frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 21 September 2021
doi: 10.3389/fevo.2021.669292

Unraveling the Hidden Diversity of the Native White Claw Crayfish in the Iberian Peninsula

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Specialty section:
This article was submitted to
Conservation and Restoration
Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 18 February 2021
Accepted: 26 August 2021
Published: 21 September 2021

Citation:
Martín-Torrijos L, Buckley D,
Doadrio I, Machordom A and
Diéguez-Uribeondo J (2021)
Unraveling the Hidden Diversity of the
Native White Claw Crayfish
in the Iberian Peninsula.
Front. Ecol. Evol. 9:669292.
doi: 10.3389/fevo.2021.669292

Several European freshwater crayfish species are currently included in one of the IUCN Red list categories. In the Iberian Peninsula, the native *Austropotamobius pallipes* species complex (the white clawed crayfish, WCC) has experienced a drastic decline since 1973. Implementing conservation management strategies for this species requires a better understanding of the patterns and structure of its genetic diversity. In this study, we assessed the levels and patterns of genetic variation in 71 populations along the whole distributional range of the WCC in the Iberian Peninsula. The two mitochondrial markers analyzed (*Cytochrome Oxidase subunit I* and *16S rRNA* genes) indicated high levels of genetic diversity, which are significantly geographically structured in three main genetic groups, two corresponding to Northern and one to Central-Eastern and the westernmost Iberian Peninsula. The diversity found included new private haplotypes, and revealed the potential effect of paleogeographic barriers and last glaciations in the population structure observed. Current conservation and management programs for the WCC in the Iberian Peninsula should consider these three phylogeographic groups as essential management units in order to preserve the remaining genetic diversity in the species.

Keywords: ***Austropotamobius pallipes*****, mitochondrial *****16S rRNA*****, mitochondrial *****COI*****, genetic diversity, phylogeographic structure, conservation**

INTRODUCTION

The status of the European crayfish represents a paradigmatic example of the worldwide freshwater biodiversity crisis. Currently, there are six native European crayfish species: the white-clawed crayfish (WCC) (*Austropotamobius pallipes* species complex, see below), the stone crayfish (SC) (*Austropotamobius torrentium*), the noble crayfish (NC) (*Astacus astacus*), the narrow-clawed crayfish (NCC) (*Pontastacus leptodactylus*), the thick-clawed crayfish (TCC) (*Pontastacus pachypus*), and the recently described *Austropotamobius bihariensis* (Pârvulescu, 2019). These crustaceans have experienced a rapid decimation due to overexploitation, water pollution, flow modification, habitat destruction, habitat and populations fragmentation, invasive species, and especially the emerging crayfish plague disease caused by the pathogen *Aphanomyces astaci* (Jussila et al., 2021 for review).

As a consequence of this decline, several crayfish species are included in one of the IUCN Red list categories (e.g., WCC and NC are listed as endangered and vulnerable, respectively) with a declining population trend (IUCN, 2021). Thus, there has been a recent interest in studying the patterns of genetic diversity in the European crayfish species to establish sound management and conservation plans (Schubart and Huber, 2006; Akhan et al., 2014; Jelić et al., 2016; Bláha et al., 2017; Schrimpf et al., 2017; Lovrenčić et al., 2020). This is especially true for the WCC in the Iberian Peninsula since this species has experienced a drastic decline for the last 45 years (Füreder et al., 2010). The taxonomic status of WCC, furthermore, is complex. Initial analyses based on nuclear DNA supported the existence of one species (Chiesa et al., 2011; Scalici and Bravi, 2012) while analyses based on mitochondrial DNA (mtDNA) and allozymes suggested that *Austrapotamobius pallipes* actually represents a species complex. This complex was proposed to comprise two species, *A. pallipes sensu stricto* and *A. italicus*, which in turn includes four subspecies: *A. i. carsicus, A. i. meridionalis, A. i. italicus* and *A. i. carinthiacus* (Fratini et al., 2005).

The WCC populations of the Iberian Peninsula have been referred as the westernmost part of the *A. i. italicus* lineage. They were thought as an introduction from the Italian Peninsula (Vedia and Miranda, 2013; Clavero et al., 2016), although they are now considered of autochthonous/natural origin (e.g., Beroiz et al., 2008; Diéguez-Uribeondo et al., 2008; Pedraza-Lara et al., 2010; Matallanas et al., 2011, 2016). Historically, its distributional range in the Iberian territory covered most part of this area until the introduction of two North American invasive crayfish, *Procambarus clarkii* and *Pacifastacus leniusculus*, which are chronic carriers of the crayfish plague pathogen (Alonso et al., 2000). Since the introduction of this pathogen, at least 80% of the original European populations have disappeared (Füreder et al., 2010), and the trend of the remaining Iberian populations is still decreasing (Aldabe et al., 1991; Temiño and Sáez-Royuela, 1998). The remaining Iberian WCC populations, and their genetic diversity, thrive in isolated mountainous creeks or in inaccessible brooks. Initial studies analyzing the genetic diversity of the Iberian WCC were based on mitochondrial regions, e.g., *16S rRNA* and *Cytochrome Oxidase Subunit I* (*COI*). These investigations did not find significant levels of genetic diversity among the populations studied (Santucci et al., 1997; Grandjean et al., 2000, 2001, 2002; Trontelj et al., 2005). However, subsequent studies evidenced higher levels of genetic diversity and a perceptible genetic structure by increasing the number of populations and sample size (Beroiz et al., 2008; Diéguez-Uribeondo et al., 2008), especially when new markers were designed. These studies indicated a strong geographical structure and confirmed the existence of high genetic diversity, similar to the levels found in other European crayfish populations (Pedraza-Lara et al., 2010; Matallanas et al., 2011, 2016). In these studies, however, the distributional range of WCC in the Iberian Peninsula was not fully covered. Although this species does not appear to be naturally distributed in some areas of the Western Iberia peninsula (is absent or very rare in the acid rock areas of Portugal, Galicia, Extremadura and West of Andalucía) (Alonso et al., 2000), there was an overlooked gap in the Central-Western area, still under-sampled and unstudied. The fact that WCC has not been studied within its whole range of distribution, could bias the estimates for overall genetic patterns of diversity for the species in the Iberian Peninsula. Therefore, a study considering the whole range of this species in the Iberian Peninsula is in need, and will provide new insights that will also help clarifying previous claims of a non-native origin of this species in the Iberian Peninsula (Vedia and Miranda, 2013; Clavero et al., 2016).

Thus, the aim of this study was to unravel the potential hidden genetic diversity within the unexplored Iberian WCC populations and to identify patterns of the genetic variation and structure. For this purpose, we have significantly increased the number of sampled populations from previous studies along a wider distributional range in the Iberian Peninsula, applying two highly informative mitochondrial DNA regions, the *16S rRNA* and *COI* genes. Approaching the true diversity and identifying patterns of genetic diversity of the native WCC in the Iberian Peninsula is crucial for maintaining the genetic pool of this endangered species. Moreover, these results will improve the design of conservation programs for the species.

MATERIALS AND METHODS

Crayfish Sampling

A total of 265 specimens of WCC were collected from 47 populations throughout the geographical distribution of the WCC in the Iberian Peninsula (**Table 1**). Due to the conservation status of some populations, and variability in populations size, the number of specimens per population included in the study was unequal. Crayfish were captured using nets and by hand in collaboration with the environmental officers of each of the localities. In addition, and to cover the geographical range of the WCC distribution, we included specimens from the "Crayfish Collection" of the RJB-CSIC in Madrid (set of historical samples collected and preserved since 1998). A walking leg from each individual was excised and preserved in 96% ethanol in a 2 ml tube until the molecular analysis. All crayfish were returned to their habitat alive.

DNA Extraction, Amplification and Sequencing

Samples were first rinsed with TE buffer (Tris 10 mM/EDTA 1 mM, pH 8) to remove the preserving ethanol. Each walking leg was cleaned up to three times with TE and left overnight in the buffer. Each sample was then transferred to a 2 ml tube, which was frozen at −80°C and afterward lyophilized in a freeze dryer VirTis BenchTop K for 24 h (≤−50°C; ≤ 20 mTorr) to facilitate the grinding of the genetic material by mechanical rupture using a TissueLyser (QIAGEN).

Genomic DNA was extracted with an E.Z.N.A. ®Insect DNA Kit (Omega bio-tek, Norcross, Atlanta, United States). The election of the mitochondrial markers used in this study was made trying to maximized the information obtained from the sequences as well as the compatibility with the information available in GenBank. Therefore, we selected the mitochondrial *16S rRNA* and *COI* genes. The primers pair used to amplify the

TABLE 1 | Populations and locations of the white-clawed crayfish analyzed in the present work (**Figure 3**).

