



# Micro-Computed Tomography Reveals a Remarkable Twin Intromittent Organ in Spiders – A Novelty for Arachnids With Direct Sperm Transfer

Michael G. Rix<sup>1,2\*</sup>, Hannah M. Wood<sup>3†</sup>, Mark S. Harvey<sup>2†</sup> and Peter Michalik<sup>4†</sup>

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### \*Correspondence:

Michael G. Rix  
michael.rix@qm.qld.gov.au

### †ORCID:

Michael G. Rix  
[orcid.org/0000-0001-5086-3638](https://orcid.org/0000-0001-5086-3638)  
Hannah M. Wood  
[orcid.org/0000-0003-0453-2699](https://orcid.org/0000-0003-0453-2699)  
Mark S. Harvey  
[orcid.org/0000-0003-1482-0109](https://orcid.org/0000-0003-1482-0109)  
Peter Michalik  
[orcid.org/0000-0003-2459-9153](https://orcid.org/0000-0003-2459-9153)

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<sup>1</sup> Biodiversity and Geosciences Program, Queensland Museum, Brisbane, QLD, Australia, <sup>2</sup> Collections and Research Centre, Western Australian Museum, Perth, WA, Australia, <sup>3</sup> Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, DC, United States, <sup>4</sup> Zoologisches Institut und Museum, Universität Greifswald, Greifswald, Germany

The modification of male pedipalps into secondary sexual intromittent organs is one of the hallmark characteristics of spiders, yet understanding the development and evolution of male genitalia across the order remains a challenging prospect. The embolus – the sclerite bearing the efferent spermatic duct or spermophor, and used to deliver sperm directly to the female genitalia during copulation – has always been considered the single unambiguously homologous palpal sclerite shared by all spider species, fundamental to the bauplan of the order and to the evolution and functional morphology of spider reproductive systems. Indeed, after two centuries of comparative research on spider reproduction, the presence of a single spermophor and embolus on each of a male spider's two pedipalps remains a central tenet of evolutionary arachnology. Our findings challenge this premise, and reveal a remarkable twin intromittent organ sperm transfer system in a lineage of Australian palpimanoid spiders, characterized by a bifurcate spermophor and the presence of two efferent ducts leading to a pair of embolic sclerites on each pedipalp. This is the first time such a remarkable conformation has been observed in any group of arachnids with direct sperm transfer, complicating our understanding of palpal sclerite homologies, and challenging ideas about the evolution of spider genitalia.

**Keywords:** Archaeidae, haplogyne, morphology, palpal organ, Palpimanoidea, phylogeny

## INTRODUCTION

Sperm transfer in Arachnida is achieved either indirectly with spermatophores (e.g., in Amblypygi, Scorpiones, and Pseudoscorpiones), or directly using a penis (e.g., in Opiliones), the chelicerae (e.g., in Solifugae) or modified appendages (e.g., in Araneae and Ricinulei). Most prominent are the male intromittent organs of spiders, the so-called palpal organs or genital bulbs situated on the pedipalps, which directly transfer sperm to the female gonopore during copulation (Coddington and Levi, 1991; Coddington et al., 2004). To do so, the male spider must first release sperm

