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Letter to the Editor

## There is no evidence that Podoctidae carry eggs of their own species: Reply to Machado and Wolff (2017)

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## ABSTRACT

In our recent publication (Sharma et al., 2017), we tested the hypothesis that eggs attached to the legs of male Podoctidae (Opiliones, Laniatores) constituted a case of paternal care, using molecular sequence data in tandem with multiple sequence alignments to test the prediction that sequences of the eggs and the adults that carried them would indicate conspecific identity. We discovered that the sequences of the eggs belonged to spiders, and thus rejected the paternal care hypothesis for these species. Machado and Wolff (2017) recently critiqued our work, which they regarded as a non-critical interpretation and over-reliance on molecular sequence data, and defended the traditional argument that the eggs attached to podoctids are in fact harvestman eggs. Here we show that additional molecular sequence data also refute the identity of the eggs as conspecific harvestman eggs, using molecular cloning techniques to rule out contamination. We show that individual gene trees consistently and reliably place the egg and adult sequences in disparate parts of the tree topology. Phylogenetic methods consistently place all egg sequences within the order Araneae (spiders). We submit that evidence for the paternal care hypothesis based on behavioral, morphological, and natural history approaches is either absent or insufficient for concluding that the eggs of podoctids are conspecific.

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"I assume that the eggs attached to the legs of the males of *Leytpodocis oviger* n. sp. are correctly identified as eggs of that species."

[Martens (1993), p. 101 (italics ours)]

## 1. Overview

Podoctidae is a small family of armored harvestman (Opiliones) that is perhaps best known for its reproductive behavior. Males of this group have been found carrying eggs, attached to their walking legs by adhesive secretions (Martens, 1993). While there are

several cases of parental, and specifically, paternal care, in the arachnid order Opiliones (reviewed by Machado and Macías-Ordóñez (2007) and Buzatto et al. (2014)), only in Podoctidae are eggs attached to adults. As podoctids are poorly studied, little else is known about their reproductive biology, beyond what can be discerned from preserved specimens.

In the course of sorting numerous museum collections and several collecting campaigns in Australasia over the last ten years to construct a molecular phylogeny for these animals, we encountered two specimens of Podoctidae with egg masses attached to their legs (Fig. 1B and C of Sharma et al. (2017)), as described by Martens (1993). Given our research program on evolutionary developmental biology of arachnids (e.g., Sharma et al., 2012a, 2014, 2015), we initially examined the eggs by manually removing the chorion and vitelline membrane, and using the fluorophore Hoechst 33342 to identify relevant embryonic structures, but

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found the eggs to be early stages (early cleavage and germ disc stages) that were not conducive to identification as harvestmen (PPS, personal communication). To establish their identity, we therefore sequenced eggs and the adult podocetid males to which they were attached for a locus (28S rRNA) used for the phylogenetic analyses, toward using one data class (molecular sequence data) to test inferences based on another (natural history/embryology). Multiple sequence alignments indicated that the eggs were not conspecific, and BLAST searches indicated that the best matches were to spider, not harvestman, sequences. We treated these data as a falsification of the paternal care hypothesis in Podocetidae. We suggested, as alternative interpretations, that the eggs could have become attached accidentally, or perhaps were attached deliberately by female spiders, as a form of egg parasitism.

In a critique of our results, Machado and Wolff (2017) contend that our work was based on a non-critical interpretation of molecular data. Their critique can be summarized as follows: (1) the eggs attached to podocetid legs, as well as the cementing secretions, have the hallmarks of harvestman eggs; (2) the attachments are unlikely to be accidental; (3) attached eggs are consistently found on male podocetids only; (4) spiders lack the means to attach eggs in the same manner, and must make use of silk for attachment; and (5) egg parasitism is rare in arachnids. They noted, non-controversially, that molecular methods are not infallible and “do not render classical behavioral, morphological, and natural history approaches obsolete or useless”, and that the most parsimonious hypothesis is that Podocetidae carry the eggs of their species as a form of paternal care (Machado and Wolff, 2017).