Population	Location	Catchment	SAMOVA	*n*	S	H	Hd	π	D	Fs	Collection
AL1	Altube/Álava	Ebro	G3	10	0	1 (H24)	0.000	0.0000	n/c	n/c	#21# Matallanas et al., 2016
AS1	Cangas de Onis/Asturias	Sella	G2	2	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
AS2	Cangas de Onis/Asturias	Sella	G2	10	0	1 (H30)	0.000	0.0000	n/c	n/c	#3# Matallanas et al., 2016
AS3	Cangas de Onis/Asturias	Sella	G2	10	0	1 (H30)	0.000	0.0000	n/c	n/c	#15# Matallanas et al., 2016
AV1	Sanchorreja/Ávila	Duero	G1	9	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
AV2	Santa María del Cubillo/Ávila	Duero	G1	8	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
AV3	Sanchorreja/Ávila	Duero	G1	9	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
BU4	Rebolledo de la Torre/Burgos	Duero	G2	10	1	2 (H30, H32)	0.356	0.0001	0.015	0.417	This study
BU7	Santa María del Campo/Burgos	Duero	G3	3	0	1 (H24)	0.000	0.0000	n/c	n/c	This study
BU22	Fuentenebro/Burgos	Duero	G1	9	3	4 (H1, H9, H13, and H15)	0.833	0.0005	0.794	−0.450	This study
BU34	Hontoria de Valdearados/Burgos	Duero	G1	6	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
BU53	Santo Domingo de Silos//Burgos	Duero	G3	7	4	4 (H24, H26, H27, and H28)	0.810	0.0006	−0.319	−0.655	This study
BU58	Santo Domingo de Silos/Burgos	Duero	G2	10	1	2 (H24, H30)	0.533	0.0002	1.303	1.029	This study
BU64	Arauzo de Miel/Burgos	Ebro	G1	9	1	2 (H1, H13)	0.500	0.0002	0.986	0.849	This study
BU82	San Zadornil/Burgos	Ebro	G2	2	1	2 (H29, H30)	1.000	0.0004	0.000	0.000	This study
BU83	San Zadornil/Burgos	Ebro	G2	4	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
BU84	San Zadornil/Burgos	Ebro	G2	5	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
BU85	San Zadornil/Burgos	Ebro	G2	3	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
BU86	San Zadornil/Burgos	Ebro	G2	5	2	3 (H30, H34, and H35)	0.700	0.0004	0.243	−0.475	This study
BU98	Padrones de Burela/Burgos	Ebro	G2	10	1	2 (H30, H31)	0.200	0.0001	−1.112	−0.339	#11# Matallanas et al., 2016
BU99	Rebolledo Traspeña/Burgos	Duero	G2	10	6	2 (H21, H30)	0.200	0.0005	−1.796	2.607	#16# Matallanas et al., 2016
CAS1	Lucena del Cid/Castellon	Júcar	G1	10	0	1 (H1)	0.000	0.0000	0.000	n/c	#18# Matallanas et al., 2016
CAS2	La Pobla de Benifassa/Castellon	Júcar	G1	10	2	3 (H1, H5, and H16)	0.644	0.0004	1.743	0.643	#23# Matallanas et al., 2016
CR2	Pozuelo de Calatrava/Ciudad Real	Guadiana	G1	4	1	2 (H1, H16)	0.667	0.0003	1.633	0.540	This study
CU1	Almagraro/Cuenca	Júcar	G1	2	1	2 (H1, H16)	1.000	0.0004	0.000	0.000	This study
CU2	Las Truchas/Cuenca	Tajo	G1	3	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
CU3	Pedregoso/Cuenca	Júcar	G1	3	1	2 (H16, H19)	0.667	0.0003	0.000	0.201	This study
CU4	Pozuelo/Cuenca	Tajo	G1	3	3	3 (H1, H16, and H24)	1.000	0.0008	0.000	−0.693	This study
CU5	Valmelero/Cuenca	Tajo	G1	4	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
CU6	Vaquerizas/Cuenca	Tajo	G1	3	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
CU7	Huerta de Obispalia/Cuenca	Guadiana	G1	10	1	2 (H16, H17)	0.200	0.0001	−1.112	−0.339	#5# Matallanas et al., 2016
CU8	Pozuelo/Cuenca	Tajo	G1	10	0	1 (H16)	0.000	0.0000	0.000	n/c	#20# Matallanas et al., 2016
CU9	Valdemoro/Cuenca	Júcar	G1	10	2	3 (H1, H4, and H16)	0.600	0.0003	0.120	−0.101	#24# Matallanas et al., 2016
GIR1	Escaramat/Gerona	Cataluña	G1	3	3	2 (H14, H16)	0.667	0.0008	0.000	1.609	This study
GIR2	Falgars/Gerona	Cataluña	G1	11	1	2 (H1, H2)	0.327	0.0001	−0.100	0.356	This study

(Continued)

TABLE 1 | (Continued)

Population	Location	Catchment	SAMOVA	*n*	S	H	Hd	π	D	Fs	Collection
GIR3	La Fabrega/Gerona	Cataluña	G1	12	1	2 (H1, H16)	0.485	0.0002	1.066	1.003	This study
GIR4	La Plana/Gerona	Cataluña	G1	11	3	3 (H1, H10, and H20)	0.346	0.0002	−1.600	0.885	This study
GIR6	Santa Llucía//Gerona	Cataluña	G1	11	2	3 (H1, H2, and H16)	0.564	0.0003	0.036	−0.113	This study
GIR7	Olot/Gerona	Cataluña	G1	10	1	2 (H1, H6)	0.200	0.0001	−1.112	−0.339	This study
GRA1	Albuñuelas/Granada	Guadalquivir	G1	10	0	1 (H1)	0.000	0.0000	n/c	n/c	#9# Matallanas et al., 2016
GU1	Chaparrillo/Guadalajara	Tajo	G1	5	1	2 (H1, H16)	0.600	0.0002	1.225	0.626	This study
GU2	Río Gallo/Guadalajara	Tajo	G1	10	1	2 (H1, H16)	0.556	0.0002	1.464	1.096	#22# Matallanas et al., 2016
HU1	Barranco Villano/Huesca	Ebro	G1	5	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
HU2	Formiga/Huesca	Ebro	G1	2	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
HU3	Casbas/Huesca	Ebro	G1	10	7	4 (H1, H3, H12, and H24)	0.711	0.0008	−0.926	0.517	#2# Matallanas et al., 2016
JA1	Cazorla/Jaen	Guadalquivir	G1	2	0	1 (H1)	0.000	0.0000	0.000	0.000	This study
LE1	Lugán/León	Duero	G3	10	3	3 (H1, H24, and H30)	0.600	0.0004	−0.658	0.206	#14# Matallanas et al., 2016
LE3	Garrafe de Torios/León	Duero	G1	11	1	2 (H1, H13)	0.436	0.0002	0.671	0.779	This study
LER1	Pont de Suert/Lérida	Ebro	G1	10	2	3 (H16, H22, and H23)	0.622	0.0003	0.019	−0.156	#1# Matallanas et al., 2016
LU1	Pol/Lugo	Miño	G1	10	1	2 (H1, H8)	0.200	0.0001	−1.112	−0.339	#4# Matallanas et al., 2016
LU2	Castro de Rei/Lugo	Miño	G1	10	0	1 (H1)	0.000	0.0000	n/c	n/c	#13# Matallanas et al., 2016
NA2	Doneztebe/Navarra	Bidasoa	G3	3	1	2 (H24, H30)	0.667	0.0003	0.000	0.201	This study
NA3	Aoiz51/Artanga/Navarra	Ebro	G3	3	3	2 (H16, H24)	0.667	0.0008	0.000	1.609	This study
NA4	Bidaurreta/Ultzama-Araquil/Navarra	Ebro	G3	4	0	1 (H24)	0.000	0.0000	n/c	n/c	This study
NA5	Leurtza/Navarra	Bidasoa	G3	4	1	2 (H24, H30)	0.500	0.0002	−0.612	0.172	This study
NA7	Sunbilla/Navarra	Bidasoa	G2	2	1	2 (H24, H30)	1.000	0.0004	0.000	0.000	This study
NA8	Estella/Navarra	Ebro	G3	10	3	2 (H11, H24)	0.200	0.0002	−1.562	1.225	#19# Matallanas et al., 2016
PA1	Herrera del Pisuerga/Palencia	Duero	G1	4	4	2 (H16, H30)	0.667	0.0011	2.080	2.719	This study
SO1	Navaceno/Soria	Duero	G1	10	1	2 (H1, H16)	0.356	0.0001	0.015	0.417	This study
SO2	Navaceno/Soria	Duero	G1	9	1	2 (H1, H16)	0.500	0.0002	0.983	0.849	This study
SO3	Mont Vicarias/Soria	Ebro	G1	2	1	2 (H1, H16)	1.000	0.0004	0.000	0.000	This study
SO8	Almarza/Soria	Duero	G1	9	2	3 (H1, H13, and H16)	0.556	0.0003	−0.583	−0.532	This study
SO15	Devanos/Soria	Ebro	G3	9	0	1 (H24)	0.000	0.0000	n/c	n/c	This study
TE1	Valderrobles/Teruel	Ebro	G1	2	1	2 (H1, H7)	1.000	0.0004	0.000	0.000	This study
TE2	Beceite/Teruel	Ebro	G1	10	0	1 (H1)	0.000	0.0000	n/c	n/c	#6# Matallanas et al., 2016
TE3	Castellote/Teruel	Ebro	G1	10	1	2 (H1, H16)	0.467	0.0002	0.819	0.818	#10# Matallanas et al., 2016
TE5	Cucalon/Teruel	Ebro	G1	10	7	5 (H1, H8, H16, H18, and H33)	0.800	0.0008	−1.002	−0.733	#12# Matallanas et al., 2016
VA1	Utiel/Valencia	Júcar	G1	10	4	2 (H16, H25)	0.356	0.0006	0.023	3.025	#17# Matallanas et al., 2016
VALL1	Adalia/Valladolid	Duero	G1	6	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
VALL2	Adalia/Valladolid	Duero	G1	2	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
ZA1	Santa Eulalia de Gállego/Zaragoza	Ebro	G1	10	3	3 (H1, H16, and H24)	0.689	0.0005	0.775	0.985	#7# Matallanas et al., 2016