from his gonopore onto a sperm web, after which the sperm is taken up into the palpal organ. The palpal organs of adult male spiders vary enormously in their morphology, complexity, and ontogeny (e.g., Coddington, 1990; Griswold et al., 2005; Dederichs et al., 2019), thought to be originally derived from a tarsal claw (Coddington, 1990; Quade et al., 2019) and consisting at their simplest of an undivided piriform sclerite (the “bulb”) with a terminal embolus (Magalhães and Ramírez, 2017; Michalik et al., 2019), the latter enclosing an efferent duct (the spermatic duct or “spermophor”) which is gradually enlarged proximally (Foelix, 2011). In most spider taxa the palpal organ is more complex – spectacularly so in some lineages, e.g., Linyphiidae (Hormiga, 1994, 2000) – with divided bulb (i.e., tegular and subtegular) sclerites, inflatable membranous elements (haematodochae) that rely on hydraulic pressure, and multiple tegular apophyses in addition to the ubiquitous internal spermophor and sclerotized embolus bearing a terminal sperm pore (Griswold et al., 2005). These palpal sclerites function in concert during copulation, engaging and inter-locking with the female genitalia (e.g., Uhl et al., 2007; Mouginit et al., 2015; Poy et al., 2020), which may explain the diversity of species-specific morphologies that occur in these structures. Given this morphological complexity, and the importance of male palpal morphology to spider reproduction and systematics, ontological and comparative morphological research has long focused on trying to determine sclerite homologies across and within spider taxa (Comstock, 1910; Shear, 1967; Kraus, 1978; Raven, 1985; Coddington, 1990; Ramírez, 2014). In all such studies it has consistently been shown that the embolus is the only primary homologous palpal sclerite shared by all known spider species, with other putatively symplesiomorphic sclerites (e.g., the conductor and median apophysis) prone to fusion or loss. By extension, the internal spermophor is also homologous across all spiders, a proposition borne out by ontogeny (Coddington, 1990) and over two centuries of taxonomic research.

Spiders of the superfamily Palpimanoidea have long been of interest to arachnologists, due to their phylogenetic antiquity (Rix and Harvey, 2012c; Wood et al., 2012, 2013, 2014), highly modified somatic morphology (Wood et al., 2012; Wood and Parkinson, 2019) and phylogenetic position as sister clade to the Entelegynae (Ramírez et al., 2021) – the latter a highly diverse lineage containing the bulk of spider diversity, with a modified “flow-through” female genitalic system bearing distinct copulatory and fertilization ducts (Griswold et al., 2005; Wheeler et al., 2017; Michalik et al., 2019). With five unambiguously monophyletic families, a remarkably rich fossil record, and an evolutionary origin on the Pangaeian supercontinent (Wood et al., 2013, 2018), the Palpimanoidea are a rare example of an arthropod lineage of putatively ancient origin to have a traditionally vicariant biogeographical hypothesis now strongly supported by dated phylogenetics (Wood et al., 2013). “Assassin” or “pelican” spiders of the family Archaeidae are a lineage of Palpimanoidea with a particularly fragmented modern distribution in Australia and the Old World Afrotropics (southern Africa and Madagascar), temporally and geographically consistent with the breakup of East and West Gondwana (Wood et al., 2013). However, perhaps unsurprisingly

for a lineage with an extant crown group of Jurassic age (Wood et al., 2013, 2014), comparative morphological work on the five extant genera of Archaeidae is challenging given certain divergent character systems that exist within and between lineages. Unlike in their diverse sister-group, the Entelegynae, the female genitalia of palpimanoidea are haplogyne, exhibiting a cul-de-sac system with only one duct connecting to the spermathecae (Michalik et al., 2019). Indeed, despite having rather simple haplogyne female genitalia, the male genitalic morphology of Archaeidae is surprisingly complex, sometimes with a functional tegular and subtegular division of the male palpal bulb, and always with expandable basal and/or distal haematodochae, and highly flexible tegular sclerites in various taxa (Rix and Harvey, 2011, 2012a; **Figure 1**). In life, most of these complex structures are sunken and partially enclosed within the distal rim of the bulb (except during copulation, when the bulb greatly expands), making comparative morphological assessment especially difficult in the unexpanded state. Thus, despite the highly derived yet nonetheless geologically conserved somatic morphology and ecology of both Afrotropical and Australian Archaeidae, a consensus on palpal sclerite homologies within the family is yet to be achieved (Wood, 2008; Rix and Harvey, 2011, 2012a,b; Wood and Scharff, 2018).