Machado and Wolff (2017) offer no data or analysis beyond their discursive criticism. They also offer no explanation for the non-identity of the podocetid eggs' sequences and those of the adults that carry them—a simple and straightforward prediction of their favored paternal care hypothesis. Their remark that, “molecular methods are not infallible and can have multiple sources of errors”, seemed to us to imply (1) that our data were contaminated, (2) that incorrect identification had occurred due to deposition of erroneous sequences in GenBank, and/or (3) that the BLASTn search method was inadequate to establish taxonomic identity of our sequence data.

Toward demonstrating the replicability and robustness of our result, we undertook the following analyses.

First, to rule out contaminations, we cloned fragments of the diagnostic loci 16S rRNA, 28S rRNA, and histone H3 for eggs attached to both of the specimens shown in Fig. 1 of Sharma et al. (2017). To rule out operator error, molecular work was performed by a different individual (JTO) not associated with the previous study. To rule out reagent contamination, entirely different reagents were used for every step of the protocol, including new primer stocks synthesized by a different vendor (IDT). After standard PCR amplification (primers and procedures listed in Sharma et al. (2017)), the TOPO TA cloning kit (ThermoFisher) was used to clone amplicons, following manufacturer's protocols. After bacterial transformation, 16 colonies were picked for colony PCR and sequencing using M13 universal primers. Each amplicon underwent a BLASTn search and multiple sequence alignment to verify identity.

Second, to test the replicability of the molecular sequence data in establishing the ordinal identity of the egg sequences, we conducted maximum likelihood searches for alignments of each locus individually. Alignments were constructed using a fusion of the spider phylogeny dataset of Dimitrov et al. (2012), the harvestman dataset of Sharma and Giribet (2011), and the Podocetidae dataset of Sharma et al. (2017). Finally, eggs' sequences were added to the multiple sequence alignments.

## 2. Cloning for sequencing confirms on-target recovery of egg sequences

Subsequent to gel electrophoresis, 10–15 individual amplicons with a band size indicating successful transformation were isolated for each target fragment. Regarding the 28S fragments previously reported by us (Sharma et al., 2017), identical or nearly identical sequences were obtained 10 times (14 amplicons sequenced) for eggs attached to specimen Pd038; and once (out of 14 amplicons sequenced) for eggs attached to specimen Pd034. The remaining sequences, which corresponded to shorter bands than the target fragments in gel electrophoresis, consistently matched with environmental fungal samples and/or nematodes upon BLASTn searches. In every case of successful cloning of an arachnid 28S, the retrieved sequence matched spider sequences, not harvestmen, in BLASTn searches.

As a separate test for ruling out contamination, we successfully amplified the unlinked loci 16S rRNA (for eggs of specimen Pd034) and histone H3 (for eggs of specimen Pd038). On-target recovery of 16S rRNA sequence was thereafter confirmed by cloning (nine times out of 11 trials). Due to its shorter band size (327 bp), histone H3 could not be reliably diagnosed using gel electrophoresis and cloning yielded mostly fungal and nematode sequences of similar length. Hence, this locus was recovered only once for eggs of Pd038. All cloned sequences are available in [Supplementary File S1](#). Aliquots of DNA extractions are available from PPS upon request.