Catchment, hydrogeographic catchment; SAMOVA, grouping structure assigned by SAMOVA; n, sample size; S, number of polymorphic sites; H, number and haplotypes found and their reference code between brackets; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs.
The "n/c" means not calculated.

mitochondrial *16S rRNA* gene, 1472 (Crandall and Fitzpatrick, 1996) and Tor12sc (Largiadèr et al., 2000) amplified a fragment that included partial sequences of the *12S rRNA*, the *16S rRNA* and the *val-tRNA*. From hereafter, the combination of the *12S rRNA*, *val-tRNA* and *16S rRNA* regions will be referred as *16S*. The primers pair used to amplify the mitochondrial *COI* gene was C/N 2769 (Gopurenko et al., 1999) and LCO1490 (Folmer et al., 1994). Both were used in a single round PCR following the protocols in Matallanas et al. (2016). Negative controls containing no DNA were included in all single round PCR for both primer pairs.

We checked for positive amplicons by running an electrophoresis with 3-μl aliquots of the amplification product in 1% agarose TAE gels stained with SBYR-Safe (Thermo Fisher Scientific). Amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany). Double strand PCR positive products were sequenced using an automated sequencer (Applied Biosystems 3730xl DNA, Macrogen, Netherlands).

Sequence Data

Both mtDNA sequence strands were assembled and analyzed using the program Geneious v10.0.2 (Kearse et al., 2012). We ran BLAST searches to check the nature of the generated sequences. We revised the sequences chromatograms for double-peaks and performed the alignments using the MAFFT algorithm (Katoh et al., 2002). The final alignments included sequences of 1,317 base pairs (bp) for *16S* gene and 1,151 bp for *COI* gene.

Additionally, we downloaded a total of 748 sequences for the *16S* gene and 669 sequences for the *COI* gene from GenBank from previous studies (**Supplementary Appendix 1**). Moreover, the sequences for the *16S* and *COI* genes from the genome of *Austropotamobius torrentium* were also downloaded from GenBank (accession numbers NC_033504), as well as the sequences for the *16S* and *COI* for *A. italicus carsicus* (accession numbers KX370126 and KX369706, respectively) and were used as outgroups in the phylogenetic analyses (**Supplementary Appendix 1**).

Data Sets

We designed three different data sets to take advantage of the genetic information in previous studies. Data Set 1 was designed to frame the samples from this study within the last phylogenetic scenario proposed by Jelić et al. (2016). Data Set 2 was designed to reconstruct the phylogenetic relationships of lineages within the Iberian Peninsula. Data Set 3 was designed to estimate the genetic diversity and the population structure of the existing populations in the Iberian Peninsula.

Data Set 1 comprised all the sequences from the range of distribution of WCC in Europe, including three subsets: (i) *16S* with a total of 1,013 sequences, (ii) *COI* with a total of 934 sequences, and (iii) concatenated *16S* and *COI* genes from the specimens that had both genes sequenced with a total of 934 specimens. *Austropotamobius torrentium* was used as an outgroup in all the subsets.

The Data Set 2 comprised all the sequences from the distributional range of the WCC in the Iberian Peninsula, including three subsets: (i) *16S* with a total of 706 sequences, (ii) *COI* with a total of 706, and (iii) concatenated *16S* and *COI* genes from the specimens that had both genes sequenced with a total of 706 specimens. One specimen from the sister clade of the Iberian WCC populations (*A. italicus carsicus*) was used as an outgroup in all the subsets.

The Data Set 3 comprised a total of 505 sequences with the largest base pair length for the three defined subsets: (i) *16S*, (ii) *COI* and (iii) concatenated *16S* and *COI* genes, of which 265 sequences were obtained from the 47 populations of this study (GenBank accession numbers MW317197-MW317461 for *16S* and MW325345-MW325609 for *COI*). The remaining 240 sequences belong to 24 populations spanning the distributional range of the *A. pallipes* complex in the Iberian Peninsula (Matallanas et al., 2016) (**Table 1**). This data set was used to determine both genetic diversity and genetic structure of the existing populations in the Iberian Peninsula.

Phylogenetic Relationships

Phylogenetic relationships were analyzed for the Data Set 1 and Data Set 2. We identified the best model of nucleotide substitution and best partition schemes for the *16S* and the *COI* genes for each of the data sets in Partition Finder v2.1.1 (Lanfear et al., 2016), using the Bayesian Information criterion (BIC). The base frequencies were estimated using maximum likelihood (+X) rather than empirically (+F) for the implemented models.

Phylogenetic analyses for the *16S*, the *COI* and the concatenated *16S* and *COI* genes were run under Bayesian inference (BI) and maximum likelihood (ML). The BI analysis was performed in MrBayes v.3.2.6 software (Ronquist et al., 2012) using the default MCMCMC search algorithm with 100,000,000 generations, three runs (eight chains per run) with a burn-in of 25% generations. Nodes with posterior probability (pp) values $\geq$ 0.95 were considered as supported. Tracer v1.6.0 (Rambaut et al., 2014) was used to check for convergence and stationarity of the three runs. The ML analysis was performed in RAxML v.8 (Stamatakis, 2014) as implemented in raxmlGUI v1.5b1 (Silvestro and Michalak, 2012), with 100 independent replicates and 1,000 rapid bootstraps. Nodes with bootstrap values $\geq$ 75 were considered as supported. The resulting trees from the BI and ML analyses were visualized in FigTree v1.4.2 (Rambaut, 2012).

Genetic Diversity and Genetic Structure

We examined the genetic structure of the WCC in the Iberian Peninsula using the Data Set 3 with a Spatial Analysis of the Molecular Variance (SAMOVA v2.0) (Dupanloup et al., 2002). This method defines groups of populations (k) that are genetically and geographically homogeneous and maximally differentiated from each other (it maximizes the proportion of total genetic variance, F_{CT}) to identify genetic barriers. Moreover, it also defines groups of populations that are maximally differentiated from each other, without constraint for the geographic composition of the groups. We run SAMOVA v2.0 from $k = 2$ to $k = 20$ and each run was with 1,000 simulated annealing processes.

We used TCS v.1.21 (Clement et al., 2000) to represent the mutational changes between the sequences throughout

the most parsimonious haplotype network and the genealogical relationships were visualized using PopArt v1.7.2 (Leigh and Bryant, 2015).

To further dissect the patterns of genetic diversity and the genetic structure of the WCC populations in the Iberian Peninsula, we defined three grouping strategies of the Data Set 3: (I) populations as independent units, (II) populations grouped in hydrogeographic areas, and (III) populations grouped in the phylogeographic areas determined by SAMOVA (**Table 1**). Due to the unequal number of the samples conforming each sampled population, we carried out the rarefaction of the data in the populations grouped in hydrogeographic areas and in the populations grouped by phylogeographic areas. For this, we selected randomly n* individuals, subsampled without replacement from the larger of the original samples, and equaled the size of the smaller original sample ($n = n^* = 4$) (Magurran and McGill, 2011).

