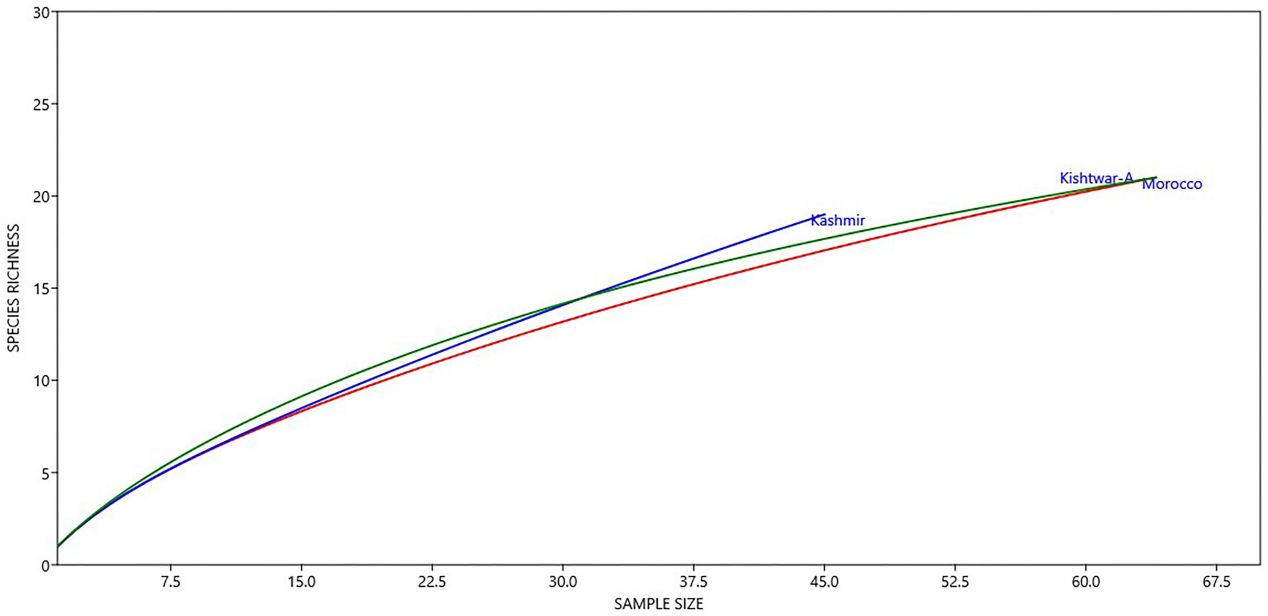

FIGURE 12 | Diversity of different genera across geographical locations (Kishtwar-A, Kashmir, and Morocco). Kashmir samples were more enriched as depicted by higher number of species in Kashmir followed by Kishtwar-A and Morocco.

reported as PGPB (plant growth promoting bacteria) (Wang et al., 2020). Core microbiome in saffron was 32.8% of the total microbiome, which was comparatively less than *Salvia miltiorrhiza*. Similar to core microbiome in rice and *Salvia miltiorrhiza* (seed), saffron's core microbiome was also enriched with *Pseudomonas*, *Rhizobium*, *Sphingomonas*, *Xanthomonas*, and *Methylobacterium*. In addition, 22 bacterial genera (out of 24) representing the core microbiome of the *C. sativus* in the present study were also reported previously in Saffron grown in Kashmir, India, by our group, out of which 19 are PGPB in saffron and other plants (Ambardar and Vakhlu, 2013; Kour et al., 2018; Umadevi et al., 2018; Vergani et al., 2019) (**Supplementary Material 3**). *Agrobacterium*, *Bacillus*, *Delftia*, *Luteibacter*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Rhizobium*, and *Variovorax* from rhizosphere and *Agrobacterium*, *Pseudomonas*, *Rhizobium*, and *Variovorax* from cormosphere of saffron grown in Morocco using cultivation dependent methods have been reported earlier as well (Chamkhi et al., 2018). Core microbiome enriched with plant growth promoting bacteria indicates that the plant selects the core microbiome based on their function (**Supplementary Material 3**). To conclude, the saffron cormosphere core microbiome is conserved across two continents, Asia and Africa, as per this study.

Microbial Genera Specific to Geographical Location

Variation in the microbiome of *C. sativus* across different geographical locations was confirmed by PCA plots, wherein surprisingly Morocco and Kashmir were clustered away from Kishtwar-A, although Kashmir is geographically closer to Kishtwar (**Figure 11**). These findings were further complemented by Shannon alpha diversity indices, which were maximum in Kashmir (507) compared to Kishtwar-A (354) and Morocco (415). The Shannon alpha diversity index indicates that microbial communities of each sample have significant differences, though they share core microbiome. There are plenty of reports on the effect of temperature on the diversity and abundance of soil microbes, though those studies have been conducted in reference to climate change (Jansson and Hofmockel, 2020).

The effect of geographical location on the microbiome has been discovered in other plants such as barley (Terrazas et al., 2020) and *Gymnadenia conopsea* (Lin et al., 2020), and significant variation has been reported. Each location in the present study harbored unique bacteria that could be used as a geographical indicator. The cormosphere of Kashmir had five unique bacteria, namely, *Marivirga*, *Planctomyces*, *Plesiocystis*, *Candidatus koribacter*, and *Microscilla*, whereas seven bacteria, namely, *Rahnella*, *Chryseobacterium*, *Bdellovibrio*, *Algoriphagus*, *Methylovorus*, *Geodermatophilus*, and *Aeromicrobium*, were specific to Morocco and five (*Rhodococcus*, *Kribella*, *Streptosporangium*, *Saccharopolyspora*, and *Amycolatopsis*) were specific to Kishtwar (**Figure 13**). These unique genera have also been reported as PGPB in other plants (**Supplementary Material 4**).

The bacterial genera (8) that have been reported for the first time from saffron underground parts were *Marivirga*, *Microscilla*, *Starkeya*, *Actinosynnema*, *Saccharopolyspora*, *Bdellovibrio*, *Algoriphagus*, and *Methylovorus*, whereas 11 bacterial genera (*Planctomyces*, *Plesiocystis*, *Candidatus koribacter*, *Chryseobacterium*, *Rhodococcus*, *Rahnella*

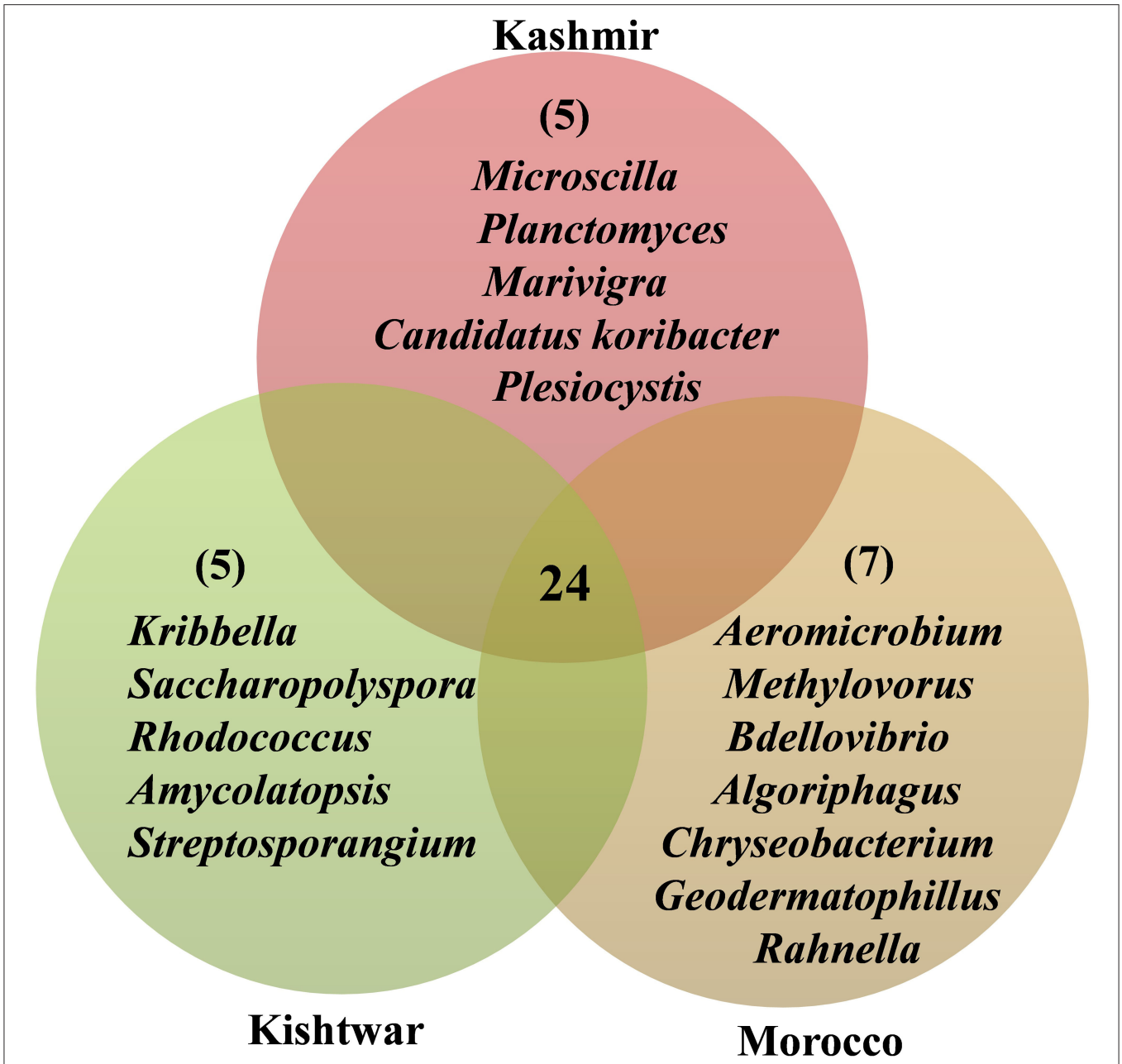

FIGURE 13 | Representing the number of common and unique genera identified in three geographical locations. Twenty-four common genera in all the three geographical locations are referred as the core microbiome of saffron. Five genera were unique to Kishtwar, seven to Morocco, and five to Kashmir.

Amycolatopsis, *Kribbella*, *Streptosporangium*, *Geodermatophilus*, and *Aeromicrobium*) have been reported earlier from saffron rhizosphere in our previous studies from Kashmir and Kishtwar in India and Taliouine in Morocco (Ambardar et al., 2021 unpublished data; Chamkhi et al., 2018). However, the bacteria reported from saffron for the first time have been reported from other plants. *Marivirga* has been reported from *Panax ginseng* using 16S rRNA sequencing (Nguyen et al., 2016), *Microscilla* from *Tuber melanosporum* (Deveau et al., 2016), *Starkeya* from root nodules of legume plants (Zakhia et al., 2006), *Actinosynnema* from beech rhizosphere (Colin et al., 2017), *Saccharopolyspora* from *Colocasia* rhizosphere (Intra et al., 2019), *Rahnella* from *Oryza sativa* (Sun et al., 2020), *Chryseobacterium* from sand dune plant rhizosphere (Cho et al., 2010), *Algoriphagus* from mangrove rhizosphere (Song et al., 2020), and *Bdellovibrio* and *Methylovorus* from grassland (Macey et al., 2020). *Marivirga*, *Microscilla*, *Actinosynnema*, and *Bdellovibrio* have been identified using cultivation independent approach (16S rRNA sequencing), whereas *Saccharopolyspora*, *Chryseobacterium*, and *Algoriphagus* have been identified

using cultivation based approaches but their PGP activities have not been estimated yet. However, *Starkeya* and *Rahnella* have been reported as PGPRs and isolated using cultivation dependent approaches, whereas *Methylovorus* is reported to be methylotrophs (Zakhia et al., 2006; Cho et al., 2010; Deveau et al., 2016; Nguyen et al., 2016; Colin et al., 2017; Intra et al., 2019; Macey et al., 2020; Song et al., 2020; Sun et al., 2020).

The preliminary study suggests that saffron across three geographical locations share common core cormosphere microbiome but have certain unique bacteria genera specific for each location. The unique genera specific to a particular location can be used as the geographical indicators and molecular markers in saffron. The study needs to be complemented further by analyzing the microbiome of saffron grown across various geographical locations, as saffron is cultivated now in both the hemispheres. It also needs to be validated by in-depth sequencing and frequent sampling for a couple of consecutive years.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA705068].

AUTHOR CONTRIBUTIONS

JV, MH, and LS gave plan of study. SS, NB, DT, RZ, and JV designed the experiments. SS, NB, SA, and DT conducted the experiments and analyzed the sequencing data. SS, SA, NB, and SR wrote the manuscript and analyzed the taxonomy data. JV, MH, and LS finalized the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

Sincere thanks and gratitude to Tahir Ali (Ph.D. Scholar, School of Biotechnology, University of Jammu, Jammu, J&K, India), helping in preparing map showing sample collection sites on the world map and to Mr. Ajmad Malik (Saffron development officer) from Kishtwar (J&K) for providing data on saffron yield. Authors are thankful to UGC for funding the project UGC [42-168/2013 (SR)] and Indo-Austrian project of OeAD GmbH and DST under call number IN2022 to carry out this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.688393/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fsufs.2021.700642

Phenolic Characterization of Cabernet Sauvignon Wines From Different Geographical Indications of Mendoza, Argentina: Effects of Plant Material and Environment

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The chemical and sensory characteristics of the wines are related to the geographical origin of the grape, as a result of the interplay between the plant material (G), its acclimatization to the environment (E) and the human factor that influences both the vineyard and the winery. The range of phenotypes that a single genotype can express depending on its environment is known as phenotypic plasticity and is the result of G×E interaction. The present study evaluated the independent and interactive effects of Cabernet Sauvignon plant materials (G: Clone 7 and Mount Eden) implanted in different geographical indications of Mendoza, Argentina (E: Agrelo, Pampa El Cepillo, Altamira and Gualtallary) according to fruit yield and phenolic profiles of wines. The experiment was carried out during 2018 and 2019 vintages using a multifactorial design. When berries reached 24 °Brix, the clusters were harvested, analyzed and wines elaborated by a standardized procedure. Then, the anthocyanin and non-anthocyanin phenolic profiles of wines were determined by high-performance liquid chromatography with diode array and fluorescence detection (HPLC-DAD–FLD). The results revealed significant G×E interactions for yield traits, including the number of clusters per plant. Differential chemical composition and quality parameters of the resulting wines, markedly affected by E, were observed; that is the geographical location of the vineyards. There were similarities in the phenolic composition between Pampa El Cepillo and Altamira, while larger differences between Agrelo and Gualtallary were observed. Gualtallary presented the highest levels of anthocyanins, quercetin and *trans*-resveratrol. The increased amount of these compounds in Gualtallary was associated with an increased UV-B exposure of plants at this high altitude environment. This is the first report that characterizes the effects of plant material and environment for Cabernet Sauvignon. These results are of oenological and viticulture interest for the wine industry demonstrating that the selection of the plant material and the vineyard location for Cabernet Sauvignon can considerably affect the quality attributes of wines.

Keywords: classification, geographical discrimination, grapevine, phenolic compounds, phenotypic plasticity, terroir

OPEN ACCESS

Edited by:
Micha Horacek, HBLFA Francisco Josephinum, BLT Wieselburg, Austria

Reviewed by:
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Specialty section:
This article was submitted to Crop Biology and Sustainability, a section of the journal Frontiers in Sustainable Food Systems

Received: *26 April 2021*
Accepted: *23 June 2021*
Published: *20 July 2021*

Citation:
Muñoz F, Urvieta R, Buscema F, Rasse M, Fontana A and Berli F (2021) Phenolic Characterization of Cabernet Sauvignon Wines From Different Geographical Indications of Mendoza, Argentina: Effects of Plant Material and Environment. Front. Sustain. Food Syst. 5:700642. doi: 10.3389/fsufs.2021.700642

INTRODUCTION

Cabernet Sauvignon is one of the best-known and most widespread red wine varieties in the world, with a high tannins concentration, within the existing red varieties (Togores, 2003). In Argentina there are planted 14,279 ha of Cabernet Sauvignon and the 76.3% of the total surface is located in the province of Mendoza (Instituto Nacional de Vitivinicultura, 2019). Within Mendoza, the largest areas of Cabernet Sauvignon are distributed in Luján de Cuyo (19.8%) and the Uco Valley (27.4%); both regions reputed for their high-quality wines. The Uco Valley is characterized by high-altitude vineyards, high ultraviolet-B solar radiation, rocky and permeable soils, and high thermal amplitudes. Meanwhile, Luján de Cuyo has vineyards at lower altitudes with deeper soils and slightly warmer temperatures.

Grapevine adapt to a wide range of environments (Van Leeuwen and Seguin, 2006; Gianoli and Valladares, 2012) with a broad phenotypic plasticity (Keller, 2010; Dal Santo et al., 2016). The phenotypic plasticity is the capacity of a genotype to express different phenotypes (including differential wine sensory attributes) when exposed to different environments (Pigliucci, 2005; Nicotra and Davidson, 2010); and plays an important role in the acclimatization and adaptation to climate change (Weiner, 2004; Van Kleunen and Fischer, 2005). Furthermore, it is known that the responses to the environment vary depending on the plant material (Schultz, 2003), i.e., some genotypes might be more suitable for a particular environment than others.

In viticulture and oenology, it is expected that the combination of genotype (plant material), microbiome (fungi and bacteria), environmental (soil and climate) and human (vineyard management and winemaking) factors affect the quality of grapes and their corresponding wines. This interaction is usually referred as the "terroir" and is finally expressed in the wine (Organización Internacional de la Viña y el Vino, 2010; Senyard et al., 2011; Anesi et al., 2015; Marlowe and Lee, 2018).

From the consumer point of view, there is much research that reveals the importance of the terroir concept when choosing a wine (Cross et al., 2011; Versari et al., 2014). Wine consumers have learned to trust provenance to predict wine quality and are willing to pay extra for it (Urvieta et al., 2021). In fact, different wine regions have exploited this concept of exclusive parcels capable of giving unique wines as an approach to add economic value to their wines. In this way, understanding phenotypic plasticity can also play an important role in the attempt to obtain differentiated wines as a commercial strategy. Phenolic compounds (PCs) are of great importance in the production of red wines, being secondary metabolites present in the vacuoles of berry skin and extracted during the winemaking process. PCs are originated in the grape berries and are essential for wines sensory characteristics, such as color, astringency, bitterness and aging capacity (Jaffré et al., 2009). These compounds are also important to evaluate the effect of the grapes environment in the wines. For example, Urvieta et al. (2021) evaluated phenolic composition of Malbec wines across multiple sites in Mendoza and was able to discriminate different GIs.

There are some studies that have shown the relevance of the grapes origin in the chemical composition of the Cabernet Sauvignon wines. Ranaweera et al. (2021) classified Australian Cabernet Sauvignon wines from three wine regions based on spectrofluorometric and multi-element analyses. Others authors evaluated Cabernet Sauvignon wines from China, and found that PCs profiles and concentrations were dependent of the vineyards locations (Li et al., 2011; Jin et al., 2017; Jiang and Sun, 2019). Another study carried out on 5 different varieties, including Cabernet Sauvignon and cultured with the same environmental conditions, demonstrated the effect of the genotype in the grape phenotype (Aleixandre et al., 2015). Pajović et al. (2014) performed a varietal differentiation of grapes from Montenegro, including Cabernet Sauvignon according to their phenolic composition. Regarding Cabernet Sauvignon wines from Argentina, there is only one study that evaluated the chemical differences. Di Paola-Naranjo et al. (2011) analyzed wines from three provinces (Mendoza, San Juan and Córdoba) and concluded that both elemental and isotopic composition allow a good differentiation among these wide regions. It should be taken into account that the wines used in the mentioned study were from different wineries, elaborated without standardized winemaking conditions, and also with different plant material (within the Cabernet Sauvignon variety).

In the present study, two Cabernet Sauvignon plant materials, implanted in four different GIs, were evaluated during two growing seasons. The PC profiles (anthocyanins and non-anthocyanins) of wines were determined by high performance liquid chromatography with a photodiode array and fluorescence detection (HPLC-DAD-FLD) with the aim to evaluate the independent and interactive effects of Cabernet Sauvignon plant materials (G: Clone 7 and Mount Eden).

MATERIALS AND METHODS

Standards Preparation and Solvents

Standards of gallic acid (99%), 3-hydroxytyrosol (≥99.5%), (−)-gallocatechin gallate (≥99%), caftaric acid (≥97%), (−)-epigallocatechin (≥95%), (+)-procyanidin B1 (≥90%), (+)-catechin (≥99%), procyanidin B2 (≥90%), (−)-epicatechin (≥95%), caffeic acid (99%), syringic acid (≥95%), *p*-coumaric acid (99%), *trans*-resveratrol (≥99%), quercetin hydrate (95%), kaempferol-3-glucoside (≥99%), myricetin (≥96%) and malvidin-3-*O*-glucoside chloride (≥95%) used for identification and quantification of PCs were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard of 2-(4-hydroxyphenyl) ethanol (tyrosol) (≥99.5%) was obtained from Fluka (Buchs, Switzerland). Individual stock solutions of compounds were prepared in MeOH at concentration levels ranging from 400 to 2,000 mg L^{-1}. Further dilutions and mixtures of compounds were prepared monthly in MeOH and stored in dark-glass bottles at −20°C. Calibration standards and mixtures of analytes used for calibration purposes were prepared in the initial mobile phase of each chromatographic method (anthocyanins and non-anthocyanins) and used within 3 days after their preparation. HPLC-grade FA and MeCN were acquired from Mallinckrodt Baker Inc. (Pillispsburg, NJ, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

FIGURE 1 | Geographical location of individual parcels in the study area. The images were created using QGIS.org, 2021. QGIS Geographic Information System. QGIS Association. http://www.qgis.org.

Plant Material and Experimental Design

The experiment was carried out during the 2017–2018 (2018) and 2018–2019 (2019) growing seasons with vines from two selected plant materials of *Vitis vinifera* L. cv. Cabernet Sauvignon. Genotypes (G) were Clone 7 (C7) and Mount Eden (ME). The Cabernet Sauvignon Mount Eden originated at Mount Eden in Santa Cruz Mountains, CA, USA (Foundation Plant Services Grape, 2011) and Clone 7 from De Latour nursery in Beaulieu, CA, USA (Caldwell, 2002). Both genotypes were chosen based on availability, fruit quatlilty and yield characteristics. Four environments (E) as parcels containing both G were selected in Mendoza province, distributed in four GIs at the main viticulture zones; First Zone (GI: Agrelo) and Uco Valley (GIs: Pampa El Cepillo, Altamira and Gualtallary) (see map of the studied area in **Figure 1**). **Table 1** shows the vineyard location, soil classification, planting year, and density of each parcel. In common for all four sites, the vines were planted on their own roots, formed in a vertical trellis system, arranged in north-south oriented rows, and drip-watered. The irrigation criteria were based on the soil characteristics and evapotranspiration measurements within each parcel. The plants were subjected to a weak water stress level after veraison, maintaining stem water potentials (Ψs) at midday between −0.6 and −0.9 Mpa. These four locations were selected because are the most representative in terms of production and quality of wines for the Cabernet Sauvignon in Mendoza. In addition, because of the possibility to have the two vegetative materials in places with different climatic conditions. All the vineyards have anti-hail nets, except for Agrelo GI.

A multifactorial experimental design with 9 replicates was used. Within each G×E combination treatment, nine representative rows were selected (each one containing 100 plants). After veraison, berry maturation was followed by weekly determinations of total soluble substances in 200 randomly sampled berries with a Pocket PAL-1 digital hand-held refractometer (Atago Co., Ltd., Tokyo, Japan).

Meteorological Data

The air temperatures (daily maximum, minimum and thermal amplitude) and rainfall data for each site were registered during the 2018 and 2019 growing seasons, between September and April, i.e., from grapevine budburst to harvest. Data were obtained from the database of the Catena Institute

TABLE 1 | Description of the Cabernet Sauvignon vineyards.

Geographical indication	Location	Altitude (m a.s.l.)	Soil type	Year of plantation	Density (plants ha^{-1})	Harvest date			
						2018		2019	
						Mount Eden	Clone 7	Mount Eden	Clone 7
Agrelo (AGR)	S33° 09.827′ W68° 54.921′	950	Sandy loam (0–30 cm) Clay (30–60 cm)	1997	4,000	22 March	22 March	20 March	20 March
Pampa El Cepillo (PEC)	S33° 48.398′ W69° 09.697′	1,073	Sandy loam	2005	5,555	27 March	27 March	25 March	25 March
Altamira (ALT)	S33° 45.267′ W69° 10.251′	1,085	Sandy loam	1995	5,555	3 April	3 April	28 March	28 March
Gualtallary (GUA)	S33° 23.752′ W69° 15.451′	1,350	Sand	1998	4,000	15 April	15 April	10 April	10 April

of Wine (CIW) and the Department of Agriculture and Climate Contingencies of Mendoza, with an automatic weather station (iMetos ag, model IMT 300; Pessl Instruments, Weiz, Austria), located close to the experimental sites. Measurements were registered every 15 min for 24 h a day and the daily means were calculated. **Supplementary Table 1** summarize the meteorological data of the studied parcels.

Measurements of Fruit Yield and Sampling of Clusters

At commercial maturity, when the berries of each G×E combination reached 24 ± 1 ° Brix, one plant was randomly selected per experimental row ($n = 9$). The first GI to be harvested was Agrelo, then Pampa El Cepillo and Altamira (1 week later) and finally Gualtallary with a difference of 3 weeks with respect to Agrelo. Two clusters were sampled from the same shoot, and all remaining clusters from the plant were harvested and weighed in the field as fruit yield. Furthermore, the number of berries per cluster, the fresh weight of the berries in the sampled clusters and the dry weight of the berry skin were determined (dried at 60°C in an oven to a constant weight).

Winemaking Procedure and Wine Analysis

A total of 24 individual wines were elaborated according to a standardized procedure (Urvieta et al., 2018) in order to obtain three replicates for each G×E combination. At commercial maturity, all the grapes from the experimental rows were manually harvested, transported to the winery, macerated for 24 h and microfermented at 25°C in 800 L plastic vessels with the addition of active dry yeast (20 g L^{-1}; Lavin EC-1118, Lallemand Inc., Montréal, Canada). After alcoholic fermentation and 10 days of maceration (post-fermentation), 50 L of drained wine were transferred to stainless steel tanks and inoculated. with malolactic bacteria (1 g L^{-1}; Lavin VP41, Lallemand Inc., Montreal, Canada). When the malic acid content was below 0.2 g L^{-1} as assessed by Oeno Foss (FOSS Analytical A/S, Hillerod, Denmark), decantation was carried out to remove thick lees. Afterwards, SO_2 was added as $K_2S_2O_5$ (Laffort Oenologie, France) at a final concentration of 35 mg L^{-1} free SO_2. Then, wines were filtered with a nominal filter with a 0.2–0.4 μm cellulose plate. Wines were stored for 3 months in 50 L stainless steel tanks at 13–15°C. No tartaric stabilization was carried out during aging. Finally, wines were fractionated in green-glass bottles (750 mL volume) of each replicate (three per parcel) and stored at 15 °C until analysis.

Phenolic Compounds Profile of Wines

An HPLC-DAD-FLD (Dionex Ultimate 3000 system, Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) and a reversed-phase Kinetex C_{18} column (3.0×100 mm, 2.6 mm; Phenomenex, Torrance, CA, USA) was used. The determination of anthocyanins was performed according to Urvieta et al. (2018), with minor modifications. Briefly, 1 mL aliquot of wine samples was evaporated to dryness by vacuum centrifugation and dissolved with 1 mL of initial mobile phase prior HPLC-DAD-FLD analysis. The different anthocyanins were separated in a reversed-phase Kinetex C_{18} column (3.0× 100 mm, 2.6 μm) Phenomenex (Torrance, CA, USA). The mobile phase consisted of ultrapure H_2O:FA:MeCN (87:10:3, v/v/v; eluent A) and ultrapure H_2O:FA:MeCN (40:10:50, v/v/v; eluent B). The separation gradient was: 0 min, 10% B; 06 min, 25% B; 610 min, 31% B; 1,011 min, 40% B; 11–14 min, 50% B; 14–15 min, 100% B; 15–17 min, 10% B; 17–21 min, 10% B. The mobile phase flow was 1 mL min^{-1}, column temperature 25°C and injection volume 5 μL. Quantification was carried out by measuring peak area at 520 nm and the content of each anthocyanin was expressed as malvidin-3-glucoside equivalents using an external standard calibration curve (1–250 mg L^{-1}, $r^2 = 0.997$). The identity of detected anthocyanins was confirmed by comparison with the elution profile and identification of analytes realized in our previous research (Antoniolli et al., 2015). The compounds that were determined were the following: delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, delphinidin 3-O-acetylglucoside, petunidin 3-O-acetylglucoside, peonidin 3-O-acetylglucoside, malvidin 3-O-acetylglucoside,

cyanidin 3-O-p-coumaroylglucoside, petunidin 3-O-p-coumaroylglucoside, peonidin 3-O-p-coumaroylglucoside and malvidin 3-O-p-coumaroylglucoside.

For non-anthocyanins compounds, wine samples were analyzed according to Ferreyra et al. (2021). A reversed-phase Kinetex C_{18} column (3.0 × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) was used for the chromatographic separation. The mobile phases were an aqueous solution of 0.1% FA (solvent A) and MeCN (solvent B). The gradient was as follows: 0–1.7 min, 5% B; 1.7–10 min, 30% B; 10–13.5 min, 95% B; 13.5–15 min, 95% B; 15–16 min, 5% B; 16–19, 5% B. The total flow rate was set at 0.8 mL min^{-1}. The column temperature was 35°C and the injection volume was 5 μL for calibration standards and wine samples. The analytical flow cell for DAD was set to scan from 200 nm to 400 nm. A data collection rate of 5 Hz, a band width of 4 nm and a response time of 1.000 s were used. Wavelengths of 254, 280, 320 and 370 nm were used depending on the analytes maximum absorbance for DAD, while an excitation wavelength (Ex) of 290 nm and a monitored emission (Em) responses of 315, 360 and 400 nm were used depending on the targeted analytes for FLD. A data collection rate of 10 Hz (peak width of 0.04 min corresponding to a response time of 0.8 s and a photomultiplier gain of 5 units) was used for FLD. The identification of non-anthocyanins in studied wines were based on the comparison of the retention times of samples with those of authentic standards. In addition, some wine samples were spiked with known concentrations of standards in order to ensure a correct quantification and identification to finally select an external calibration with pure standards as quantification technique. Linear ranges between 0.05 and 40 mg L^{-1} with coefficient of determination (r^2) higher than 0.993 were obtained for quantified compounds.

Statistical Analysis

Multifactorial analyses and Fisher's least significant difference (LSD) test, with a significance level of $P \leq 0.05$ were performed to evaluate the effect of genotype (G), environment (E) and the G×E interaction. Principal component analysis (PCA) throughout biplot graphics and standardized (centered and variance-scaled) were used to find association of the variables with the classification patterns. For anthocyanin and non-anthocyanin compounds, the matrix for the PCA consisted of 8 cases (G×E combinations) as classification criteria, with the wine phenolic compounds as variables. For meteorological data, the PCA matrix consisted of 8 cases (vineyards and growing seasons) as classification criteria. All the statistical analyses were performed with InfoStat Software (InfoStat version 2020; Grupo InfoStat, Córdoba, Argentina).

RESULTS

Meteorological Data

Figure 2 shows that the vineyard in Agrelo (AGR) had higher maximum and minimum temperatures during both growing seasons (September–April), while the environment in Gualtallary (GUA) was the coldest as compared to the other GIs. The vineyard in Pampa El Cepillo (PEC) had the maximum accumulated rainfall for 2019 growing season and the thermal amplitude increased in AGR and Altamira (ALT) for 2018. In general, the air temperatures were similar for both growing seasons, while the 2019 was rainier than the 2018.

Fruit Yield

All the variables measured were affected by E, with significant G×E interactions for yield and the number of clusters per plant during the 2018 season (**Figure 3A**). The fruit yield in 2018 was higher in AGR, with differences between G (3 kg $plant^{-1}$ for Clone 7 and 4 kg $plant^{-1}$ for Mount Eden), and it was lower for PEC, ALT and GUA, respectively (significant G×E interaction; **Figure 3A**). The number of clusters per plant for 2018 was affected by E and G×E interaction, being higher in AGR for Mount Eden (**Figure 3B**). For the 2019 growing season, the AGR vineyard was affected by a hail storm that affected the plants canopy near the harvest date. In AGR, the yield (weight of the clusters and number of clusters per plant) was reduced for both G in 2019 (**Figures 3A,B**).

Significant G×E interactions were found for berries fresh weight in both seasons (**Figure 3C**). During 2018, the dry weight of the berry skin increased in GUA for both genotypes and more markedly for Clone 7 [$P_{(G\times E)} = 0.0935$; **Figure 3D**].

PCs Characterization of Wines

The anthocyanins detected were the same in all GIs, regardless of the year and genotype and their concentrations were mainly affected by E (**Table 3**). The predominant anthocyanin was malvidin-3-*O*-glucoside (386 mg L^{-1}) for all GIs, followed by peonidin-3-*O*-glucoside (250 mg L^{-1}) and petunidine-3-*O*-glucoside (187 mg L^{-1}). These maximum concentrations belong to GUA for both G. Di-hydroxylated (cyanidin and peonidin) and tri-hydroxylated (delphinidin, petunidin and malvidin) anthocyanins were higher in GUA with 873 mg L^{-1} and decreased to 756, 765 and 300 mg L^{-1} for ALT, PEC and AGR, respectively (**Table 2**).

For non-acylated anthocyanins, the GUA interact with Mount Eden and increased the total concentration with respect to the other interactions (468 mg L^{-1}; **Table 2**). AGR showed low concentration of total acetylated (82 mg L^{-1}) and total coumarylated (17 mg L^{-1}) anthocyanins, irrespectively of the G (**Table 2**). Methylated anthocyanins increased in ALT (415 mg L^{-1}), especially for ME plant material.