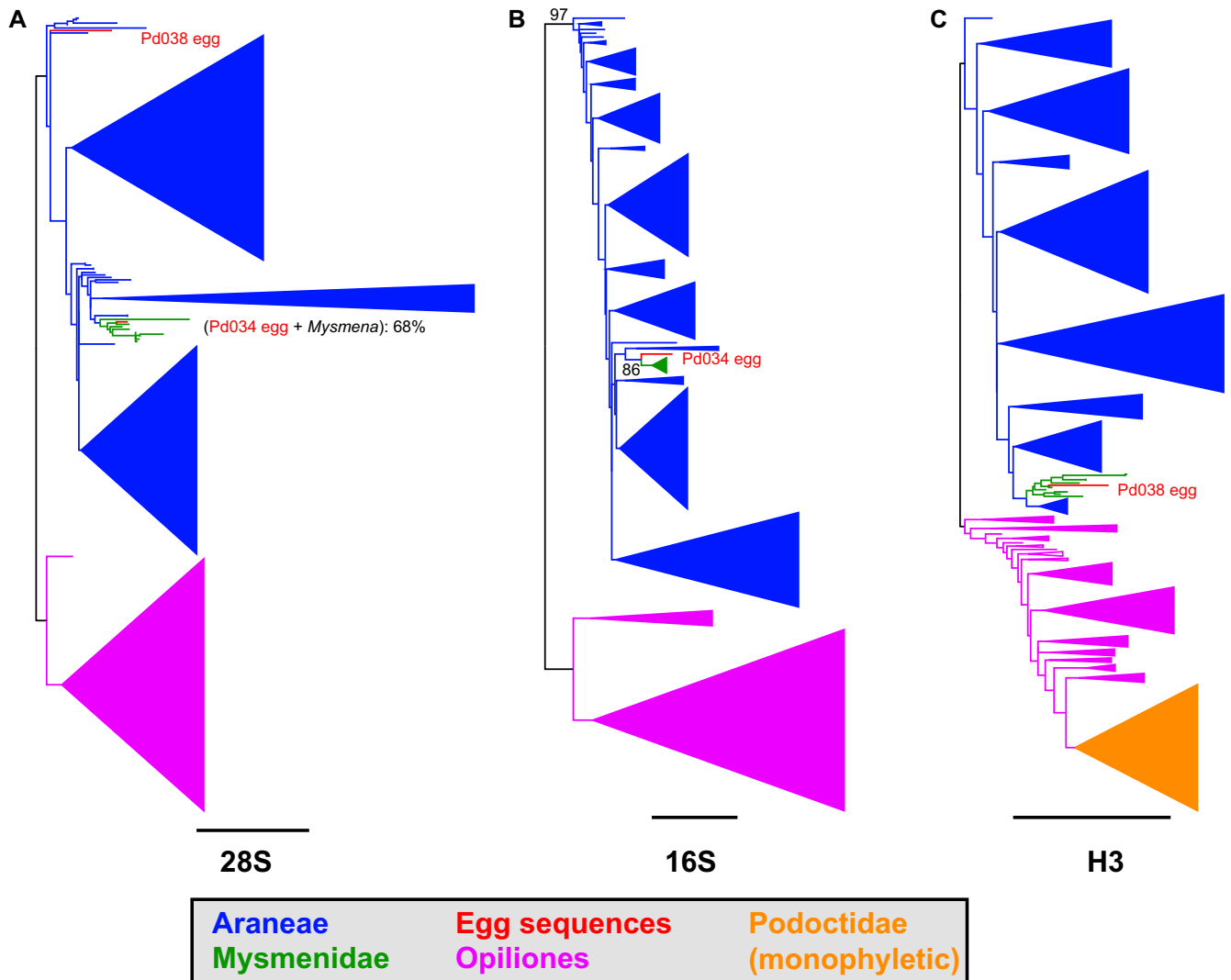
## 3. Gene tree analysis repeatedly places egg sequences within Araneae for multiple unlinked loci

Subsequent to multiple sequence alignment and masking of ambiguously aligned regions (as in Sharma et al. (2017)), maximum likelihood analysis was conducted using RAxML v. 8.0 (Stamatakis, 2006), with heuristics as described in Sharma et al. (2017). All alignments and tree topology files are available as [supplementary material](#).

The 28S rRNA alignment of spider, harvestman, and egg sequences consisted of 323 taxa and 276 conserved sites. In the 28S rRNA gene tree topology, Araneae (spiders) and Opiliones (harvestmen) formed two reciprocally monophyletic groups (Fig. 1A). The egg sequence of Pd034 was recovered as nested within the ground-dwelling spider subfamily Mysmeninae, and specifically as sister group to a Thai species of *Mysmena* (GenBank accession number GU456858; Lopardo et al., 2011), with bootstrap resampling frequency of 68%, indicating relationship (but not identity) to the sequenced Thai *Mysmena*. The placement of the eggs attached to Pd038 was ambiguous, forming a polytomy close to the root of the spider clade among the haplogynes, with no nodal support.

The 16S rRNA alignment of spider, harvestman, and egg sequences consisted of 211 taxa and 336 sites, and spiders and harvestmen were recovered as reciprocally monophyletic groups (97% bootstrap resampling frequency). This gene tree placed the eggs of Pd034 in a clade with Mysmenidae (86% bootstrap resampling frequency), corroborating the result observed in the 28S rRNA gene tree (Fig. 1B).