We performed two independent analyses for the two grouping structures (hydrogeographic areas and the phylogeographic areas), first for the raw data and then for the rarefied data. We estimated the number of polymorphic (segregating) sites (S), the number of haplotypes (H), the haplotype diversity (Hd), the average number of nucleotide differences (k) and the nucleotide diversity (π) using the program DNAsp v.5.10.01 (Librado and Rozas, 2009). We estimated the haplotypes frequencies and the genetic diversity indices (Tajima's D and Fu's Fs) with the software Arlequin v3.5.2.2 (Excoffier et al., 2005). The patterns of genetic variation with the analysis of the molecular variance (AMOVA) were analyzed in Arlequin v3.5.2.2 (Excoffier et al., 2005). Significance values ($p < 0.05$) were assessed by using 10,000 permutations.

RESULTS

Phylogenetic Relationships

The phylogenetic analyses of the independent *16S* and *COI* genes subsets (Data Set 1) provided congruent trees. The concatenated mtDNA fragments conforming Data Set 1 were divided in three partitions. First partition included the *12S rRNA* and *val-tRNA* genes (155 pb) with a JC substitution model (Jukes and Cantor, 1969), a second partition included the *16S rRNA* gene (1,162 pb) with a HKY + I + X substitution model (Hasegawa et al., 1985) and a third partition included the *COI* gene (1,151 pb) with a HKY + G + X substitution model (Hasegawa et al., 1985). The analyses (BI and ML) of the concatenated data set showed a clear differentiation between *A. pallipes* and *A. italicus* (**Figure 1**). Within *A. italicus*, sequences attributed to *A. i. italicus*, *A. i. carinthiacus*, and *A. i. carsicus* formed a well-supported Clade (Clade I). The remaining samples corresponding to *A. i. meridionalis* did not form a monophyletic group, as defined in Fratini et al. (2005) (**Figure 1**). The samples of *A. i. italicus* and *A. i. carinthiacus* from Austria, Italy, France, and the Iberian Peninsula grouped in a well-supported sub-clade, sister to the samples of *A. i. carsicus* (**Figure 1**).

The analyses of the Data Set 2 showed that the independent analysis of the two subsets of *16S* and *COI* genes provided congruent trees. PartitionFinder subdivided the concatenated Data Set 2 into three partitions. The first partition included *12S rRNA* and *val-tRNA* genes (155 pb) with a JC substitution model (Jukes and Cantor, 1969), a second partition included the *16S rRNA* gene (1,162 pb) with a HKY + G substitution model (Hasegawa et al., 1985), and a third partition included the *COI* gene (1,151 pb) with a HKY + G substitution model (Hasegawa et al., 1985).

The phylogenetic analyses (BI and ML) resulting from this concatenated dataset showed no clear relationships among Iberian *A. i. italicus* populations. Some samples grouped together with high support but sometimes with no clear geographic correspondence (**Figure 2**).

Genetic Diversity and Genetic Structure

The SAMOVA analysis resolved three main groups ($K = 3$) out of the 71 populations of WCC in the Iberian Peninsula: the Central-Eastern (Group 1), the North-Western Group (Group 2) and the North-Central Group (Group 3) (**Figure 3**), representing the Iberian populations with and without a geographical constraint (equal values for complete data and rarefied samples, $F_{CT} = F_{CT}^* = 0.73$). Group 1 included 48 populations, Group 2 included 13, and Group 3 included 10 populations (**Table 1**).

The most parsimonious haplotype network showed 35 haplotypes in the Iberian Peninsula (**Figure 4**). Four of them (H1, H16, H24, and H30) were the most represented in the area. The two haplotypes H1 and H16 covered the Center, South, East and the westernmost populations of the Iberian Peninsula (**Figure 3**) (Group1 defined by SAMOVA), haplotype H30 covered the North-Western of the Iberian Peninsula (Group2 defined by SAMOVA), and the haplotype H24 covered the North-Center of the Iberian Peninsula (Group3 defined by SAMOVA). We recovered six shared haplotypes (H1, H2, H13, H16, H24, and H30) among different populations, presenting medium-high frequencies, while the 29 remaining haplotypes appeared as unique from one specific population, conforming private haplotypes with low-medium frequencies (**Table 2**).

The 71 Iberian WCC populations hosted 35 haplotypes, representing a noteworthy mean haplotype diversity (Hd = 0.775), but low nucleotide diversity ($\pi = 0.00073$). We found that 27 out of the 71 populations were monomorphic for one of four different haplotypes (H1, H16, H24, or H30). The highest number of haplotypes per population was found in TE5 with five haplotypes (H1, H8, H16, H18, and H33), followed by other three populations hosting four different haplotypes each: BU22 (H1, H9, H13, and H15), BU53 (H24, H26, H27, and H28), and HU3 (H1, H3, H12, and H24) (**Table 1**). These four populations hosted a high haplotype diversity ($Hd_{TE5} = 0.800$; $Hd_{BU22} = 0.833$; $Hd_{BU53} = 0.810$, and $Hd_{HU3} = 0.711$), and medium-high nucleotide diversity ($\pi_{TE5} = 0.0008$, $\pi_{BU22} = 0.0005$, $\pi_{BU53} = 0.0006$, and $\pi_{HU3} = 0.0008$) (**Table 1**).

Following the Iberian hydrogeographic river basins, we defined 10 hydrogeographic areas (**Table 1**). The results for the raw data set showed differences between the hydrogeographic areas, with the Ebro area standing out by hosting the largest number of haplotypes (18 out of 35 haplotypes). The Ebro river basin also presented the highest haplotype

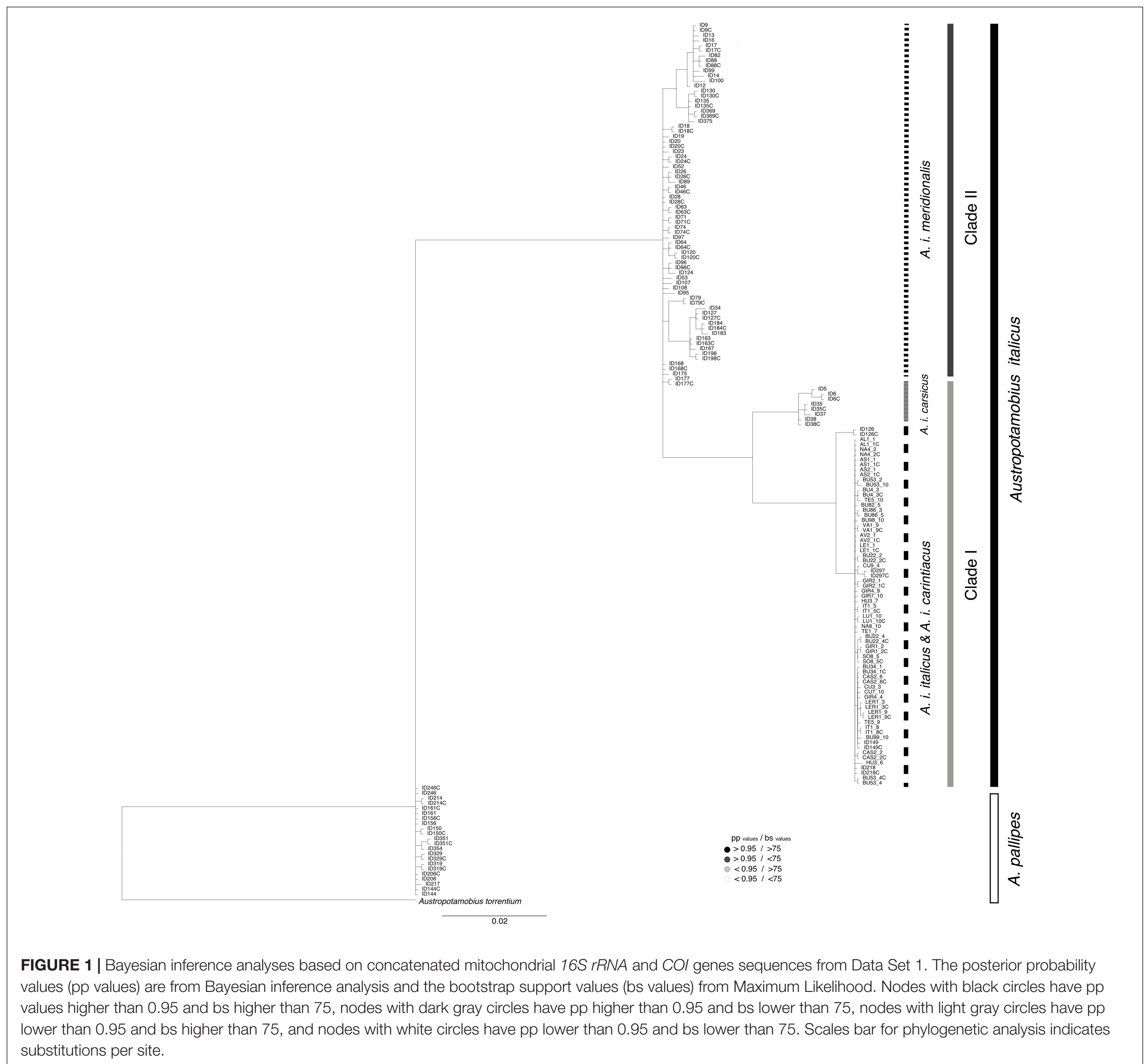

FIGURE 1 | Bayesian inference analyses based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences from Data Set 1. The posterior probability values (pp values) are from Bayesian inference analysis and the bootstrap support values (bs values) from Maximum Likelihood. Nodes with black circles have pp values higher than 0.95 and bs higher than 75, nodes with dark gray circles have pp higher than 0.95 and bs lower than 75, nodes with light gray circles have pp lower than 0.95 and bs higher than 75, and nodes with white circles have pp lower than 0.95 and bs lower than 75. Scales bar for phylogenetic analysis indicates substitutions per site.