In this study, our initial aim was to use the trajectory of the spermophor to identify the embolus in lineages of Palpimanoidea, especially in Archaeidae from the Wet Tropics of north-eastern Queensland (Australia), for which the identification of the embolus was unexpectedly problematic (Rix and Harvey, 2012b). In doing so, we revealed a remarkable bifurcate conformation of the spermophor and a twin intromittent organ in the archaeid genera from Australia. Our secondary aims, therefore, were to understand the morphology of the palpal organ with a focus on the sperm transferring parts in the Afrotropical archaeid genera and the remaining palpimanoidea families, and to pose a number of hypotheses that could be used to understand possible evolutionary or ontogenetic drivers in the context of correlated female genitalic morphology and sexual selection.

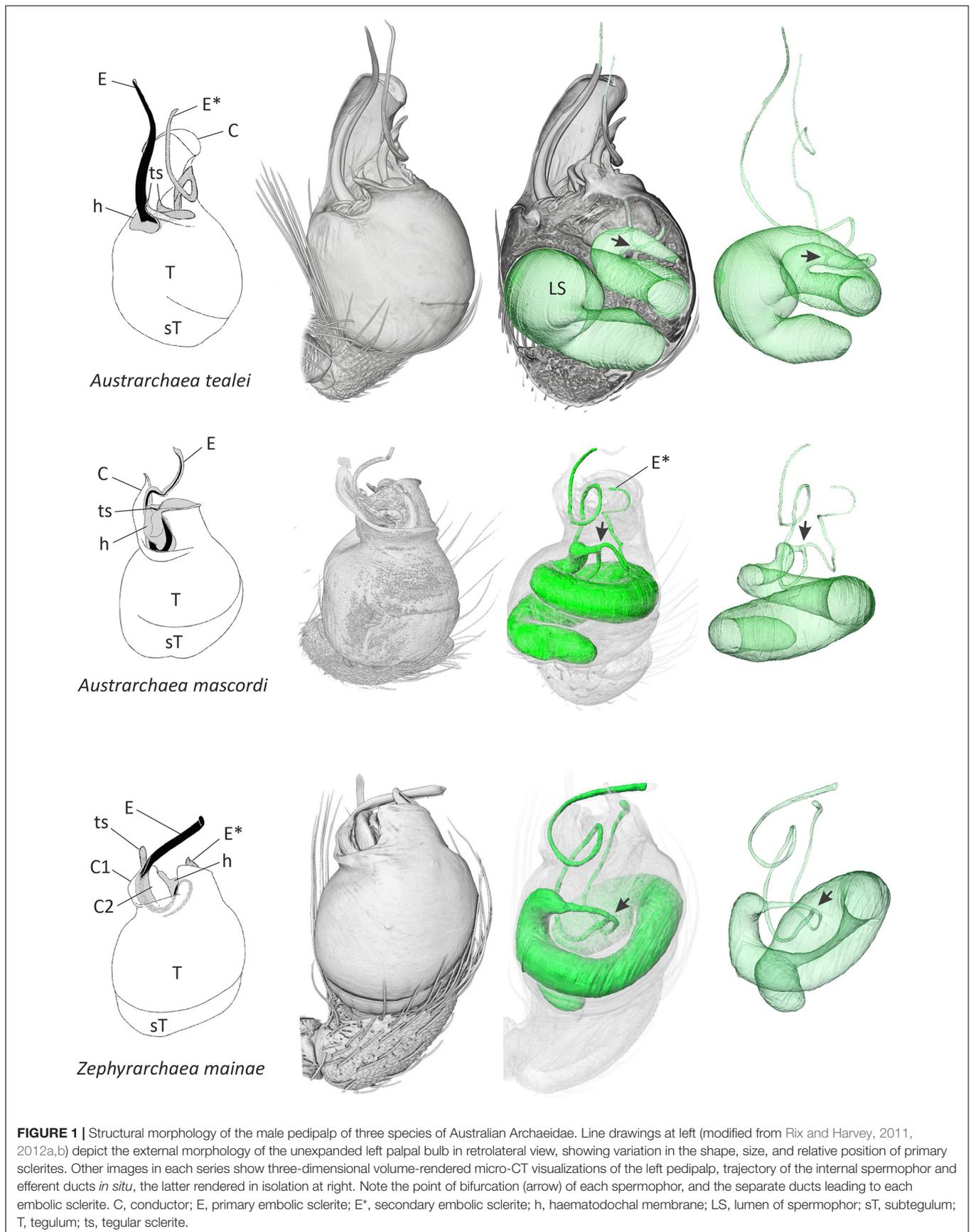
## METHODS

### Specimens

Ten exemplar male specimens from each of the five palpimanoidea families, and from all five genera of Archaeidae, were sourced from collections held at the California Academy of Sciences (San Francisco, CA, United States), the Western Australian Museum (Perth, WA, Australia) and the Zoologisches Institut und Museum (Greifswald, Germany) (**Table 1**). Animals were preserved in either 70 or 95% ethanol, prior to stereo and compound microscopy, and micro-computed tomography (micro-CT) analysis.

### Micro-CT Analysis

Preserved male pedipalps were dehydrated in graded ethanol and stained with a 1% iodine solution overnight. Subsequently, the samples were washed in absolute ethanol and critical-point dried



**TABLE 1** | Exemplar male specimens examined for micro-computed tomography (micro-CT) analysis.

Family	Species	Collection locality	Voucher
Archaeidae	<i>Austrarchaea mascordi</i> Rix and Harvey, 2011	Australia: New South Wales: Coolah Tops N.P., Breeza Lookout	WAM T112565