Finally, the histone H3 alignment consisted of 359 taxa and 327 sites. Spiders and harvestmen were again recovered as reciprocally monophyletic groups, with a monophyletic Podocetidae nested within Opiliones. The eggs of Pd038 were recovered as nested within Mysmenidae, and specifically as sister group to another Thai species of *Mysmena* (GenBank accession number GU456859; Lopardo et al., 2011) (Fig. 1C).



**Fig. 1.** Maximum likelihood gene tree topologies for 28S rRNA (A), 16S rRNA (B), and histone H3 (C). Colors in branches correspond to lineages, as indicated in the legend. Numbers on nodes represent bootstrap resampling frequencies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In summary, our molecular sequence data and gene tree analyses demonstrate that the eggs attached to our podoctid specimens' legs do not belong to Podoctidae or any other harvestmen.

#### 4. The paternal care hypothesis in Podoctidae relies exclusively on untested assumptions

Machado and Wolff (2017) dispute the idea that the attachment of eggs to podoctid appendages is accidental, as well as the notion that spiders may be attaching eggs to podoctid males deliberately as a form of egg parasitism. We regret that they interpreted those ideas as actual alternative “hypotheses”, *sensu stricto*. We were rather aiming to demonstrate the ease of constructing stories to reconcile an observation when few other data are available. It may be tempting to draw inferences or some sense of corroboration based on the geographic proximity of Borneo (collecting locality of Pd034), Luzon (collecting locality of Pd038), and Thailand (collecting locality of the two *Mysmena* species most closely related to the egg sequences). There is certainly a correspondence between the morphology of mysmenid egg sacs (which mysmenid females attach to their webs with a single thread; Lopardo and

Hormiga, 2015), and the appearance of the egg masses observed on our podoctid specimens (Fig. 1 of Sharma et al. (2017)). One could even construct an appealing narrative invoking the natural history of *Mysmena*, which inhabit leaf litter and ground cavities, where they build their foraging webs (Hormiga and Griswold, 2014; Lopardo and Hormiga, 2015), placing Mysmenidae and Podoctidae in close physical proximity within their microhabitats in Southeast Asia. We do not, however, regard the notions of either accidental attachment or egg parasitism by spiders any more seriously than the hypothesis of paternal care; any of these are pure conjectures in the absence of actual observational data. To us, conjectures are of course valuable waypoints in the construction of hypotheses, but they are no substitute for evidence. Our evidence is sufficient to reject conspecific identity of attached eggs and adults.

In light of that precept, a reexamination of the paternal care hypothesis will show that this traditional understanding is not based on any direct evidence. Machado and Wolff (2017) seem to imply that classical behavioral, morphological, and natural history approaches have substantiated the paternal care hypothesis. While we agree that all of these data classes are vital to understanding the biology of harvestmen, considering each in turn as it relates

to the present case, we note that there is no behavioral evidence whatsoever that Podocitidae engage in paternal care. To our knowledge, no one has observed podocitid mating and egg-laying behavior, and thus conspecific egg identity is, at most, an assumption, as originally stated by Martens (1993).

Similarly, there is no evidence from natural history approaches either in this regard—to our knowledge, no one has ever cultivated a podocitid colony or reared the attached eggs on wild-caught adult males to hatching. Thus, there is no natural history basis for inferring that the eggs are those of podocitids. This is not a case of conflict between behavioral/natural history data and molecular sequence data, as implied by Machado and Wolff (2017). Rather, we implemented molecular sequence data to redress a gap where behavioral and natural history observations are completely lacking.

Machado and Wolff (2017) do make a compelling case that the eggs attached to the specimens they have examined (N.B., not our specimens) are eggs of harvestmen. This reasoning is based on the description of the developing limb-bud stage embryos (Martens, 1993; in-text personal communications of Machado and Wolff), the ultrastructure of the chorion, and the nature of the attachment. They also reason that the attachment of eggs exclusively to males of a podocitid collection from the Solomon Islands (six out of eight males; zero out of seven females), is strongly suggestive of paternal care; they attribute this datum to an original report by Kury and Machado (2003).