diversity (Hd = 0.803) and nucleotide diversity (π = 0.00082) (**Table 3**). Only two hydrogeographic areas (Guadalquivir and Sella) were monomorphic for two different haplotypes (H1 and H30, respectively) (**Table 3**). The rarefied data set for the 10 hydrogeographic areas included nine individuals per area (**Table 3**). There were again differences among the hydrogeographic areas, with the Ebro and Duero areas hosting five different haplotypes. Rarefied samples from the Ebro area hosted haplotypes H1, H30, H11, H16, and H22, and Duero hosted haplotypes H1, H9, H16, H24, and H30. Both presented the highest haplotype (Hd_{Ebro} = 0.861 and Hd_{Duero} = 0.806) and nucleotide (π_{Ebro} = 0.001 and π_{Duero} = 0.0007) diversities. Three hydrogeographic areas were monomorphic for two different haplotypes H1 for Guadalquivir and Miño areas, and H30 for Sella area (**Table 3**). Tajima's D and Fu's Fs were non-significant ($p > 0.05$) for the 10 hydrogeographic areas in both raw and rarefied data, indicating no evidence of recent demographic expansion within these grouping structures (**Table 3**).

Results of AMOVA analysis using the 10 hydrogeographic areas with the raw data suggested more genetic differentiation among populations within hydrogeographic areas (52.58% of variation, $p < 0.0001$) than between the hydrogeographic areas (20.46% of variation, $p < 0.0001$). The rarefied data suggested on the other hand more genetic differentiation between hydrogeographic areas (51.96% of variation, $p < 0.0001$), although they also showed high genetic differentiation among populations within hydrogeographic areas (31.08% of variation, $p < 0.03$). Fixation indices F_{ST} and F_{SC} presented medium/high

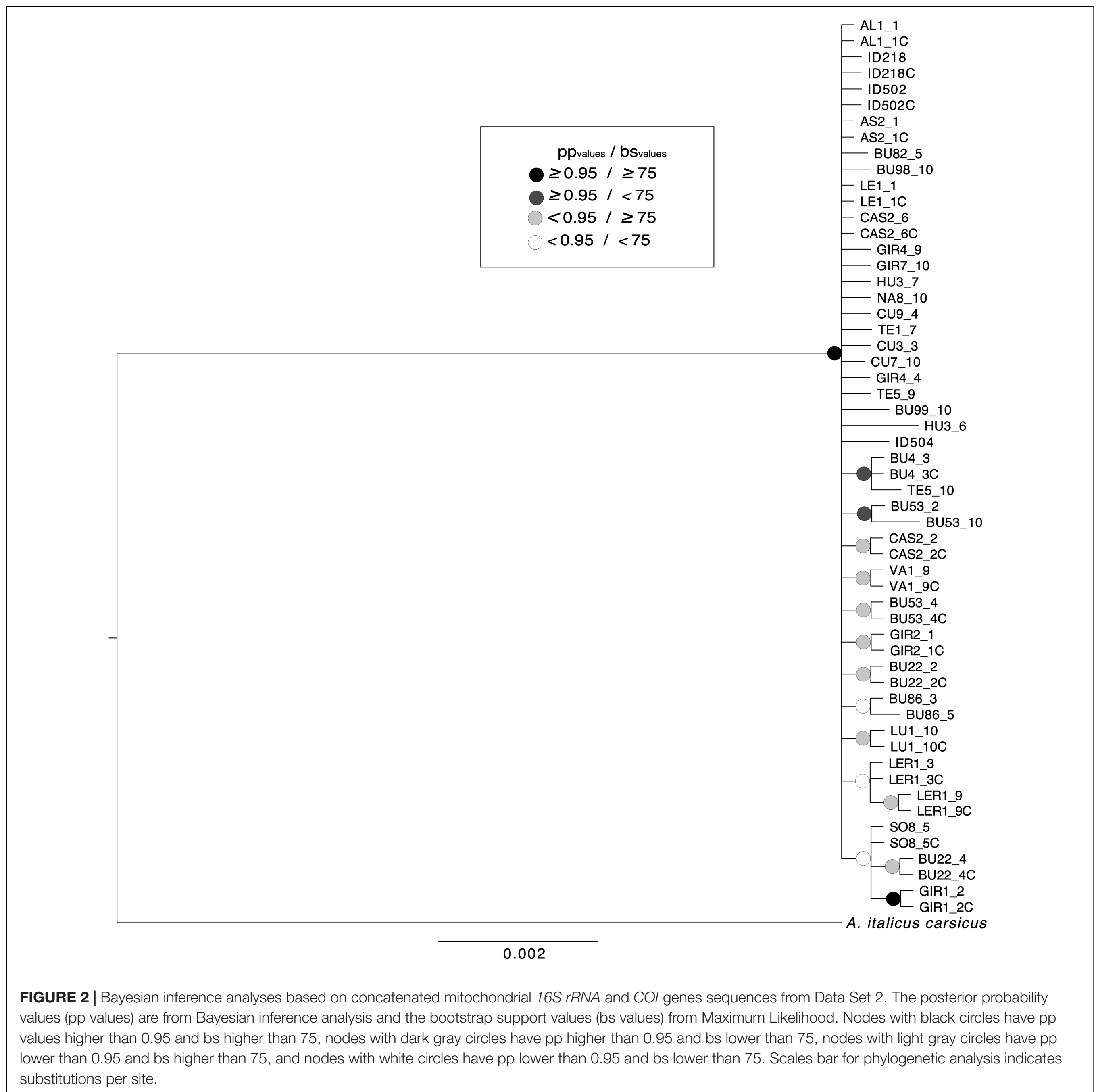

FIGURE 2 | Bayesian inference analyses based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences from Data Set 2. The posterior probability values (pp values) are from Bayesian inference analysis and the bootstrap support values (bs values) from Maximum Likelihood. Nodes with black circles have pp values higher than 0.95 and bs higher than 75, nodes with dark gray circles have pp higher than 0.95 and bs lower than 75, nodes with light gray circles have pp lower than 0.95 and bs higher than 75, and nodes with white circles have pp lower than 0.95 and bs lower than 75. Scales bar for phylogenetic analysis indicates substitutions per site.

scores for the raw data (F_{ST} = 0.73032 and F_{SC} = 0.66096) and rarefied data (F_{ST} = 0.83034 and F_{SC} = 0.64684) showing visible genetic differentiation within populations and among populations within hydrogeographic areas. On the other hand, the fixation indices found no noteworthy differentiation among hydrogeographic areas, being bigger for the rarefied than for the raw data (F_{CT-RAW} = 0.20456 and $F_{CT-RAREFIED}$ = 0.51959).

According to the third grouping structure, we defined three phylogeographic areas, one grouping most of the localities from the Center and East of the Iberian Peninsula (Group 1), and two in the North (Group 2 and Group 3) (**Figure 3**). The results for the raw data set showed differences among three phylogeographic areas, with the Group 1 hosting 28 out of 35 haplotypes [haplotype diversity (Hd) = 0.636, and nucleotide diversity (π) = 0.00038, and no monomorphic groups for any of the locations] (**Table 4**). The rarefied data set for each of the three phylogeographic areas included 62 individuals per basin (**Table 4**). None of the groups were monomorphic, and there were differences among them, with the Group 1 also hosting the largest set of haplotypes (9 out of a total of 18), with the highest haplotype (Hd = 0.646) and nucleotide (π = 0.00039) diversities. Values for Tajima's D and Fu's Fs were significant ($p < 0.05$) for