The PCA with anthocyanins shows that PC1 explain 84.9% of the variance and PC2 10.6% (**Figure 4**). Also, that the main differences for anthocyanin concentration are between AGR and GUA, being the concentrations higher for GUA (AGR was associated with tri/di-hydroxylated anthocyanins ratio). It can be also observed from **Table 2** that both ALT and PEC have similarities in the anthocyanin profile as peonidin-3-*O*-glucoside (193 mg L^{-1} for ALT and 212 mg L^{-1} for PEC), petunidin-3-*O*-glucoside (168 mg L^{-1} for ALT and 178 mg L^{-1} for PEC) and cyanidin-3-*O*-glucoside (5 mg L^{-1} for ALT and 5 mg L^{-1} for PEC).

Tables 3, **4** shows the non-anthocyanins phenolic profiles of the wines and that the most abundant compounds were gallic acid, caftaric acid and epigallocatechin gallate with

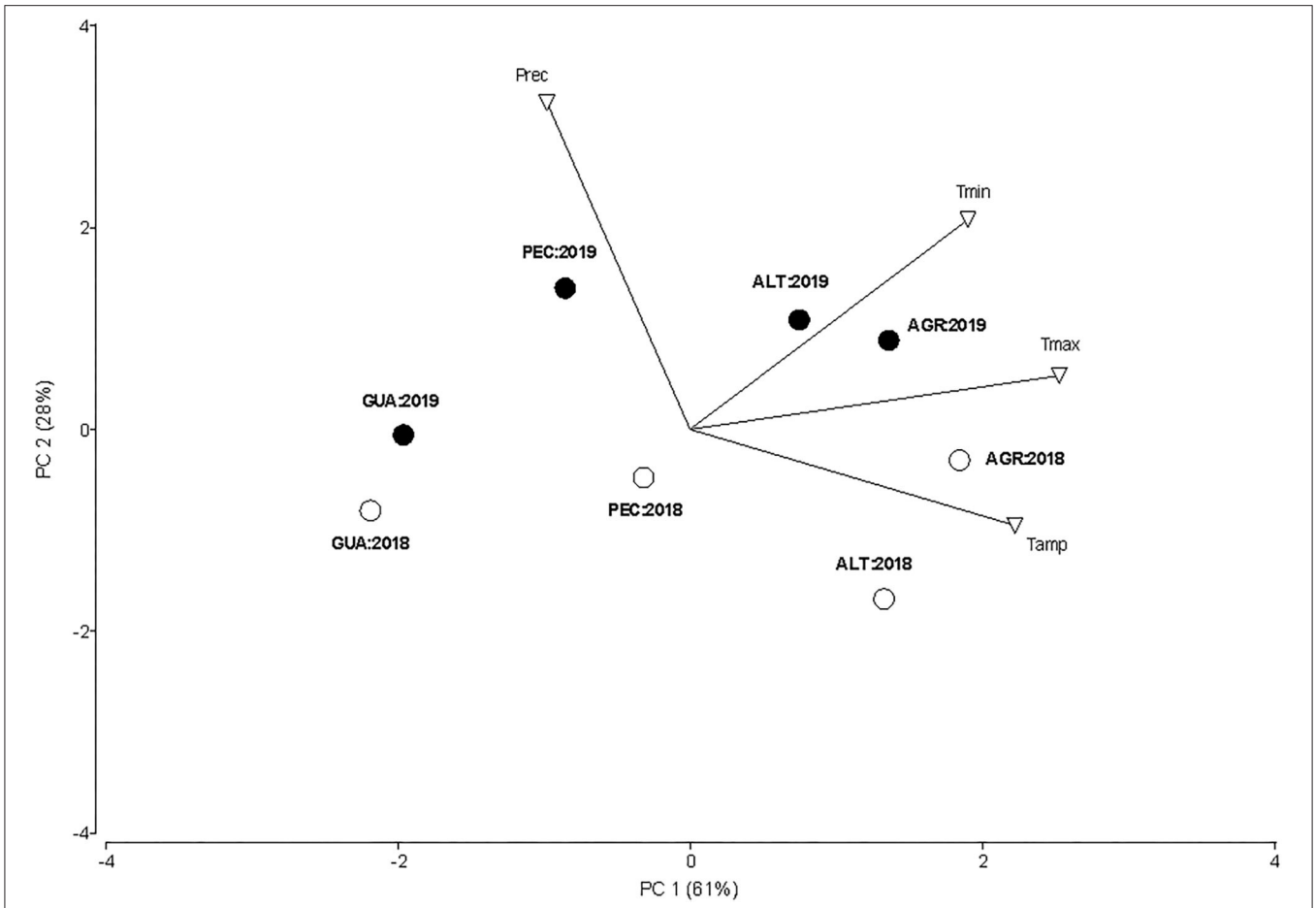

FIGURE 2 | PCA of minimum and maximum daily air temperatures, thermal amplitude and precipitation measured in the AGR, PEC, ALT and GUA vineyards during the 2018 and 2019 growing seasons (September–April). daily maximum (Tmax; °C), minimum (Tmin), thermal amplitude (Tamp) and precipitation (Prec).

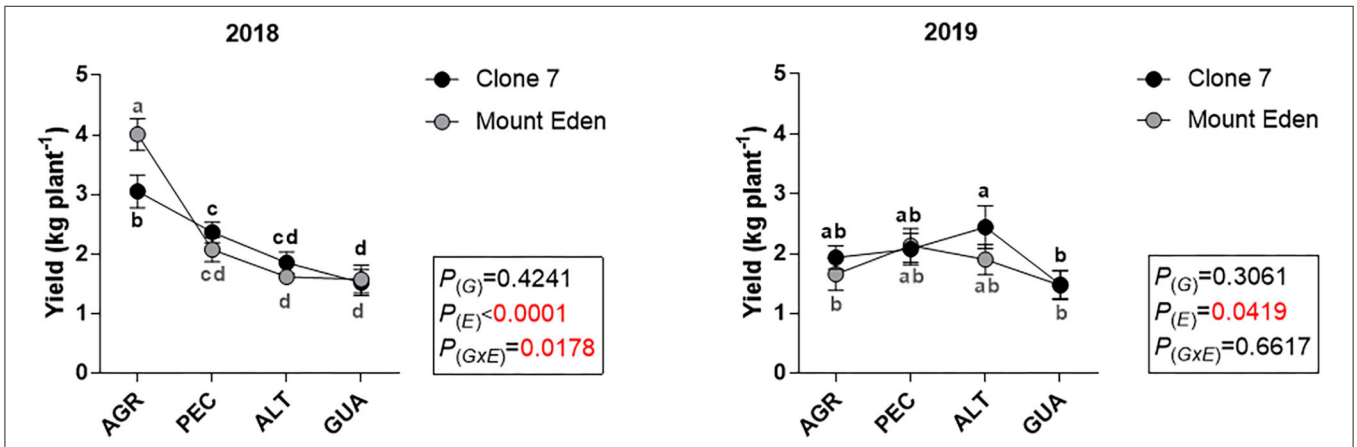

FIGURE 3 | Fruit yield indicators, measured during 2018 and 2019 growing season for two Cabernet Sauvignon clones (Clone 7 and Mount Eden) in four vineyards (AGR, PEC, ALT and GUA). Fruit yield **(A)**, number of clusters per plant **(B)**, Berry fresh weight **(C)** and berry skin dry weight **(D)**. Values are means ($n = 9$) and different letters indicate statistically significant differences (LSD Fisher; $P \leq 0.05$). $P_{(E)}$, $P_{(G)}$ and $P_{(GxE)}$: effects of environment, genotype and their interaction, respectively. For the 2019 growing season, the AGR vineyard was affected by a hail storm that affected the plants canopy near the harvest date. Supplementary data in **Supplementary Tables 2–4**.

TABLE 2 | Levels of anthocyanin PCs in wines of Cabernet Sauvignon Clone 7 (C7) and Mount Eden (ME) from four vineyards (AGR, PEC, ALT and GUA).

	Total anthocyanins		Di-hydroxylated				Tri-hydroxylated						Total non-acylated		Total acetylated		Total coumarylated		Total methylated		Di-hydroxylated		Tri-hydroxylated	
			Cyanidin		Peonidin		Delphinidin		Petunidin		Malvidin													
E																								
AGR	300	b	3	c	16	b	14	b	25	b	242	b	200	c	82	b	18	b	286	c	19	b	281	b
PEC	765	a	5	b	212	a	14	b	178	a	355	a	389	b	145	a	230	a	325	bc	218	a	547	a
ALT	758	a	5	bc	193	a	11	b	168	a	381	a	413	ab	133	a	212	a	415	a	198	a	560	a
GUA	873	a	13	a	250	a	36	a	187	a	386	a	459	a	152	a	262	a	377	ab	264	a	609	a
G																								
ME	667	a	6	a	163	a	19	a	136	a	350	a	362	a	129	a	176	a	363	a	169	a	498	a
C7	681	a	7	a	174	a	18	a	143	a	339	a	368	a	127	a	185	a	339	a	180	a	500	a
G×E																								
AGR/ME	325	b	3	c	16	b	15	b	27	b	264	b	212	c	93	bc	20	b	316	cd	19	b	306	b
AGR/C7	275	b	3	c	16	b	13	b	23	b	221	b	188	c	72	c	15	b	256	d	19	b	265	b
PEC/ME	711	a	5	bc	192	a	15	b	162	a	338	a	364	b	139	a	208	a	334	bcd	197	a	514	a
PEC/C7	819	a	6	b	233	a	13	b	194	a	373	a	414	ab	152	a	252	a	316	cd	238	a	580	a
ALT/ME	739	a	4	bc	186	a	11	b	163	a	375	a	405	ab	129	ab	205	a	418	a	191	a	548	a
ALT/C7	777	a	5	bc	201	a	11	b	173	a	388	a	421	ab	137	a	219	a	411	ab	205	a	572	a
GUA/ME	893	a	13	a	256	a	35	a	194	a	395	a	468	a	156	a	270	a	382	abc	269	a	624	a
GUA/C7	852	a	14	a	245	a	37	a	180	a	377	a	450	ab	148	a	254	a	372	abc	258	a	594	a
ANOVA																								
$P_{(E)}$	**<0.0001**		**<0.0001**		**<0.0001**		**<0.0001**		**<0.0001**		**0.0009**		**<0.0001**		**<0.0001**		**<0.0001**		**0.0009**		**<0.0001**		**<0.0001**	
$P_{(G)}$	**0.8248**		**0.4814**		**0.7090**		**0.8514**		**0.7625**		**0.2209**		**0.7018**		**0.8486**		**0.7639**		**0.2209**		**0.7018**		**0.9443**	
$P_{(GxE)}$	**0.7757**		**0.9608**		**0.9208**		**0.9380**		**0.8691**		**0.7399**		**0.9287**		**0.5487**		**0.9129**		**0.7399**		**0.9287**		**0.5970**	

Values are means (mg L^{-1} wine) of three replicates ($n = 3$) and different letters within a column indicate statistically significant differences between extracts (LSD Fisher test, $P < 0.05$). $P_{(E)}$, $P_{(G)}$ and $P_{(G\times E)}$: effects of environment, genotype and their interaction, respectively.

TABLE 3 | Levels of non-anthocyanin PCs in wines of Cabernet Sauvignon Clone 7 (C7) and Mount Eden (ME) from four vineyards (AGR, PEC, ALT and GUA).

	Total non-anthocyanins		Hydroxybenzoic acids						Hydroxycinnamic acids								Flavonols							
			Gallic acid		Syringic acid		Total		Caftaric acid		Caffeic acid		Coumaric acid		Total		Quercetin		Myricetin		Kaempferol-3-G		Total	
E																								
AGR	208	a	22	a	7	bc	29	a	7	a	4	a	4	c	15	b	18	a	8	a	3	b	29	ab
PEC	127	c	8	b	8	b	16	b	5	ab	2	b	11	b	13	b	10	b	3	c	2	c	16	c
ALT	156	b	8	b	10	a	18	ab	5	b	4	a	26	a	22	a	13	b	6	b	3	c	21	b
GUA	156	bc	8	b	6	c	13	ab	4	b	1	b	1	d	5	c	20	a	7	b	5	a	31	a
G																								
ME	159	a	12	a	7	a	19	a	6	a	3	a	10	a	14	a	15	a	6	a	3	a	24	a
C7	165	a	11	a	8	a	19	a	5	a	3	a	11	a	13	a	15	a	6	a	3	a	25	a
G×E																								
AGR/ME	209	a	25	a	7	bc	33	a	8	a	4	a	5	c	17	ab	19	a	8	a	3	c	31	bc
AGR/C7	208	a	19	b	7	bc	26	ab	6	ab	4	a	4	cd	13	bc	16	ab	9	a	3	cd	27	ab
PEC/ME	107	c	6	c	7	bc	14	b	4	bc	4	a	9	b	11	cd	10	c	3	d	2	d	15	d
PEC/C7	146	bc	10	c	8	b	18	ab	7	ab	4	a	12	b	15	bc	11	bc	4	cd	2	d	17	cd
ALT/ME	160	b	9	c	9	ab	18	ab	5	abc	2	bc	25	a	22	a	12	bc	6	bc	2	d	20	bcd
ALT/C7	149	b	8	c	11	a	18	ab	4	bc	3	ab	26	a	21	a	13	bc	6	ab	3	cd	22	bcd
GUA/ME	158	b	8	c	5	c	13	b	5	bc	1	bc	0	d	7	de	18	ab	6	abc	4	b	28	ab
GUA/C7	156	b	8	c	6	c	14	ab	3	c	1	c	1	d	4	e	21	a	7	ab	6	a	34	a
ANOVA																								
$P_{(E)}$	**<0.0001**		**<0.0001**		**<0.0001**		**0.0700**		**0.0121**		**<0.0001**		**<0.0001**		**<0.0001**		**0.0002**		**<0.0001**		**<0.0001**		**<0.0001**	
$P_{(G)}$	**0.4037**		**0.4894**		**0.1832**		**0.6548**		**0.4992**		**0.9979**		**0.6054**		**0.5169**		**0.7692**		**0.2594**		**0.3453**		**0.1830**	
$P_{(GxE)}$	**0.3536**		**0.1727**		**0.6737**		**0.2956**		**0.1101**		**0.6180**		**0.2095**		**0.2540**		**0.3677**		**0.9170**		**0.0922**		**0.8925**	

Values are means (mg L^{-1} wine) of three replicates ($n = 3$) and different letters within a column indicate statistically significant differences (Fisher's LSD test, $P < 0.05$). $P_{(E)}$, $P_{(G)}$ and $P_{(GxE)}$: effects of environment, genotype and their interaction, respectively.

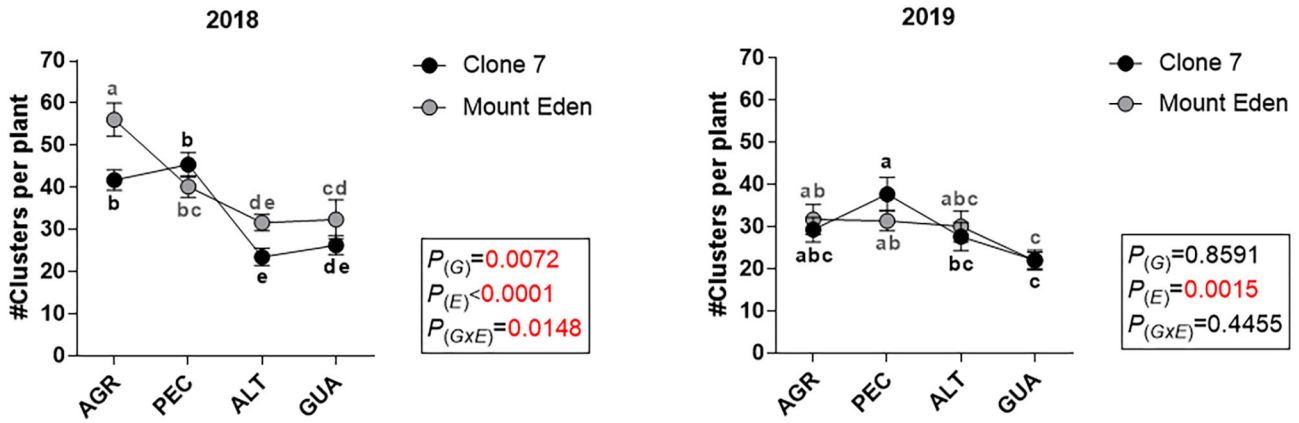

FIGURE 4 | PCA of main anthocyanins in Cabernet Sauvignon Clone 7 (C7) and Mount Eden (ME) wines, from vineyards in AGR, PEC, ALT and GUA.

concentrations of 22, 7 and 22 mg L^{-1}, respectively. These compounds were significantly higher in AGR. The highest accumulation of total non-anthocyanins was found for AGR/Clone 7 [$P_{(G\times E)} = 0.3536$], mainly through the effect of procyanidins (significant G×E interaction). Gallic acid, caffeic acid, epigallocatechin gallate and tyrosol stood out in the AGR/ME interaction at concentrations of 25, 8, 24 and 70 mg L^{-1}, respectively.

The stilbene *trans*-resveratrol increased markedly in GUA (3 mg L^{-1}), followed by ALT (2 mg L^{-1}). The *trans*-resveratrol levels in GUA were between 32 and 55% higher than those of the other environments. The GUA/C7 interaction stood out from the other G×E with a concentration of 3 mg L^{-1}. In comparison with the other GIs, the flavanol quercetin was also found in higher concentration in GUA (20 mg L^{-1}).

The PCA for non-anthocyanins PCs (**Figure 5**) explains 74.80 % of the data variability, where there is a differentiation of non-anthocyanin compounds based on G×E interactions. GUA grouped together *trans*-resveratrol (3 mg L^{-1}), quercetin (20 mg L^{-1}), procyanidin B1 (5 mg L^{-1}). For AGR the associated compounds were epigallocatechin gallate (22 mg L^{-1}), caftaric acid (7 mg L^{-1}) and tyrosol (67 mg L^{-1}), among others. With respect to C7 in ALT and PEC, there are some common compounds such as *p*-coumaric acid (25 mg L^{-1} for ALT and 11 mg L^{-1} for PEC) and syringic acid (11 mg L^{-1} for ALT and 8 mg L^{-1} for PEC).

DISCUSSION

Mendoza has a temperate arid continental climate with scarce rainfall (200–300 mm per year), so it is necessary to irrigate for the normal growth and development of the vines. Also, the vineyards are located in an altitudinal gradient that markedly modify the environmental conditions. The year of implantation and density of the Cabernet Sauvignon Clone 7 and Mount Eden at AGR and GUA are the same, but the parcels are located at different altitudes (see **Table 1**). Between the two parcels, there is a linear distance of 41 km and an altitude difference of 400 m, maximizing the differences in environmental conditions. There are differences in the soil types, being sandy loam with clay at 0.3–0.6 m depth in AGR and sandy in GUA.

Also, higher levels of ultraviolet-B (UV-B) radiation are registered in GUA in relation with AGR (Berli et al., 2010), with lower average air temperature (Varela et al., 2021). Previous data support that a lower temperature tends to increase color in red wines, while warmer temperatures during the day and night tend to reduce the content of anthocyanins (Drappier et al., 2019). Additionally, other research has shown that lower nighttime temperatures do not reverse the effects of higher daytime temperatures. Optimal anthocyanin accumulation occurs when grapes are exposed to cool nights (15°C) and moderate daytime temperatures (25°C) during ripening (Schultz, 2000). Low anthocyanin concentrations were correlated with warmer regions, which is climatically consistent with some studies revealing that high temperatures decreased the total anthocyanin content in Cabernet Sauvignon berries by 50% (Mori et al., 2007). These results agree with our results where the low levels of anthocyanins were associated to the lower altitude AGR. GUA presented nearly three times higher levels of total anthocyanins than AGR.

The classification of anthocyanins used is based on their biosynthetic pathway, where tri-hydroxylated anthocyanins derived from flavonoid 3′,5′-hydroxylase (F3′5′H), i.e., delphinidin-, petunidin- and malvidin-derived. Whereas, di-hydroxylated anthocyanins derived from flavonoid 3′-hydroxylase (F3′H), being cyanidin- and peonidin-derived (Castellarin et al., 2007). Within this classification criteria, we can separate methylated anthocyanins (malvidin-, petunidin- and peonidin-derived) from non-methylated anthocyanins (delphinidin- and cyanidin-derived). Also, we can group the anthocyanins by acylation as non-acylated, acetylated or coumarylated.

Although the anthocyanin profile is primarily a genetic characteristic unique to each grapevine cultivar, we previously found that stressful environmental conditions as high UV-B levels reduced the proportion of tri-hydroxylated anthocyanins,

TABLE 4 | Levels of non-anthocyanin PCs in wines of Cabernet Sauvignon Clone 7 (C7) and Mount Eden (ME) from four vineyards (AGR, PEC, ALT and GUA).

	Stilbene		**Flavanols**												**Phenyl ethanol derivatives**					
	***trans*-resveratrol**		**Procyanidin B1**		**Catechin**		**Epicatechin**		**Gallocatechin gallate**		**Epigallocatechin gallate**		**Procyanidin B2**		**Total**		**OH-Tyrosol**		**Tyrosol**		**Total**	
E																						
AGR	2	bc	2	b	27	a	7	b	4	ab	22	a	2	c	63	a	2	a	68	a	69	a
PEC	2	c	2	b	12	b	12	ab	4	a	12	b	1	c	44	a	2	a	35	c	37	b
ALT	3	ab	2	b	12	b	16	a	1	b	11	b	5	a	46	a	2	a	45	b	47	ab
GUA	3	a	5	a	18	b	18	a	3	ab	12	b	3	b	58	a	2	a	42	bc	45	ab
G																						
ME	2	a	3	a	15	a	13	a	3	a	14	a	2	a	49	a	2	a	48	a	50	a
C7	3	a	3	a	19	a	14	a	3	a	15	a	3	a	56	a	2	a	47	a	49	a
G×E																						
AGR/ME	2	abc	1	b	20	b	5	c	3	a	24	a	2	cd	54	ab	2	b	71	a	72	a
AGR/C7	2	bc	3	ab	33	a	10	abc	4	a	20	a	2	cd	72	ab	2	ab	65	a	66	a
PEC/ME	1	c	3	ab	7	c	8	bc	4	a	10	b	1	d	33	b	1	b	33	c	34	a
PEC/C7	2	bc	2	ab	17	bc	16	ab	5	a	14	b	2	cd	55	ab	2	ab	37	bc	39	a
ALT/ME	3	ab	2	ab	14	bc	19	a	1	a	12	b	3	bcd	51	ab	2	b	44	bc	46	a
ALT/C7	3	abc	1	b	10	bc	12	abc	1	a	11	b	3	ab	37	b	1	b	46	b	48	a
GUA/ME	4	a	4	a	20	b	19	ab	4	a	10	b	4	ab	60	ab	2	ab	45	b	47	a
GUA/C7	3	ab	5	a	17	bc	17	ab	2	a	14	b	5	a	60	a	3	a	40	bc	42	a
ANOVA																						
$P_{(E)}$	**0.0034**		**0.0252**		**0.0011**		**0.0324**		**0.2021**		**<0.0001**		**<0.0001**		**0.3409**		**0.1322**		**<0.0001**		**0.1347**	
$P_{(G)}$	**0.6357**		**0.3747**		**0.1559**		**0.7349**		**0.8649**		**0.6000**		**0.2706**		**0.1175**		**0.1658**		**0.7619**		**0.4877**	
$P_{(GxE)}$	**0.8251**		**0.3786**		**0.0992**		**0.1863**		**0.6410**		**0.2200**		**0.8775**		**0.3756**		**0.4569**		**0.4913**		**0.4204**	

Values are means (mg L^{-1} wine) of three replicates ($n = 3$) and different letters within a column indicate statistically significant differences (Fisher's LSD test, $P < 0.05$). $P_{(E)}$, $P_{(G)}$ and $P_{(GxE)}$: effects of environment, genotype and their interaction, respectively.

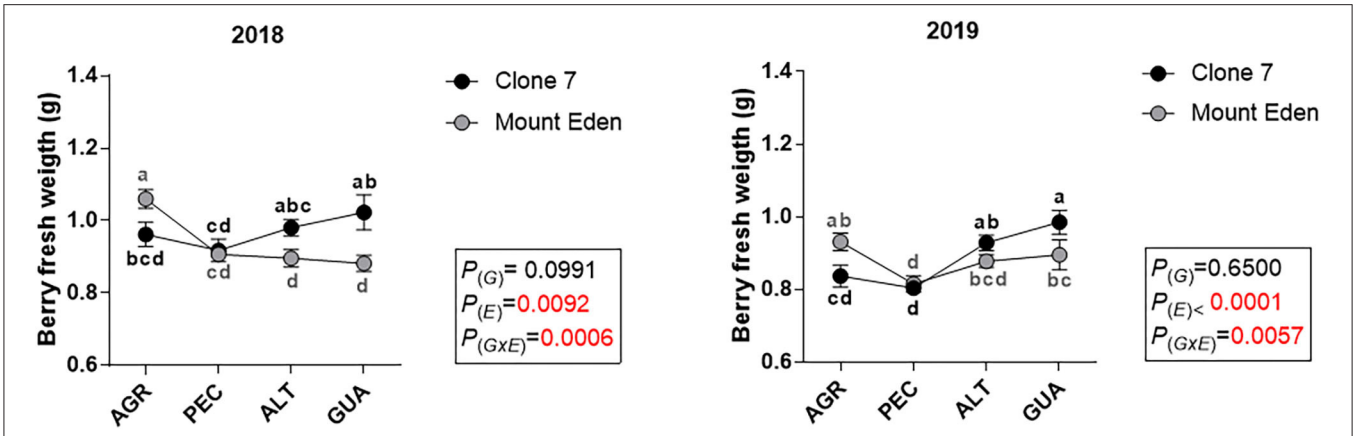

FIGURE 5 | PCA of non-anthocyanin compounds in Cabernet Sauvignon Clone 7 (C7) and Mount Eden (ME) wines, from vineyards in AGR, PEC, ALT and GUA.

as acclimation shift in berry skin anthocyanin composition (Berli et al., 2011; Alonso et al., 2016), possibly since the most oxidized anthocyanins are less antioxidant (Wang et al., 1997).

Considering the different UV-B irradiance at different altitudes for several of the studied GIs, particularly those of the Uco Valley, this is an additional factor related to the differential composition of PCs that is observed in the present study. In China, a similar behavior was found with respect to PCs for Cabernet Sauvignon wines made with grapes grown at high altitudes and that had large differences between day and night temperatures (Li et al., 2011). In Argentina, Urvieta et al. (2021) also highlighted the importance of these "terroir" related conditions (elevation and temperatures of the places where vineyards are located) on the PCs composition of Malbec variety. Particularly, anthocyanins and quercetin showed high levels in GUA, in correspondence with the present work.

In relation to the anthocyanins of wines, higher differences between AGR and GUA were also found, while ALT and PEC showed similarities, particularly for petunidin, malvidin and methylated derivatives.

Particularly for the di-hydroxylated anthocyanins, they increased in the GI of high altitude and colder temperatures, in agreement with previous works for other varieties (Yamane et al., 2006; Berli et al., 2011; Gil et al., 2013; Alonso et al., 2016). Two effects could be associated to these results observed for the Uco Valley GIs. Previous research showed that anthocyanin accumulation in grapes depends on both, low temperatures and light (Azuma et al., 2012). The high altitude climatic characteristics could be responsible of the stimulation of the increased carbon flow to the F3′H branch pathway (Li et al., 2011). Therefore, cyanidin-derived (di-hydroxylated) anthocyanins might increase in the berries of GIs located at the high altitudes of Uco Valley, and related to the high levels of those compounds in GUA elaborated wines. We previously found that the high solar UV-B irradiances registered in GUA increased total anthocyanins and the relative abundance of di-hydroxylated anthocyanins (i.e., reducing the proportion of tri-hydroxylated relatives) in Malbec berry skins (Berli et al., 2011; Alonso et al., 2016). On the other side, AGR wines showed a lower concentration of anthocyanins, and associated with a higher ratio of tri/di-hydroxylated anthocyanins, that is, the proportion of tri-substituted anthocyanins (particularly malvidin derivatives) was more abundant than their di-substituted counterparts. These results are in agreement with previous reports in Sangiovese grapes where the effects of two different temperature regimes on the accumulation of mRNAs and enzymes controlling berry skin anthocyanins was evaluated (Movahed et al., 2016). They observed an anthocyanins biosynthesis reduction at both, transcriptional and enzymatic levels. Besides, the peroxidase activity was higher in berries ripened under high temperatures (36°C), a similar condition as AGR. In fact, the low anthocyanin levels were the result of a combined reduction of biosynthesis and an increase of their degradation by the direct action of peroxidases (Movahed et al., 2016). This may contribute to the contrasting results observed between AGR and GUA. The PEC and ALT GIs presented similar concentrations of individual anthocyanins which could be related to the geographical proximity and very similar climatic condition i.e., both GIs are located at similar altitudes (1073–1085 m a.s.l.).

ALT had the highest Tamp during the 2018 and 2019 seasons. The effects on berry PCs were low (data not shown), but after analyzing the wines, ALT increased the total anthocyanin content, particularly derivatives of malvidin, syringic acid and *trans*-resveratrol. These data are in agreement with the results observed for the other high-altitude GIs.

Among the non-anthocyanins, the ALT and PEC wines did not show significant differences, denoting the syringic and *p*-coumaric acids as the most representative compounds. AGR had higher amounts of caftaric and gallic acids, while GUA had higher amounts of quercetin, *trans*-resveratrol, OH-tyrosol and tannin derivatives. The concentration of these particular non-anthocyanins PCs depends on the elevation of vineyards, because at higher altitudes GIs, such as GUA, the levels of UV-B radiation increase and the average temperatures decrease. This stressful condition for plants stimulates the synthesis of high antioxidant compounds such as those found in GUA at higher

concentrations. These compounds were those responsible of the separation of this GI from others in **Figure 5**. The increased synthesis and accumulation of these high antioxidant PCs has been also previously correlated with the increase of altitude of vineyards in different regions of the world (Tao et al., 2009; Jin et al., 2017; Urvieta et al., 2018, 2021).

In the anthocyanin profile of wines, the relative amounts of di-hydroxylated and tri-hydroxylated anthocyanins increased in GUA in comparison to the other studied GIs, possibly due to GUA lower air temperatures and higher solar UV-B levels (Berli et al., 2010). In addition, *trans*-resveratrol, an antioxidant compound known to increase with UV-B exposure in plants located at high altitudes (Haselgrove et al., 2000; Berli et al., 2008), increased significantly in GUA wines when compared to other GIs. Another compound that increased in this GI was quercetin, in correspondence with previous reports for Malbec leaves (Berli et al., 2010), berries (Berli et al., 2008, 2011) and wines (Urvieta et al., 2018, 2021). Price et al. (1995), determined that the concentration of flavonols such as quercetin-3-glucoside can play a fundamental role in co-pigmentation, helping to stabilize the color of the wine. Previous research showed that solar irradiation, water deficits, and higher temperature differences between day and night could positively regulate gene expression related to metabolism of flavonoids and, therefore, significantly increase the flavonoid content (Gollop et al., 2002; Yamane et al., 2006).

The presented and discussed results indicate that the fruit yields (number of clusters per plant) and the phenolic composition of wines are influenced by environment, plant material and their interaction. The environment has a direct relation with the climatic conditions in the studied GIs, highlighting the higher concentration of some specific PCs or families at lower temperatures and higher altitude GIs. The results suggest the possibility of identifying Cabernet Sauvignon wines of different origins of Mendoza that are associated with different plant material. The data presented is of interest for the wine industry because is the first time that Cabernet Sauvignon wines from different GIs of Argentina are chemically characterized. As well, to our knowledge this is the first comparison of the Clone 7 and Mount Eden plant material. The results contribute to characterize the terroir of the regions and to take oenological decisions by the selection of vegetative material, expanding also the current knowledge of Cabernet Sauvignon wines, and its geographical origin.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FM: conceptualization, methodology, investigation, formal analysis, data curation, writing—original draft, and writing—review and editing. RU: conceptualization, methodology, investigation, and writing—review and editing. FB: investigation and writing—review and editing. MR: investigation, methodology, investigation, formal analysis, and writing—review and editing. AF and FB: conceptualization, methodology, formal analysis, investigation, resources, data curation, visualization, supervision, project administration, funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Agency for Scientific and Technological Promotion (ANPCyT), Argentina (Grant Number: Banco Interamericano de Desarrollo (BID)-Proyectos de Investigación Científica y Tecnológica (PICT) N° 2016-0486), Secretaría de Investigación, Internacionales y Posgrado from Universidad Nacional de Cuyo (SIIP-UNCuyo, Grant Number: A688) and Catena Zapata Winery. FM is a scholar of CONICET. RU and FB are researchers at CIW. AF and FB are fellows of CONICET.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.700642/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
published: 28 July 2021
doi: 10.3389/fsufs.2021.657412

Differentiation Between Argentine and Austrian Red and White Wines Based on Isotopic and Multi-Elemental Composition

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In this work, the characterization of white and red wines from Austria and Argentina was carried out based on the isotopic and multi-elemental profile data. They were determined using vanguard techniques such as isotope ratio mass spectrometry and inductively coupled plasma mass spectrometry. In particular, Al, As, B, Ca, Co, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Sr, V, Zn, $\delta^{18}O$, and $\delta^{13}C$ were determined. The results show that the samples of wines from Argentina generally present higher concentrations of the elements analyzed compared to Austrian wines. $\delta^{18}O$ values from wine water were characteristic of each country, while $\delta^{13}C$ values from ethanol did not present any geographical distinction. Linear discriminant analysis using isotopes and elements allowed us to classify 100% of the wines according to the origin and additionally, 98.4% when separately investigating red and white wines. The elements Sr, Li, V, Pb, B, Mn, Co, Rb, As, Na, Mg, Zn, and $\delta^{18}O$ were identified as sensitive indicators capable of differentiate wines according to their production origin. Furthermore, Sr, Li, Na, $\delta^{13}C$, $\delta^{18}O$, Ca, B, Fe, Mn, V, Mg, Co, and Zn contributed to the differentiation of wines according to origin and color. To our knowledge, it is the first work that involves the measurement of a wide range of elements and stable isotopes in white and red wines in Argentina, as well as in Austria. This research highlights the power of the application of stable isotopes and multi-element data in multivariate statistical analysis, in order to obtain an accurate differentiation of wines origin.