Regrettably, these observations are presented in the published literature for the first time in the critique of Machado and Wolff itself. The work cited as Kury and Machado (2003) corresponds to the abstract of a scientific meeting, and thus the only actual published data on podocitid eggs are ours (Sharma et al., 2017; this study) and the original report of Martens (1993). A PDF document of the slides from the Kury and Machado (2003) presentation were generously provided to PPS by G. Machado and A.B. Kury prior to the writing of Sharma et al. (2017). In the presentation corresponding to the Kury and Machado (2003) reference we found no textual mention of the sex ratio data, nor image data detailing the egg chorion morphology and the limb bud stage embryos as described by Machado and Wolff (2017). No illustrations (including line drawings) of podocitid limb bud stage embryos are given in the published literature at all (ref. Fig. 2 of Martens (1993)). Given the monumental expertise of A.B. Kury in harvestman biology, we do not question the authenticity of the unpublished findings, but rather, we observe that the embryonic morphology and sex ratio data that Machado and Wolff (2017) describe simply do not exist in published form at the time of this writing.

But let us grant, for the sake of expeditious and theoretical argument, that the attached eggs Machado and Wolff have examined are indubitably harvestman eggs, with complete epistemological certainty. Even complete confidence in this regard would not substantiate the paternal care hypothesis. The identity of those eggs as Opiliones eggs is a *necessary* condition of the paternal care hypothesis, but it is not a *sufficient* condition to support the paternal care hypothesis. The precise requirement of the paternal care hypothesis is that the eggs are *conspecific*, not simply that they belong to one of the >6400 species of the order Opiliones. (It was exactly for this reason that we opted to test this hypothesis using molecular data, as sequence data are dispositive of conspecific identity, whereas egg/embryonic morphological data are not.) At the point where no morphological (or any other type of) data exist in support of conspecific identity of egg and carrier, the paternal care hypothesis is, at best, an inference grounded in untested assumptions derived from unpublished anecdotes. Certainly, as Machado and Wolff point out, egg parasitism is rare in arthropods, but the discovery of a true case of egg parasitism would be no rarer within harvestmen than egg attachment to conspecific males—either

phenomenon, if validated, would be restricted within Opiliones to Podocitidae.

Machado and Wolff (2017) are welcome to conclude that “the most plausible and parsimonious explanation is that podocitid males are caring for their own offspring”, but this explanation (1) does not redress major evidentiary gaps in its construction, (2) does not reconcile the discrepancies revealed by our molecular sequence data, (3) does not meet our standard of evidence for establishing a sound hypothesis, and (4) is now neither parsimonious nor plausible for every known case of egg attachment in podocitid males, given these new data. To us, the practice of dismissing an entire data class (in this case, molecular sequence data) that stands in opposition to preconceived ideas or beliefs is the essence of confirmation bias.

## 5. Conclusion

Machado and Wolff (2017) term our work a non-critical “assassination” of the paternal care hypothesis. Their narrative exercise does not, however, compensate for the number of inferential leaps and untested assumptions of the traditional paternal care hypothesis. An examination of their favored scenario reveals an absence of direct evidence that Podocitidae are carrying eggs of their own species, a simple *sine qua non* requirement of their hypothesis. In the particular case of the specimens sequenced by us, the paternal care hypothesis clearly and repeatedly failed a straightforward litmus test.

We thus reiterate our previous conclusions: Until *bona fide* evidence of conspecific paternal care can be established in this lineage (be it observations of behavior, a natural history study with captive animals, or sequencing and phylogenetic analysis of eggs and adults), there is no justification for accepting the paternal care hypothesis over any other explanatory vehicle. We welcome, and are demonstrably committed to, the integration of different data classes and a broad array of evidentiary devices, and particularly so the fusion of morphological, behavioral, developmental, computational, bioinformatic, and natural history approaches with empirical molecular methods, toward better understanding the biology of harvestmen (Boyer et al., 2007, 2015; Clouse et al., 2009, 2016; Garwood et al., 2014; Sharma and Wheeler, 2014; Sharma et al., 2012a, 2012b, 2013a, 2015), as well as other invertebrates (Janda et al., 2004; Clouse et al., 2005; Boyer et al., 2011; Sharma et al., 2013b).

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympbev.2017.03.026>.

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