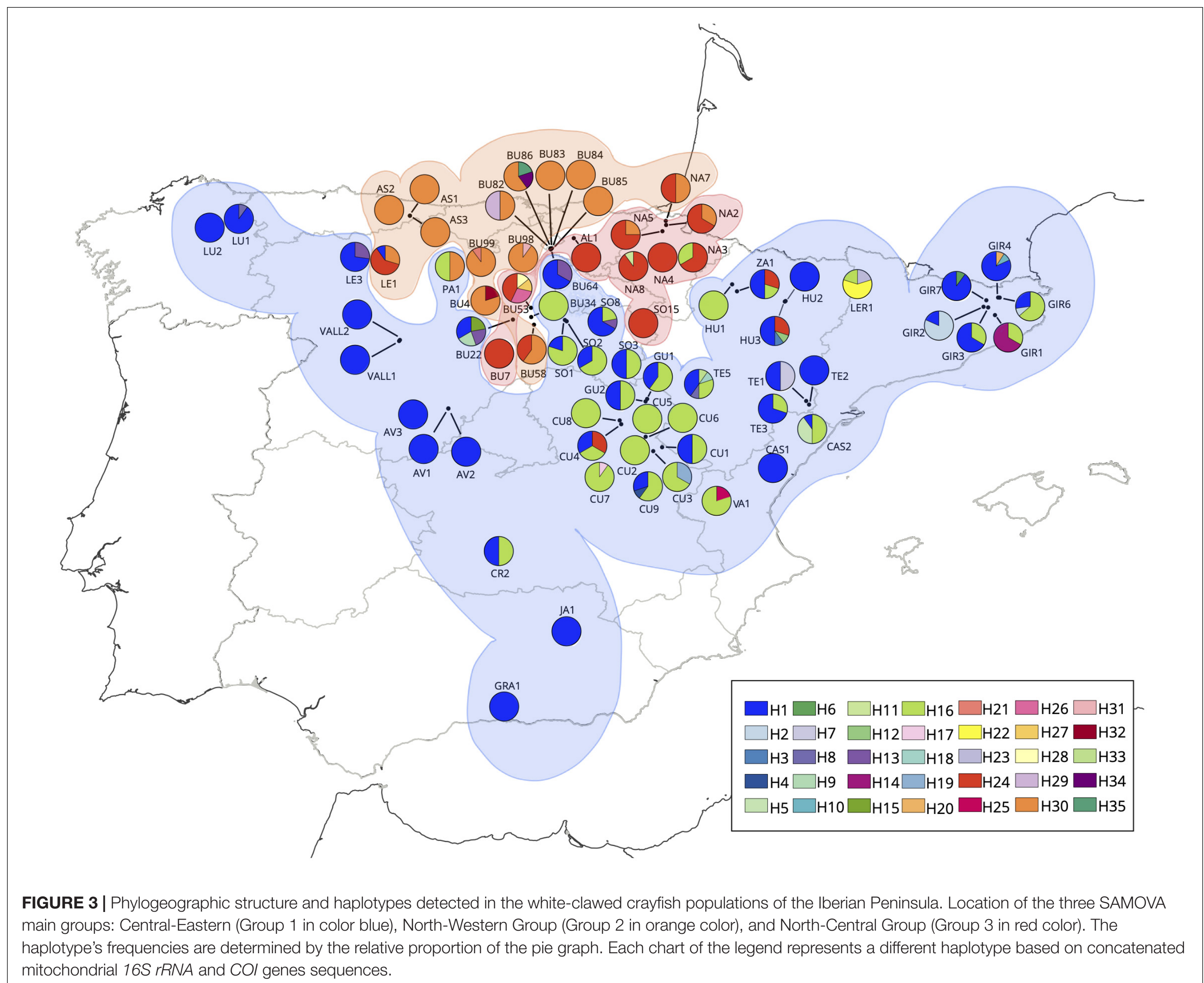

FIGURE 3 | Phylogeographic structure and haplotypes detected in the white-clawed crayfish populations of the Iberian Peninsula. Location of the three SAMOVA main groups: Central-Eastern (Group 1 in color blue), North-Western Group (Group 2 in orange color), and North-Central Group (Group 3 in red color). The haplotype's frequencies are determined by the relative proportion of the pie graph. Each chart of the legend represents a different haplotype based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences.

the three phylogeographic areas for both raw and rarefied data, with the exception of Tajima's D on rarefied data from Group 1. These significances indicated an evidence of recent demographic expansions within each of these three groups.

Results of the AMOVA analysis using the three phylogeographic areas suggested more genetic differentiation among phylogeographic areas for both raw and rarefied data (73.01% of variation, $p < 0.0001$ for raw data, and 73.80% of variation, $p < 0.0001$ for rarefied data). Moreover, the variation within populations was noticeable (16.91% of variation, $p < 0.0001$ for raw data and 18.23% of variation, $p < 0.0001$ for rarefied data). Fixation indices differed, presenting F_{ST} high values for the raw data ($F_{ST} = 0.83091$) and rarefied data ($F_{ST} = 0.81768$), but lower F_{SC} values for the raw data ($F_{SC} = 0.37358$) and rarefied data ($F_{SC} = 0.30425$), showing a visible genetic differentiation within populations but not among populations within three phylogeographic areas. On the other hand, the fixation indices found a statistically significant differentiation ($p < 0.05$) among the three phylogeographic areas for both raw and rarefied data ($F_{CT-RAW} = 0.73007$ and $F_{CT-RAREFIED} = 0.73796$).

DISCUSSION

As far as we know, this work represents the most complete and updated approximation of the WCC genetic diversity in the Iberian Peninsula. The results obtained complete and confirm the historical scenario proposed by previous studies on the genetic diversity of the WCC (Pedraza-Lara et al., 2010; Jelić et al., 2016; Matallanas et al., 2016), but also reveal new patterns of genetic diversity and phylogeographic structure. The phylogeographic approach used here, which incorporates samples from previously unexplored areas, has allowed us to find a higher genetic variation in the WCC than previously reported and also identifies new private haplotypes in the Iberian Peninsula. Moreover, the results regarding the origin of the genetic diversity and its phylogeographic structure do not support the hypothesis of an

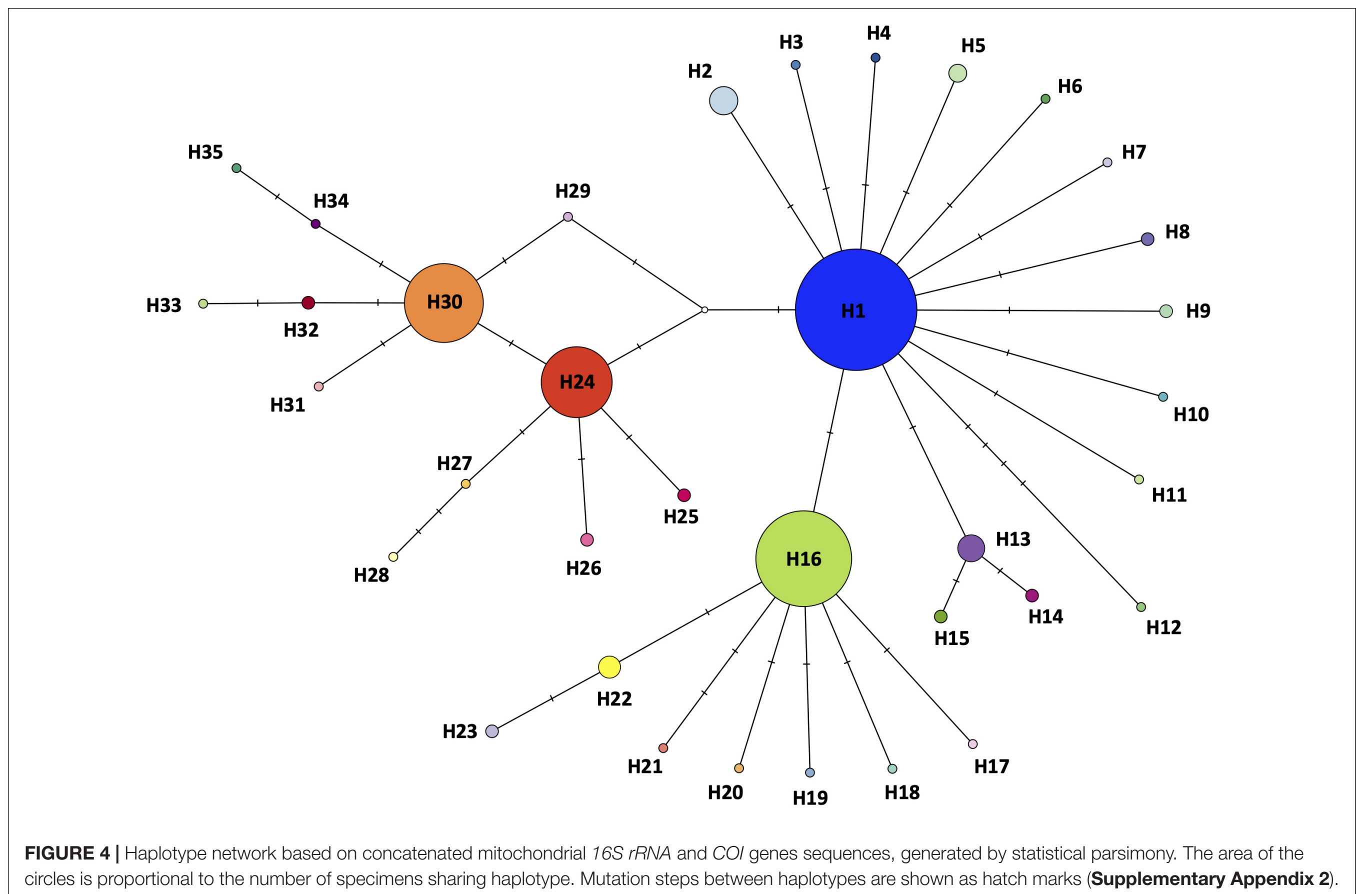

FIGURE 4 | Haplotype network based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences, generated by statistical parsimony. The area of the circles is proportional to the number of specimens sharing haplotype. Mutation steps between haplotypes are shown as hatch marks (**Supplementary Appendix 2**).

introduction from Italy in the 17th century (Vedia and Miranda, 2013; Clavero et al., 2016), and, instead, strongly suggest a native origin of the WCC in the Iberian Peninsula.