Keywords: chemical fingerprint, multi-elemental analysis, stable isotopes, discriminant analysis, geographical origin

OPEN ACCESS

Edited by:
Autar Krishen Mattoo, Agricultural Research Service (USDA), United States

Reviewed by:
Mark Paul Running, University of Louisville, United States
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Specialty section:
This article was submitted to Crop Biology and Sustainability, a section of the journal Frontiers in Sustainable Food Systems

Received: *22 January 2021*
Accepted: *24 June 2021*
Published: *28 July 2021*

Citation:
Griboff J, Horacek M, Wunderlin DA and Monferrán MV (2021) Differentiation Between Argentine and Austrian Red and White Wines Based on Isotopic and Multi-Elemental Composition. Front. Sustain. Food Syst. 5:657412. doi: 10.3389/fsufs.2021.657412

INTRODUCTION

Wine is a unique and widely-consumed alcoholic beverage worldwide since early civilization (Vystavna et al., 2014). Wine production is a major economic agricultural activity and its commercial value is derived significantly by the geographic area, year of grape production, cultivation and quality (Raco et al., 2015). It is viewed as a luxury product, which makes it highly susceptible to fraud and adulteration (Ranaweera et al., 2021). Wine counterfeiting or adulteration refers to the illegal techniques that have the goal to substitute one valuable component with a cheaper one to increase profit, thus deceiving the consumer (Geana et al., 2016). Manipulations can be ascribed to both its intrinsic (e.g., dilution of wines with water, addition

of alcohol, sugar, coloring, or flavoring substances) and its extrinsic properties (e.g., fraudulent misrepresentation of the cultivar, producer, and geographic origin) (Villano et al., 2017).

Special emphasis is placed on certifying the origin of wine since the geographic origin (terroir) has a fundamental impact on the wine profile and quality (Bejjani et al., 2014). In recent times, stable isotope analysis has been applied in numerous studies to determine the geographical origin of wines (Magdas et al., 2012; Camin et al., 2015; Durante et al., 2016, Horacek et al., 2019a,b; Horacek et al., 2021). Isotopic fractionation is related to the type of plant and area of growth, which are in correlation with climatic factors (humidity, temperature, amount of precipitation, etc.), as well as geographical factors (distance from the sea or other evaporation source, elevation, altitude, latitude) (Dordevic, 2015). The stable isotopes of oxygen ($\delta^{18}O$) values of water of the grape are modified during ripening and harvest with the processes of evaporation and condensation of the water cycle (Dutra et al., 2013). The specific climatic conditions of a location, which already influence the isotopic composition of the precipitation, additionally modify the transpiration of leaves and fruit, resulting in the $\delta^{18}O$ of water of wines reflect the location where the product comes from Roßmann et al. (1999) and Raco et al. (2015). The stable isotopes of carbon ($\delta^{13}C$) value of wine ethanol are closely related to the origin of fermented sugar and can also be affected by climatic factors, especially drought stress (Roßmann et al., 1996, Horacek et al., 2015).

Furthermore, the fingerprinting of the content of metals in wines is a valuable method to authenticate their geographical origin (da Costa et al., 2020). The mineral composition of wine is influenced by endogenous sources, such as elements naturally present in the soil where grapes are grown, or climatic conditions; and exogenous sources, associated with external impurities that reach wine, such as the use of pesticides/fertilizer, machinery in the winery, or use of fining agents (Preti, 2019, Viviers et al., 2013). In particular, white and red wines are produced by different winemaking procedures and consequently, a differential metal uptake may result (Haseeb et al., 2019; Dumitriu et al., 2021). Moreover, knowledge of the elemental composition of wine is also very important due to their toxic effect on the human body in case of excessive intake and the impact on the quality (Płotka-Wasylka et al., 2018). Metals play an important role in the stability, color and clarity of wines that affect their organoleptic characteristics (Bimpilas et al., 2015).

In order to enhance the ability to assure wine authenticity, the outputs derived from analytical techniques have been combined with multivariate statistical analysis to differentiate samples according to the country/region they come from, variety, or other properties (Fan et al., 2018; Yamashita et al., 2019). The main goal of this work was to study whether the wines from Argentina (South America) and Austria (Europe) could be characterized and differentiated according to the isotopic and multi-elemental profile. Therefore, the analysis of 18 elements, $\delta^{18}O$ and $\delta^{13}C$ was carried out in 62 wine samples, 37 from Argentina, and 25 from Austria. Subsequently, chemometric analysis (linear discriminant analysis) was used to find the most discriminative variables to distinguish among countries and color of wine.

FIGURE 1 | World map in which the countries under study are highlighted; Argentina (South America) and Austria (Europe).

MATERIALS AND METHODS

Wine Samples

A set of 62 wines purchased from the major wine-producing regions of Argentina (32 red and 5 white samples) and Austria (6 red and 19 white samples) were subject of this study (**Figure 1**). However, it is noteworthy that in statistical terms, some categories are smaller than the other and for more accurate results it will be necessary to analyses a large number of samples. The wine samples were collected from the vintage years of 2008, 2009, 2012, 2013, 2014, and 2015. Different wine varieties were collected, which included the main grape varieties in the different countries. The grape varieties under study were Cabernet Sauvignon, Malbec, Merlot, Pinot Noir, and Syrah for red wines and Chardonnay, Torrontés, and Viognier for white wines from Argentina and Chardonnay, gruener Veltliner, Rhine Riesling, Welschriesling, Pinot blanc (Weissburgunder), Müller Thurgau, and Sauvignon blanc for white and Zweigelt, Blaufränkisch, and blue Wildbacher (Blauer Wildbacher) for red wines from Austria. Wines were sampled from freshly opened bottles and transferred to polypropylene flasks for isotopic and multi-element analysis. The plastic containers used to place the wine samples for element analysis were previously cleaned by washing with 10 % HNO_3 (Merck, ultrapure grade) for at least 24 h, and then rinsed copiously with ultra-pure water (Vrček et al., 2011).

Multi-Element Analysis

Al, As, B, Ca, Co, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Sr, V, and Zn were measured using an Agilent 7500cx Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Agilent Technologies, CA-USA), equipped with an ASX-100 autosampler (CETAC Technologies, Omaha, NE-USA). Wine samples were digested using Teflon tubes. First, ∼2.5 mL of wine sample was digested using 3 mL of HNO_3 (sub-boiling) in closed Teflon tubes on heating plates set to 220°C, during 6 h. Digested samples were quantitatively transferred to 10 mL volumetric flasks, completing

the volume with HNO_3 2%, followed by filtration using 0.45 μm filters. This process was done in triplicate. All samples were stored at 4°C until analysis. Moreover, three samples were spiked to verify recovery percentages of elements. Variable amounts of mixed standard solutions, containing all elements analyzed were added to the wine sample prior to sample digestion, to double the starting concentration for each element. The rest of the procedure was the same as used for non-spiked samples. The average recovery was 98 ± 7%. In addition, the accuracy of measurements of the ICP-MS was checked through the analysis of a standard reference material (NIST 1643e, Freshwater). The recoveries of the reference and spiked samples for tested elements are shown in **Supplementary Table 1**.

Isotopic Analysis

The determination of $^{18}O/^{16}O$ isotopic ratio from the water extracted from wine and of $^{13}C/^{12}C$ isotopic ratio in the ethanol extracted from wine was performed. Prior to analysis, the wine samples were distilled using an automated distillation control system (Eurofins/Nantes, France) in order to extract the ethanol and water. The samples were measured at the BLT Wieselburg stable isotope facility. Carbon isotopes were measured by injecting the alcohol in a Flash HT elemental analyzer (ThermoFisher, Bremen, Germany). The produced CO_2 gas is flushed by continuous helium flow into a Delta V IRMS (ThermoFisher, Bremen, Germany) and analyzed for their stable isotope ratio. Oxygen isotopes were measured by equilibration method in a gas bench (ThermoFisher, Bremen, Germany) and the equilibrated gas (mixture 0.36% CO_2 in Helium 5.0) is flushed by continuous gas flow into a Delta V IRMS (ThermoFisher, Bremen, Germany) for stable isotope ratio determination.

The results are expressed in the conventional δ-notation in ‰ with respect to the V-SMOW (Vienna-Standard Mean Ocean Water) and to the V-PDB (Vienna-PeeDee Belemnite) standards for oxygen and carbon, respectively. The enlarged reproducibility of measurements of $\delta^{13}C$ and $\delta^{18}O$ were better than ± 0.1 and ± 0.2‰, respectively. For quality control certified standards and reference materials were analyzed together with the wine samples. Among the measured standards are V-SMOW, SLAP (both provided by the International Atomic Energy Agency (IAEA) Vienna), BCR 660, BCR 656 [both produced by the Institute of Reference Materials and Measurements (IRMM) from Geel, Belgium].

Statistical Analysis

The results were expressed as maximum, minimum, mean values and standard deviations and were processed according to four wine categories. The groups were defined as follows: Arg- white and Arg-red (white and red wines from Argentina, respectively) and Aut-white and Aut-red (white and red wines from Austria, respectively). Shapiro–Wilks and Levene tests were used to assess normality and homogeneity of variances, respectively. The data obtained satisfy these assumptions and were processed statistically by analysis of variance (ANOVA), to examine the difference between groups of wines, using DGC at $p < 0.05$ to compare means (Balzarini et al., 2015). Linear discriminant analysis (LDA, stepwise mode) was executed in order to differentiate countries and wine types by multivariate models. All the statistical analyses were performed using InfoStat (V1.1) and Statistica 7.0 Software.

RESULTS AND DISCUSSION

Elemental Profiling of Four Wine Groups

Table 1 summarizes the results obtained for the set of Argentine and Austrian samples, according to red and white wines. Regarding the mean concentrations, the elements were divided into "macro elements" (concentration ≥1 mg L^{-1}) and "trace elements" (concentration <1 mg L^{-1}). Among the results, the variability of element concentration in each group of samples is considerable, showing a wide range of values for each element even in wines from the same geographical area and type. It should be noted that the results presented correspond to average values of samples of wines from different varietals, producers and years. Elemental wine composition is influenced by many factors, including the mineral composition of soil, viticultural practices, environmental factors, fermentation process, and the procedure of storage condition (Gutiérrez et al., 2017).

Despite this, the wines from Argentina showed higher concentrations for most of the elements, except for of Al, Ni, Rb, and Zn, which showed no significant differences between the countries under study and the wine color. Considering that some elements exhibit a good correspondence between its content in both soil and wine, the results obtained would indicate that the geochemical or soil composition is very different between countries, and presumably, the Argentine soil has higher levels of metals than the Austrian soil. The values adopted by these elements are within the range reported in the literature for wines from different countries (Kment et al., 2005; Galgano et al., 2008; Jurado et al., 2012; Espinoza Cruz et al., 2020). A few elements were statistically differentiated in the four groups; Sr and Li (the latter not detected in Aut-red). Strontium is considered a very interesting parameter in the study on the geographical origin of wines because it is an element less prone to being altered by anthropogenic influences (Gremaud et al., 2004).

Considering the Argentine samples, Ca, B, K, Mg, As, Co, and V did not show statistically significant differences between white and red wines. However, Cu, Mn, and Sr were found in higher concentration in red wines and Fe, Na, Li, and Pb in white wines. The results received for Argentinean wines were comparable with those reported in the literature available about this country. It should be noted that to our knowledge, it is the first work that involves the measurement of a wide range of elements in white and red wines in Argentina, as well as in Austria. Azcarate et al. (2015) analyzed the elemental composition of 57 Argentine white wines, those results were similar on As, Cu, Li, Rb, and Sr concentrations found in Arg-white. Instead, in the present study Co, Mn, Ni, and V were found in lower concentration and Pb in higher concentration. Pb may come from fungicidal treatments, sealed or corroded containers, or from pollution (Potortí et al., 2017). On the other hand, Di Paola-Naranjo et al. (2011) analyzed several elements in typical Argentinean red

TABLE 1 | Means and standard deviations of measured elements and stable isotopes corresponding to Arg-white, Arg-red, Aut-white, and Aut-red samples.

	Arg-white		Arg-red		Aut-white		Aut-red	
	Minimum–maximum	Mean ± SD	Minimum–maximum	Mean ± SD	Minimum–maximum	Mean ± SD	Minimum–maximum	Mean ± SD
Macro elements (mg L^{-1})								
B	4–10	7 ± 2^{A}	3–18	8 ± 4^{A}	1–12	3 ± 2^{B}	3–8	5 ± 2^{B}
Ca	53–114	79 ± 23^{A}	43–96	69 ± 13^{A}	40–86	54 ± 11^{B}	45–62	54 ± 7^{B}
Fe	1–6	4 ± 2^{A}	1–41	2 ± 1^{B}	0.2–1.7	1.0 ± 0.4^{C}	0.3–1.6	1.1 ± 0.5^{C}
K	687–1,584	$1{,}243 \pm 337^{A}$	790–1,606	$1{,}182 \pm 191^{A}$	384–1,780	644 ± 354^{C}	658–1,349	925 ± 236^{B}
Mg	66–131	91 ± 28^{A}	77–118	100 ± 9^{A}	58–105	78 ± 10^{B}	56–103	81 ± 16^{B}
Na	52–200	120 ± 68^{A}	17–200	51 ± 40^{B}	5–16	8 ± 3^{C}	4–15	7 ± 4^{C}
Trace elements (μg L^{-1})								
Al	275–1,388	947 ± 444^{A}	n.d.–12,260	$729 \pm 2{,}135^{A}$	n.d.–169	50 ± 57^{A}	n.d.–156	99 ± 57^{A}
As	n.d.–19	11 ± 9^{A}	n.d.–32	11 ± 7^{A}	<LOD		<LOD	
Co	0.7–3.4	2.5 ± 1.1^{A}	0.3–9.8	2.1 ± 1.6^{A}	0.3–1.8	1.0 ± 0.4^{B}	0.7–1.7	1.1 ± 0.4^{B}
Cu	34–137	95 ± 42^{B}	18–869	191 ± 208^{A}	5–198	57 ± 50^{B}	52–82	67 ± 11^{B}
Li	115–267	187 ± 67^{A}	n.q.–250	100 ± 54^{B}	n.q.–13	6 ± 5^{C}	< LOD	
Mn	584–981	830 ± 149^{B}	599–1,291	996 ± 174^{A}	267–953	485 ± 187^{C}	467–938	729 ± 198^{B}
Ni	3–22	12 ± 7^{A}	1–262	17 ± 46^{A}	3–16	8 ± 4^{A}	7–22	13 ± 6^{A}
Pb	4–33	20 ± 11^{A}	n.q.–30	11 ± 7^{B}	2–6	4 ± 1^{C}	2–4	3 ± 1^{C}
Rb	423–1,316	810 ± 381^{A}	585–1,669	970 ± 286^{A}	260–1,564	706 ± 395^{A}	154–1,878	894 ± 605^{A}
Sr	742–1,178	911 ± 162^{B}	684–1,613	$1{,}215 \pm 245^{A}$	93–195	142 ± 31^{D}	233–446	364 ± 86^{C}
V	1–56	29 ± 21^{A}	1–131	40 ± 34^{A}	0.3–5.3	1.3 ± 1.1^{B}	0.3–2.9	0.8 ± 1.0^{B}
Zn	161–655	367 ± 221^{A}	1–2,161	495 ± 355^{A}	75–642	318 ± 140^{A}	242–441	352 ± 73^{A}
Stable isotopes								
$\delta^{13}C$	−27.30 to −25.71	-26.21 ± 0.70^{A}	−29.7 to −26.5	-27.56 ± 0.63^{A}	−29.08 to −25.61	-27.64 ± 1.02^{A}	−28.72 to −25.45	-26.91 ± 1.23^{A}
$\delta^{18}O$	2.20–2.80	2.50 ± 0.42^{A}	0.08–6.47	2.60 ± 1.68^{A}	0.03–3.13	1.12 ± 0.85^{B}	0.48–1.82	1.43 ± 0.64^{B}

n.d., not determined; <LOD, below limit of detection. LODs: Al 1.02 μg L^{-1}; As 0.09 μg L^{-1}. n.q., not quantified; <LOQ, below limit of quantification. LOQs: Li 0.06 μg L^{-1}; Pb 0.05 μg L^{-1}. Different letters in the same row indicate significant differences among the four categories $P < 0.05$.

wines and the concentrations were compatible with those found in this study.

Regarding Austrian samples, for the majority of elements, there are no significant differences between the red and white wines, except for K, Mn, and Sr, which are present in higher concentrations in red wines and Li in white wines. A study conducted by Gruber et al. (2006) determined the concentration of Ca, Fe, K, Mn, Rb, and Sr in Austrian red and white wine. The informed values are similar to those obtained in this study, generally with the minimum concentration reported. It is noteworthy that a harmful element such as As was not detected in any wine sample from Austria, while in the Argentine ones it was. Arsenic is one of the most toxic pollutants abundantly present in the earth's crust (Litter et al., 2019). Argentina is one of the most affected countries by the natural presence of high concentrations of this element in soil and groundwater (Bardach et al., 2015).

Knowledge of the mineral content in wine is of considerable interest because of their repercussions in organoleptic, hygienic, and nutritional characteristics as well as their toxicological implications (Gutiérrez et al., 2017). In moderate quantities, the consumption of wine contributes to obtain elements that are essential to the human organism (such as Ca, Co, Cu, K, Fe, Mg, Mn, Ni, and Zn), while others must be controlled due to their potential toxicity (such as As, Cd, Cr, Hg, and Pb) (Rocha et al., 2019). In accordance with the maximum acceptable limits of B, As, Cu, Pb, and Zn, defined by the International Organization of Vine and Wine—OIV (International Code of Oenological Practices., 2020), none of the element concentrations analyzed in this work exceeds the risk levels reported by this intergovernmental organization, of which Argentina and Austria are member countries.

Values of $\delta^{18}O$ of Wine Water and $\delta^{13}C$ of Ethanol

The studied samples were characterized by $\delta^{18}O$ of water in the range of 2.20–2.80, 0.08–6.47, 0.03–3.13, and 0.48–1.82‰ for Arg-white, Arg-red, Aut-white, and Aut-red, respectively (**Table 1** and **Figure 2A**). Among the studied countries, significant differences were observed in the results of $\delta^{18}O$ of water, with lower oxygen isotope values for the Austrian wines. Wines from each country did not differ between white and red. The value of this parameter has a strong relationship with location and the origin of the water the vines accessed, and climatic conditions (Fan et al., 2018). Differences in the isotopic ratio of precipitation are present in hot and dry areas compared

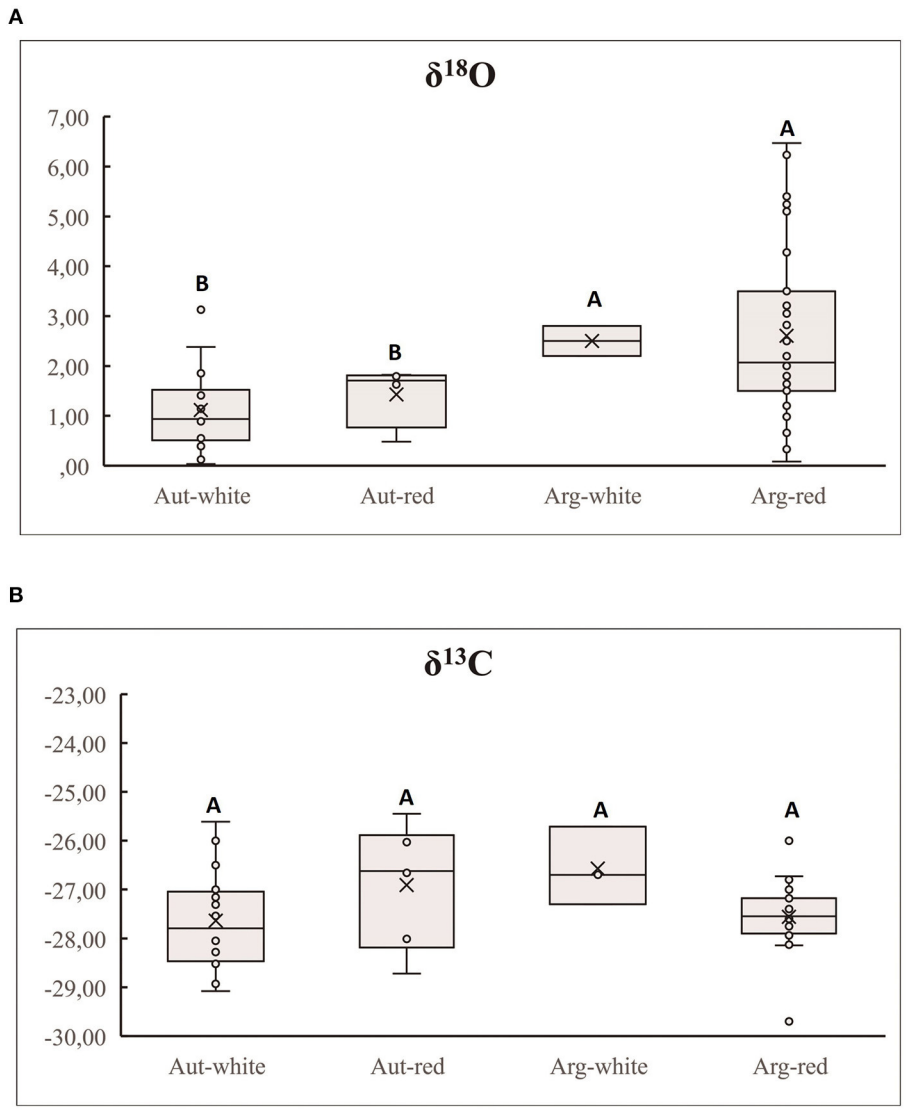

FIGURE 2 | Comparison of the $\delta^{18}O$ values of wine water **(A)** and the $\delta^{13}C$ values of wine ethanol **(B)** among Arg-white, Arg-red, Aut-white, and Aut-red wines. Different letters indicate significant differences among the four categories $P < 0.05$.

to wet and cold areas (Dutra et al., 2013). Usually, more positive values of $\delta^{18}O$ indicate lower altitude, higher temperature and/or lower rainfall and/or coastal environment (Magdas et al., 2012; Wu et al., 2019; Horacek et al., 2021; Leder et al., 2021). Based on this, and the specific characteristics of each country, the more enriched values of the Argentine wines can be dominantly explained by the warmer climate they are produced in with respect to the Austrian wines. However, this difference in $\delta^{18}O$ between Austrian and Argentine is reduced (lower than to be assumed), as in Argentine most wine regions widely irrigate their vineyards using water coming from high-altitude mountainous areas. The origin of this irrigation water, possessing a low $\delta^{18}O$ value, which in turn is transferred into the vines, thus resulting in depleted $\delta^{18}O$-values of wines from these regions (Horacek et al., 2021).

TABLE 2 | Result of classification using linear discriminant analysis in the four group of samples.

	Percent correct	Aut-white	Aut-red	Arg-white	Arg-red
Aut-white	100.00	19	0	0	0
Aut-red	83.33	1	5	0	0
Arg-white	100.00	0	0	5	0
Arg-red	100.00	0	0	0	32
Total	98.39	20	5	5	32

The range of values of $\delta^{18}O$ found in the Austrian samples was similar to the range found in Austrian wines previously (Philipp et al., 2018) and in wines from Germany (Monakhova et al., 2014), but showing less variation with respect to those

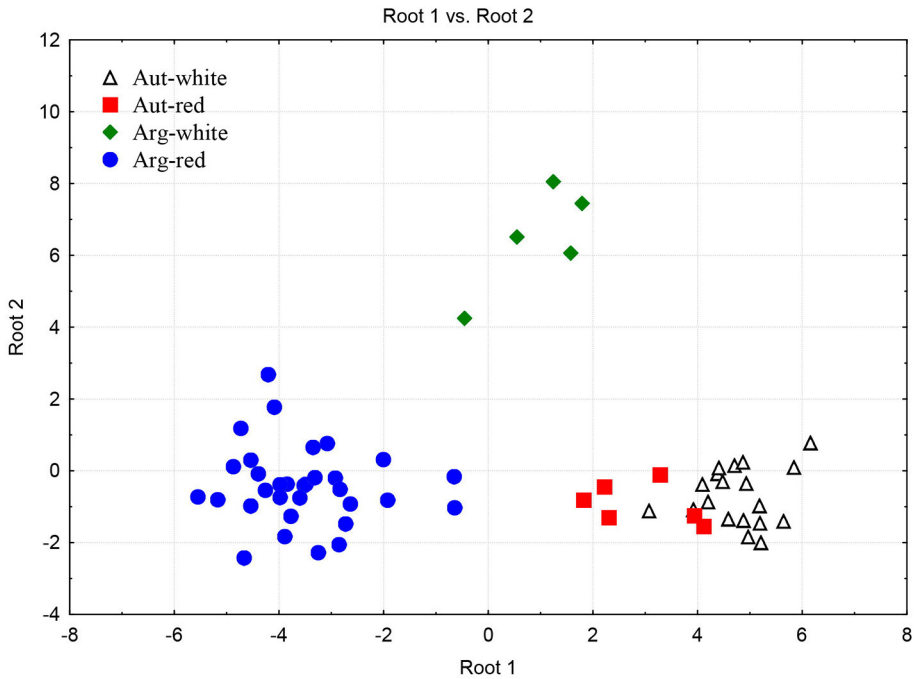

FIGURE 3 | Discriminant analysis (canonical roots) of wine samples according to the country and color.

found in studies conducted in other neighboring countries like Switzerland (Gremaud et al., 2004) and Italy (Raco et al., 2015). Conversely, the range of isotopic values of Argentine wines is wider than those found in the neighboring country Brazil (Adami et al., 2010; Dutra et al., 2013).

The $\delta^{13}C$ ethanol values of the studied wines range from −27.3 to −25.7‰, −29.7 to −26.0‰, −29.1 to −25.6‰ and −28.7 to −25.5‰ for Arg-white, Arg-red, Aut-white, and Aut-red, respectively (**Figure 2B**). No significant differences were observed between the analyzed samples from Argentina and Austria in terms of $\delta^{13}C$. Considering that grape vines are C3 plants and assume typical values between −29 and −24‰, all the samples show expected $\delta^{13}C$ ethanol values (Wu et al., 2019). This would indicate that the oenological practices carried out are similar for wine production in both countries, according to the non-addition of C4 sugar (cane or maize sugars) (Geana et al., 2016) and the absence of clear drought stress, as the latter results in an increase of the $\delta^{13}C$-value (Ballantyne et al., 2011; Horacek et al., 2015).

The values reported in the present study are similar to the range found in Austrian wines (Philipp et al., 2018) and in different European countries; for example, Italy where the range was −28.9 to −25.7% (Bonello et al., 2018), France where it was from −29.5 to −24.1% (Wu et al., 2021), and Romania where it was from −29.2 to −25.2% (Geana et al., 2016).

Verification of Wine Origin

Linear discriminant analysis (LDA) is one of the most widely used statistical methods for classification purposes in the field of food analysis (Magdas et al., 2021). The wine samples from Argentina and Austria were subjected to Forward stepwise LDA, providing a mathematical model to classify and identify the particular fingerprints, regarding to the isotopic and multi-elemental profile, according to their origins. The analysis of the data set allows distinguishing between the countries with 100% certainty on the basis of 13 variables out of 20: Sr, Li, $\delta^{18}O$, V, Pb, B, Mn, Co, Rb, As, Na, Mg, and Zn. In similar studies, some of these selected variables have been reported for statistical analysis in LDA models as contributing to the differentiation of milk in Argentina (Griboff et al., 2019) and carrots in Austria (Jandric et al., 2020), demonstrating the usefulness of multivariate statistical approaches, that apply chemical data, for the characterization, and differentiation of food from different countries.

Another LDA was executed to discriminate the four wine groups (Arg-white, Arg-red, Aut-white, and Aut-red) using elements and stable isotopes. **Table 2** present the classification results of the Forward stepwise LDA performed. Classification of the wine samples by the LDA model showed 98.4% accuracy (**Figure 3**). In other words, 61 of the 62 samples were correctly predicted and only one sample belonging to the Aut-red group, could not be classified correctly. As can be seen from **Figure 3**, white and red wines originating in Argentina clearly differ according to their multi-element and isotopic profile. However, for white and red wines from Austria a complete differentiation is not achieved. Thirteen elements and both stable isotopes contributed to the differentiation of the four groups: Sr, Li, Na, $\delta^{13}C$, $\delta^{18}O$, Ca, B, Fe, Mn, V, Mg, Co, and

Zn. Similar variables that made the strongest contribution to the differentiation of wine were reported by Di Paola-Naranjo et al. (2011) in Argentina, Horacek et al. (2019b) in Central Europe, Wu et al. (2021) in France and Shimizu et al. (2018) in Japan.

CONCLUSIONS

To our knowledge, this is the first report for a wide variety of elements and stable isotopes, from both Argentinean and Austrian origins, in white and red wines. Furthermore, the execution of the multivariate statistical analysis using elemental concentrations and stable isotopes proved to be a powerful strategy to differentiate the origin of wine on a global scale. On the one hand, the metal content was remarkably different between Austrian and Argentine wines, where the latter has generally higher concentrations. On the other hand, $\delta^{18}O$ values from wine water were shown to be efficient in differentiating wines according to the country of origin. However, the $\delta^{13}C$ values from ethanol did not allow the differentiation of wines according to their origin or color. Moreover, LDA models using isotopes and minerals have shown that good wine origin/color classification can be reached. In this work, the proposed LDAs showed a great potential ability for wine discrimination, with the accuracy rate of 100 and 98.4% classifying wines from Argentina and Austria, and the four groups, respectively. Therefore, we estimate that using the merged data from these techniques in combination with multivariate analysis is worthwhile and effective in wine traceability.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JG: methodology, investigation, and writing—original draft. MH: conceptualization, methodology, writing—review and editing, and funding acquisition. DW: conceptualization, writing—review and editing, and funding acquisition. MM: conceptualization, methodology, and writing—review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This project was partially funded by a bilateral grant of the OEAD (Österreichischer Austauschdienst) and the Ministerio de Ciencia Tecnología e Innovación Productiva (Dirección Nacional de Relaciones Internacionales) to MH and DW, respectively. Stable isotope analyses have been carried out by Elisabeth Riegler, which is thankfully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.657412/full#supplementary-material

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frontiers in Sustainable Food Systems

ORIGINAL RESEARCH
published: 10 December 2021
doi: 10.3389/fsufs.2021.781158

Storage Changes Stable Isotope Composition of Cucumbers

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Vegetable food stuff produced under controlled and identical conditions from one farm of identical "age" (batch) has a similar isotopic composition. This fact can be used to control the origin of vegetables. This question is of special relevance when food-contaminations have to be traced back to the producer, or certain production claims have to be controlled. However, as vegetables are harvested, brought to whole-sale merchants and to retail shops, where they remain until being bought by the consumer, one has to consider possible changes in isotopic composition during this transfer period, when comparing vegetables of questioned origin with reference samples taken directly from the field/producer. We investigated changes in the isotope composition of vegetables during storage by studying as an example cucumbers from one batch. We stored the cucumbers in a vegetable storage under controlled conditions and removed one sample every day and analyzed its isotopic composition. We found changes in the $\delta^{15}N$ and $\delta^{18}O$ isotope values over the investigated period of 21 days, with both parameters showing positive linear correlations, and maximum enrichments with time of more than 1.5‰ for $\delta^{15}N$ and more than 2‰ for $\delta^{18}O$. However, within the interval the samples remained in a saleable condition the isotope variations remained more or less within the variability of the sample batch. Our study demonstrates that changes in the isotopic signature in vegetables might occur after harvest during storage and have to be taken into account when (commercial) samples collected in a market are investigated.

Keywords: shelf life, oxygen isotopes, carbon isotopes, nitrogen isotopes, transpiration, maturation processes

OPEN ACCESS

Edited by:
Matteo Balderacchi, Independent Researcher, Piacenza, Italy

Reviewed by:
Weiguo Liu, Institute of Earth Environment, Chinese Academy of Sciences (CAS), China
Vlastimil Novak, Lawrence Berkeley National Laboratory, United States

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Specialty section:
This article was submitted to Crop Biology and Sustainability, a section of the journal Frontiers in Sustainable Food Systems

Received: *22 September 2021*
Accepted: *19 November 2021*
Published: *10 December 2021*

Citation:
Horacek M and Papesch W (2021) Storage Changes Stable Isotope Composition of Cucumbers. Front. Sustain. Food Syst. 5:781158. doi: 10.3389/fsufs.2021.781158

INTRODUCTION

The control of the declaration of origin of food becomes a topic of increasing importance. One reason is the willingness of consumers to pay more for products from a certain region or of a certain brand. As this might lead to incorrect labelling of goods to increase the profit, there exists the need to control declared origins. A special relevance has the control of geographic origin of food, when products with contaminations are found in the market. Then it is absolutely essential to be able to trace these products back to the respective producers. In 2011 there was an outbreak of a new strain of *Escherichia coli* in Germany and western Europe and the suspicion of having been caused by contaminated Spanish cucumbers (among other fresh vegetables as tomatoes and lettuce) which lateron proved incorrect and organic sprouts were regarded as source of contamination instead (https://en.wikipedia.org/wiki/2011_Germany_E._coli_O104:H4_outbreak, last accessed 24.6.2021). Other, requests and needs for (back-) tracing of authenticity of vegetables (and generally food) are control of the geographical and agricultural origin of food, fertilization strategies and type of production system (organic/conventional). Such incidents

require the means for quick back-tracing of food for the protection of consumer health and the control of accusations and claims.