Archaeidae	<i>Austrarchaea tealei</i> Rix and Harvey, 2011	Australia: Queensland: Daintree N.P., Mossman Gorge	CASENT 9028385
Archaeidae	<i>Afrarchaea woodae</i> Lotz, 2006	South Africa: Eastern Cape: Kei Mouth	CASENT 9018994
Archaeidae	<i>Eriauchenius workmani</i> O. P.-Cambridge, 1881	Madagascar: Fianarantsoa: Ranomafana N.P., Vatoharanana River	CASENT 9018968
Archaeidae	<i>Madagascarchaea lavatenda</i> (Wood, 2008)	Madagascar: Antsiranana: SW of Sakaramy	CASENT 9006802
Archaeidae	<i>Zephyrarchaea mainae</i> (Platnick, 1991)	Australia: Western Australia: Sand Patch Beach Reserve	WAM T89582
Huttoniidae	<i>Huttonia palpimanoides</i> O. P.-Cambridge, 1880	New Zealand: Otago: Dunedin: Leith Saddle	ZIMG II-29999
Mecysmaucheniidae	<i>Mecysmauchenius segmentatus</i> Simon, 1884	Chile: Magellanes: Isla Grande de Tierra del Fuego	CASENT 9036216
Palpimanidae	<i>Diaphorocellus</i> sp.	Madagascar: Toliara: Andohahela N.P., Forêt d'Ambohibory	CASENT 9012203
Stenochilidae	<i>Colopea</i> sp.	Brunei Darussalam: Temburong: Kuala Belalong Field Center	CASENT 9035143

CASENT, California Academy of Sciences; WAM, Western Australian Museum; ZIMG, Zoologisches Institut und Museum, Universität Greifswald.

using a Leica CPD300 automated dryer (Leica Microsystems GmbH, Wetzlar, Germany). After mounting on a plastic rod, each pedipalp was scanned using an XRadia MicroXCT-200 X-Ray imaging system (Carl Zeiss Microscopy GmbH, Pleasanton, CA, United States). The tomography projections were reconstructed using the XMReconstructor software (Carl Zeiss Microscopy GmbH, Pleasanton, CA, United States). The volume rendering of the image stacks and the segmentation of the spermophor was performed by Amira 6.4 (Thermo Fisher Scientific Inc., Waltham, CA, United States). Post-processing of images was performed in Corel PaintShop Pro 2020 and Corel Draw 2020.