Genetic variation found within Iberian WCC populations is strongly structured geographically. The results from this phylogenetic approach, also supports the scenario proposed by Pedraza-Lara et al. (2010) and Jelić et al. (2016) for the Iberian *A. i. italicus* populations within the European area. In these scenarios, the Iberian lineages grouped together with populations from Austria, Italy and South France. This group is closely related to the *A. i. carsicus*, which occurs in Croatia and Italy, and phylogenetically separated from the Central European WCC populations. Following these previous studies and the results in here, the existing genetic differentiation between Central and Southern Europe WCC populations would be of Pleistocenic/Holocenic origin, and might be related to the climate oscillations and glaciations, including the phylogeographic and demographic effects of the ice sheet presence during and after the Last Glacial Maximum (LGM) (Hewitt, 2000, 2004). The fact that Central regions of Europe were glaciated during the LGM led many species to remain isolated in the Southern glacial refugia, i.e., the Iberian, Italian, and Balkan Peninsulas. Besides the presence of the ice itself, the LGM also entailed drastic changes in temperature, droughts, desertification, and large drops in the sea levels changes, that might have shaped the range area in WCC. The distribution of *A. i. italicus* follows a Circum-Mediterranean distribution. This distribution is also found in other freshwater species (Perea et al., 2010) associated with the isolation in glacial refugia during the LGM, and/or with ancient paleogeography events. For instance, the Alps orogenesis during the late Miocene until the Pleistocene had isolated the Iberian Peninsula from Central Europe and prevented most Mediterranean freshwater species, such as the European cyprinids, to move northward (Zardoya and Doadrio, 1999; Perea et al., 2010).

The phylogenetic analyses for the Iberian WCC populations showed a basal polytomy, indicating non-solved phylogenetic relationships among them. Although some well-supported grouping are presented, more molecular markers would be necessary to identify evolutionary lineages and their relationships. These results agree with the previous assignation of Pedraza-Lara et al. (2010) and Matallanas et al. (2016) for the Iberian populations. On the other hand, we confirmed the utility of these mitochondrial markers that revealed variability patterns not found in previous studies (Toon et al., 2009). The combination of a greater sampling effort and the use of these two regions revealed a total of 35 haplotypes, 16 of which were new for the Iberian Peninsula, while Matallanas et al. (2016) found 19 haplotypes using the same *16S* and *COI* genes.

In addition, we found a strong phylogeographic structure in the Iberian populations. Previous studies designated two phylogeographic areas, while we found three genetically and geographically differentiated areas. Thus, we found two areas in

TABLE 2 | Frequency of the haplotypes found in the 71 analyzed populations (the population's code is detailed in **Table 1**).

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35
AL1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
AS1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
AS2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
AS3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
AV1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
AV2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
AV3	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU22	0.33	·	·	·	·	·	·	·	0.22	·	·	·	0.22	·	0.22	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU34	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.80	·	0.20	·	·	·
BU53	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.43	·	0.29	0.14	0.14	·	·	·	·	·	·	·
BU58	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.40	·	·	·	·	·	0.60	·	·	·	·	·
BU64	0.67	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
BU82	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	0.50	·	·	·	·	·
BU83	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
BU84	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
BU85	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
BU86	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	0.20	0.20
BU98	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.90	0.10	·	·	·	·
BU99	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	0.90	·	·	·	·	·
CAS1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CAS2	0.10	·	·	·	0.40	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CR2	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU1	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU4	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·
CU5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU6	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.90	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU9	0.30	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR1	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR2	0.18	·	·	0.82	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR3	0.67	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

(Continued)

TABLE 2 | (Continued)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35
GIR4	0.82	·	·	·	·	·	·	·	·	0.09	·	·	·	·	·	·	·	·	·	0.09	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR6	0.27	0.09	·	·	·	·	·	·	·	·	·	·	·	·	·	0.64	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR7	0.90	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GRA1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GU1	0.40	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GU2	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HU1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HU2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HU3	0.50	·	0.10	·	·	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	0.30	·	·	·	·	·	·	·	·	·	·	·
JA1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LE1	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	·	·	0.30	·	·	·	·	·
LE3	0.73	·	·	·	·	·	·	·	·	·	·	·	0.27	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LER1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.20	·	·	·	·	·	0.60	0.20	·	·	·	·	·	·	·	·	·	·	·	·
LU1	0.90	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LU2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
NA2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	·	·	·	·	0.33	·	·	·	·	·
NA3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	0.67	·	·	·	·	·	·	·	·	·	·	·
NA4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
NA5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.75	·	·	·	·	·	0.25	·	·	·	·	·
NA7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	0.50	·	·	·	·	·
NA8	·	·	·	·	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	0.90	·	·	·	·	·	·	·	·	·	·	·
PA1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·
SO1	0.20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.80	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
SO15	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
SO2	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
SO3	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
SO8	0.67	·	·	·	·	·	·	·	·	·	·	·	0.11	·	·	0.22	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE1	0.50	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE3	0.70	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.30	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE5	0.40	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	0.30	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.10	·	·
VA1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.80	·	·	·	·	·	·	·	·	0.20	·	·	·	·	·	·	·	·	·	·
VALL1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
VALL2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ZA1	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.20	·	·	·	·	·	·	·	0.30	·	·	·	·	·	·	·	·	·	·	·

The symbol "·" means zero frequency.

TABLE 3 | Genetic diversity within white-clawed crayfish hydrogeographic catchments in the Iberian Peninsula for the raw and rarefied data.

	n	S	H	Hd	π	D	Fs
Raw data							
Bidasoa	9	1	2	0.5	0.0002	0.98627	0.849
Ebro	145	20	18	0.80316	0.00082	−1.22384	−6.389
Cataluña	58	7	7	0.65094	0.00036	−1.06969	−2.232
Júcar	45	7	6	0.65354	0.00042	−0.96976	−1.075
Guadalquivir	12	0	1	0	0	n/c	n/c
Guadiana	14	2	3	0.38462	0.00016	−0.95919	−0.855
Tajo	38	3	3	0.38265	0.00019	−0.73449	0.115
Duero	142	14	12	0.76136	0.00075	−0.70395	−1.964
Miño	20	1	2	0.1	0.00004	−1.16439	−0.879
Sella	22	0	1	0	0	n/c	n/c
Rarefied data							
Bidasoa	9	1	2	0.5	0.0002	0.98627	0.849
Ebro	9	6	5	0.861	0.00101	0.57782	−0.354
Cataluña	9	2	3	0.667	0.00032	0.1959	−0.108
Júcar	9	1	2	0.556	0.00023	1.40117	1.015
Guadalquivir	9	0	1	0	0	n/c	n/c
Guadiana	9	2	3	0.417	0.00018	−1.3624	−1.081
Tajo	9	1	2	0.389	0.00016	0.15647	0.477
Duero	9	5	5	0.806	0.00068	−0.39837	−1.26
Miño	9	0	1	0	0	n/c	n/c
Sella	9	0	1	0	0	n/c	n/c

n, sample size; S, number of polymorphic sites; H, number of haplotypes found; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs.
The "n/c" means not calculated.