Research about the geographic origin of food products has been carried out on a wide variety of food products, as wine (e.g. Christoph et al., 2004, 2015; Griboff et al., 2021; Horacek et al., 2021; Leder et al., 2021), meat (e.g., Boner and Förstel, 2004; Camin et al., 2007; Horacek and Min, 2010), coffee (e.g., Serra et al., 2005; Rodrigues et al., 2009), vegetables and crops (e.g., Horacek et al., 2010, 2015; Bontempo et al., 2011; Goitom Asfaha et al., 2011; Opatić et al., 2018; Kongsri et al., 2021) among many others.

Usually, stable isotope analysis is the preferred method of choice for the control of geographic origin of food (see references mentioned above). However, to be able to reliably track back the geographic origin, or to control the fertilization strategy, production system, or other declared qualities, one has to know if storage of a product after the harvest can significantly alter the isotope signature, or if the isotope pattern remains unchanged within the period the respective food commodity is in a saleable condition. A few studies on this topic have been carried out for meat by investigating the meat water of meat samples. Thiem et al. (2005) report an increase in $\delta^{18}O$ of meat samples stored in a refrigerator, while Horacek et al. (2009) report no significant changes in $\delta^{18}O$ and δD with time for meat samples stored in a slaughter house cool storage. However, to our knowledge, no such investigations have been carried out for plant food materials. Therefore, in the present study we investigate the changes in the isotope composition of cucumbers (*Cucumis sativus*) during storage. Botanically, cucumbers belong to the Cucurbitaceae family (gourd plants), conventionally/culinary they are regarded as vegetables (https://fruitorvegetable.science/cucumber, last accessed 24.6.2021). We test the hypothesis, that during storage under controlled conditions the cucumbers will not change their isotopic composition significantly.

MATERIALS AND METHODS

Fifty cucumbers from one batch from one greenhouse have been weighed and put into a vegetable storage under controlled ideal storage conditions at temperature of 10°C and humidity above 90%. Every day over period of 21 days two cucumbers were taken, weighed again, sealed in a plastic bag and freeze-stored at −16°C until processing and analysis. (Weight differences are given in **Supplementary Table 1**).

During sample processing cucumber water was extracted from the cucumbers using a kitchenware juice extractor. In the extractor the entire cucumber is minced and falls into a rotating cylindrical sieve, through which the juice passes due to the centrifugal force and is caught in a glass. The duration for a sample depends on its size but usually lasts <10 seconds. The cucumber water was then pipetted into a glass flask and quickly transferred to the oxygen isotope analysis, while the residue of the cucumber was collected in a sample container.

Oxygen isotope values of the cucumber water samples were measured using an isotope ratio mass spectrometer Finnigan Delta+XL, coupled to an automatic equilibration device equipped with pneumatic valves (Papesch and Horacek, 2009). For analysis an aliquot of 3 ml is put into the glass vessel and attached to the equilibration device at a bath temperature of 20°C. Each vessel is equipped with a magnetic stirrer to speed up initial degassing of the water sample and to attain isotope equilibrium within 4 h. The evaluation of the raw data of ratios of mass 46 to 44 to oxygen isotope values is accomplished by two laboratory standards (Adriatic sea water and Vienna tap water) which are measured alike with each batch of samples. These standards have been calibrated by means of the international standards V-SMOW and SLAP distributed by the IAEA.

The residue of the cucumber was dried at 40°C in a dry-oven and homogenized. Each sample is then weighed into a tin capsule (ca. 2 mg) for $\delta^{15}N$ and $\delta^{13}C$ analyses. The samples were introduced into an elemental analyser (Elementar) where the samples are combusted and the evolving gases transferred via a ConFlo IV (Thermo) into a Thermo/Finnigan DeltaplusXP mass spectrometer. The isotope ratio is expressed in the conventional δ-value:

$$\delta = (\text{Rsample} - \text{Rstandard})/\text{Rstandard},$$

with

$$\text{R} = \text{heavy isotope/light isotope}.$$

Long-term reproducibility of our instruments using in-house laboratory standards was better than 0.1‰ for $\delta^{18}O$ and 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$ (1σ) (Horacek et al., 2008). In this paper $\delta^{18}O_{water}$ refers to the oxygen isotope values of the extracted cucumber water samples, and $\delta^{15}N_{pulp}$ and $\delta^{13}C_{pulp}$ refers to the carbon and nitrogen isotope values of the cucumber pulp samples.

All isotope results are reported as per mil (‰) deviation vs. international standards. The $\delta^{13}C_{pulp}$, $\delta^{15}N_{pulp}$ and $\delta^{18}O_{water}$ values are calibrated vs. the VPDB standard, air standard and VSMOW standard, respectively.

Calculation of the population standard deviation was carried out using a web-tool (https://miniwebtool.com/population-standard-deviation-calculator/ last accessed 08.11.2021).

The data are statistically evaluated by the determination coefficient (R^2) and significance (p-value) of a simple linear regression using the Microsoft program Excel, which shows the strength of correlation of the respective parameters. R^2-values range between 0 and 1 with values close to 0 indicate a very weak or no correlation between the evaluated parameters and values close to 1 a very high correlation between the evaluated parameters. Significance above the 95%-level is indicated by a $p < 0.05$.

RESULTS

The cucumbers from the storage experiment yield the following results (**Supplementary Table 2**): The initial isotope composition was −6.8‰ for $\delta^{18}O_{water}$, −0.3‰ for $\delta^{15}N_{pulp}$ and −28.7‰ for $\delta^{13}C_{pulp}$ (**Figures 1A–C**). The $\delta^{13}C_{pulp}$ values show a slight increase in the averaged trend of about 0.7‰, however, the values

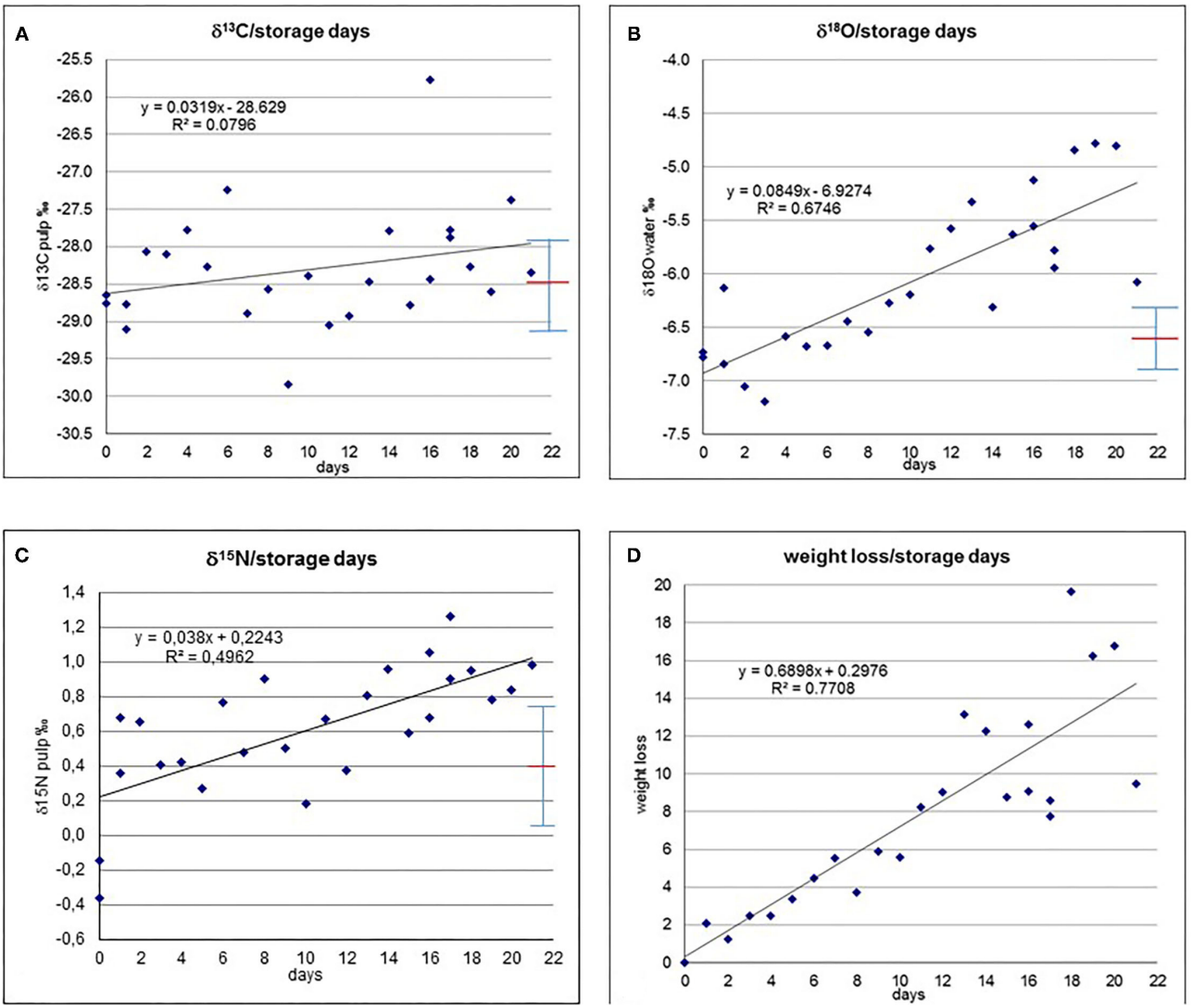

FIGURE 1 | (A) $\delta^{13}C_{pulp}$ vs. storage days. **(B)** $\delta^{15}N_{pulp}$ vs. storage days. **(C)** $\delta^{18}O_{water}$ vs. storage days. **(D)** Weight loss (weight %) vs. storage days. Red line denotes average, whiskers show population standard deviation.

are scattered and range from −29.9 to −25.8‰ (**Figure 1A**). The $\delta^{15}N_{pulp}$ values show an increase from the initial value within the observed period to a maximum value of +1.3‰ (**Figure 1B**). The values are scattered and partially deviate from the averaged trend. It is interesting to note that we observed in $\delta^{15}N_{pulp}$ a jump from day 0 to day 1 of ∼0.6 to 0.3‰. However, when we repeated this experiment by storing another batch of cucumber for 2 days, no such jump could be observed (**Supplementary Table 3**). For $\delta^{18}O_{water}$ the values increase within the 21 days of storage to a maximum value of −4.8‰ (**Figure 1C**). Also for this system, the increase is not steady but with some scatter, even showing some values within the first days that are depleted with respect to the initial value, with a minimum of −7.2‰. The weight loss with time is almost completely continuous and without much deviations from the averaged trend (**Figure 1D**; heavier cucumbers do not necessarily have a lower weight loss than lighter ones, see **Supplementary Table 1**). Only in the third week the scattering increases to some extent. A maximum weight loss of 19.7% was found. The weight loss correlates very well with the increase in $\delta^{18}O_{water}$, evidencing a direct link. The cucumbers were in saleable condition (based on the appearance of the cucumbers) until day 10 of the experiment (pers. comm. J. Hobiger). The Population Standard Deviation for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ for the first ten days are very close to the standard deviation with +/− 0.62, 0.34 and 0.3‰, respectively.

The determination coefficient is shown for the isotope parameters with respect to time and weight loss for the entire storage duration and its first 10 days (**Figures 1A–D**, **2A–C** and **Supplementary Tables 4A,B**). For the entire period the highest strength in correlation exists for $\delta^{18}O_{water}$ and weight loss, and weight loss with time (with an R^2 of ca. 0.8) and $\delta^{18}O_{water}$ and time (R^2 is ca. 0.7). Strength of correlation

TABLE 1 | (A) *p*-value for the investigated parameters $\delta^{13}C_{pulp}$, $\delta^{15}N_{pulp}$, $\delta^{18}O_{water}$ and cucumber weight loss correlated with time (days), and weight loss for the entire duration of the experiment (21 days); (B) for the first 10 days, during which the cucumbers remained in saleable condition.

(A)

21 days	***p*** **time**	***p*** **weight loss**
$\delta^{13}C$	0.162565561	0.369653499
$\delta^{15}N$	**5.8972E-05**	**0.001153983**
$\delta^{18}O$	**2.71726E-07**	**1.69767E-10**
weight loss	**3.82401E-09**	1

(B)

First 10 days	***p*** **time**	***p*** **weight loss**
$\delta^{13}C$	0.822027543	0.662438259
$\delta^{15}N$	0.159385608	0.09918314
$\delta^{18}O$	0.073343678	0.051963117
weight loss	**3.76636E-06**	1

Significant correlations are marked by bold values.

of $\delta^{15}N_{pulp}$ and time is ca. 0.5, $\delta^{15}N_{pulp}$ and weight loss ca. 0.4, and strength of correlation of $\delta^{13}C_{pulp}$ with time and weight loss is below 0.1. Strength in correlation of the investigated isotope parameters vs. time and weight loss are notably lower for the first 10 days of the experiment (**Supplementary Table 4B**), but weight loss vs. time is already quite high.

The determination of the *p*-value (significance) shows (**Table 1**) significant correlations of $\delta^{15}N$ and $\delta^{18}O$ with time (days) and weight loss over the entire experiment duration of 21 days, but no significance during the first 10 days, when the cucumbers remained in saleable condition. Weight loss shows a significant correlation with time for the 10 and 21 days intervals. $\delta^{13}C$ has neither a significance during the 21 nor the first 10 days interval.

DISCUSSION

Which effects can influence the isotopic composition of the investigated cucumbers during storage and thus time? We assume photosynthetic processes, degradation and ripening processes and water loss. Photosynthetic processes should influence the ^{13}C-signal, as the samples are cut-off from a water supply (Farquhar et al., 1982, 1989). Degradation and ripening processes should influence the ^{15}N-value with time (O'Deen, 1989; Unkovich, 2013), and water loss results in weight reduction and change in $\delta^{18}O$, due to evaporation/transpiration processes (Dansgaard, 1964; Roden et al., 2000; Yakir and Sternberg, 2000).

The R^2-values of $\delta^{13}C_{pulp}$ with respect to weight loss (**Figure 2A**) and time (**Figure 1A**) remain below 0.1, indicating that there is only a very minor correlation (if at all), or that any correlation is masked by the batch heterogeneity. Theoretically, in the harvested cucumbers photosynthetic processes might continue, which should be evidenced by an increase in $\delta^{13}C$ (Farquhar et al., 1982, 1989; Horacek et al., 2015) due to closure of the stomata because of drought stress, as the harvested cucumbers are cut off from any water supply. However, the absence of $\delta^{13}C_{pulp}$ correlating with storage time demonstrates that this process did not occur, and all variations can be regarded as dominantly due to the sample batch heterogeneity.

The $\delta^{18}O_{water}$-values show a clear trend towards higher values with time (**Figure 1C**, **Supplementary Table 4A**) and, even better correlated (evidenced by a higher R^2-value), with weight/water loss (**Figure 2C**), indicating a plausible explanation of passive enrichment in $^{18}O_{water}$ of cucumber water by evaporation/transpiration (Dansgaard, 1964; Roden et al., 2000; Yakir and Sternberg, 2000). During evaporation/transpiration the light water isotopes (^1H, ^{16}O) preferably go into the gaseous phase and evaporate (a process which is temperature dependent and gets stronger the lower the ambient temperature), thus passively enriching the remaining water with time. However, within the first ten storage days (**Supplementary Table 4B**), which was the period of saleable condition for the investigated cucumbers, evaporation/transpiration only accounts for ca. 30% of the variation in $\delta^{18}O_{water}$ (R^2 around 0.3). As within these 10 days transpiration resulted in a weight loss of <6%, this value is, on the one hand a limit for the saleable condition of the cucumbers, and a kind of threshold for transpiration having a minor, insignificant effect (**Table 1B**) on the isotope pattern with respect to the isotope variability of the batch. One might speculate that the change in $\delta^{18}O$ might be due to equilibration with the ambient water vapour. Two reasons speak against this possibility: (I) the cucumber water is confined within the cucumber and thus separated from the water vapour (and therefore this also does not happen during the time the cucumber is still attached to the plant), and (II) the almost perfect fit of $\delta^{18}O$ with weight loss (**Figure 2C**).

The $\delta^{15}N_{pulp}$-values demonstrate a trend towards increasing values with time (**Figure 1B**). As the correlation of $\delta^{15}N$ with time is better than with weight loss (**Figure 2B**), as the respective R^2-value is higher, the responsible degradation and ripening processes (O'Deen, 1989; Rodrigues et al., 2009) do not seem to be connected with transpiration. Similar to $\delta^{18}O_{water}$, within the period of saleable condition of the cucumber samples the degradation and ripening processes only account for a minor part of the variations in $^{15}N_{pulp}$ ($R^2 \sim 0.2$).

Generally, within a storage period under ideal storage conditions of 10 days, during which the cucumbers remained in saleable conditions, the isotopic changes within the cucumber with storage remained insignificant (**Table 1**). Water loss, however, shows a significant correlation with time from the beginning of the experiment. After passing a threshold of ca. 10 days under ideal conditions, storage has a notable influence on the isotope pattern of cucumbers. If the storage conditions are less favourable, which most likely will be the case in most supermarkets and food stores, this threshold will be significantly shorter.

Cucumbers that are stored in plastic foil will have a longer shelf life, as the saleable condition mainly depends on the weight

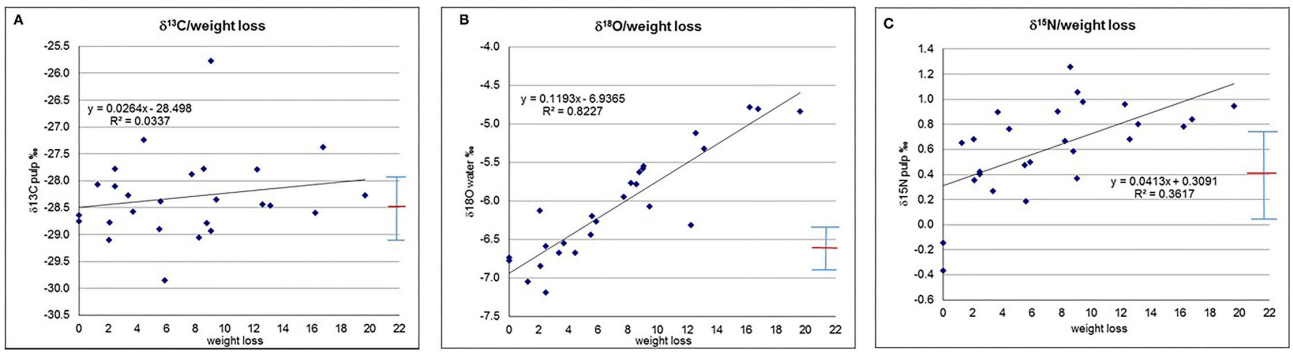

FIGURE 2 | (A) $\delta^{13}C_{pulp}$ vs. weight loss. **(B)** $\delta^{15}N_{pulp}$ vs. weight loss. **(C)** $\delta^{18}O_{water}$ vs. weight loss. Red line denotes average, whiskers show population standard deviation.

loss, which is reduced in foiled cucumbers. However, it will be necessary to investigate the behaviour of the N-isotopes, as the ripening and degradation processes should not be hampered by the foil.

CONCLUSIONS

During saleable condition (10 days, based on the appearance of the cucumber, approximately limited to a weight loss of <6% under ideal storage conditions in a cool-house) the variations in the isotope composition remain insignificant and approximately within the initial range. Samples exceeding this weight loss limit get notably enriched in $\delta^{18}O_{water}$ due to transpiration. $\delta^{15}N_{pulp}$ also tends towards higher values, but the scattering also is larger and erratic. The variation in $\delta^{13}C_{pulp}$ of the cucumber samples investigated in the present study are almost exclusively related to batch variability. Thus, our hypothesis of cucumbers remaining isotopically almost unchanged and thus can be used for control of declared provenance is correct for cucumbers in saleable condition, which means <6% weight loss, and a maximum of ten days from harvest under ideal conditions (cool-house). Under less favourable conditions shelf life will be shorter and thus the period of isotope patterns unchanged by storage. Thus, when investigating cucumbers or other vegetables for the control of (declared) geographic origin, the condition of the investigated samples have to be closely inspected and taken into account to avoid incorrect conclusions.

Cucumbers shrink-wrapped in plastic foil will have to be investigated separately to control the extent to which the correlation of $\delta^{15}N$ and weight loss will be decoupled, together with potential other influencing factors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MH developed the project design, acquired funding, performed project, and wrote the manuscript. WP analyzed the cucumber water samples and contributed to the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study received funding from LGV GmbH. The funder was not involved in the study design, analysis, interpretation of data, and the writing of this article or the decision to submit it for publication. M. Hobiger, employee of LGV GmbH determined the weight loss of the cucumbers by weighing.

ACKNOWLEDGMENTS

This study has been partially financed by the LGV GmbH, Vienna, Austria, which is gratefully acknowledged. We thank Karl Herret and Johanna Hobiger from the LGV GmbH for their support and enthusiasm. Preparation of the samples has been done by Gerhard Spitzer and Erwin Tschank. Measurement of samples has been carried out by Karl Blochberger and Peter Kostecki. This study was funded under the Independent Research Program of the Austrian Institute of Technology GmbH – AIT. We thank the two reviewers for valuable recommendations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.781158/full#supplementary-material

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Conflict of Interest: MH and WP were employed by company Austrian Institute of Technology GmbH.

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frontiers in Forests and Global Change

ORIGINAL RESEARCH
published: 27 January 2022
doi: 10.3389/ffgc.2021.650257

Stable Isotope Ratio Analysis for the Comparison of Timber From Two Forest Concessions in Gabon

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Consumers are becoming increasingly aware of the environmental impacts caused by deforestation and illegal logging and there is an increasing demand for supply chain transparency and traceability of wood products. Many importing and exporting nations have implemented regulations which aim to control the origin and species of traded timbers of high ecological importance and economic value. However, despite growing interest in method development for timber authentication purposes, many studies have been limited by insufficient numbers of authentic timber reference samples. Our aim was to address the differences in stable isotope ratio profile of bulk, homogenized wood samples collected from living or recently felled trees in two FSC concessions in Gabon, which are approximately 240 km apart, for the purposes of origin classification and protecting valuable forest commodities. Forty-seven timber samples comprising 10 genera of tropical trees were obtained using a Pickering Punch sampling device or chainsaw from two forest concessions in Gabon (Precious Woods Group and Compagnie des Bois du Gabon) during July 2019. Samples were subject to $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ stable isotope analysis using elemental analysis-isotope ratio mass spectrometry (EA-IRMS). Results show that significant differences are evident in the stable isotope ratios of *Aucoumea klaineana* between Precious Woods Group and Compagnie des Bois du Gabon forest concessions. Relationships are evident between climatic and geological variables and the stable isotope ratios of the samples suggesting that further degrees of origin classification may be achievable in Gabon. For other species, insufficient numbers meant the possibility to determine discriminating factors between the two concessions was limited though data from these samples may prove useful to contribute to the understanding of stable isotope variability in tropical timber. The data presented establish a basis for evaluating origin claims of forest products and timber from the Compagnie des Bois du Gabon and Precious Woods Group concessions and lay a foundation for future development of timber tracking technologies in Gabon. The technique can be used for purposes of due diligence or forensic investigation by law enforcement as part of demand-side regulations such as the EU Timber Regulation, Illegal Logging Prevention Act, or the Lacey Act.

Keywords: IRMS, *Aucoumea klaineana*, Gabon, 2H/1H isotope ratio analysis, illegal logging and timber trade, Lacey Act, EUTR

OPEN ACCESS

Edited by:
Micha Horacek, HBLFA Francisco Josephinum, BLT Wieselburg, Austria

Reviewed by:
Dana Alina Magdas, National Institute for Research and Development of Isotopic and Molecular Technologies, Romania
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Specialty section:
This article was submitted to Forest Ecophysiology, a section of the journal Frontiers in Forests and Global Change

Received: *06 January 2021*
Accepted: *27 September 2021*
Published: *27 January 2022*

Citation:
Watkinson CJ, Rees GO, Gwenael MC, Gasson P, Hofem S, Michely L and Boner M (2022) Stable Isotope Ratio Analysis for the Comparison of Timber From Two Forest Concessions in Gabon. Front. For. Glob. Change 4:650257. doi: 10.3389/ffgc.2021.650257

INTRODUCTION

Consumers are becoming increasingly aware of the environmental impacts caused by deforestation and illegal logging and there is an increasing demand for supply chain transparency and traceability of wood products. Many importing and exporting nations have implemented regulations which aim to control the origin and species of traded timbers of high ecological importance and economic value. One of the earliest regulations (US Lacey Act, 2008) saw the world's first ban on trade in illegally sourced wood products, and in March 2013, the European Union (EU) implemented the EU timber regulation (EUTR) which prohibits illegally sourced timber from entering EU markets. A handful of scientific methods ranging from light microscopy to DNA analysis and mass spectrometry, are being developed in laboratories around the world to help verify the legal status of traded timbers. However, despite growing interest in method development for timber authentication purposes, many studies have been limited by insufficient numbers of authentic timber reference samples. To overcome these limitations, World Forest ID was established through the collaboration of several key organizations including Defra, Royal Botanic Gardens, Kew, Forest Stewardship Council (FSC), Agroisolab and the United States Forest Service, with the aim of carrying out large scale collections of timber reference samples from some of the world's most endangered forests (Gasson et al., 2020)[1].

Gabon is a West African nation located on the Gulf of Guinea and Atlantic Ocean, bordering Cameroon, Equatorial Guinea, and the Republic of Congo. Its land mass is approximately 85% forested with deforestation rates of approximately 0.1% occurring annually (NEPcon, 2017). The timber industry in Gabon accounts for nearly 20,000 jobs (EIA, 2019) and is the country's most important export (5% of GDP) after oil (Bayol, 2002; NEPcon, 2017). Of the 22–23 million ha of forested area, about 4 million ha is protected and 14 million ha are allocated for forestry (EIA, 2019). All Gabon's forests are managed by the Ministry of Forest, Environment and Natural Resource Protection which oversees the monitoring of forest resources, including the allocation of forest concessions. Two types of permits are issued by the ministry: Concession Forestière sous Aménagement Durable (CFAD), and Permis Forestier Associé (PFA). CFAD, allows for logging by corporations in land areas of between 50,000 and 200,000 ha, whereas PFA – (which is reserved exclusively for Gabonese nationals) has a maximum size of 50,000 ha (NEPcon, 2017). As of 2017, China, followed by France, Belgium, and Italy were the largest export markets for timber sourced from Gabon (EIA, 2019).

The most important species for the Gabonese timber industry is Okoumé (*Aucoumea klaineana*) which accounts for most of the country's timber exports. The timber is commonly manufactured into plywood and veneers at plants within Gabon and Asia (EIA, 2019). A 4-year investigation by the EIA established that illegally sourced timber from Gabon has routinely entered the United States (US) for over a decade and made its way to thousands of United States consumers. An in-depth analysis of the Okoumé (*Aucoumea klaineana*) veneer imported directly into the United States from Gabon highlights the lax attitude toward legality and the complicity of United States importers (EIA, 2019). Following a corruption scandal in 2010, Gabon moved to ban the export of logs and switched to the export of sawn logs and plywood as a means of stimulating the domestic economy (Karsenty, 2019). Other economically important species for the Gabonese timber industry include Azobé, Bongossi (*Lophira alata*), Okan (*Cylicodiscus gabunensis*), Padouk d'Afrique (*Pterocarpus soyauxii*), Beli (*Julbernardia pellegriniana*), Tali (*Erythrophleum ivorense*), Missanda (*Erythrophleum suaveolens*). These timber species, and forest products derivatives, are in great need of protection.

Aucoumea klaineana is a long-lived pioneer species capable of converting savannah into rainforest (White et al., 1996; Born et al., 2006) and is the only species in its genus. It grows relatively quickly and makes up a large proportion of the trees within Gabon. The natural range of *Aucoumea klaineana* includes Gabon but also extends into Equatorial Guinea, southern Cameroon, and parts of the Republic of Congo (Born et al., 2010). Despite its relatively wide distribution in Gabon, *Aucoumea klaineana* can be difficult to grow in plantations. One study into the silviculture of young *Aucoumea klaineana* trees found that saplings grew best in the soil of their original population. The growth of the saplings could be described in terms of a function of the distance from the sites where their seeds were harvested to the research site (Koumba Zaou et al., 1998). This means that if *Aucoumea klaineana* is harvested to the point of exhaustion in one region of Gabon, it will not be a straightforward task to simply replant the trees from another region. Genotypic mechanisms were proposed as a potential explanation for this phenomenon. However, phenotypes are ultimately the product of genetic expression, of which, the available genetic sequences are only part of the story; the mechanism phenotypic variation can also be considered in terms of epigenetics or other genetic regulatory mechanisms. Even so, there are clear examples of distinct genetic populations of *Aucoumea klaineana* within Gabon; Muloko-Ntoutoume et al. (2000) revealed population differences in chloroplast DNA (cDNA), and differences in polymorphic microsatellites have also been identified (Born et al., 2006, 2008).

Humans are not the only great apes that rely on *Aucoumea klaineana* for survival; western lowland Gorillas have been observed eating the flowers of on *Aucoumea klaineana*. Though it is not a main food source for Gorillas, the availability of *Aucoumea klaineana* during times of famine caused by shortages of other food sources may ensure that Gorillas survive (Williamson et al., 1990). Nevertheless, in research where Gorillas have been observed, logging did not appear to have affected the local population at the time meaning that logging the trees can still be acceptable if done in a sustainable manner. Although Gabon constitutes a fraction of the Congo Basin, the country also shelters approximately 45,000 forest elephants, representing nearly 60% of Africa's remaining population (EIA, 2019). Protection of natural forest is vital for the survival of forest elephants.

[1] www.worldforestid.org

Aucoumea klaineana is most commonly traded as plywood and is often used for boatbuilding due to its excellent properties (Negro et al., 2011). Plywood is produced from the layering of multiple sheets (veneers) of rotary-peeled logs which are held together with thermosetting formaldehyde-urea-based resins (Desch and Dinwoodie, 1996; Negro et al., 2011). Typically, heartwood is used for veneer making. Heartwood can be defined as "*the inner layers of the wood, which, in the growing tree, have ceased to contain living cells, and in which the reserve materials (e.g., starch) have been removed or converted into heartwood substance*" (Hillis, 1987). The process of heartwood formation does not always lend itself to the preservation of genetic material either in plant cell nuclei, mitochondria, or chloroplasts. The process of veneer-making involves boiling the wood, applying steam and hot-pressing at over 100°C to cure the thermosetting resin. Though population genetics is an excellent method for timber tracking (Jolivet and Degen, 2012) and is highly suitable for verifying the origin of logs, the manufacturing process of plywood does not lend itself to ideal preservation of genetic material for later analysis and may present limitations for this technique in demand-side regulatory scenarios. Therefore, there needs to be a method capable of evaluating the origin of the veneers in plywood so supply chain stakeholders, enforcement and concerned parties can be assured of its legal origin as part of demand-side authentication. Nevertheless, population genetics is a vital technique and is suitable for addressing the origin of timber within Gabon where genetic material can be accessed for analysis.

Stable isotope ratio analysis (SIRA) is a widely accepted analytical technique, and since the beginning of the 21st century, has become established as a means of verifying the origin of food and drink (Kelly et al., 2002; Boner and Förstel, 2004; Heaton et al., 2008; Pilgrim et al., 2010; Li et al., 2015). The same principles used to authenticate food were later applied to timber provenance research (Boner et al., 2007; Keppler et al., 2007; Kagawa et al., 2008; Horacek et al., 2009; Kagawa and Leavitt, 2010; Gori et al., 2013, 2018; Rees, 2015; Watkinson et al., 2020, 2021). Verifying the origin of timber involves the comparison of an unknown sample against an authentic reference database for a region or territory. The technique is used routinely to assess legality, compliance with labeling legislation, and its use to conduct due diligence is advocated by EUTR (Regulation (EU) No 995/2010, 2010).

The ambitions of this project were to define the ranges of stable isotope ratios from multiple species of trees from two FSC forest concessions in Gabon by analyzing timber samples extracted from living trees. Our aims were to:

1. Collect and perform stable isotope analysis of authentic geo-referenced timber such as: Okoumé/*Aucoumea klaineana* (Burseraceae), Moabi/*Baillonella toxisperma* (Sapotaceae), Ebiara/*Berlinia confusa* (Leguminosae-Caesalpinioideae), Okan/*Cylicodiscus* gabunensis (Leguminosae-Caesalpinioideae), Ozigo/*Dacryodes buettneri* (Burseraceae; synonym of *Pachylobus buettneri*), Gombe/*Didelotia africana* (Leguminosae-Detarioideae), Kevazingo/*Guibourtia tessmannii* (Leguminosae-Detarioideae), Azobe/*Lophira alata* (Ochnaceae), Bilinga/*Nauclea diderrichii* (Rubiaceae), and Padouk d'Afrique/*Pterocarpus soyauxii* (Leguminosae-Papilionoideae).
2. Assess what differences occur in the stable isotope ratios of trees between and within different forest concessions within Gabon.
3. Use the data produced from samples as a baseline for evaluating the origin of timber from the two sampled concessions if the data characterize the concession well. In cases where data are insufficient to characterize the concession, the data will remain available to build data for later use.

If significant differences in the stable isotope ratios of trees from within different concessions in Gabon are evident, this may be of great benefit to audit teams wanting to meet due diligence requirements and demonstrate sustainable practices by using analysis to verify origin declarations. If the isotope ratios of trees from within different forest concessions in Gabon are relatively homogenous, this would mean that it may not be necessary to collect reference samples from all concessions to verify that the timber is from Gabon.

MATERIALS AND METHODS

During planning, Agroisolab liaised with several organizations including FSC, Royal Botanic Gardens, Kew, United States Fish and Wildlife Service, World Resource Institute (WRI) and United States Forest Service to establish a taxa priority list and reach a consensus on which locations within Gabon should be sampled. It was agreed that two forest concessions; Compagnie des Bois du Gabon (C.B.G) and Precious Woods (P.W.G) would be selected for reference sample collection. The concessions are separated by approximately 240 km and presented an opportunity to assess the variability in stable isotope ratios at higher resolution and the viability of stable isotope data to differentiate several taxa at concession level resolution.