## Phylogenetic Character-State Mapping

The phylogenetic topology used for character-state mapping against all 10 exemplar palpimanoid taxa (Figure 2) was modified from previously published phylogenetic studies. For inter-familial relationships, the latest phylogenomic topology of Ramírez et al. (2021) was applied, and for inter-generic relationships within Archaeidae, the topology followed the results of Rix and Harvey (2012c) and Wood et al. (2012, 2013, 2014, 2018).

## RESULTS

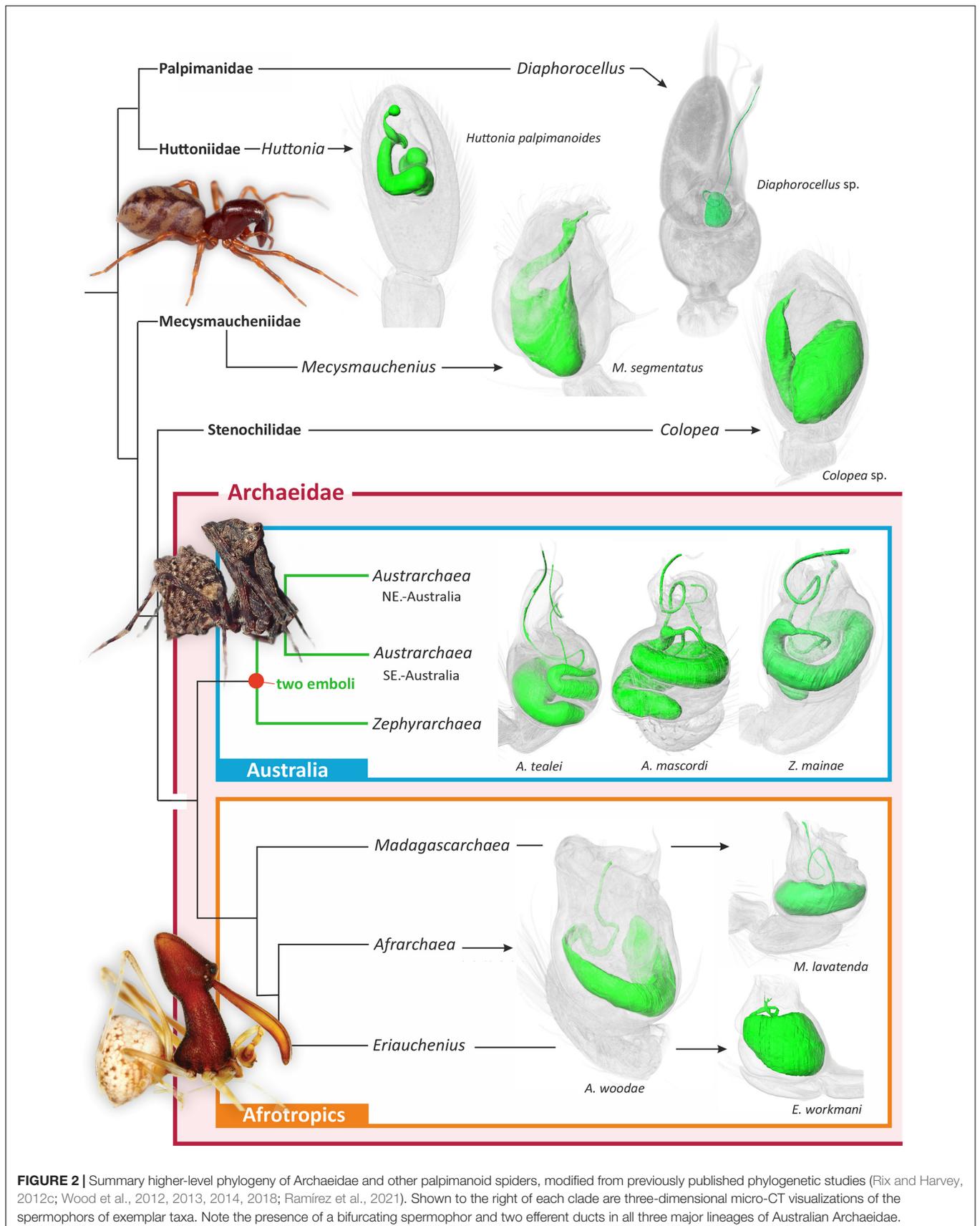
Comparative micro-CT analysis of the palpal organ across palpimanoid spider families revealed a medio-distal bifurcation of the spermophor and two efferent distal ducts in all three major monophyletic lineages of Archaeidae from Australia, i.e., in exemplar species belonging to the *Austrarchaea nodosa* species-group from south-eastern Australia (exemplar *Austrarchaea mascordi*), the *Austrarchaea daviesae* species-group from north-eastern Australia (exemplar *Austrarchaea tealei*), and the genus *Zephyrarchaea* from southern Australia (exemplar *Zephyrarchaea mainae*) (Rix and Harvey, 2012c; Figures 1, 2). In the three specimens scanned the spermophor was highly sinuous, bifurcating deep within the tegular division of the bulb and splitting into two relatively long, filiform efferent ducts (Figures 1, 2). These ducts had convoluted, looped trajectories each leading to separate embolic sclerites representing a twin intromittent organ, one of which (the primary embolic sclerite; E)