TABLE 4 | Genetic diversity within white-clawed crayfish SAMOVA groups in the Iberian Peninsula for the raw and rarefied data.

	n	S	H	Hd	π	D	Fs
Raw Data							
G1	360	28	25	0.636	0.00038	**−2.08288***	**−23.426***
G2	83	10	8	0.267	0.00017	**−2.07311***	**−6.285***
G3	62	8	7	0.32	0.0002	**−1.85126***	**−1.85126***
Rarefied data							
G1	62	12	9	0.646	0.00039	−1.77358	**−3.902***
G2	62	9	6	0.268	0.00018	**−2.07514***	**−3.479***
G3	62	8	7	0.32	0.0002	**−1.85126***	**−1.85126***

*n, sample size, S, number of polymorphic sites; H, number of haplotypes found; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs; *p < 0.05.*

the North (North-Central and North-Western) represented by two main haplotypes, H24 and H30, respectively. This matches with the differentiation and structure found in other terrestrial and aquatic species, such as *Salamandra salamandra* (García-París et al., 2003), *Ichthyosaura alpestris* (Recuero et al., 2014) or *Lissotriton helveticus* (Recuero and García-París, 2011). During the LGM, strong range shifts and bottlenecks occurred and they probably played a key role in Iberian WCC populations. Although the Northern regions of the Iberian Peninsula were mostly covered with ice, glaciers remained confined within the mountain systems and did not reach the surrounding lowlands (Oliva et al., 2019). As a consequence, a number of unglaciated habitats are believed to have acted as refugia for several species (Gómez and Lunt, 2007). The identification of private haplotypes in this area could indicate that these WCC populations were geographically limited and isolated in these regions, as it occurred to other species (Hewitt, 2000). A significant postglacial expansion of these endemic haplotypes might have occurred from several populations during favorable climate periods (Hewitt, 2004). Moreover, the genetic results and the shape of the haplotype network indicated an evidence of recent demographic expansions within these two groups. These results are consistent with those of Matallanas et al. (2016), in which molecular estimations dated last WCC population expansion back to Pleistocene.

The third phylogeographic area that comprises Central-Eastern and the westernmost Iberian populations, suggested another expansion event. The evidences from the significant results obtained in the genetic diversity analysis, as well as the star-like haplotype network showed another demographic

expansion, and also reflect the possibility of another LGM-refugium. The Central-Eastern area is also represented by two main haplotypes, H1 and H16. During the LGM, these populations could have persisted in Southern areas of the Iberian Peninsula in absence of geographical or climate barriers. As evidenced by the analyses and the samples studied, these two main haplotypes could represent ancestral haplotypes: H1 (the most frequent), and H16 (only separated by one mutational step from H1). Besides, the H1 has been found in other European populations (Jelić et al., 2016; Matallanas et al., 2016). The Central-Eastern area could have had an earlier expansion than that of the Northern areas, since this area was not covered by ice during the LGM and thus, in absence of barriers, crayfish could have expanded over long distances to some extent (Robinson et al., 2000), and a better climate would have favored it. However, the unequal sampling of the specimens within the studied populations seem to have favored higher frequencies of the H1 and H16 haplotypes, so this statement must be taken with caution. Furthermore, the low genetic diversity found within the WCC southern areas could be explained by the introduction of the North American crayfish species *P. clarkii* and *P. leniusculus* during the 1970s (Alonso et al., 2000; Martín-Torrijos et al., 2019). These North American crayfish are natural carriers of the crayfish plague pathogen *A. astaci* (Martín-Torrijos et al., 2021) and responsible for the decline of the Iberian WCC until nowadays. In particular, by the end of the 1990s the Southern WCC populations almost had disappeared in the Iberian Peninsula (Alonso et al., 2000; Martín-Torrijos et al., 2019).

The current genetic diversity found in Iberian Peninsula may have been shaped by the LGM, as suggested above and in previous studies. The extensive sampling of this study has allowed us to find the greatest haplotype and nucleotide diversities so far reported for the Iberian WCC populations (Matallanas et al., 2016). We should point out, however, that 27 populations were monomorphic. These monomorphic populations were represented by the four most common haplotypes (H1, H16, H24, and H30). This agrees with the strong phylogeographic structure found. In contrast, the most diverse phylogeographic area was the Central-Eastern. This contains populations highly diverse that are located in the provinces of Burgos, Teruel and Huesca. This high genetic variation found within these populations coincides with the historical records of dense populations of WCC described (Alonso et al., 2000). This abundance evidences the importance that crayfish fisheries used to have in local economies of rural Iberian areas. By 1964, Spanish legislation already had regulated the size, amount of crayfish and the fishing gear allowed for the fishing activities (Torre Cervigón and Rodríguez Marqués, 1964). This regulation might have helped to avoid the overexploitation of the resource, maintaining most of the genetic diversity that remains nowadays. Although recent human translocations might have influenced the current Iberian crayfish distribution, there is, still, a strong phylogeographic structure. During the past years, several authors have suggested that the origin of the Iberian populations might have been the result of an Italian crayfish translocation during the 17th century (Vedia and Miranda, 2013; Clavero et al., 2016). However, the results obtained in the present study show a greater genetic diversity than that described in previous studies, and indicate that is highly structured and difficult to attribute to a 17th century translocation from Italy. Therefore, our results support the native origin of the WCC in the Iberian Peninsula. Moreover, the rapid impact of the crayfish plague during the 1970s' dramatically reduced the number of Iberian WCC populations in less than 2 years (Alonso et al., 2000). These massive declines might have extinguished highly diverse WCC populations, and what we actually come across is a small fraction of its original genetic diversity. In addition, the enormous extinctions due to the crayfish plague may have driven Iberian WCC populations to suffer inbreeding, bottlenecks and genetic drift. Currently, the difficulty of obtaining samples due to the threatened status of the WCC made us to use different number of individuals from each of the analyzed populations. The reduced number of samples obtained from some of the populations revealed the appearance of rare private haplotypes in low frequencies. These rare haplotypes may represent a reflect of the remaining biodiversity within WCC populations.

In addition, the delimited phylogeographic areas seem to explain better the genetic diversity of the Iberian WCC populations. The AMOVA analysis showed more genetic differentiation among the three phylogeographic areas and a slight genetic differentiation among populations within these areas. This suggests substantial gene flow among populations from the same phylogeographic areas. On the other hand, the reduced population sites, the geographical distribution, and the pressure over the remaining Iberian WCC populations by the continuous threat by crayfish plague, for instance, might be favoring their isolation and hindering the gene flow between them. Thus, the unique genetic diversity represented by private haplotypes, which is found in low frequencies, would remain in the same populations instead of being transferred to proximal populations.

Current populations are a remain of what Iberian WCC populations used to be. The massive extinction events that Iberian WCC have been suffering during the past 45 years due to the introduction of North American invasive crayfish carrying the crayfish plague (Martín-Torrijos et al., 2019), and intensive harvesting, might have had irreversible effects on the Iberian WCC genetic diversity. Thus, the majority of the ancient Iberian WCC genetic variation might be already extinct. The reduction of the genetic diversity, and consequently their adaptive potential (Boulding, 2008; Jump et al., 2009), could increase the species extinction risk. To preserve the maximum genetic diversity, we recommend that current conservation and management programs for the WCC in the Iberian Peninsula should consider the patterns of genetic diversity found in this study. Thus, we propose that the three phylogeographic areas revealed in this study should be considered as essential management units to preserve the genetic diversity that characterized them. Furthermore, conservation actions that include breeding and re-stocking programs should consider for each specific area not only the most common but also the private haplotypes. This

will certainly help to preserve the unique genetic pool from the endangered Iberian WCC populations.

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 in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LM-T contributed to the design, with the laboratory work, and wrote the manuscript. DB, ID, AM, and JD-U contributed to the supervision of the manuscript and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

We would like to acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). JD-U was supported from Project CGL2016-80526-R of the Ministerio de Economía y Competitividad, Spain, and LM-T was supported LIFE MedWetRivers (LIFE11 NAT/ES/000699) and LIFE Potamo Fauna (LIFE12 NAT/ES/001091). This study was also supported by Gobierno de Navarra (20153614 and 20166383), Junta de Comunidades de Castilla-La Mancha (20153074 and 20166103), Gobierno de Aragón (20153608; 20171065 and 20171106), and La Agencia de Medio Ambiente y Agua de Andalucía (20091163).

ACKNOWLEDGMENTS

We would like to thank the assistance in providing us with crayfish samples and information to Jokin Larumbe (Gobierno de Navarra); Fernando Alonso and Javier Valenciano (Junta de Comunidades de Castilla-La Mancha), Sara Lapesa (Gobierno de Aragón), Joan Montserrat (Parque Natural La Garrotxa, Catalunya), Lluis Benejam (CEBCAT-La Balca S. L.), Antonio Pradillo (CCEDCV—EL PALMAR, Generalitat Valenciana), Juan María Herrero (Junta de Castilla y León), Jon Zulaika (Diputación de Gipuzkoa, País Vasco), Borja Nebot (Junta de Andalucía), and Javier Galindo (Agencia Medio Ambiente y Agua de Andalucía). We would also like to thank Marta Higuera and Emilio Cano from the Real Jardín Botánico–Consejo Superior de Investigaciones Científicas (RJB-CSIC) for their technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.669292/full#supplementary-material

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