Samples were taken from 47 trees in two forest concessions (C.B.G, $n = 33$ and P.W.G, $n = 14$) in Gabon during June 2019 (**Figure 1**). Between 2 and 4 pieces of timber (heartwood and sapwood) were collected from each tree. In most cases three samples were taken per tree as well as leaf and twig samples to act as herbarium vouchers. Samples of heartwood/sapwood were submitted to Agroisolab for stable isotope ratio analysis whereas the remaining material was distributed between Conservation of Biodiversity at IRET/CENAREST in Gabon and the World Forest ID collection at the Royal Botanic Gardens, Kew. A Pickering Punch (Agroisolab UK, Welburn, United Kingdom), a type of hammer-driven bore, was used to collect 45 of the 47 cores of timber 9–11 cm in length and 1.5 cm wide. One sample (*Berlinia confusa*) was smaller than the recommended size due to the hardness of the tree. Samples were then transferred from the Pickering Punch into cardboard tubes which were sealed inside 500 mL evacuated bags with silica gel to aid drying. Additionally, 2 of the 47 samples were collected with a chainsaw. Samples were dried in-transit to Conservation of Biodiversity

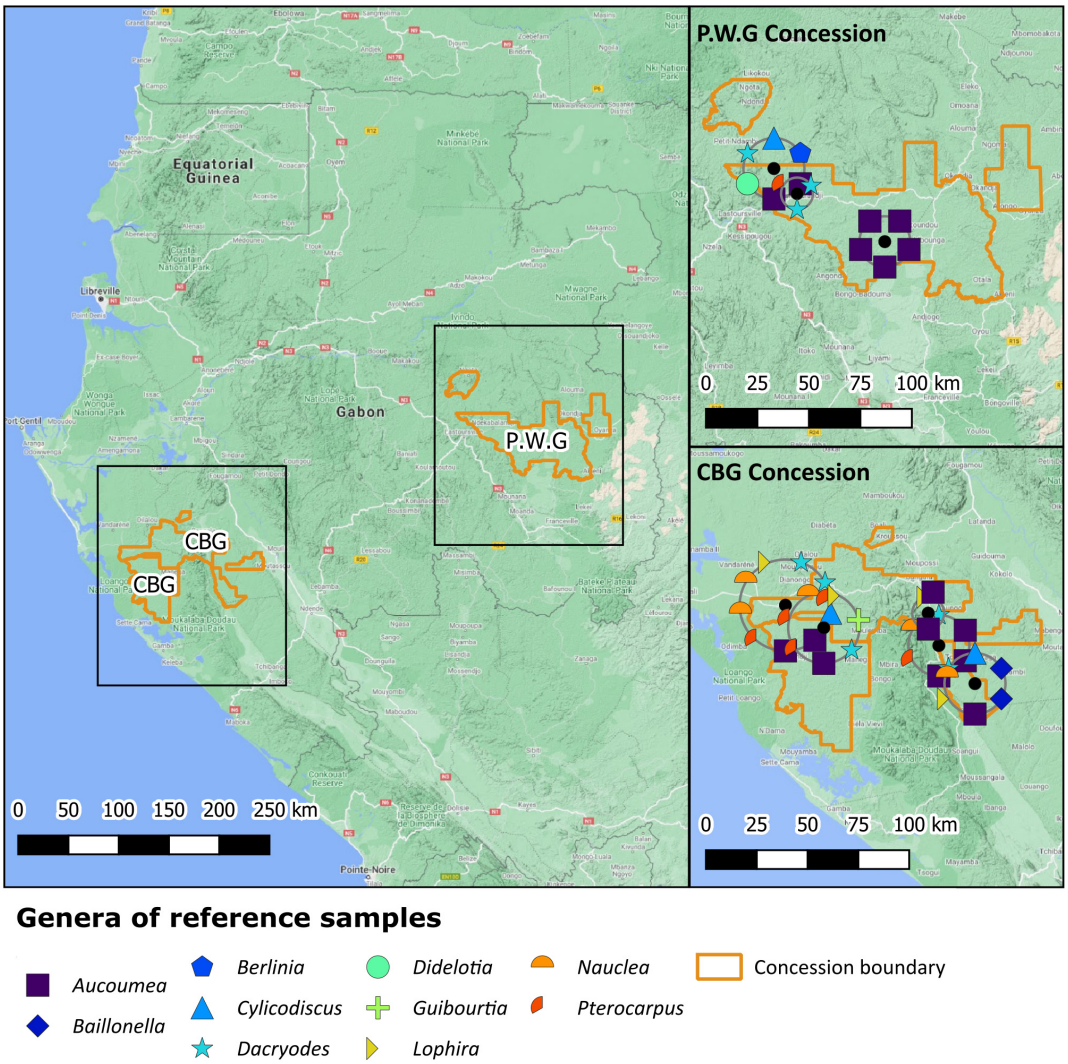

FIGURE 1 | Locations and taxa of samples collected in the C.B.G and P.W.G concessions in Gabon. The positioning of location markers is displayed using point displacement. The black dots represent the centroid of the sample cluster.

at IRET/CENAREST using 30 g silica gel per plug/core. No perforations were made in the acid-free cardboard tubes; field tests found this did not adequately facilitate drying and some cores presented with mold growth upon receipt at the Plant Quarantine Unit, Royal Botanic Gardens, Kew. To eliminate the risk of pathogenic fungi entering the United Kingdom, samples were subjected to 121°C heat at 15 psi in an autoclave for 30 min before they were dried and released into the WFID collection for analysis and storage. Prior to routine preparation mold was manually removed as it is considered that it may be source of variability for stable isotope analysis (Horacek et al., 2018; Beeckman et al., 2020).

GPS data, photographs of the trees and leaves, descriptions and comments about the sampled trees were recorded in a mobile phone application named TreeSnap (Crocker et al., 2019; TreeSnap version 1.15.3 Department of Entomology and Plant Pathology, University of Tennessee, Institute of Agriculture, Knoxville, TN, United States). The data from this collection have since been moved to the World Forest ID application (World Forest ID version 1.2.0, Knoxville, TN, United States).

There are clear environmental differences between the two concessions (**Table 1**) that may influence isotope ratios in timber. These are in addition to differences in elevation and distance to the sea. The C.B.G concession is situated next to a lagoon and approximately 20 km from the Atlantic Ocean at its closest point. P.W.G concession is approximately 380 km from the ocean at its nearest. The two concessions are approximately 240 km apart.

Measurement

Samples that presented with hyphae upon receipt at Kew had any mold physically removed as part of preparation in accordance with advice from Beeckman et al. (2020). Samples were dried at 103°C before being coarsely ground and placed into a ball-mill (Retsch MM220, Haan, Germany). The resulting fine powder

TABLE 1 | Environmental differences between the P.W.G and C.B.G concessions.

Environmental variable	Spatial resolution of gridded dataset	Compagnie des Bois du Gabon (C.B.G) concession	Precious Woods Group (P.W.G) concession
$\delta^{18}O$ Annual precipitation (Bowen and Revenaugh, 2003)	0.25° × 0.25°	−3.6‰	−4.4‰
$\delta^{2}H$ Annual precipitation (Bowen and Revenaugh, 2003)	0.25° × 0.25°	−16‰	−22‰
SO_4 tropospheric deposition, December (mean 1980–2018) (Global Modeling and Assimilation Office [GMAO], 2015)	0.5° × 0.625°	6.5 mg/m^2	7.8 mg/m^2
Precipitation, mean February 1983–2016 (multi-satellite method) (Huffman and Bolvin, 2019)	0.5° × 0.5°	200–250 mm/day	130–150 mm/day
Soil types (Jones et al., 2013)		• Dystric fluvisol • Ferralic arenosol • Ferralic cambisol • Xanthic ferralsol	• Ferralic cambisol • Xanthic ferralsol
Elevation		20–650 m	230–670 m

was extracted in a soxhlet apparatus for 6 h with non-polar (dichloromethane) and polar (methanol) solvents which were then dried in a laboratory drying cabinet for at least 1 h. Finally, the samples were stored in air-tight sample vials and weighed for analysis.

The method chosen to isolate non-exchangeable hydrogen in cellulose is outlined by Cheung (2014). Homogenized samples were nitrated using 4 mL HNO_3 (90%) and 4 mL of glacial H_2SO_4 (96%) in falcon tubes with 35 mL distilled water. As the reaction is exothermic, the solution was refrigerated for 2 h during the digest. The sample solution was agitated using an automatic shaker for 2 h allowing the cellulose to precipitate. Precipitated cellulose was then separated from the solution with an initial centrifugation for 1 min at 3,000 rpm. The supernatant was then discarded, and the precipitate was resuspended using 40 mL distilled water and subjected to another centrifugation for 1 min at 3,000 rpm. This process was repeated until a pH of 6–7 was achieved. Finally, precipitated cellulose was resuspended in 2–3 mL of distilled water, transferred to a glass vial and water decanted following centrifugation. Residual water was removed by gentle heating.

To avoid equilibration or humidity effects in the oxygen and hydrogen analysis, the weighed-in samples were equilibrated overnight in desiccators with a defined humidity of 10.6%. Afterward the samples were vacuum dried for at least 2 h.

Sample measurements were corrected against in-house standards at the beginning, middle and end of each measurement run. In-house standards are traceable back to certified reference materials enabling measurements to be reported relative to an internationally defined standard; for hydrogen and oxygen isotope ratio analysis, Vienna Standard Mean Ocean Water (VSMOW) is used. For carbon isotope ratio analysis, Vienna Pee Dee Belemnite (VPDB) is used. For sulfur isotope ratio analysis Vienna Canyon Diablo Troilite (VCDT) is used. The in-house standards used were 1,4-dihydroxyanthraquinone (Merck Schuchardt) for $\delta^{18}O$ and $\delta^{2}H$, L-leucine (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for $\delta^{13}C$ and $\delta^{15}N$, and L-cysteine (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for $\delta^{34}S$. In-house standards are also controlled in Agroisolab's free intercomparison testing platform "Agroisolab-KPT" which routinely operates with over 20 isotope laboratories. Measurements are reported in per mil (‰) and were made in accordance with processes outlined in Boner et al. (2007).

$\delta^{18}O$ and $\delta^{2}H$ measurement was performed using two Isotope Ratio Mass Spectrometers (IRMS) in a master/slave configuration (Isoprime, Elementar, Cheadle, United Kingdom) with each IRMS measuring one isotope ratio; $\delta^{18}O$ or $\delta^{2}H$. This configuration provides excellent stability because the magnetic field and accelerating voltage remain constant on each IRMS. The working temperature for pyrolysis is >1530°C and HT-PyrOH is performed with a covalently bonded silicon carbide tube (patented by Agroisolab GmbH, Jülich, Germany) filled with glassy carbon chips and coal powder.

$\delta^{13}C$ measurement was performed using an Elemental Analyzer (EA3000, Eurovector, Milan, Italy) in combination with IRMS (Nu Horizon, NU-Instruments, Wrexham, United Kingdom). The working temperatures are 1,021°C for oxidation and 600°C for reduction. Reduction is carried out in the presence of copper.

$\delta^{34}S$ measurement was performed using an Elemental Analyzer (EA3000, Eurovector, Milan, Italy) with IRMS (Isoprime, Cheadle, United Kingdom). A one tube combustion (oxidation and reduction in one tube) is used to solve issues caused by SO_3. Combustion water is directly trapped with magnesium perchlorate at the end of the tube. The working temperature is 1,000°C.

Typical values for Combined Uncertainty (u_c) measurement in timber is 0.2‰ for $\delta^{18}O$, 1.3‰ for $\delta^{2}H$, 0.1‰ for $\delta^{13}C$, and 0.2 for $\delta^{34}S$.

Data Analysis

Due to the low number of samples representing many taxa, multivariate analysis methods were not considered ideal to draw conclusions from the data but were still performed to at least make inferences. Particular attention was given to *Aucoumea klaineana* which had high enough sample numbers from both concessions. Univariate analyses were performed to show differences between distributions of means such as Student's *t*-test and ANOVA, or to assess co-variance such as regression as well as boxplot visualizations (Orange 3.24, University of Ljubljana, Slovenia; Microsoft Excel 2019 ver. 16.0.6742.2048, Redmond, WA, United States). *Aucoumea*

klaineana and *Dacryodes buettneri* were assessed in SAGA GIS 2.3.2 (Departments for Physical Geography, Hamburg and Göttingen, Germany) for spatially related patterns using Inverse Distance Weighting (IDW) to the 2nd order power and weighting was applied using all sampling points in the area. IDW is an exact spatial interpolator, therefore, isoscapes generated in this way are useful to visualize consistencies, differences, and outliers within defined areas such as forest concessions. However, IDW is not an ideal method to generate isoscapes as statistical models due to the fact it always generates an "overfit" model. We do not recommend IDW's use as a method of evaluating the authenticity of test samples.

RESULTS

Nauclea diderrichii (n = 5) was remarkably consistent in terms of its $\delta^{18}O$ isotopic composition relative to other sampled taxa, and it also has the least positive $\delta^{18}O$ on average (mean) by group. *Lophira alata* (n = 4) has the most positive and widest range of $\delta^{18}O$ (2.6‰). This seems unusual because the range $\delta^{18}O$ for *Aucoumea klaineana* was 1.1‰ in the C.B.G concession and 0.8‰ in the P.W.G concession, and the range of *Dacryodes buettneri* was 0.7‰ in C.B.G and 0.5‰ in P.W.G, yet these species were sampled in greater quantities (**Table 2**). The reason for the wide range in $\delta^{18}O$ for *Lophira alata* can be attributed to a single sample which was far more depleted in ^{18}O than others. Of the four samples of *Lophira alata* that were collected, the sample in question was the only one collected with a chainsaw as opposed to a Pickering Punch. The samplers were contacted about the result and were able to provide further corroborating information about the authenticity of the sample. We cannot explain this outlier result, but outliers exist in the dataset and must be included. There are significant differences between the $\delta^{18}O$ isotope ratios of the different taxa across the two locations (p = 0.002) as judged by ANOVA. For the interpretation of this result, it should be considered that the *Aucoumea* and *Dacryodes* were collected in two sites, whereas the other taxa were collected in just one. Based on the data from samples collected in this study, there is no strong relationship between the oxygen stable isotope ratios and the elevation of each sample. *Aucoumea klaineana* has the strongest relationship with elevation (R^2 = 0.2) although this value lacks explanatory power to be used as a sole predictor of $\delta^{18}O$ isotopic composition in *Aucoumea klaineana*.

Nauclea diderrichii has the narrowest range of δ^2H isotope ratios in comparison to other taxa that were analyzed in this study. Contrary to the trend between taxa having the most positive $\delta^{18}O$, *Nauclea* shows the least positive δ^2H (**Table 2**). The differences in the mean δ^2H of the taxa are significant (ANOVA, p = 0.009), however, there is a lot of overlap in the ranges. The range of all taxa is approximately 20‰ (between −62 and −42‰). The typical range of δ^2H in each concession is around 10‰, however, *Pterocarpus* (five samples collected in C.B.G concession, one sample collected in P.W.G concession) shows a range of 18‰ (**Table 2**). *Nauclea diderrichii* and *Pterocarpus soyauxii* appear to have strong, positive relationships with elevation (R^2 s of 0.79 and 0.55,

TABLE 2 | Stable isotope data from taxa sampled from the two concessions in Gabon.

Origin	Scientific name	N samples	$\delta^{18}O$ vs. VSMOW (‰)			δ^2H vs. VSMOW (‰)			δ^2H non-exchangeable vs. VSMOW (‰)			$\delta^{13}C$ vs. VPDB (‰)			$\delta^{15}N$ vs. Air (‰)			$\delta^{34}S$ vs. VCDT (‰)		
			μ	σ	Range	μ	σ	Range	μ	σ	Range	μ	σ	Range	μ	σ	Range	μ	σ	Range
CBG Concession, Gabon	*Aucoumea klaineana*	9	+23.6	0.3	1.1	−55.2	3.3	10.1	−16.3	4.4	10.5	−27.5	0.7	2.4	+2.8	1.6	5.1	+10.3	1.4	3.8
	Baillonella toxisperma	2	+23.6		1.2	−44.7		4.2	−2.2		0.3	−28.3		0.8	+4.9		1.1	+10.1		0.6
	Cylicodiscus gabunensis	2	+22.9		0.4	−45.4		3.5	−4.7		1.2	−26.7		0.3	+5.0		3.0	+11.3		0.3
	Dacryodes buettneri	5	+24	0.3	0.7	−50.7	6.8	14.4	−8.0	5.8	14.3	−29.2	1.4	3.3	+4.5	1.4	3.8	+11.5	1.2	3.0
	Guibourtia tessmannii	1	+25.1			−58.8			−10.8			−28.0			+4.5			+8.1		
	Lophira alata	4	+23.5	1.2	2.6	−54.6	3.9	8.8	−10.1	7.5	16.3	−28.2	0.7	1.5	+5.7	0.4	0.9	+9.7	0.9	1.9
	Nauclea diderrichii	5	+22.9	0.2	0.5	−48.6	2.7	6.5	−10.3	4.3	11.2	−27.7	0.6	1.5	+7.0	1.6	3.7	+9.7	0.4	1.0
	Pterocarpus soyauxii	5	+23.3	0.4	0.9	−51.1	7.0	18.0	−16.1	6.5	16.0	−28.2	1.5	4.0	+1.5	2.7	6.2	+12.1	0.9	2.5
Precious Woods Concession, Gabon	*Aucoumea klaineana*	7	+23.8	0.3	0.8	−57.6	4.3	10.6	−21.5	4.7	11.9	−27.3	0.8	2.0	+1.9	1.3	3.7	+7.3	0.9	2.3
	Berlinia confusa	1	+25.1			−44.8			−6.0			−28.2			+6.2					
	Cylicodiscus gabunensis	1	+23.4			−42.7			+3.3			−26.8			+7.4			+8.7		
	Dacryodes buettneri	3	+24.0	0.3	0.5	−53.0	1.9	3.7	−7.6	5.9	11.0	−30.2	0.1	0.1	+5.4	1.0	2.0	+10.5	0.7	1.2
	Didelotia africana	1	+23.2			−55.8			−26.2			−28.9			+4.7			+8.5		
	Pterocarpus soyauxii	1	+22.9			−43.2			−5.4			−27.0			+2.5			+10.6		

Ranges are given when n > 1, standard deviations (σ) are given when n > 2. Insufficient sulfur was recovered from the sample of Berlinia confusa to report its $\delta^{34}S$.

respectively). This positive proportionality suggests that their δ^2H should increase with increasing elevation if the trend can be extrapolated. *Dacryodes buettneri, Lophira alata*, and *Aucoumea klaineana* do not show strong relationships between their δ^2H and elevation.

Relative to the δ^2H ratios of the extracted wood, analysis of the non-exchangeable δ^2H isotope ratios show some interesting patterns: *Aucoumea klaineana* has the most negative δ^2H isotope ratios in both cases, the remainder of the sampled taxa seemed to have switched places entirely. **Table 2** shows that the typical ranges observed in the taxa are approximately 10‰ for δ^2H from extracted wood, whereas the ranges in the δ^2H isotope ratios for the non-exchangeable δ^2H are all greater than 10‰ for all well-sampled taxa. *Nauclea diderrichii*, *Pterocarpus soyauxii,* and *Lophira alata* appear to have positive relationships between their non-exchangeable δ^2H from cellulose and elevation with R^2 s of 0.26, 0.73, and 0.31, respectively. This positive proportionality suggests that their δ^2H isotope ratios should increase with increasing elevation if this trend persists. *Aucoumea klaineana* has a negative relationship between its non-exchangeable δ^2H from cellulose and elevation with an R of −0.43. *Dacryodes buettneri* was the only taxon without a strong relationship between its δ^2H and elevation.

Carbon isotope ratios have a range that varies between 1.5 and 4.0‰. The carbon isotope ratios of all species vary between −27 and −30.2‰ (**Table 2**). The widest range evident in this study exists in *Pterocarpus* (4‰ range).

The range of nitrogen isotope ratios in all sampled taxa was approximately between −2‰ to +8.5‰. The least positive $\delta^{15}N$ belongs to a *Pterocarpus* sample, whereas the most positive $\delta^{15}N$ belong to a *Nauclea* sample. The nitrogen isotope ratios of each taxa had an individual range of around 1–4‰. There are significant differences in the mean nitrogen isotope ratios of the taxa sampled (ANOVA, $p = 0.000$) implying a strong taxonomically related effect in the data for $\delta^{15}N$.

All taxa were enriched in ^{34}S with $\delta^{34}S$ greater than +6‰. The range of sulfur isotope ratios in all taxa range between +6 and +13.5‰ and have per-taxon ranges of approximately 2–3‰ in each concession (**Table 2**). The broadest range per taxon was evident in the *Aucoumea* samples which were collected in two concessions and were the most well-sampled timbers in this project. There are significant differences between the taxa (ANOVA, $p = 0.001$) suggesting a strong taxonomically related effect in sulfur stable isotope ratio profile. All analyzed taxa show negative relationships between sulfur isotope ratio and elevation meaning that, with increasing elevation, a decreasing sulfur isotope ratio would be expected if this trend can be extrapolated. The strongest relationship was evident in *Aucoumea klaineana* with an R of −0.79 (**Figure 2**).

Aucoumea klaineana (Okoumé)

Significant differences are evident in the mean values of non-exchangeable δ^2H (Student's t, $p = 0.036$), and the $\delta^{34}S$ (Student's t, $p = 0.000$) of the *Aucoumea klaineana* samples between the C.B.G and P.W.G concessions. **Figure 3** show that there is very little overlap between the two concessions in the sulfur isotope ratios from the two concessions.

Linear Projection (**Figure 4**) of the stable isotope ratios of *Aucoumea* from the two concession shows the two concessions are well-separated. The k-means silhouette scores from the Principal Components of the stable isotope data show that it is most likely that the data have two clusters (48% probability), but it is also possible that there are more (3 clusters = 36.5%, 4 clusters 37.3%). There is one sample of *Aucoumea* from the C.B.G concession that has stable isotope ratios that are in the range of reference samples from the P.W.G concession.

The Inverse Distance Weighting isoscape (**Figure 5**) highlights the spatial patterns in the stable isotope ratios in *Aucoumea* between and within the C.B.G and Precious Woods concessions. The color scales of the figures are set to maximize the visual differentiation in values rather than to reflect whether differences are significant or not. For example, there are no significant differences between the oxygen, hydrogen (extracted), carbon and nitrogen stable isotope ratios of *Aucoumea* between the two concessions. The scale for **Figure 5A** shows insignificant differentiation in the oxygen isotope ratios, however, the scales of the isotope ratios of carbon (B), extracted hydrogen (C), non-exchangeable δ^2H from cellulose (D), nitrogen (E), and sulfur (F) all show good ranges in their respective values. **Figure 5B** shows that carbon isotope ratios may have been a more useful classifier to each concession were it not for a single sample in the northwest of the main portion of the P.W.G concession having such an enriched carbon isotope ratio. **Figures 5C,D** do not show comparable spatial patterns in hydrogen isotope ratios, **Figure 5C** shows that, generally, the hydrogen isotope ratios of *Aucoumea* are enriched in the C.B.G concession and depleted in the Precious Woods Group concession, whereas **Figure 5D** seems to show the opposite, discounting one sample in the C.B.G concession that has an enriched hydrogen isotope ratio in both situations. **Figure 5E** shows that the C.B.G concession has some populations of $\delta^{15}N$ enriched *Aucoumea* close to $\delta^{15}N$ depleted *Aucoumea*, whereas most of the samples collected in the Precious Woods Group concession are relatively depleted in $\delta^{15}N$ with the exception of a sample in the northwest of the main body of the concession. This same sample had an enriched carbon isotope ratio. **Figure 5F** shows that the sulfur isotope ratios of *Aucoumea* within each concession are consistent. The C.B.G concession shows enriched sulfur isotope ratios whereas the P.W.G shows depleted. Only one sample in the C.B.G concession shows a relatively depleted sulfur isotope ratio.

Dacryodes buettneri (Synonym of *Pachylobus buettneri*)

Significant differences in the oxygen, hydrogen, non-exchangeable hydrogen, and nitrogen stable isotope ratios were not evident in *Dacryodes buettneri* between the C.B.G and Precious Woods concessions, however, differences between

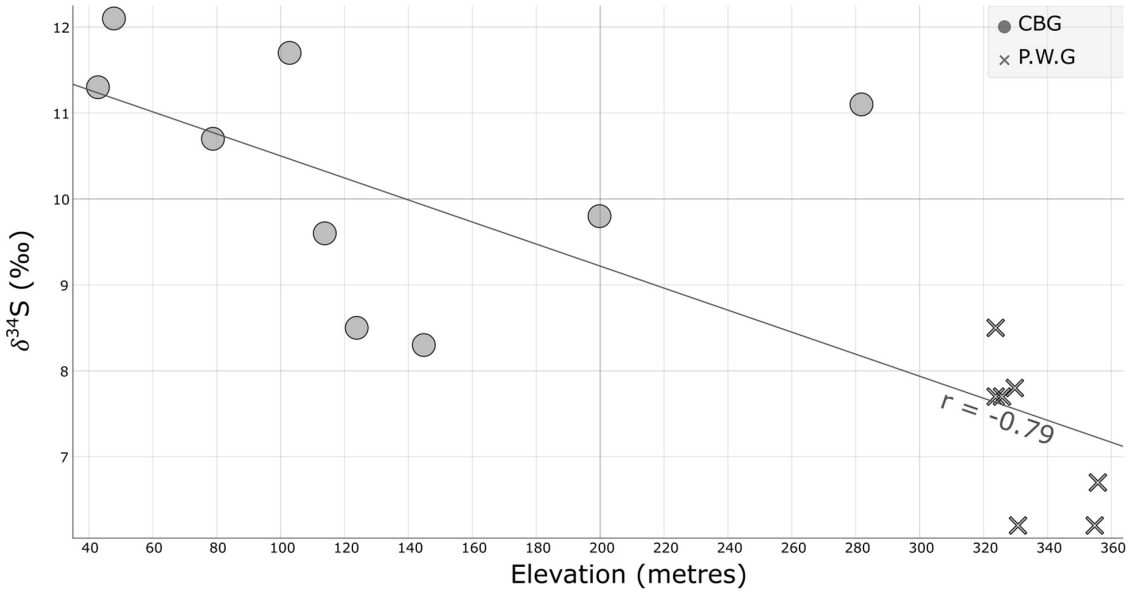

FIGURE 2 | Relationship between elevation and the $\delta^{34}S$ of *Aucoumea klaineana* sampled in the P.W.G and C.B.G concessions from Gabon.

the mean values of the carbon and sulfur stable isotope ratios between the two concessions were observed. These differences were close to being significant (p = 0.143 for carbon isotope ratios, p = 0.127 for sulfur isotope ratios **Figure 3**).

Linear projection of the stable isotope ratios of the samples of *Dacryodes buettneri* (**Figure 6**) further demonstrate that there is some apparent separation of the stable isotope data between the two concessions, but these differences are not well-defined. This is also evident when observing the k-means silhouette scores of the PCA, there is a 24% probability that there are two clusters in the data, however, there is 24.7% probability that there are 3 clusters or 4 clusters within the data.

The inverse distance weighted (IDW) isoscapes (**Figure 7**) give insight into the spatial patterns of the *Dacryodes* data between and within the two concessions. **Figure 7A** shows that there is more variation of the oxygen stable isotopes of the *Dacryodes* within each concession than there is between the two. **Figure 7B** shows that there would be good segregation of the two concessions by the carbon isotope ratios of the *Dacryodes* were it not for the high variance in the C.B.G concession perhaps explaining why the Student's *t*-test shows a nearly significant difference in carbon isotope ratios (P = 0.127). **Figures 7C,D** show that there are similar spatial patterns in the hydrogen stable isotopes of the extracted wood and of the non-exchangeable hydrogen from cellulose of the *Dacryodes*. The relatively high variation of hydrogen isotope ratios in the C.B.G concession limit the statistical separation of *Dacryodes* using the hydrogen isotope ratios alone. **Figure 7E** shows that *Dacryodes buettneri* have lower nitrogen isotope ratios in the C.B.G concession relative to the Precious Woods Group concession save for a single sample in C.B.G that was particularly enriched in $\delta^{15}N$. **Figure 7F** shows that *Dacryodes* follows a similar pattern to *Aucoumea* in its sulfur isotope ratios; the samples from the C.B.G concession are more enriched in $\delta^{34}S$ than in the Precious Woods concession save for one sample in C.B.G that was depleted in ^{34}S.

DISCUSSION

Aucoumea klaineana

Aucoumea klaineana (Okoumé) is a fast-growing, light-demanding pioneer tree (Koumba Zaou et al., 1998). In growing conditions with favorable light, *Aucoumea klaineana* can develop rapidly. In terms of photosynthesis, it can be considered that the main source of water for incorporation into sugars is likely to be primarily precipitation (Ohashi et al., 2016). It is therefore difficult to understand why there is no strong relationship between the hydrogen and oxygen isotope ratios of Okoumé, and very little trend with respect to elevation and its water hydrogen and oxygen isotope ratios. The lack of a trend may be summarized by the fact there are only relatively small differences in $\delta^{18}O$ and $\delta^{2}H$ in annual precipitation. This may be due to phenomena that work against each other such as the rainout effect and the continental effect (Ohashi et al., 2016), or that despite the fact there are differences in rainfall and elevation they are not sufficient to make a significant difference. Perhaps there is little meaningful difference in relative humidity between the P.W.G and C.B.G concessions that may also counter the anticipated relationship between $\delta^{18}O$ and $\delta^{2}H$ (Fritts et al., 1971; Roden et al., 2000).