was enlarged and supported by a tegular conductor or conductor-like analog (Figure 1). The secondary embolic sclerite (E\*) differed markedly in size between taxa, from relatively small and partially concealed in *Z. mainae* and *A. mascordi*, to extremely large, whip-like and exposed in *A. tealei* (Figure 1).

The morphology of the spermophor in all other examined palpimanoid exemplars was unmodified and symplesiomorphic in structure relative to *Austrarchaea* and *Zephyrarchaea*, including in those representatives of the three extant Afrotropical archaeid genera (Figure 2). Exemplar species of *Afrarchaea*, *Eriauchenius*, and *Madagascarchaea* all had spermophors which were shorter and less sinuous than those of Australian archaeid species, and there was also no evidence in Afrotropical taxa for a division of the palpal organ into a tegulum and subtegulum (cf. Figure 1). Similarly, in *Huttonia* (Huttoniidae), *Mecysmauchenius* (Mecysmaucheniidae), *Diaphorocellus* (Palpimanidae), and *Colopea* (Stenochilidae), there was no evidence of a bifurcate spermophor, despite the presence of an unusual divided embolic sclerite in *Diaphorocellus*. Mapping of the bifurcate state onto the phylogeny of Archaeidae and other Palpimanoidea thus revealed it to be an unambiguous synapomorphy of the Australian archaeid clade [i.e., (*Austrarchaea* + *Zephyrarchaea*)] (Figure 2).

## DISCUSSION

The embolus – enclosing the efferent spermatic duct or spermophor, and used to deliver sperm to the female genitalia during copulation – has always been considered the single unambiguously homologous sclerite on a male spider's pedipalp, shared by all known species and fundamental to the bauplan of the order and to the evolution, architecture and functional morphology of spider reproductive systems. Indeed, despite extraordinary variation in the structure and complexity of spider palpal sclerites (Hormiga, 1994, 2000; Griswold et al., 2005), the presence of a single embolus on each of a male spider's two pedipalps remains a central tenet of evolutionary arachnology. Even after two centuries of taxonomic, ontological and comparative morphological research, no species has ever been found with multiple “emboli,” and by extension, no



**FIGURE 2 |** Summary higher-level phylogeny of Archaeidae and other palpimanoid spiders, modified from previously published phylogenetic studies (Rix and Harvey, 2012c; Wood et al., 2012, 2013, 2014, 2018; Ramírez et al., 2021). Shown to the right of each clade are three-dimensional micro-CT visualizations of the spermophors of exemplar taxa. Note the presence of a bifurcating spermophor and two efferent ducts in all three major lineages of Australian Archaeidae.

spermophor has ever been recorded as having more than one efferent duct. Our findings challenge this premise, as in this study we report a lineage of palpimanoid spiders with a remarkable twin intromittent organ, whereby males possess a uniquely bifurcate spermophor with two efferent distal ducts leading to a pair of prominent embolic sclerites on each palpal bulb (Figures 1, 2). This “double-embolic” morphology (Figures 1, 2) was found to occur in all studied archaetid species from Australia, and optimizes as a unique and unambiguous synapomorphy of the “Australian clade” (Figure 2).