One of the most important activities of rainforests is regulating the temperature of the atmosphere by removing CO_2 and moderating rainfall. Provided that excessive evaporation does not occur, precipitation can be expected to follow the Global Meteoric Water Line (Craig and Boato, 1955; Craig, 1961). Evapotranspiration in rainforests produces rain for the rainforest

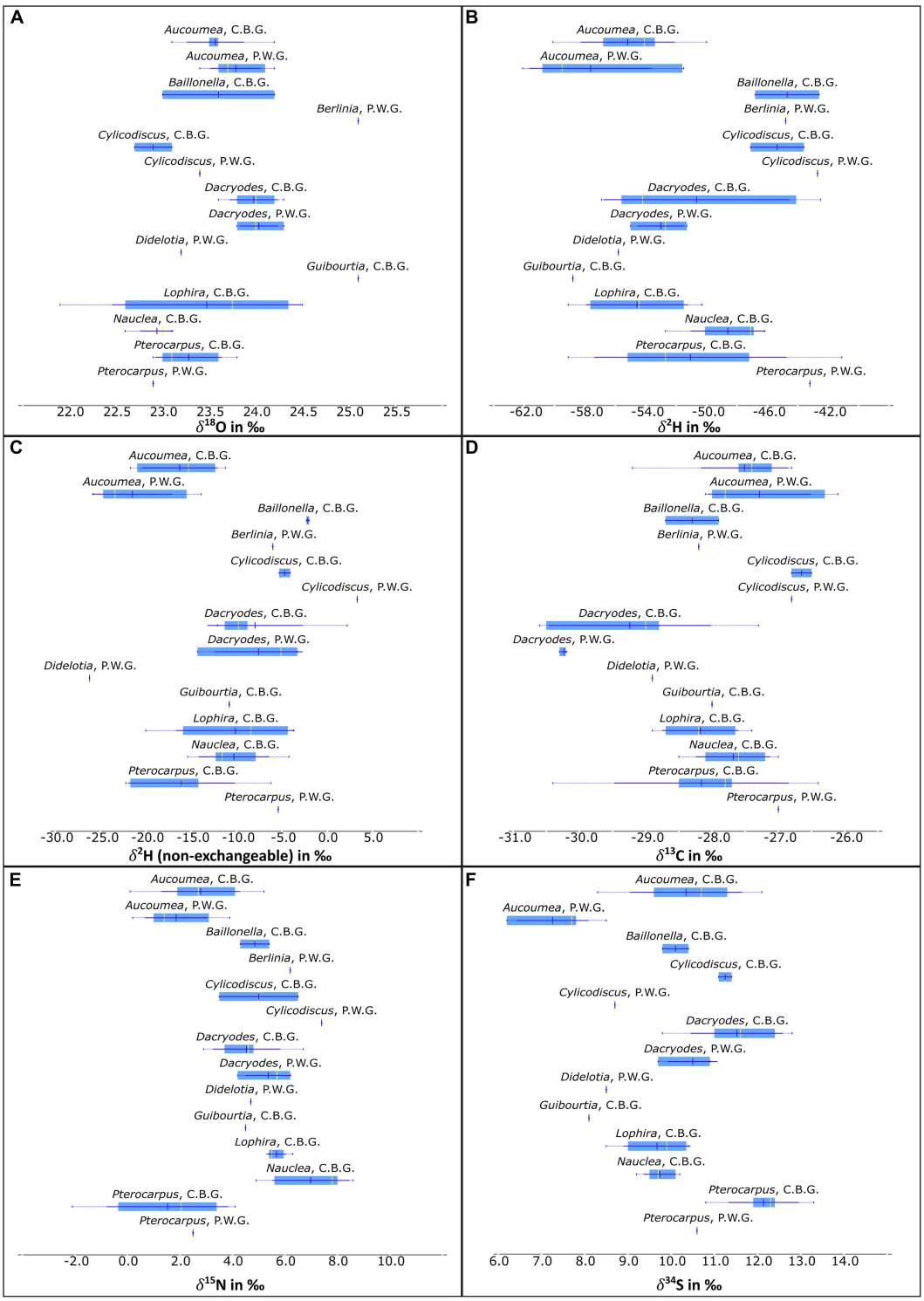

FIGURE 3 | Boxplots of **(A)** $\delta^{18}O$, **(B)** $\delta^{2}H$, **(C)** $\delta^{2}H$ (non-exchangeable), **(D)** $\delta^{13}C$, **(E)** $\delta^{15}N$, and **(F)** $\delta^{34}S$ of the different genera from each concession (C.B.G. and P.W.G.).

and contributes to atmospheric cooling. However, this cyclical evapotranspiration may be considered as "excessive evaporation" meaning that the precipitation tropical trees receive is effectively not meteoric precipitation (Marryanna et al., 2017). One idea to investigate this further would be to sample *Aucoumea klaineana* under very different conditions (i.e., not just within Gabon, but

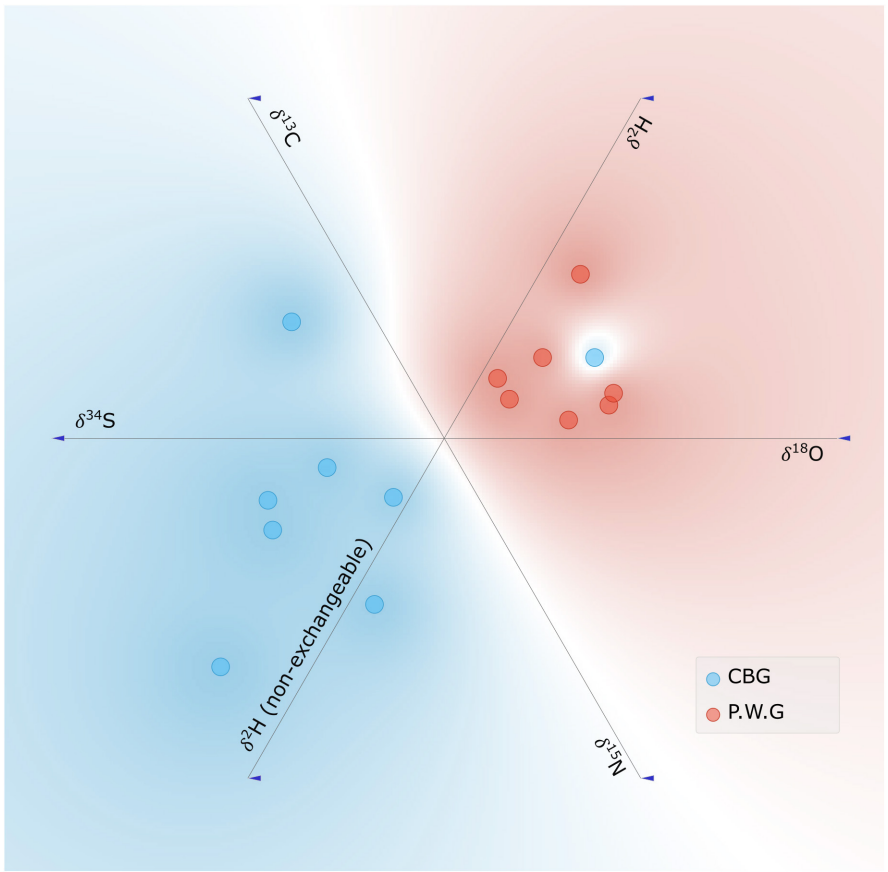

FIGURE 4 | Linear projection of the stable isotope ratios of *Aucoumea klaineana* samples from the C.B.G (n = 9) and P.W.G (n = 7) concessions.

elsewhere in the world), however, this is marred by the fact that *Aucoumea klaineana* primarily grows in Gabon and Equatorial Guinea with a small extent in Republic of Congo (Born et al., 2010). There are few plantations outside this range. Differences are evident in the nitrogen isotope ratios of the *Aucoumea klaineana* samples from the P.W.G and C.B.G concessions. Nitrogen isotope ratios are expected to increase with the age of a tree. Hietz et al. (2011) showed that a 2‰ increase in nitrogen isotope ratio was evident over an 80-year chronology of three tropical timbers. Variation in age of trees that were sampled may have played a factor in the nitrogen isotope ratio that was measured. Nitrogen deposition is increasing in the tropics (Högberg and Johannisson, 1993; Hietz et al., 2010), one of the suggested means is by tropospheric NO_x deposition. It is not inconceivable that the differences between the two concessions may be partially explained by this, however, there are insufficient samples and conditions to be able to assess if this was the case in this study. Adding nitrogen to trees is associated with losses of NO_3^{2-} from a tree and is a mechanism by which trees can become more enriched in ^{15}N (Högberg and Johannisson, 1993), this phenomenon is also related to cation loss from soil and increase in soil acidity. Different soils can be more resistant to change perhaps explaining why significant differences were observed in the nitrogen isotope ratios of *Aucoumea klaineana* on the three different soil types. It also must be considered that segregating the *Aucoumea klaineana* samples by soil type rather than by concession may, on one hand, suggest that there is a predictable overall pattern within Gabon that may aid the origin classification of unknown samples of Okoumé; on the other, it may be an arbitrary segregation and the significant differences that were observed are the result of the fact that very few samples of *Aucoumea klaineana* have been examined in this study (n = 16). Though *Aucoumea klaineana* was the best-sampled species the data only portray a snapshot of reality. Greater sampling of *Aucoumea klaineana* in the Precious Woods concession would give spatial models more certainty. Sampling other concessions and countries will give a better idea of the differentiation and classification that is possible with Okoumé. One challenge to using nitrogen isotope ratios as an origin classifier is that *Aucoumea klaineana* exists as plywood in western markets. Veneers in plywood are typically glued using formaldehyde-urea-based resins (Desch and Dinwoodie, 1996; Negro et al., 2011), some also include melamine. Urea and melamine contain nitrogen, this exogenous nitrogen must be removed for the natural nitrogen to be accessed for comparison. The method of sample preparation posed in this study is also

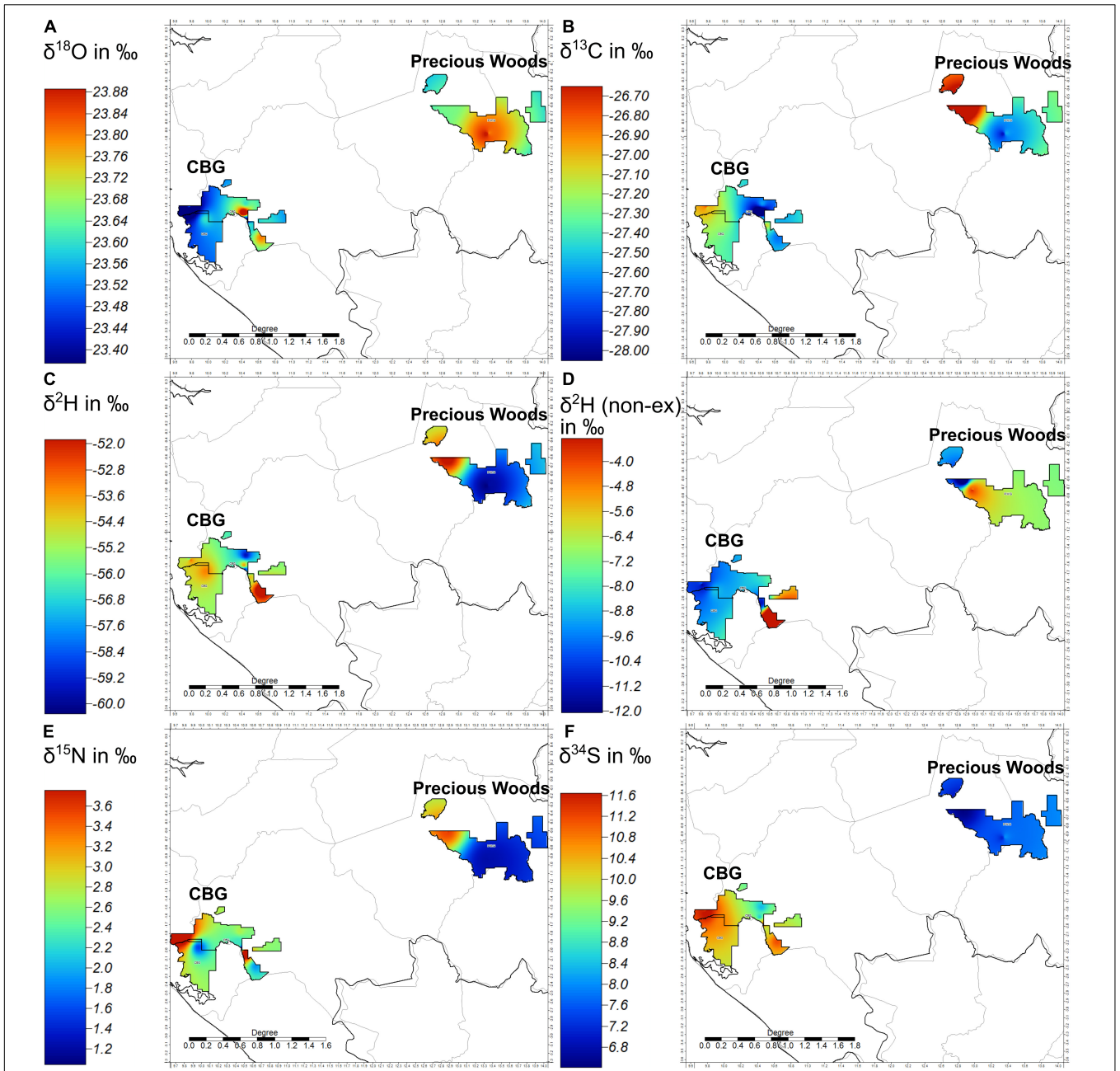

FIGURE 5 | Inverse distance weighting (global) isoscapes of *Aucoumea klaineana* stable isotope data from samples collected in the C.B.G and P.W.G concessions. **(A)** $\delta^{18}O$, **(B)** $\delta^{13}C$, **(C)** $\delta^{2}H$, **(D)** non-exchangeable $\delta^{2}H$ from cellulose, **(E)** $\delta^{15}N$, **(F)** $\delta^{34}S$.

intended to mitigate the effect of resins and other secondary metabolic compounds and therefore is a practical solution to the problems that glues present in plywood.

Sulfur isotope ratios were the best discriminator of *Aucoumea klaineana* between the two concessions. There are clear differences between C.B.G and P.W.G concessions in terms of distance from the sea, tropospheric sulfate deposition (Novák et al., 1996; Global Modeling and Assimilation Office [GMAO], 2015) and elevation that may explain the differences in observed values either individually or in combination. Soil type may also be important in separating ranges of sulfur isotope ratios in *Aucoumea klaineana* as different soils contain varying concentrations of sulfate (Jones et al., 2013). If this is the case patterns in $\delta^{34}S$ in Gabon would follow the patterns in soil type. All these potential predictors suggest that there is a higher spatial structure of sulfur isotope ratios in *Aucoumea klaineana* across Gabon that is likely to be useful at identifying the origin of Okoumé.

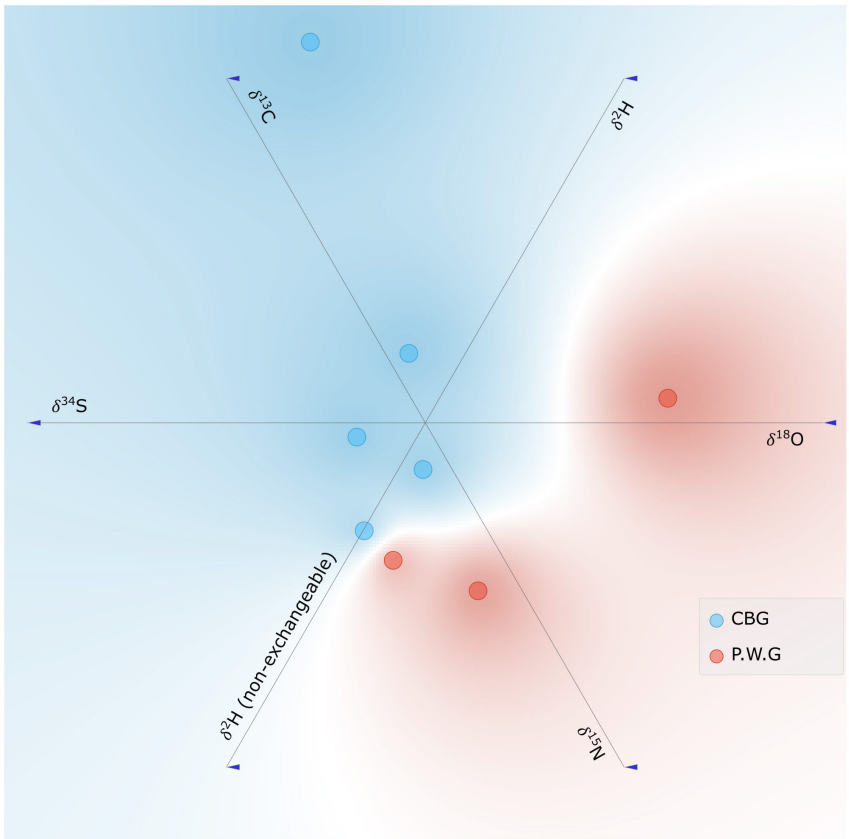

FIGURE 6 | Linear projection of *Dacryodes buettneri* stable isotope data.

Based on the results, we believe that the ranges of stable isotope ratios for *Aucoumea klaineana* for the two concessions are defined well-enough. Leavitt (2010) shows that temperate trees growing on the same site may vary between 1 and 4‰ for $\delta^{18}O$, 5 and 30‰ for $\delta^{2}H$, and 1 and 3‰ for $\delta^{13}C$. In the C.B.G concession, *Aucoumea klaineana* ranged 1.1‰ for $\delta^{18}O$, 10.1‰ for $\delta^{2}H$, 10.5‰ for $\delta^{2}H$ (non-exchangeable), and 2.4‰ for $\delta^{13}C$. In the P.W.G concession, *Aucoumea klaineana* ranged 0.8‰ for $\delta^{18}O$, 10.6‰ for $\delta^{2}H$, 11.9‰ for $\delta^{2}H$ (non-exchangeable), and 2.0‰ for $\delta^{13}C$. It is not clear what proportion of sites had greater ranges in $\delta^{18}O$, $\delta^{2}H$, and $\delta^{13}C$ in the Leavitt (2010) review or what the concession ranges from *Aucoumea klaineana* mean in this context, the isotopic ranges recorded in this study are on the lower end of the variability scale. Perhaps this means that *Aucoumea klaineana* is relatively homogenous in its isotopic composition in its growing sites, and the comparison of tropical trees with temperate trees is not ideal. Van der Sleen et al. (2017) conducted a review of tropical timber stable isotope variability. The review mainly focuses on inter-ring stable isotope variability rather than inter-tree (on the same site) stable isotope variability. The review discusses that inter-ring variation of 1–2‰ for $\delta^{13}C$, in some cases up to 3‰ is often found, and ranges of 1–5‰ for $\delta^{18}O$ are not uncommon. The review also demonstrated one extreme case of 9‰ $\delta^{18}O$ variability within a tree ring. Similar values for inter-ring variability are discussed by Leavitt (2010). Though it may be a bit of leap to try to infer that because inter-ring stable isotope variability is similar in tropical and temperate trees, inter-tree variability should be similar too, the lack of a good comparison shows the importance of publishing stable isotope data for tropical trees so that Reviews and comparisons can be made in future. It is recommended that sample quantity per site should be compared to the range of stable isotope ratios for many species and many origins to observe the relationship between the two variables. We hypothesize that this relationship is horizontal asymptotic where having a small quantity of samples per site gives the greatest uplift in knowledge (from no knowledge) about the variability or range of site initially with the benefit of increased sampling yielding diminishing returns after a certain threshold is reached. This is one of the reasons for analyzing opportunistically collected samples in low quantities (e.g., *Baillonella toxisperma, Cylicodiscus gabunensis, Guibourtia tessmannii, Lophira alata, Nauclea diderrichii, Pterocarpus soyauxii, Berlinia confusa*, and *Didelotia africana*).

Dacryodes buettneri (Synonym of *Pachylobus buettneri*)

Eight samples of *Dacryodes buettneri* were sampled in the Precious Woods (n = 3) and C.B.G (n = 5) concessions. The

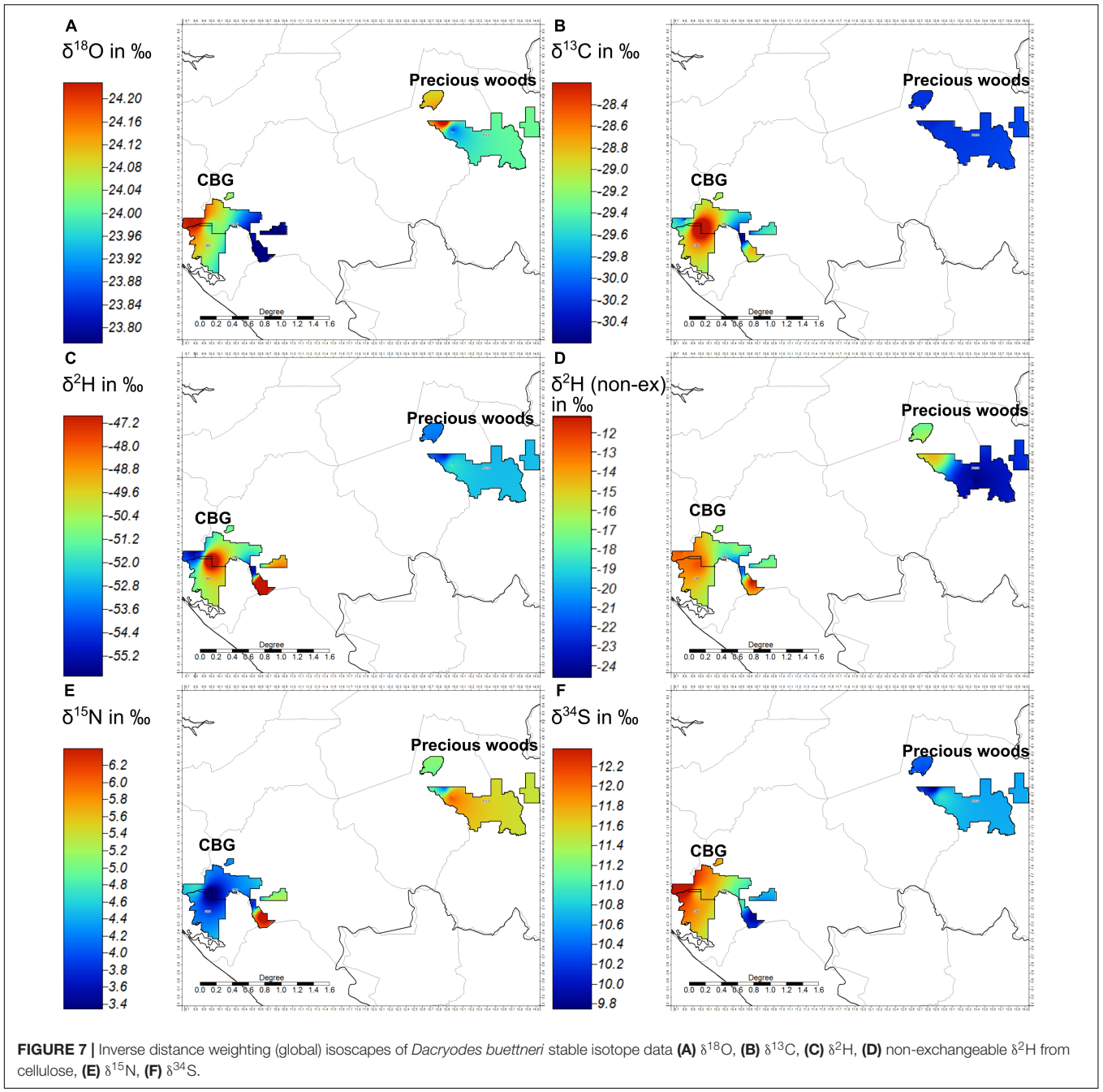

FIGURE 7 | Inverse distance weighting (global) isoscapes of *Dacryodes buettneri* stable isotope data **(A)** $\delta^{18}O$, **(B)** $\delta^{13}C$, **(C)** $\delta^{2}H$, **(D)** non-exchangeable $\delta^{2}H$ from cellulose, **(E)** $\delta^{15}N$, **(F)** $\delta^{34}S$.

range of stable isotope variability is well-defined enough for the C.B.G concession, but more samples are needed for the P.W.G concession. *Dacryodes* ranged 0.7‰ for $\delta^{18}O$, 14.4‰ for $\delta^{2}H$, 14.3‰ for $\delta^{2}H$ (non-exchangeable), and 3.3‰ for $\delta^{13}C$ in the C.B.G concession which is comparable to the ranges expected on a site as discussed by Leavitt (2010). It is likely collecting more samples of *Dacryodes* would elucidate the true differentiation possibilities between the two origins. Though multiple multivariate models were attempted with the *Dacryodes* data, it is statistically inappropriate to attempt to use three to six variables to classify eight samples from two origins. The Principal Component Analysis and the silhouette scores of the data show that there is not any real clustering of the data, this is because there aren't enough samples to define clusters. Further interpretation of the differentiation possibilities of *Dacryodes buettneri* using stable isotope ratio measurements is beyond the scope of this paper. Nonetheless, it is interesting that the sulfur isotope ratios of *Dacryodes buettneri* appear to follow a similar trend to those of the *Aucoumea klaineana* reference samples. Further collection of this timber should reveal a higher spatial structure within Gabon that can be used to classify the origin of *Dacryodes* timber.

Baillonella toxisperma, Cylicodiscus gabunensis, Guibourtia tessmannii, Lophira alata, Nauclea diderrichii, Pterocarpus soyauxii, Berlinia confusa, and *Didelotia africana*

Data presented in this report provide a first glimpse at the stable isotopic variation of the species from the concessions where they were collected from within Gabon. However, little can be stated about what overtly the data means for collections with an $n < 3$ until a point where collections are large enough to permit thorough analysis and interpretation. Ideally, collections of species/genera from concession areas should be at least five samples and as many as 10–20, perhaps more depending on the size of the concession. There are clear, significant differences between these species even though they were collected mostly on the same sites, suggesting that data from one species cannot easily be applied to another at this stage. As collections of samples and data grow, it is likely that higher-order patterns will become evident and this may permit interpretation of the origin of various species using data from another species such as using Dunbar lines (Dunbar and Wilson, 1983) to convert between datasets, or using mechanistic models to forecast data (Roden et al., 2000; Cueni et al., 2021).

Of all sampled timbers, *Pterocarpus soyauxii* showed the most negative nitrogen stable isotope ratios. This was expected as *Pterocarpus soyauxii* is a nitrogen-fixing tree and perhaps acts as a primary nitrogen source in the areas where it was sampled. The results are supported by Hietz et al. (2011) who demonstrated that leaves of leguminous trees had more negative $\delta^{15}N$ relative to non-leguminous trees. Variability in nitrogen isotope composition was also demonstrated by Hietz et al. (2011) to be influenced by sun/shade. Results from nitrogen fixers are important from an ecological perspective as they are one of the nitrogen sources for other nearby trees due to the nitrogen they fix in the soil and the distribution of the nitrogen by mycorrhiza. Mycorrhizal type varies with climate, such as arbuscular mycorrhiza which are more abundant in tropical areas such as Gabon. Steidinger et al. (2019) show that varying proportions of these arbuscular mycorrhiza exist across Gabon and between the two concessions that were sampled in this project. This may give rise to geographic variation in nitrogen isotope ratios of *Pterocarpus* and other flora and fauna that source nitrogen through mycorrhizal networks (Williamson et al., 1990). However, *Berlinia confusa* and *Cylicodiscus gabunensis* are also from the Fabaceae family yet have much more positive $\delta^{15}N$ than the reference samples of *Pterocarpus soyauxii* (**Figure 3**). If the explanation for the $\delta^{15}N$ in Fabaceae timbers is a simple as nitrogen fixing trees have lower $\delta^{15}N$ then the results from the *Berlinia confusa* and *Cylicodiscus gabunensis* suggest there is more to $\delta^{15}N$ variance in tropical timber than simply local nitrogen fixation.

CONCLUSION

Despite the limited quantity of samples and species, the data acquired establish a basis of evaluation for assessing geographic origin claims of certain forest products including plywood and veneers from Gabon. The differences in the sulfur isotope ratios of *Aucoumea* and *Dacryodes* reference samples from Precious Woods Group (P.W.G) and Compagnie des Bois du Gabon (C.B.G) forest concessions suggest that regional scale origin classification may be realized with high enough frequency of reference sample collection. Furthermore, higher resolution sampling of target species including *Aucoumea* and *Dacryodes* will address this much needed comparison as it may enable more efficient allocation of reference sampling resources. Further sampling of Burseraceae family timbers in the tropics may permit a global model for verifying their geographic origin.

FUTURE WORK

Expanding the collection of reference samples will be necessary to investigate regional classification further. More sample data will improve discrimination between concessions or regions, as well as allowing for comparison of stable isotopes both within a single sampling site and single taxa. A higher frequency of reference sample collection will also enable assessment of the suitability of specific taxa to act as isotopic proxies for other species. Natural variability of isotopic distribution within a site is still not fully understood, and it is anticipated that large scale sample collections for specific taxa will also enable a better understanding of this. So far, the study has focused on whole wood and cellulose components of timber reference samples. Several alternative analytical methods detail procedures for analyzing alternative metabolic fractions including lignin and proteins in the form of amino acids. Furthermore, isotope methods are already being used to measure the stable isotopes of position-specific atoms within a selection of molecules such as ethanol, providing higher resolution information on the source water incorporated during metabolism. Analysis of alternative fractions and position-specific isotope ratios within a molecule such as glucose or lignin may yield higher quality results and aid the discrimination of reference samples taken from concessions within the same country.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CW was repsonsible for sample planning and co-ordination with FSC. CW and GR were responsible for interpretation and production of manuscript. MG was responsible for sample collection and organization in Gabon as well as contribution of contextual information. PG was responsible for co-ordinating curation of samples as well as editing and advising on content and contributed significantly to disussions and content related to the relation of phylogenetics, wood structure and stable isotope

results. SH, LM, and MB were responsible for sample preparation and analysis and contributed the analytical method section.

ACKNOWLEDGMENTS

Thanks go out to the following people and organizations, as this research would not have been made possible without their input, collaboration, and expertise: The United States Department of State and United States Department of Agriculture for funding this research. Elizabeth Lebow, Alex Moad, and their respective colleagues at the United States Forest Service for overseeing the World Forest ID project. This work published here has been made possible by RBG Kew's Defra Plant Health License 2149/194627/5 which enables the safe import, keeping and use of plant materials which are normally prohibited due to biosecurity concerns. FSC, World Forest ID, Phil Guillery, Emily Crumley, and Nathalie Bouville organizing and collecting samples in Gabon. Staff of Precious Woods Group and Compagnie des Bois du Gabon for permitting and facilitating the collection of reference samples. Meaghan Parker-Forney and her respective colleagues at the World Resources Institute for overseeing the World Forest ID project. Jade Saunders of Forest Trends for her advisory and coordination support to the World Forest ID project. Isabella Miles-Bunch and Sara Redstone at the Royal Botanic Gardens, Kew for assisting with sample shipment documentation, receipt of samples in quarantine and their expert help in verifying declared information of field collectors. Tim Fox, Jackie Borrows, and Matt Watkinson for spending 100 s of collective hours assembling sample collection packs. Agroisolab GmbH staff for spending 100 s of collective hours preparing and measuring samples. Meg Staton, Abdullah Almsaeed et al. for developing the World Forest ID app which tracks samples from the field through Kew to analysis labs. We gratefully acknowledge funding via Defra-ICF: Building and Enhancing the Evidence Base for Nature Based Solutions to Climate Change. Project 29084. Driving innovation in forest protection and enforcement monitoring. Tackling illegal logging: creating a timber reference library to support enforcement (World Forest ID at Kew). Google Maps Terrain basemap (accessed 21.06.2021) was used to help create **Figure 1**.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2021.650257/full#supplementary-material

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Conflict of Interest: CW and GR were employed by company Agroisolab UK Ltd. SH, LM, and MB were employed by company Agroisolab GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Forests and Global Change

ORIGINAL RESEARCH
published: 08 February 2022
doi: 10.3389/ffgc.2021.645222

A Case Study to Establish a Basis for Evaluating Geographic Origin Claims of Timber From the Solomon Islands Using Stable Isotope Ratio Analysis

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Global demand for low-cost forest products is leading manufacturers and traders to source timber and wood products from vulnerable nations and delicate ecosystems. One small island nation, the Solomon Islands, is seeing exploitation of natural resources accelerating to such a point that its natural forests may be exhausted by 2036. The main causes of natural forest loss on the archipelago are unsustainable or illegal logging practices. Various laws in consumer countries require that members of industry ensure that only legally sourced timber is placed onto their respective national markets. Those that break these laws or fail to act in a way that is compliant may be subject to harsh penalties. This study aims to establish scientific data to evaluate claims that timber has originated from the Solomon Islands. This will enable Operators to carry out due diligence analysis and permit members of Law Enforcement to conduct forensic investigations. Eighty timber core samples comprising 13 different genera of tropical trees were obtained from mature trees in two sites in the Solomon Islands (Guadalcanal and Kolombangara islands) during the period August 2019 to November 2019 using a Pickering Punch sampling device. Homogenised core samples were subject to $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$, and $\delta^{34}S$ stable isotope analysis using elemental analysis-isotope ratio mass spectrometry. Additional stable isotope data from relevant taxa and geographic origins (elevation, geographic co-ordinates) were also included in this research as an initial assessment of differences in stable isotope ratios between countries. Results show that significant differences are evident in the stable isotope ratios of the sampled taxa within the Solomon Islands (Guadalcanal and Kolombangara Islands) and between other countries. These data can be used as a basis of evaluation to evaluate origin claims of timber or wood products from the Solomon Islands, particularly Kolombangara Island. Furthermore, in the right context, these data can also be used to establish whether timber or wood products declared to be from origins other than the Solomon Islands have stable isotope ratios that are consistent with data from the Solomon Islands. If not, this would suggest foreign timber/forest products are from elsewhere and are being passed-off as originating from the Solomon Islands.

Keywords: stable isotopes, Solomon Islands, tropical timber, EUTR, Lacey Act amendment, EA-IRMS

OPEN ACCESS

Edited by:
Micha Horacek, HBLFA Francisco Josephinum, BLT Wieselburg, Austria

Reviewed by:
Jessica Young-Robertson, University of Alaska Fairbanks, United States
Bernd Degen, Thünen Institute of Forest Genetics, Germany

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Specialty section:
This article was submitted to Forest Ecophysiology, a section of the journal Frontiers in Forests and Global Change

Received: *22 December 2020*
Accepted: *27 May 2021*
Published: *08 February 2022*

Citation:
Watkinson CJ, Rees GO, Hofem S, Michely L, Gasson P and Boner M (2022) A Case Study to Establish a Basis for Evaluating Geographic Origin Claims of Timber From the Solomon Islands Using Stable Isotope Ratio Analysis. Front. For. Glob. Change 4:645222. doi: 10.3389/ffgc.2021.645222

INTRODUCTION

The Solomon Islands is an archipelago comprising seven major islands: Guadalcanal, Choiseul, Santa Isabel, New Georgia, Malaita, and Makira (or San Cristobal), and a host of smaller ones. They are extensively forested with 88% of the country under forest cover. More than 80% of the country is under high rainforest with the remaining 8% mainly swamp forest, including mangroves, and upland forests. The rainforest has generally fewer timber species than surrounding countries, with around 60 reaching large sizes. The major species harvested are *Pometia pinnata*, *Calophyllum* sp., and a mixture of "whitewoods" (FAO and Pauku, 2009).

The Solomon Islands is China's second largest source of tropical timber by country (after Papua New Guinea) (Smyth, 2018), yet the landmass of the entire country is only twice the size of Beijing. By comparison, the largest source of tropical logs, Papua New Guinea constitutes roughly half of the landmass of New Guinea, the world's second largest Island at 785,753 km^2. The heavy reliance on such a small territory for timber is rapidly depleting the Solomon Islands' forest cover, biodiversity, and natural resources at rates several NGOs describe as "unsustainable." If logging continues at its current rate, the island nation is expected to be deforested by 2036 (Rarawa, 2012). Given the magnitude of the situation, there are serious questions considering how this can be happening in a way that is compliant with law. One NGO report presents evidence that the extraction of timber from the Solomon Islands is not happening legally (Global Witness, 2018).

In 2015 over 2.5 million cubic metres of logs were exported from the Solomon Islands (Global Witness, 2018), this is over 10 times the annual sustainable yield. It is difficult to perceive how such a small nation could place such a large volume of timber on the global market. There is speculation that declared imports from the Solomon Islands may have originated elsewhere, such as Papua New Guinea, and that this occurs to launder illegal timber into the global supply. However, declared import and export data for the Solomon Islands do not support this hypothesis (Global Witness, 2018). Unless timber traders who engage in illegal practices are shipping logs to the Solomon Islands for re-export, it seems the major concern is that timber originating from the Solomon Islands is often unsustainably harvested and possibly illegally harvested rather than mislabelled with respect to its geographic origin.

Much of the timber exported from the Solomon Islands is destined to be turned into plywood or other composite products for export and is not necessarily consumed domestically in China. European Union Timber Regulation (EUTR) (Regulation (EU) No 995/2010, 2016) and the Lacey Act (2008) both demand that timber and timber products are obtained legally before they are placed on the respective EU and United States markets. EUTR places penalties against companies that are the first to place timber products on the EU market if they do not enact a due diligence/due care system to ensure the timber is from a legal source. The Lacey Act may be used in criminal and civil cases to enact penalties against violators that place illegally sourced timber on the United States market.

The main islands/regions for logging in the Solomon Islands are: Western Province (includes the islands of Vella Lavella, Kolombangara, New Georgia, Rendova, Vangunu, and Ngatokae), Choiseul Province, Isabel Province, Guadalcanal Province, Malaita Province, and Makira Province (FAO and Pauku, 2009). Kolombangara Island is the base of operations of Kolombangara Forest Products Ltd. (KFPL) and, to date, this is the only Forest Stewardship Council (FSC) certified source of timber within the Solomon Islands. With FSC's mandatory chain of custody, sustainability requirements, regular audits, GPS mapping of individual trees amongst many other facets, FSC certified timber from KFPL has negligible risk of illegality. This is contrary to timber from other islands within the Solomon Islands which have elevated risks of illegality. Therefore, being able to verify that timber is from Kolombangara Island is a way to demonstrate that the timber is likely legal.

The ambitions of this research were to define the ranges of stable isotope ratios from multiple species of trees from Kolombangara Island by analysing timber samples extracted from living trees. Data from samples collected from origins other than Kolombangara Island allow for the demonstration of differences which is useful to imply further potential. Analysis of the data helps us to:

- Assess what differences are available in the stable isotope ratios of trees from different provinces within the Solomon Islands and outside of the Solomon Islands.
- Utilise these methods to check whether the declared origin of the Solomon Islands timber is consistent with data from the Solomon Islands.

If significant differences in the stable isotope ratios of trees from different provinces within the Solomon Islands are evident, this will be of great benefit to legal investigation teams in demonstrating that seized timber is illegal or at risk of being illegal. If the isotope ratios of trees from within different provinces of the Solomon Islands are relatively homogenous, this would mean that it may not be necessary to collect reference samples from all provinces to establish that the timber is from the Solomon Islands. However, it could mean that to question its legality, further evidence would be required to demonstrate that timber is not from Kolombangara Island.

Stable isotope analysis is a widely accepted analytical discipline and has a long history, with many laboratories adopting the technology around the world. Since the beginning of the 21st century, the technique has become well established as a means of verifying the origin of food and drink (Boner and Förstel, 2004; Kelly et al., 2005; Heaton et al., 2008; Pilgrim et al., 2010; Li et al., 2015) and is established as an analytical technique that can be used in legal cases (Camin et al., 2017). The same principles used to authenticate food were later applied to timber provenance research (Boner et al., 2007; Keppler et al., 2007; Horacek et al., 2009; Kagawa and Leavitt, 2010; Gori et al., 2012, 2018; Rees, 2015; Watkinson et al., 2020). Verifying the origin of timber typically depends on comparing an unknown sample against an authentic reference database for a region or territory. The technique is used routinely to assess legality, compliance with

labelling legislation (DEFRA, 2018; Forest Trends, 2019), and its use to conduct due diligence is advocated by EUTR (Regulation (EU) No 995/2010, 2016). The United Kingdom Office for Product Safety and Standards (OPSS) within the Department for Business, Energy and Industrial Strategy (BEIS) use this technique to ascertain the veracity of timber origin claims.

MATERIALS AND METHODS

Sampling

Kolombangara Forest Products Ltd., provided FSC with a list of species ranked in importance in terms of their own supply and importance to the Solomon Islands. This list was comparable to the list of economically important species published in the Solomon Islands Forestry Outlook (FAO and Pauku, 2009). The top 10 species intended for sampling were a mix of native and introduced trees including:

- *Eucalyptus deglupta*
- *Gmelina arborea*
- *Campnosperma brevipetiolatum*
- *Swietenia macrophylla*
- *Terminalia calamansanai* and *Terminalia brassii*
- *Agathis* sp.
- *Tectona grandis*
- *Acacia* sp.
- *Calophyllum inophyllum*
- *Palaquium* sp.

In addition to this, Solomon Islands Law prohibits the export of unprocessed logs of *Vitex cofassus, Intsia bijuga, Gmelina moluccana*, and *Pterocarpus indicus* (Australian Government, 2014; Global Witness, 2018) meaning that samples of these trees would also prove valuable.

Sixty-eight samples of timber were collected on Kolombangara Island in August, September, and November of 2019. Twelve samples of timber were also taken from Guadalcanal Island in September and November 2019 for analysis. Samples were collected by trained collectors who were able to identify the species or genus of the tree in the field. Herbarium voucher material was collected for some specimens, but not all.

A Pickering Punch (Agroisolab UK, Welburn, United Kingdom), a type of hammer-driven bore, was used to collect cores of timber 12–18 cm in length and 1.3 cm wide from living trees. Samples were then transferred from the Pickering Punch into cardboard tubes along with silica gel which in turn, were sealed inside 500 ml evacuated bags to aid drying and protect the sample from the humid air in the local environment. August and September samples were collected using 30 g silica gel per plug and no perforations were made in the acid-free cardboard tubes, however, field tests found this did not adequately facilitate drying, and some cores presented with hyphae upon receipt at the Plant Quarantine Unit at the Royal Botanic Gardens, Kew, United Kingdom. Extensive rot is a concern with respect to stable isotope analysis, Filley et al. (2002) demonstrates that brown rot fungi can cause significant differences in the $\delta^{13}C$ in lignin and polysaccharides in spruce sapwood over a sufficient timespan, therefore it was necessary to treat samples to prevent unwanted degradation and re-consider collection packs.

Collecting samples of biological material, such as wood, from the tropics without spoilage is challenging due to the humidity and biodiversity of tropical rainforest. Controlling for mould was therefore equally challenging. To prevent mould growth, collection packs were redesigned for the November collection to allow for better drying without the need to recycle the silica gel, this included 30–40 perforations 6 mm in diameter in each cardboard tube and increasing the mass of silica gel to 100 g per plug, as well as drying and replacing/recharging silica gel prior to sample collection. Samples were shipped to the Royal Botanic Gardens at Kew, destined for the World Forest ID (WFID) collection (Gasson et al., 2020), and were held in quarantine pending approval. To eliminate the risk of pathogenic fungi entering the United Kingdom, samples were heated to 121°C at 15 psi for 30 min before they were released into the WFID collection for storage and analysis.

GPS data, photographs of the trees and leaves, descriptions, and comments about the sampled trees were recorded in a mobile phone application named TreeSnap (Crocker et al., 2019; TreeSnap version 1.15.3 Department of Entomology and Plant Pathology, University of Tennessee, Institute of Agriculture, Knoxville, TN, United States). The data from this collection have since been moved to the WFID application (World-ForestID version 1.2.0, Department of Entomology and Plant Pathology, University of Tennessee, Institute of Agriculture, Knoxville, TN, United States). **Figure 1** shows the locations of the samples that were collected from Kolombangara Island.

Additionally, this report contains data from: four samples of *T. grandis* from Papua New Guinea which were collected using an electric drill (Makita BDF 451, Nagoya, Japan) and a 20 cm long 10 mm diameter auger in April 2012 near the Adelbert mountain range, 10 samples of *Calophyllum* sp. from Kolombangara Island that were collected by KFPL in 2015 as part of an FSC project, 20 samples of *Calophyllum* sp. from Yang Zai Forestry in southern Taiwan that were collected by FSC in November 2017, two samples of *P. pinnata* from New Britain (Papua New Guinea, Bismarck Archipelago) in May 2020, and six samples of *Pterocarpus soyauxii* that were obtained from the Precious Woods Group (PWG) and Compagnie des Bois du Gabon (CBG) concessions in Gabon in June 2019 (**Figure 2**). Samples from Gabon were collected as part of an effort to define the stable isotope ratios of various species in two FSC concessions and are also evaluated in Watkinson et al. (2021). These data are to be used as a preliminary assessment of differences between geographic origins. At present, the collections of reference samples are not extensive, and any inferences drawn from their evaluation can only be appraised in the context of their current limitations.

Measurement

Any samples that were affected by hyphae had the mould removed prior to preparation in accordance with advice in Horacek et al. (2018) and Beeckman et al. (2020). Samples were initially dried at 103°C before grinding/drilling. Samples

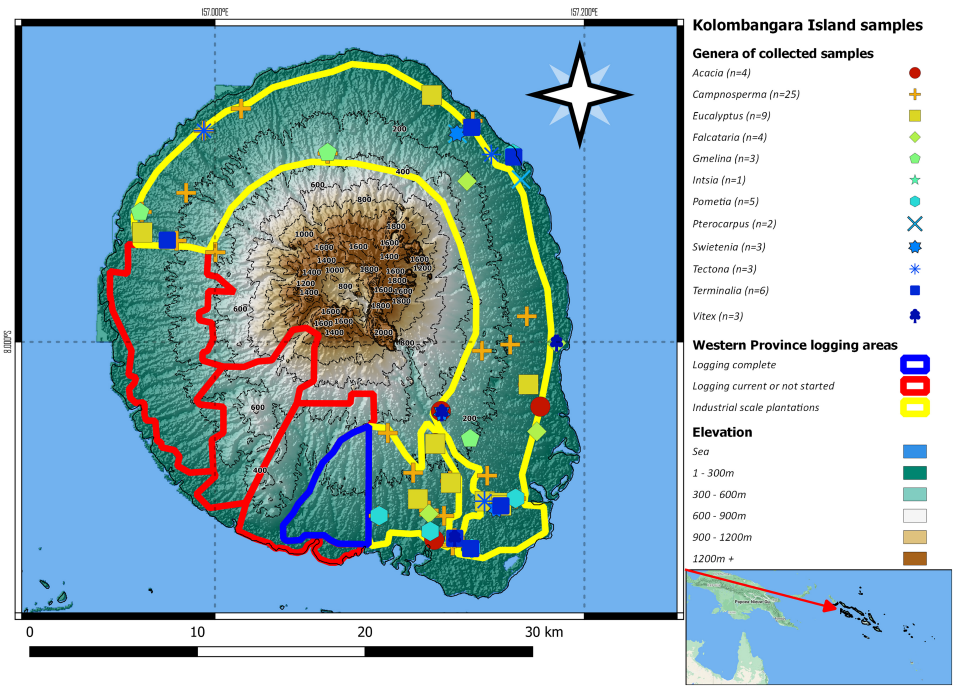

FIGURE 1 | Map of Kolombangara Island with the locations and taxa of samples that were collected during the period September 2019–November 2019. Spatial data sources: Global Administrative Dataset (GADM), ASTER GDEM (ASTER GDEM is a product of METI and NASA), FAO and Pauku (2009), and Google Terrain.

were drilled using a 7 mm drill bit to the depth of 10–13 cm from the bark side to the pith side to grind them to a coarse powder. All materials were subsequently milled into a fine powder using a ball-mill (Retsch MM220–Haan, Germany). The powder was extracted in a soxhlet apparatus over 6 h with non-polar (dichloromethane) and polar (methanol) solvents which were then dried in a laboratory-type drying cabinet for at least 1 h. Finally, the samples were stored in air-tight sample vials and weighed for analysis.

To avoid equilibration or humidity effects in the oxygen and hydrogen analysis, the weighed-in samples were equilibrated overnight in desiccators with a defined humidity of 10.6%. Afterwards the samples were vacuum dried for at least 2 h.

Sample measurements were corrected against in-house standards at the beginning, middle, and end of each measurement run. In-house standards are traceable back to certified reference materials enabling measurements to be reported relative to an internationally defined standard; for hydrogen and oxygen isotope ratio analysis, Vienna Standard Mean Ocean Water (VSMOW) is used. For carbon isotope ratio analysis, Vienna Pee Dee Belemnite (VPDB) is used. For sulphur isotope ratio analysis Vienna Canyon Diablo Troilite (VCDT) is used. The in-house standards used were 1,4-Dihydroxyanthraquinone (Merch-Schuckhardt) for $\delta^{18}O$ and $\delta^{2}H$, L-leucine (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for $\delta^{13}C$ and $\delta^{15}N$, and L-cysteine (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for $\delta^{34}S$. In-house standards are also controlled in Agroisolab's free intercomparison testing platform "Agroisolab-KPT" which routinely operates with over 20 isotope laboratories. Measurements are reported in per mil (‰) and were made in accordance with processes outlined in Boner et al. (2007).

$\delta^{18}O$ and $\delta^{2}H$ measurement was performed using two Isotope Ratio Mass Spectrometers (IRMS) in a master/slave configuration (Isoprime, Elementar, Cheadle, United Kingdom) with each IRMS measuring one isotope ratio; $\delta^{18}O$ or $\delta^{2}H$. This configuration provides excellent stability because the magnetic field and accelerating voltage remain constant on each IRMS. The working temperature for pyrolysis is >1530°C and HT-PyrOH is performed with a covalently bonded silicon carbide tube (patented by Agroisolab GmbH, Julich, Germany) filled with glassy carbon chips and coal powder. The mode Combined Uncertainty (u_c) for $\delta^{18}O$ was 0.2‰ and for $\delta^{2}H$ was 1.3‰.

$\delta^{13}C$ measurement was performed using an Elemental Analyser (EA3000, Eurovector, Milano, Italy) in combination with IRMS (Nu Horizon, NU-Instruments—Wrexham, Wales). The working temperatures are 1021°C for oxidation and 600°C for reduction. Reduction is carried out in the presence of copper. The mode Combined Uncertainty (u_c) for $\delta^{13}C$ was 0.1‰.

$\delta^{34}S$ measurement was performed using an Elemental Analyser (EA3000, Eurovector, Milano, Italy) with IRMS (Isoprime, Cheadle, United Kingdom). A one tube combustion (oxidation and reduction in one tube) is used to solve issues caused by SO_3. Combustion water is directly trapped with magnesium perchlorate at the end of the tube. The working temperature is 1000°C. The mode Combined Uncertainty (u_c) for $\delta^{34}S$ was 0.2‰.

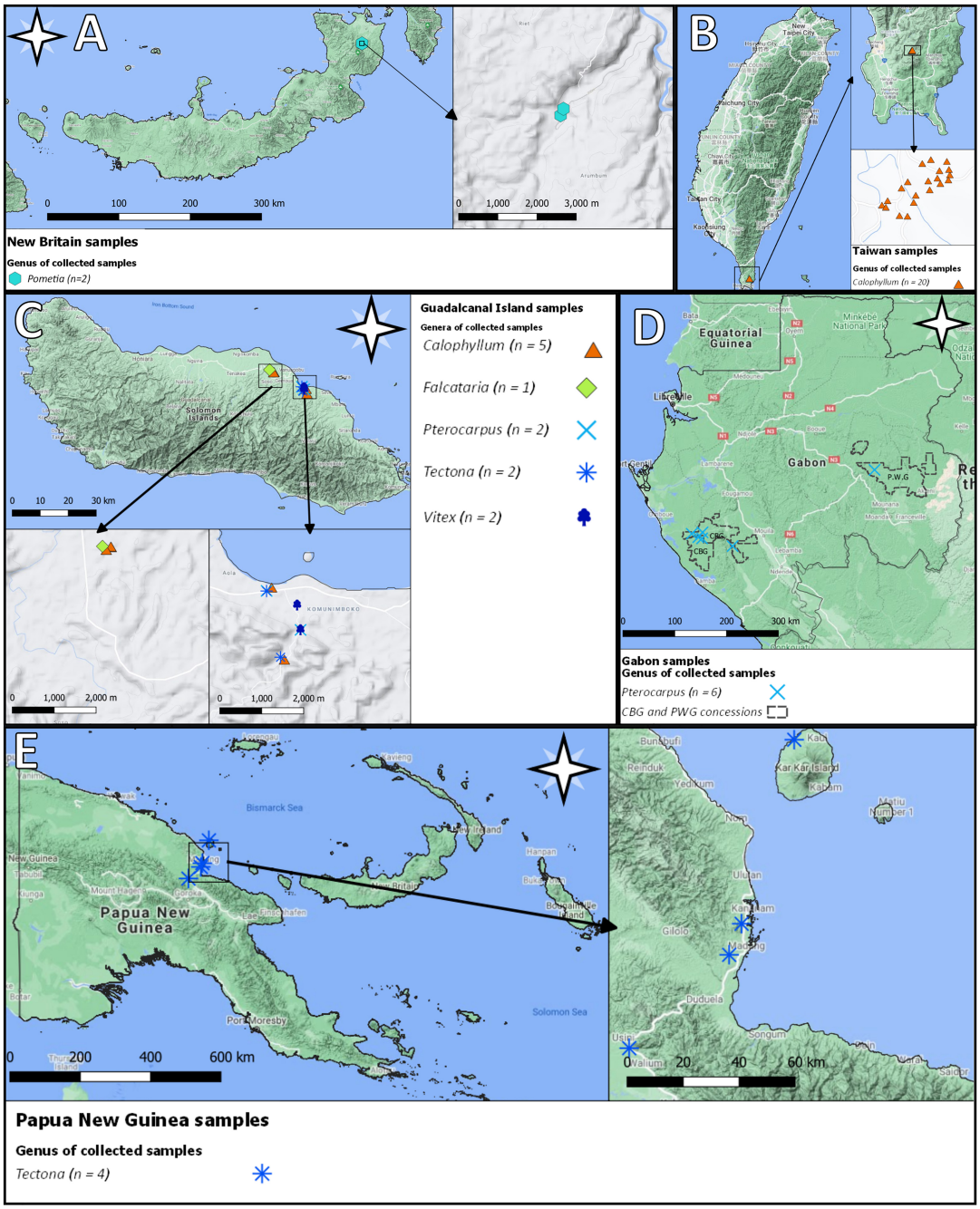

FIGURE 2 | Locations of reference samples in **(A)** New Britain (Papua New Guinea), **(B)** Taiwan, **(C)** Guadalcanal Island (Solomon Islands), **(D)** Gabon, and **(E)** Papua New Guinea. Spatial data sources: Global Administrative Dataset (GADM), and Google Terrain.

Data Analysis

As many of the taxa analysed in this project had not been previously analysed by Agroisolab or other laboratories, and the quantities of samples were relatively low, multi-variate analysis methods were not used to help draw conclusions from the data. Instead, univariate analyses were performed to show differences between distributions of means such as Student's *T*-test and ANOVA, or to assess co-variance such as regression as well as boxplot visualisations (Microsoft Excel 2016, Microsoft Corporation, Redmond, WA, United States). Three well-sampled taxa (*E. deglupta*, *Terminalia* spp., and *C. brevipetiolatum*) were assessed in SAGA GIS 2.3.2 (Departments for Physical Geography, Hamburg and Göttingen, Germany) for spatially related patterns in their data using variogram analysis (variance/distance) and representing interpolated data using Universal Kriging (Global). This method was used to

describe variance/distance related patterns in the data *via* graphical representation.

RESULTS

In most analysed taxa, oxygen stable isotope ratios ranged between approximately 19.5 and 21.5‰ (**Table 1**). There are differences in the mean values of the oxygen isotope ratios of the taxa, ANOVA has a low *P*-value (<0.05) indicating that the differences are significant. However, some results show similar means such as *Vitex* and *Eucalyptus* which are not significantly different as the means are nearly identical. *Acacia* stands out as being significantly enriched in ^{18}O relative to most other sampled timbers in the dataset. The greatest differences in oxygen isotope ratios exist between *Acacia*, *Vitex,* and *Eucalyptus* (2.4‰ difference between means). A wide range of hydrogen stable isotope ratios is evident among the different taxa indicating that there appears to be species effect in the data, i.e., different taxa in the same region may have significantly different stable isotope ratios.

Acacia samples from Kolombangara Island had remarkably negative carbon stable isotope ratios in comparison to other analysed taxa from the island. Several ranges of $\delta^{13}C$ isotope ratios are evident among the different samples of timber, further supporting the hypothesis that different trees on the same site can have significantly different stable isotope ratios. Reference samples of *Acacia* show two extremes relative to the dataset, significantly negative $\delta^{13}C$ stable isotope ratios and significantly enriched $\delta^{18}O$ isotope ratios.

A remarkably wide range of sulphur isotope ratios was evident among the sampled taxa ranging from approximately +6‰ in *Calophyllum* to +15‰ (11‰ range) in *Campnosperma*. Even taking the range into context, all samples can be said to be enriched in ^{34}S. The wide ranges of values between different taxa can also be found in the $\delta^{2}H$ and $\delta^{13}C$ isotope ratios of the samples. ANOVA of *Acacia*, *Pometia*, *Eucalyptus*, *Vitex*, *Calophyllum*, *Tectona*, *Terminalia*, *Campnosperma*, and *Falcataria* $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$, and $\delta^{34}S$ yielded P values less than

TABLE 1 | Stable isotope ratio data from taxa sampled in the Solomon Islands, Gabon, Taiwan, Papua New Guinea, and New Britain.

Origin	Taxa	*N* samples	$\delta^{18}O$ vs. VSMOW (‰)			$\delta^{2}H$ vs. VSMOW (‰)			$\delta^{13}C$ vs. PDB (‰)			$\delta^{34}S$ vs. CDT (‰)		
			Mean	σ	Range	Mean	σ	Range	Mean	σ	Range	Mean	σ	Range
Kolombangara Island														
	Acacia mangium	4	+22.3	0.5	1.2	−81.5	9.7	22.9	−31.1	0.4	0.9	+10.0	0.8	1.7
	Campnosperma sp.	25	+20.6	0.4	1.7	−82.8	3.5	13.0	−28.3	0.7	2.7	+11.3	2.3	8.4
	Eucalyptus deglupta	9	+19.9	0.4	1.2	−104.7	8.7	25.3	−29.2	0.5	1.5	+7.4	1.1	3.1
	Falcataria sp.	4	+20.2	0.3	0.6	−98.2	2.6	5.3	−27.9	0.3	0.7	+12.6	0.5	1.1
	Gmelina sp.	3	+21.0	0.2	0.4	−82.8	8.0	15.1	−28.5	0.6	1.2	+7.9	1.2	2.3
	Intsia bijuga	1	+21.1			−75.9			−27.2			+7.4		
	Pometia pinnata	5	+20.5	0.5	1.1	−84.2	3.6	8.1	−29.6	0.5	1.2	+8.9	0.9	2.2
	Pterocarpus indicus	2	+21.3		0.2	−67.4		5.7	−27.0		0.3	+11.8		0.1
	Swietenia macrophylla	3	+21.5	0.5	0.9	−70.8	4.5	8.9	−29.1	0.9	1.8	+9.2	0.9	1.6
	Tectona grandis	3	+20.3	0.4	0.7	−68.8	8.5	16.8	−28.5	0.7	1.4	+10.3	1.1	2.2
	Terminalia sp.	6	+20.4	0.4	1.2	−69.1	13.8	37.7 (19.7)*	−28.3	0.8	2.1	+10.8	2.4	6.6
	Vitex sp.	3	+19.9	0.2	0.3	−79.8	4.6	8.3	−29.0	0.3	0.6	+11.2	0.6	1.0
	Calophyllum sp.	10	+20.6	0.3	0.8	−67.0	5.1	14.1	−28.5	0.7	2.1	+7.0	0.8	2.7
Guadalcanal Island														
	Calophyllum sp.	5	+21.2	0.2	0.7	−75.7	6.1	15.9	−28.4	0.7	1.9	+5.6	3.8	10.3 (2.0)*
	Falcataria sp.	1	+22.0			−98.1			−27.3			+12.0		
	Pterocarpus indicus	2	+21.7		0.2	−72.0		1.3	−26.8		0.7	+7.8		1.3
	Tectona grandis	2	+20.9		1.2	−63.9		9.9	−28.2		1.6	+7.5		1.6
	Vitex sp.	2	+20.0		0.1	−75.9		0.3	−29.5		0.1	+9.4		0.5
Gabon														
	Pterocarpus soyauxii	6	+23.2	0.4	0.9	−49.8	7.1	18.0	−28.0	1.4	4.0	+11.9	1.0	2.7
Taiwan														
	Calophyllum sp.	20	+21.7	0.3	1.0	−56.2	5.0	15.5	−28.3	1.4	4.0	+8.4	0.9	1.8
Papua New Guinea														
	Tectona grandis	4	+18.2	1.0	2.2	−88.6	16.3	36.5	−28.7	0.6	1.2	+4.1	3.7	7.3
New Britain														
	Pometia pinnata	2	+19.6		0.1	−89.6		7.0	−28.0		1.8	+3.2		0.9

**Values within brackets are the ranges if outlier values are removed.*

0.05 in all cases and indicates that there is a significant difference between the mean values of each stable isotope ratio of each genus. On its own, this is not conclusive as the sample size for each genus was relatively small but is indicative that different taxa may be expected to have different stable isotope ratio profiles even on an island as small as Kolombangara. It should also be noted that the mean range of sulphur isotope ratios in each genus from Kolombangara Island is not so wide (3.0 ± 2.35‰).

$\delta^{18}O$ and $\delta^{2}H$ are expected to be well correlated systems where excessive evaporation does not occur Craig (1961). One may reason that this trend should be observable in $\delta^{18}O$ and $\delta^{2}H$ in wood given that oxygen and hydrogen in timber originate from water. In some sampled taxa this trend is evident, however, in *Eucalyptus* sp. (Pearson's R = 0.03), *T. grandis* (Pearson's R = −0.40), *Falcataria* sp. (Pearson's R = 0.30), and *Campnosperma* sp. (Pearson's R = −0.06), this relationship is either weak or not currently evident. This may be due to the low level of sampling, however, 25 samples of *Campnosperma* sp. were obtained from Kolombangara Island. It is more likely that the relationship cannot be established due to the lack of an adequate range of values being present in the reference samples. To evaluate these relationships further in these taxa, it will be necessary to obtain and analyse more samples from origins that are far from the Solomon Islands (i.e., where different ranges of values are more likely to occur). This may mean sampling timbers from countries where the species is not of concern with respect to legality (e.g., plantation *Eucalyptus*) to develop better models for evaluation.

Though the values of the stable isotope ratios of the four taxa shown in **Figure 3** are different to one another, there appears to be an offset between species in the oxygen and hydrogen stable isotope ratios. This may be of use as it could enable development of statistical models built around a single taxon primarily with lower sampling occurring on other taxa and simply relating the two to one another if this trend is further evaluated. These relationships have been referred to as "Dunbar lines" (Dunbar and Wilson, 1983). If the offset between the taxa can be controlled over a sufficiently wide geographic range and range of values with at least 30 locations per taxa, it should be possible to calculate $\delta^{18}O$ and $\delta^{2}H$ between different types of wood. This is advantageous as it would alleviate the heavy burden of comprehensively sampling all concerned species in every possible location.

There are significant differences in the $\delta^{18}O$, $\delta^{2}H$, and $\delta^{34}S$ stable isotope ratios of *Calophyllum* from regions that were sampled in Kolombangara Island, Guadalcanal Island, and Taiwan as judged by ANOVA ($P < 0.05$). This is promising as not only can a differentiation be established between *Calophyllum* spp. from two countries (Taiwan and Solomon Islands), but the data suggest that there is a chance there may be significant differentiation in timbers from different islands within the Solomon Islands (**Figure 4**). It would only be possible to discover this trend with further sampling of other islands that are important for the logging industry within the Solomon Islands, however, a method that is able to differentiate timber on an island basis would likely be very useful to support questions of legality on timber products that originated from the Solomon Islands.

Campnosperma brevipetiolatum Volkens

Campnosperma brevipetiolatum Volkens was the most frequently sampled species in the project with 25 samples collected from different parts of Kolombangara Island. $\delta^{18}O$, $\delta^{2}H$, and $\delta^{34}S$ isotope data for *Campnosperma* sp. have the widest ranges of all sampled taxa. This may be because the data paint a more realistic depiction of the variance that occurs on the island and perhaps should be used as a template in that regard. **Figure 5** shows there are very weak trends in the stable isotope ratios of the trees across the island judging by the patterns and ranges evident in

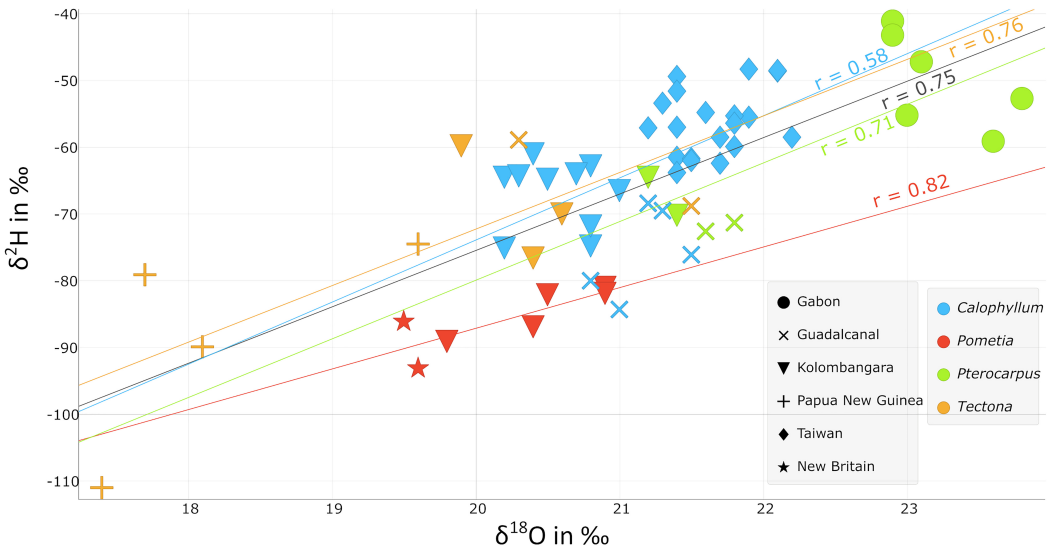

FIGURE 3 | Scatter plots of the $\delta^{18}O$ and $\delta^{2}H$ stable isotope ratios of *Calophyllum* spp. (*n* = 35), *Pometia pinnata* (*n* = 7), *Pterocarpus* spp. (*n* = 10), and *Tectona grandis* (*n* = 9) from the Solomon Islands, Papua New Guinea, Taiwan, and Gabon.

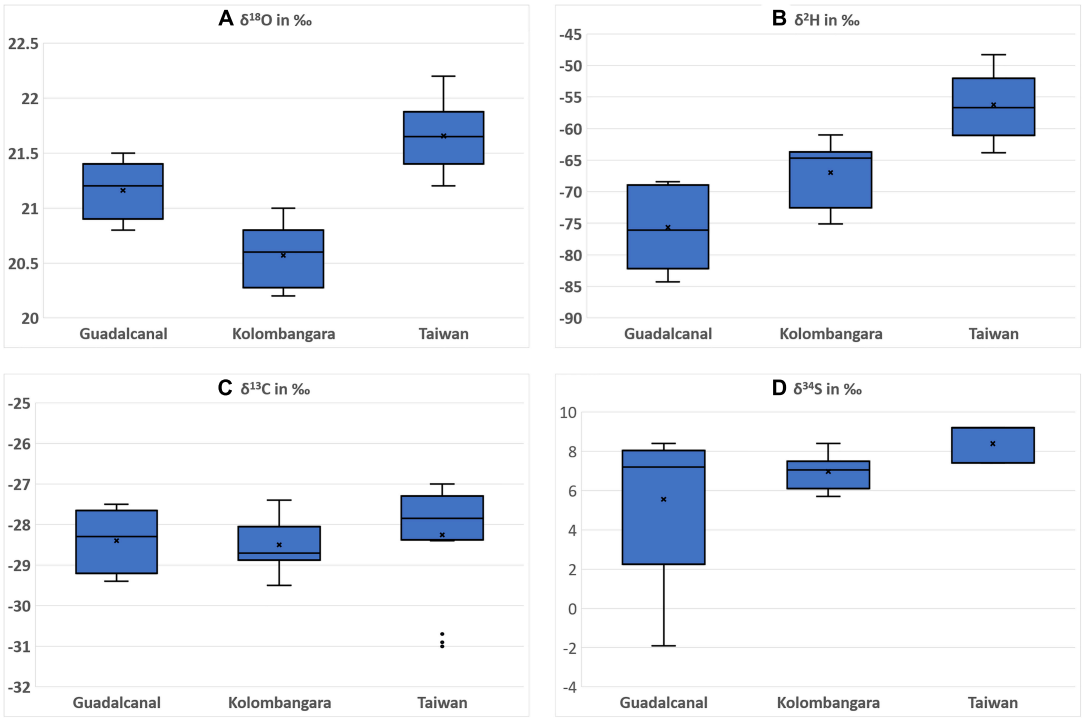

FIGURE 4 | Boxplots of the **(A)** oxygen stable isotope ratios, **(B)** hydrogen stable isotope ratios, **(C)** carbon stable isotope ratios, and **(D)** sulphur stable isotope ratios of *Calophyllum* spp. from Guadalcanal Island (SI) (n = 5), Kolombangara Island (SI) (n = 10), and Taiwan (n = 20).