Understanding how, and especially why, stem-group archaetids of the Australian clade evolved such an extraordinary deviation from the normal spider bauplan is difficult to explain, both evolutionarily and ontogenetically. Post-copulatory sexual selection (Eberhard, 1985, 2004; Simmons, 2001, 2013) may be invoked as a possible evolutionary driver, as the female genitalia of Australian assassin spiders are unique among Archaetidae in possessing multiple aciniform clusters of defined spermathecae (Rix and Harvey, 2011, 2012a,b). Indeed, with so many potential sperm reservoirs (>20 in some taxa) in each female, it would reasonably be expected that sperm competition would favor males with functional access to the most spermathecae. Other Afrotropical Archaetidae, in contrast, share a relatively simple female genital system, consisting of a haplogyne bursa bearing clusters of secretory pores on modified “poreplates” (Wood and Scharff, 2018). The “double-embolic” state is documented here to have evolved one time in spiders, and as such, we are restricted to the simple observation that female genitalia differ markedly in Australian taxa relative to their Afrotropical relatives. However, within the Australian clade, significant inter-specific variation in the morphology of the secondary embolus (E\*; see Figure 1) does lend itself to evolutionary analysis. For example, future studies could examine whether the size, length or shape of E\* across multiple species in both genera is phylogenetically correlated to the number of spermathecae in females, the length of the female bursa, or the length of the spermathecal stalks. Similarly, intra-specific studies could examine whether paternity success is related to embolic morphometrics (with implications for post-copulatory sexual selection), as has been examined in other terrestrial arthropods with secondary sexual intromittent organs (e.g., Wojcieszek and Simmons, 2011).

Ontogenetically, a morphological shift of this magnitude seems harder to explain. The spider spermophor forms from an invagination of cells, with the duct anlage developing from the distal tip of the pedipalp and later spiraling proximally (Coddington, 1990; Quade et al., 2019). The embolus itself is one of the last sclerites to differentiate in the apical division, surrounding the distal spermophor and sperm pore. At present, the developmental genetic architecture required for such a fundamental transition of this conserved phenotypic system – and the complexity or otherwise of this transition – are unknown, although the trait has seemingly evolved just once in the long evolutionary history of spiders, sometime between the mid-late Mesozoic and early-mid Cenozoic (during which time Australian stem-group archaetids diverged from Old World relatives; Wood et al., 2013, 2014). Understanding the ontogenetic architecture

underlying this transition would therefore be a particularly valuable avenue for future research, not least because it may provide insight into the sort of evolutionary genetic shift required for bauplan modification more generally. Our results highlight the need for ongoing functional genomic, ontogenetic, morphometric, and sexual selection studies of Archaetidae and related Palpimanoidea, and highlight the importance of understanding the internal morphology of the palpal organ as it relates to the external functional morphology of spider pedipalps.

## DATA AVAILABILITY STATEMENT

Data are available from the Morphobank Digital Repository: <http://morphobank.org/permalink/?P4100>.

## ETHICS STATEMENT

Fieldwork was conducted under the following permits in: Queensland, Australia (No. WITK03859806); New South Wales, Australia (Nos. S13035 and XX48918); Western Australia, Australia (Nos. SF005357, SF005814, SF006247, and SF006821); Chile (No. 01/2008); and New Zealand (No. CA-30389-FAU). Specimens from Brunei Darussalam, Madagascar, and South Africa were sourced from existing museum material.

## AUTHOR CONTRIBUTIONS

MR, HW, MH, and PM performed the study and wrote the manuscript. PM conducted micro-CT analysis. All authors agreed to be held accountable for the content therein and approved the final version of the manuscript.

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