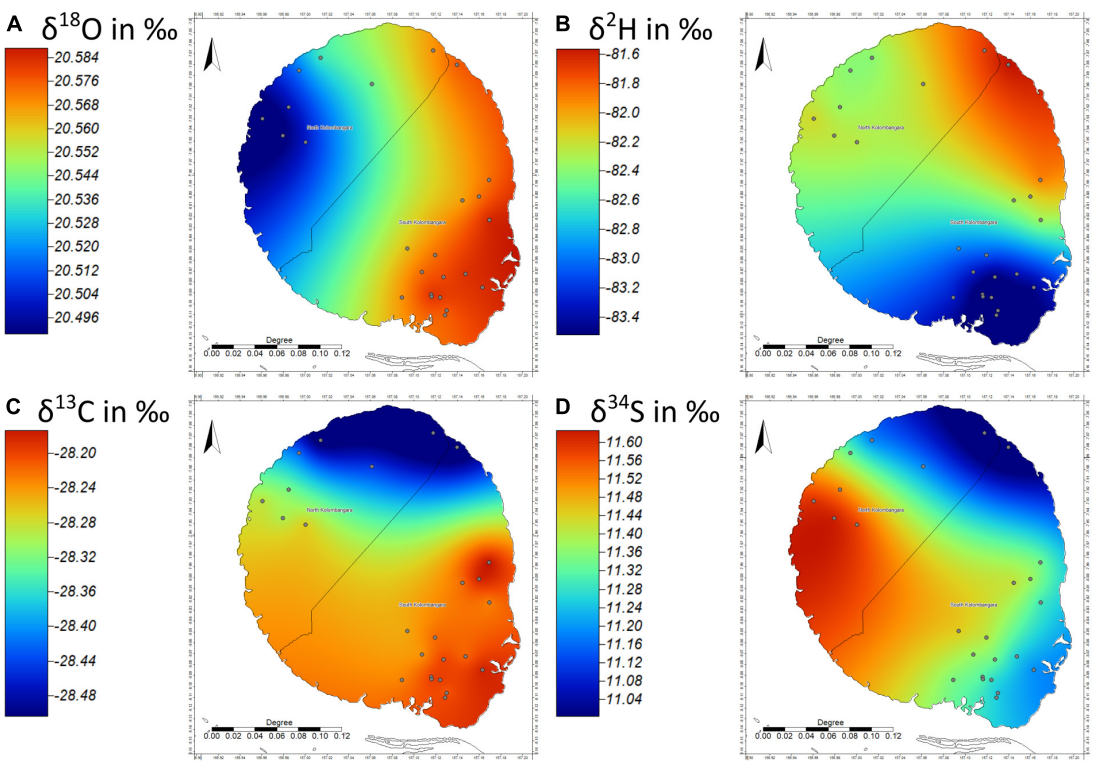

FIGURE 5 | Universal Kriging Isoscapes of **(A)** $\delta^{18}O$, **(B)** $\delta^{2}H$, **(C)** $\delta^{13}C$, **(D)** and $\delta^{34}S$ of *Campnosperma brevipetiolatum* from Kolombangara Island (n = 25), Solomon Islands. Note that the range displayed in the *color* scales is within the u_c for $\delta^{18}O$, $\delta^{13}C$, and $\delta^{34}S$ and is illustrative of a lack of significant difference across the island.

the isoscapes. The overall spatial trend in the data as represented by the Universal Kriging models demonstrates that the ranges of $\delta^{18}O$, $\delta^{13}C$, and $\delta^{34}S$ are within the Combined Uncertainty (u_c). Empirical variogram analysis shows that differences in the values do not appear to be strongly related to distance as regressions of the $\delta^{18}O$ and $\delta^{2}H$ isotope ratios with the elevation of the samples yield Pearson's R scores of −0.24 and −0.02, respectively. This means that distance between sampling sites and/or elevation of the sampled trees does not describe much of the observable variation in the *Campnosperma* data suggesting that the observed variance is due to a combination of random natural variation and measurement uncertainty in *Campnosperma* sp. from Kolombangara Island. If the variation is due to natural variation, the ranges of values in the $\delta^{18}O$, $\delta^{2}H$, and $\delta^{13}C$ isotope ratios of the samples, 1.7, 13, and 2.7‰, respectively, are comparable to ranges of variation in stable isotope ratios between different trees on the same site (intra-site variation) as reviewed by Leavitt (2010). This is of benefit as it illustrates that an *n* of 25 samples in an area as large as Kolombangara Island appears sufficient to characterise stable isotope variation in accordance with the methods used.

Eucalyptus sp.

Eucalyptus was the third most sampled taxon in this project (*n* = 9). Overall, *Eucalyptus* samples had the most negative $\delta^{2}H$ isotope ratios (mean = −104.7 ± 8.7‰) and the second most negative $\delta^{34}S$ isotope ratios (**Figure 6**). Empirical Variogram analysis of the *Eucalyptus* data shows that the distance between samples can explain up to 44% of the variation in the data of the $\delta^{2}H$ isotope ratios and 12% in the $\delta^{18}O$ isotope ratios. Variation in hydrogen isotope ratios is often linked to elevation (Bowen and Revenaugh, 2003), however, the Pearson's R for $\delta^{18}O$ and $\delta^{2}H$ with elevation was −0.29 and −0.47, respectively, suggesting that elevation explains only a small portion of the observable variance in the data. The fact that the Pearson's R had a negative result in both cases is also surprising as this suggests that elevation and $\delta^{2}H$ or $\delta^{18}O$ are inversely related on Kolombangara Island. This contrasts with expectations as normally $\delta^{2}H$ or $\delta^{18}O$ become more negative with increasing elevation rather than becoming more enriched.

Pometia pinnata

The samples of *P. pinnata* from New Britain are more depleted in ^{18}O and ^{2}H than samples from Kolombangara Island (**Figure 7**). It does not appear that the reason for this difference is due to the elevation of where the trees grew as the samples from New Britain were collected at approximately 200 m elevation whereas the samples from Kolombangara were collected between 100 and 200 m elevation. The $\delta^{18}O$ and $\delta^{2}H$ are well-correlated and have a Pearson's R of 0.82 (**Figure 3**), this is expected

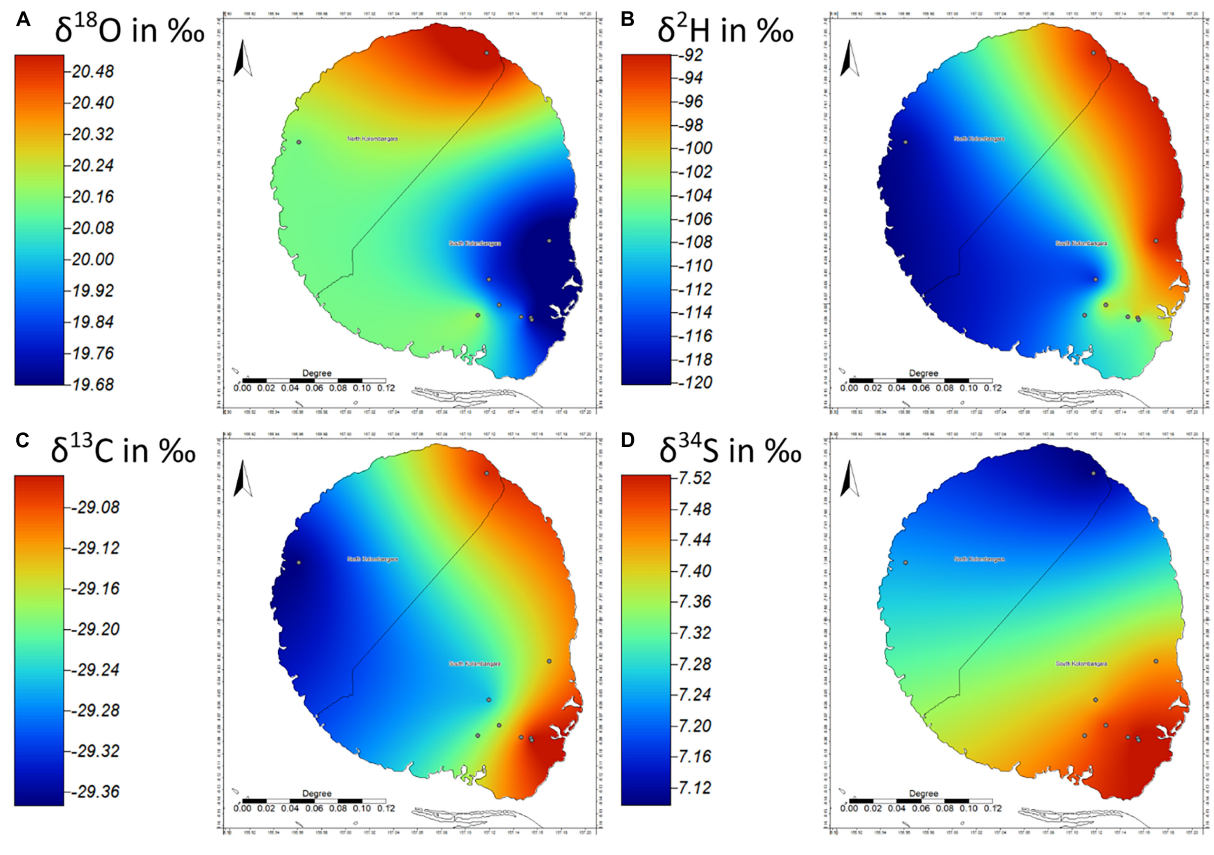

FIGURE 6 | Isoscapes of **(A)** $\delta^{18}O$, **(B)** $\delta^{2}H$, **(C)** $\delta^{13}C$, and **(D)** $\delta^{34}S$ of *Eucalyptus* sp. from Kolombangara Island (*n* = 9), Solomon Islands.

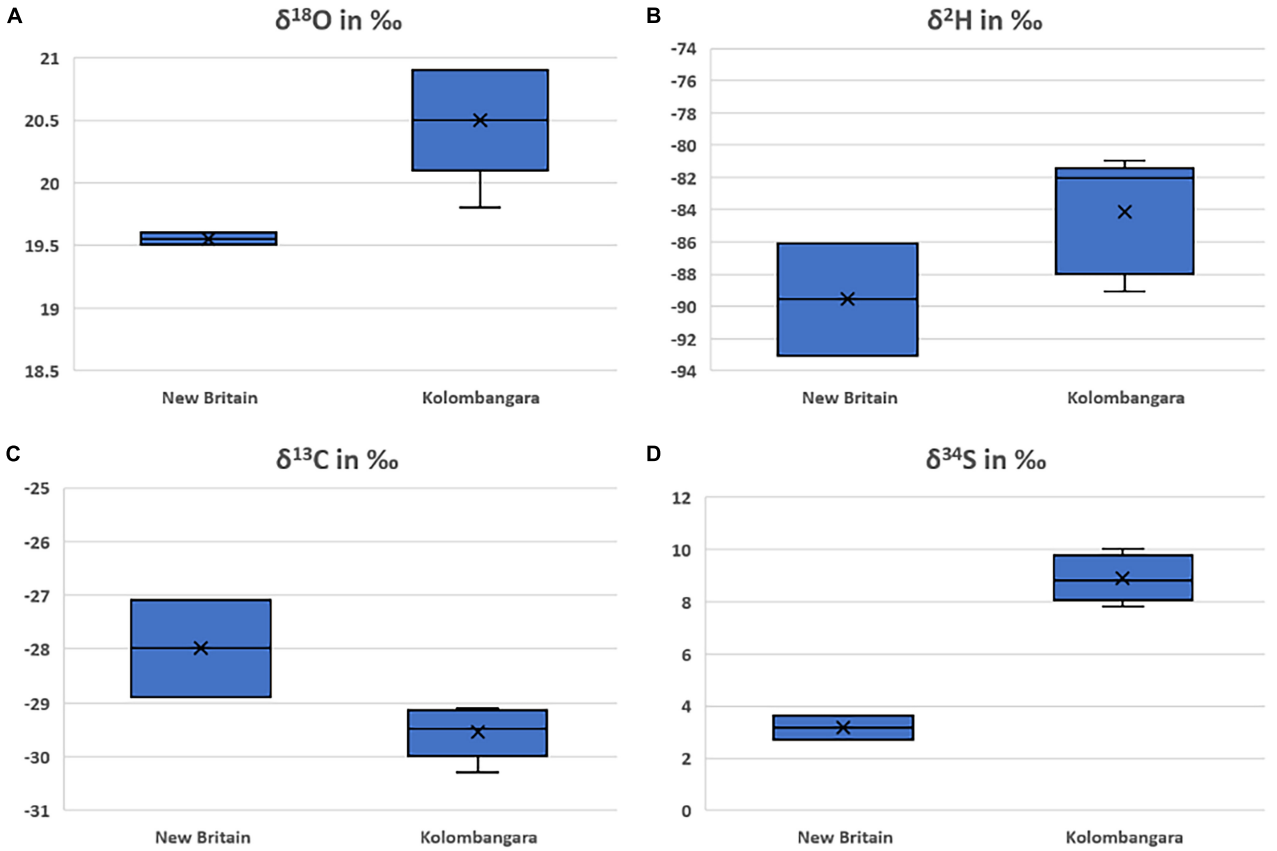

FIGURE 7 | Boxplots of the **(A)** oxygen stable isotope ratios, **(B)** hydrogen stable isotope ratios, **(C)** carbon stable isotope ratios, and **(D)** sulphur stable isotope ratios of *Pometia pinnata* from New Britain, Papua New Guinea (n = 2), and Kolombangara Island (SI) (n = 5).

as $\delta^{18}O$ and $\delta^{2}H$ are correlated in meteoric precipitation. The *P. pinnata* from New Britain are more enriched in ^{13}C than samples from Kolombangara Island whereas samples from Kolombangara Island are more enriched in ^{34}S than the two samples from New Britain.

Pterocarpus spp.

Though the overall quantities of *Pterocarpus* spp. that have been analysed and presented within this report are small, there are significant differences in the $\delta^{18}O$, $\delta^{2}H$, and $\delta^{34}S$ ($P < 0.05$ in all cases) between specimens from locations that were sampled in Kolombangara Island, Guadalcanal Island, and Gabon as judged by ANOVA. This is promising as it demonstrates the potential for differentiation within the Solomon Islands provinces and between different nations that produce *Pterocarpus* spp. given that it is pantropical and is subject to overharvesting.

Tectona grandis

Significant differences between the $\delta^{18}O$ ($P < 0.05$) and $\delta^{2}H$ ($P < 0.05$) isotope ratios of *T. grandis* from Guadalcanal Island, Kolombangara Island, and Papua New Guinea are evident (**Table 1**). This result is promising for future sampling and to be able to address the question of whether exported/imported timber from the Solomon Islands is truly from its declared origin, or whether the timber is from another origin that is a producer of Teak (e.g., Papua New Guinea, Myanmar, and Indonesia). The question of origin is especially important for *T. grandis* as it is one of the most widely used tropical hardwoods in plantations around the world.

Terminalia sp.

Terminalia sp. was the third most sampled timber from Kolombangara Island ($n = 6$) and thus was subjected to variogram analysis to investigate spatial patterns in the data. Empirical Variogram analysis shows that an insignificant (<1%) amount of variance of the data for all analysed stable isotope ratios can be explained by the distance between sampling sites. This is also evident in **Figure 8** as there appears to be very little in terms of the range of values of the stable isotope ratios across the island with the exception of the $\delta^{34}S$ (**Figure 8D**). In this case the Empirical Variogram shows the distance between samples explains 0.13% of the variance in $\delta^{34}S$; this appears to contradict the north to south trend evident in **Figure 8D**. The reason for the insignificant correlation is that the sample on the north-eastern edge of the island is an outlier relative to the other samples and the Empirical Variogram treats it as such. This suggests that there is either no strong spatial structure to the data for *Terminalia* sp. on Kolombangara Island, the variance is random, or that

there were too few samples to adequately describe the variance across the island. Given the atypical Empirical Variogram result it may be a combination of factors. The ranges for the $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$, and $\delta^{34}S$ isotope ratios are 1.2, 19.7‰ (after removal of an outlier result), 2.1, and 6.6‰, respectively. The ranges of carbon, oxygen, and hydrogen stable isotope ratios are comparable to the ranges investigated in a multi-species review by Leavitt (2010). Kolombangara Island could be considered as a single "site" as the variation of the stable isotope data is in the natural range and there is no strong spatial structure evident at this time (**Figure 8**). However, the presence of the outlier could suggest the island may be considered as several sites, though the lack of spatial structure to the data does not strongly support this hypothesis. Further sampling of *Terminalia* in the region of the outlier sample may answer this question.

Vitex sp.

Though few samples of this genus were obtained and analysed, there are no significant differences in the $\delta^{18}O$, $\delta^{2}H$, and $\delta^{13}C$ isotope ratios of *Vitex* sp. between Kolombangara and Guadalcanal Islands. Only the $\delta^{34}S$ isotope ratios show significant differences between the two origins, with the samples from Kolombangara showing significantly more enrichment in ^{34}S (Student's *T*-test $P < 0.05$) than the samples from Guadalcanal Island. This may be meaningful as other taxa sampled in this project did show significant differences in the $\delta^{18}O$, and $\delta^{2}H$ isotope ratios between the two islands (e.g., *Calophyllum* sp., *P. indicus*, and *T. grandis*).

DISCUSSION

Though some taxa have been minimally sampled, we believe it important to include their data so that others may have access to it for use in further dendroprovenancing research using stable isotope analysis. Sampling in tropical regions comes with many challenges, rain makes it hard to collect dry samples, biodiversity is high and important trees are often difficult to find (e.g., *P. indicus*), tropical wood is often dense, and it is hard to collect many samples in a short space of time. Despite all of this, a great many samples were collected overall, and we hope to build on their data over time throughout the WFID project with this piece of research laying the foundation for data, ideas, and improvements.

We believe that stable isotope data from *Campnosperma* and *Calophyllum* characterise Kolombangara Island well. *Eucalyptus* is characterised well-enough for evaluative purposes, but we are interested in gaining more samples from different parts of the island if *Eucalyptus* can be found in all areas of interest to further investigate the spatial trend that was observed. An *n* of 6 for *Terminalia*, given the outlier we observed, is close to the minimum for characterisation and it may be necessary to either explore the outlier sample more or eliminate it from the dataset. These findings are in keeping with guidelines from the Leavitt (2010) review which shows "*sampling 4–6 trees at a site while avoiding juvenile effects in rings near the pith*" is often sufficient for characterising site variability. However, the Leavitt (2010) review considers stable isotope variation in sampling sites for the purposes of characterising tree-ring variability and can involve taking many more measurements per tree than in this research. The value of 4–6 trees at a site from studies used by Leavitt (2010) is based on using Expressed Population Signal (EPS) calculations which are prone to misinterpretation according to Buras (2017). EPS is often misunderstood to be a measure for the suitability of tree ring data for climate reconstruction, whereas according to Buras (2017, p. 2) "*In theory, EPS is the amount of variance of a population chronology (infinitely replicated) explained by a finite subsample*." Therefore, it will be necessary to produce a more appropriate system for evaluating site variability and characterisation that is directly applicable to the methods presented in this research rather than rely on criteria outlined by Leavitt (2010).

Significant differences were found in numerous parameters of stable isotope data of multiple taxa between islands in the Solomon Islands (*Calophyllum* sp., *Pterocarpus* sp., *T. grandis*, and *Vitex* sp.). Though the overall quantities of each sampled taxa were relatively low, they were enough to establish ranges of values that can be used for the future evaluation of samples declared to have originated from the Solomon Islands. What is particularly promising is if the trend in the data collection continues, reliable island-level differentiation may be possible if more islands are sampled in the Solomon Islands archipelago. The results demonstrate that there is now a way to perform an evaluation to check whether a sample of FSC timber from the Solomon Islands (which must originate from Kolombangara Island) is from Kolombangara Island or not. Should a tested sample be inconsistent with data from Kolombangara Island, this could indicate that Kolombangara Island was not the origin of the timber and therefore should be subject to further scrutiny.

Differences in the ranges of stable isotope ratios were also evident in different taxa on the same site (assuming Kolombangara Island can be treated as a single site from a stable isotope perspective). On one hand this is disadvantageous because it highlights the necessity to sample as many different taxa as possible to defend any given origin and therefore will increase the cost of projects aimed at protecting the origin of taxa. On the other hand, four taxa (*Calophyllum* spp., *Pometia* sp., *Pterocarpus* spp., and *T. grandis*) show relationships in their $\delta^{18}O$ and $\delta^{2}H$ isotope ratios that can be compared to one another. To investigate this further, sampling 10 different taxa in the same site in 30 global locations would allow investigation into these relationships. A practical use of this information would be to establish a "Master" taxon for sampling that other taxa can be compared to in a model on a global scale.

Though there is a wide range in the $\delta^{34}S$ of samples collected on Kolombangara Island (11‰) all samples were enriched in ^{34}S. The sulphur in the timber is expected to have originated from soil sulphur sources which in turn are expected to have originated from the volcano. Little has been published on the geology of the Solomon Islands, let alone Kolombangara Island save for "The British Solomon Islands Geological Record 1957 – 1958" (The Government of The British Solomon Islands, 1960) which reduces the ability for detailed interpretation of sulphur isotopes somewhat. Studies related to volcanic rocks have found

that volcanic rocks are enriched in ^{34}S relative to CDT (or VCDT) and ocean floor basalts (Sakai et al., 1982; Ueda and Sakai, 1984) which appears to be consistent with the findings from the Kolombangara Island samples.

Only one of the three investigated taxa (*Eucalyptus* sp.) showed any spatially predictable trends in variance within Kolombangara Island (**Figure 9**). While this is useful to make the case that a 30 km diameter area in the Solomon Islands can be considered as a single site for classification, the trends in *Eucalyptus* should be further investigated. It may be possible that the differences in *Eucalyptus* on the island can be explained by random chance, or it may be that a prevailing wind may influence the isotopic composition of precipitation even within such a small area. Climate reports for the island state that the northern side of the island is more sheltered whereas the southern side of the island is more exposed. Whitmore (1969) also documented that there is a "10 cm increase in rainfall for every 30 m increase in elevation up to 1500 m" on Kolombangara Island (likely meaning a 100 mm increase in annual rainfall for every 30 m increase in elevation). Gat et al. (2003) discusses that air-sea interactions near the coast in conditions where there is a deficit in humidity can result in a large deuterium-excess. It is possible that the gradient in wind and precipitation is responsible for the difference in stable isotopic composition of *Eucalyptus* sp. across the island. However, it is also worth considering that while the 25 samples of *C. brevipetiolatum* that were analysed did not show a spatially predictable trend across the island, the pattern in δ^2H is similar across the island (**Figure 5B**) and may contribute to supporting the prevailing wind and precipitation hypothesis. This poses further questions, for example, why is the range in δ^2H only 13‰ for *Campnosperma* whereas it is 25.3‰ for *Eucalyptus*? Both woods are diffuse porous and can lack distinct growth ring boundaries. However, *C. brevipetiolatum* Volkens has 5–20 and 20–40 vessels/mm^2 whereas *E. deglupta* Blume has 5–20 vessels/mm^2 suggesting that water may move in greater rates in *C. brevipetiolatum*, though *E. deglupta* has vasicentric tracheids which conduct water albeit at lower rates. If the Peclet effect, the mixing of water from xylem and evaporative sites as a place for isotopic fractionation (Farquhar and Lloyd, 1993; Ferrio et al., 2011), is to be considered, these are important considerations for explaining differences in the isotopic composition of cellulose in the two types of tree. Given that there appear to be some differences in the ability of each tree to supply water to its leaves, this may explain some of the differences that have been observed. However, a greater difference between the trees appears to be in terms of the size of the leaves. *E. deglupta* leaves are approximately 7.5–20 cm long by 5.7–10 cm wide (Chippendale, 1972; Soerianegara and Lemmens, 1993), whereas *C. brevipetiolatum* leaves are much larger being over 30 cm long and up to 17.5 cm wide (Havel, 1963; Soerianegara and Lemmens, 1993). Perhaps the difference in size of the leaves also reflects that one is less susceptible to changing its stomatal conductance than

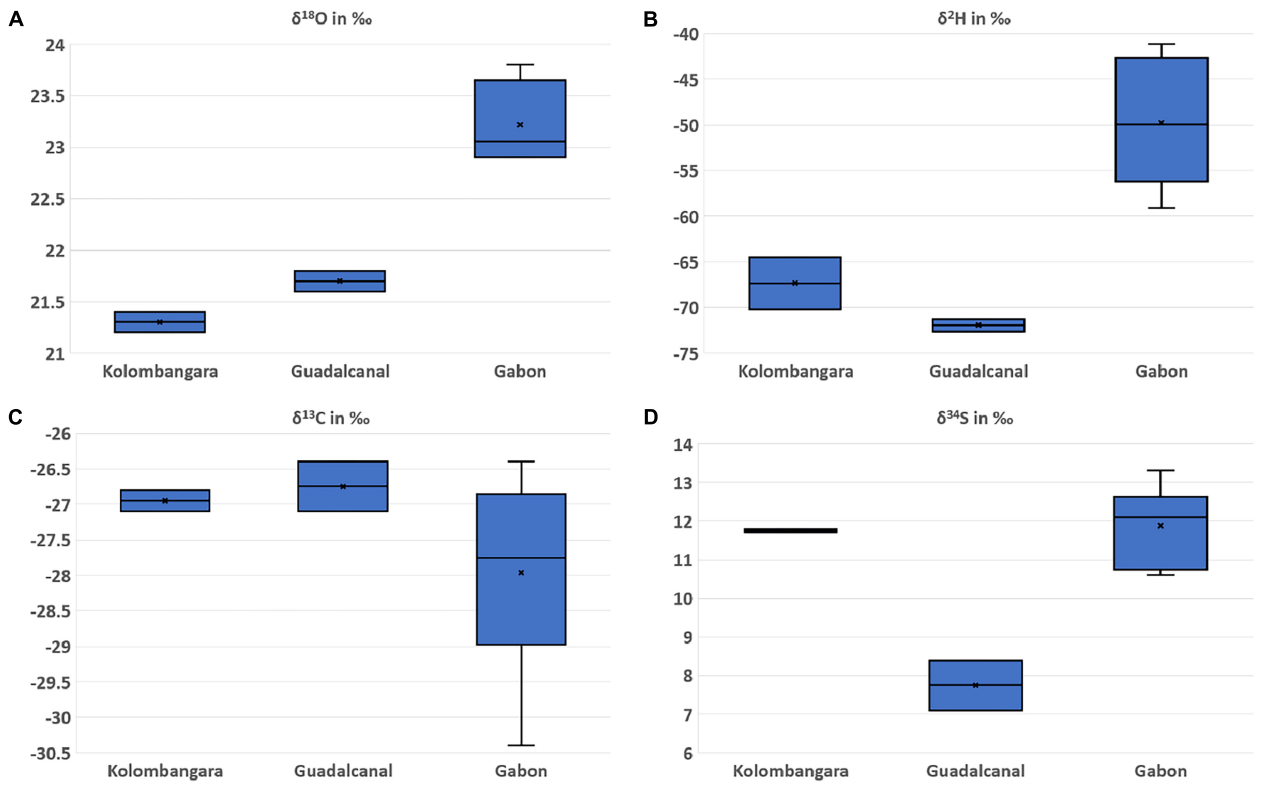

FIGURE 8 | Boxplots of the **(A)** oxygen stable isotope ratios, **(B)** hydrogen stable isotope ratios, **(C)** carbon stable isotope ratios, and **(D)** sulphur stable isotope ratios of *Pterocarpus* spp. from Kolombangara Island (SI) (n = 2), Guadalcanal Island (SI) (n = 2), and Gabon (n = 6).

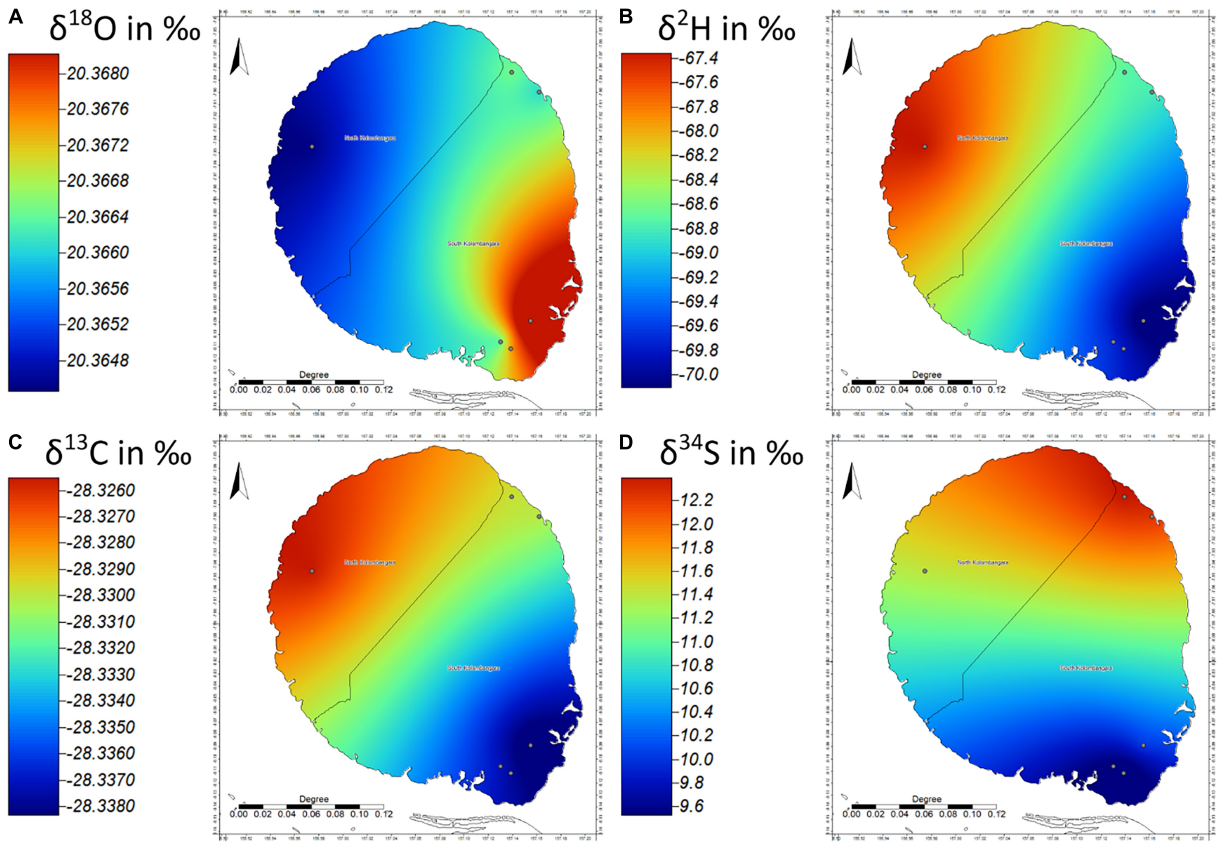

FIGURE 9 | Isoscapes of **(A)** $\delta^{18}O$, **(B)** $\delta^{2}H$, **(C)** $\delta^{13}C$, and **(D)** $\delta^{34}S$ of *Terminalia* sp. from Kolombangara Island (n = 6), Solomon Islands. Note that the apparent range in $\delta^{18}O$ and $\delta^{13}C$ are within the range of Combined Uncertainty and exhibit no practical difference across the island.

the other and may explain why one tree has a more homogenous isotopic composition than the other on the same island. Further investigation into this topic would yield results that may greatly inform how the differences in isotopic composition of different taxa can be calculated or considered.

The method of homogenising a core for analysis is appropriate to real-world testing of timber products. Homogenisation of multiple years of growth of a sample creates a robust mean value that is not heavily influenced by years that may have contained climatic anomalies (Leavitt, 2010). As all measured stable isotope ratios can be influenced by climate in some manner, this must be considered. A large proportion of the logs that are exported from the Solomon Islands to China are manufactured into plywood. As the veneers in plywood are created by rotary peeling of logs, a veneer sample subject to origin analysis may be influenced by climatic anomalies. However, as the data from the core reference sample represents this, the range of values in the reference samples should adequately cover this. However, one disadvantage to this approach appears to be that a degree of information is lost in the homogenisation of the sample and manifests only in terms of model uncertainty. $\delta^{13}C$ stable isotope ratios appear to be the most affected by this compromise; Kagawa and Leavitt (2010) demonstrated that high-level resolution of geographic origin assignment is achievable if multiple ring-widths of timber are subject to $\delta^{13}C$ stable isotope ratio analysis. $\delta^{13}C$ stable isotope ratios are most significantly influenced by water-stress in C3 plants (Farquhar et al., 1989), therefore, building up a profile of years of variation in local climatic conditions related to water-stress can allow for excellent potential for origin assignment. Nevertheless, the disadvantage of this method is that one must know the year corresponding to the ring width of the test sample to make best use of this method for origin assignment. In plywood, or even sawn timber, the way to obtain this information does not seem obvious, hence the compromised utility of carbon isotope ratio analysis that is put forward in this project. The approach used here may be limited in some terms but is designed for practical use.

However, further work to validate the ability of these ranges to verify Kolombangara Island provenance is advisable. Good practice in data analysis often involves building datasets that are large enough to be partitioned into "training" and "test" sections so that an evaluative model can be constructed using the "training" dataset with its performance being assessed by techniques such as k-fold or leave one out cross validation (LOOCV), then subsequently validated using the "test" portion of the dataset. It may also be advisable to collect further samples of known origin and perform a "blind-test" evaluation. This case study has set out to establish a basis for evaluating origin claims

of timber from the Solomon Islands using stable isotope analysis and hopefully further work will permit validations of these sorts to be performed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CW was responsible for data analysis and much of the introduction and discussion. GR was responsible for quality checking the interpretation and the introduction. SH and LM were jointly responsible for sample preparation and data preparation. PG was responsible for discussing the relationship between wood anatomy and the stable isotope results. MB was responsible for the scientific methods, data analysis, and overall management of the project. All authors contributed to the article and approved the submitted version.

FUNDING

We gratefully acknowledge funding *via* The US Department of State, US Department of Agriculture, and Defra-ICF: Building and Enhancing the Evidence Base for Nature Based Solutions to Climate Change. Project 29084. Driving innovation in forest protection and enforcement monitoring. Tackling illegal logging: creating a timber reference library to support enforcement (World Forest ID at Kew).

ACKNOWLEDGMENTS

A very deep and heartfelt thanks to the following, without whom this work would not have been possible: Elizabeth Lebow, Alex Moad, and their respective colleagues at the United States Forest Service for overseeing the World Forest ID project. FSC, Phil and Tatai Guillery for collecting samples in the Solomon Islands. FSC, Emily Crumley and Cynel Moundounga for collecting samples in Gabon. Dan Raymond, Wilson Tiubule, KFPL and the communities of Kolombangara and North East Guadalcanal for collecting samples and supporting collection efforts in the Solomon Islands. Meaghan Parker-Forney and her respective colleagues at the World Resources Institute for overseeing the World Forest ID project. Jade Saunders of Forest Trends for her advisory and coordination support to the World Forest ID project. Isabella Miles-Bunch and Sara Redstone at the Royal Botanic Gardens at Kew for assisting with sample shipment documentation, receipt of samples in quarantine and their expert help in verifying declared information of field collectors. Tim Fox, Jackie Borrows, and Matt Watkinson for spending hundreds of collective hours assembling sample collection packs. Agroisolab GmbH staff for spending hundred's of collective hours preparing and measuring samples. Meg Staton, Abdullah Almsaeed et al. for developing the WorldForestID app which tracks samples from the field through Kew to analysis labs.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2021.645222/full#supplementary-material

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Conflict of Interest: CW and GR were employed by company Agroisolab UK Ltd., Welburn, United Kingdom. SH, LM, and MB were employed by company Agroisolab GmbH.

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