Received: 26 March 2019 | Revised: 25 August 2019 | Accepted: 26 August 2019

DOI: 10.1111/zsc.12390

ORIGINAL ARTICLE

Zoologica Scripta | WILEY

Molecular phylogeny places the enigmatic subfamily Masoninae within the Ichneumonidae, not the Braconidae

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Funding information
Australian Biological Resources Study, Grant/Award Number: RF214-16 and CT215-08; Ratchadaphiseksomphot Fund; Chulalongkorn University, Grant/Award Number: CU-GR_62_06_23_03

Abstract

The Masoninae (type genus *Masona* van Achterberg) is a widespread but seldom collected group of morphologically aberrant tiny (body length 2 mm or less) wasps that have always been considered as a subfamily of Braconidae, albeit on little supporting evidence. Discovery of a fully winged female of *Masona* from remote Australia has enabled a reassessment of its relationships. This specimen yielded sequence data for the nuclear 28S and 18S rDNA genes, the barcoding fragment of mitochondrial cytochrome oxidase subunit 1 and the mitochondrial 16S rDNA gene. These were analysed separately and together, along with representatives of Braconidae and Ichneumonidae. Maximum likelihood analyses of all four genes separately and combined concur that *Masona* is nested within the Ichneumonidae and, therefore, is not a member of Braconidae. This is also supported by the position of the sternaulus low on mesopleuron, absence of fore wing vein (RS + M)a (though the great reduction in wing venation makes further interpretation problematic) and the articulated junction of metasomal tergites 2 and 3. On balance, molecular analyses place *Masona* basally among the ophioniformes lineage of Ichneumonidae. Formal description of the Australian species as *Masona timpaynei* Quicke sp. n. and a revised diagnosis of Masoninae are provided in S1 together with illustration of the holotype.

KEYWORDS

Braconidae, Ichneumonidae, *Masona*, Masoninae, molecular phylogenetic analysis

1 | INTRODUCTION

The Ichneumonoidea currently comprises two extant families, the Braconidae and Ichneumonidae, which collectively comprise more than 44,000 described extant species (Quicke, 2015; Yu, van Achterberg, & Horstmann, 2012) and undoubtedly many times more. The extinct Praeichneumonidae are also treated tentatively as members of the superfamily. Several other taxa now placed in one or other of the two extant families of Ichneumonoidea have at various times been afforded family-level status largely because of aberrant biology or conspicuous character states that are now interpreted as autapomorphies, notably the extinct Eoichneumoninae and the extant Aphidiinae, Apozyginae and Hybrizontinae (=Paxylommatinae) (Quicke, 2015). Apart from the extinct grade taxon Eoichneumoninae and the rare Chilean Apozyginae which has yet to be sequenced, molecular data now firmly place all of these among the extant ichneumonids or braconids.

The genus *Masona* was erected for a small group of highly aberrant parasitoid wasps with prognathous heads in the

females and with body lengths between 1.1 and 1.8 mm. The four described extant *Masona* species are known only from Australia (Queensland) and south-east United States (Georgia and Florida, including the Key Islands), but an undescribed East African species was also noted by van Achterberg (1995) and one of us (DQ) has recently been made aware of its occurrence also in Asia (Cambodia) and South America (Brazil; Quicke, Chaul & Butcher, 2019). Two fossils of Masoninae from the New World have been described from Miocene Dominican amber, one in the extant genus *Masona* (van Achterberg, 2001) and one in the probably extinct genus *Anoblepsis* Engel & Bennett, 2008.

Van Achterberg (1995) named the genus *Masona* after Bill [W.R.M.] Mason (1911–1981) who was going to describe it as a new family of Hymenoptera because of its peculiar morphology and in particular its large gular sclerite. In describing Masoninae as a new subfamily of Braconidae, van Achterberg (1995) noted as the only putative synapomorphies the 'united second and third metasomal tergites and the reduction of veins of fore wing'. Its wide [disjunct] geographic distribution further led van Achterberg to suggest that it was most likely an archaic group. The subfamily Masoninae itself was originally described to include a second tribe, the Mannokeraiini, but this has since been shown to belong to the braconid subfamily Euphorinae (Belshaw & Quicke, 2002; Sharanowski et al., 2011; Stigenberg et al., 2015).

Up until now, all known female *Masona* species are apterous, but males are winged with very reduced venation (van Achterberg, 1995: Figures 1 and 2); the fore wing of *Masona prognatha* van Achterberg, 1995, having only weakly pigmented indications of the pterostigma and veins M + CU, CU, 1-M and 1-1A, and the hind wing completely lacking veins.

Here, we present DNA sequence data for four gene fragments together with a range of braconids and ichneumonids to re-assess the relationships of the Masoninae. Additionally, we describe new morphological characters pertinent to the relationships of Masoninae from re-examination of paratypes of the Australian *Masona similis* van Achterberg, 1995 (Figure 1a–e), a new Australian species described here as *Masona timpaynei* Quicke sp. n. (Appendices S1 and S2), and from *Masona popeye* Quicke & Chaul (Figure 1f). As a result of both molecular analyses and morphological considerations, we show that Masoninae are not members of the Braconidae but are actually ichneumonids, probably derived near the base of the informal 'ophioniformes' clade.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

Approximately half of our molecular data are derived from publicly available sequences on GenBank. In addition, new sequence data were generated for *M. timpaynei* Quicke sp. n., as well as a large number of principally ichneumonid species. The diverse historical molecular phylogenetic studies from which most of the analysed DNA sequence data originated mean that very few braconid and ichneumonid taxa are represented in GenBank by all the gene fragments employed. As relatively little molecular phylogenetic research has been carried out on the Ichneumonidae apart from detailed studies of a few subfamilies, we generated new sequence data, particularly for the 16S rDNA gene, for a taxonomically dispersed set of taxa. We have treated genera as the basic taxonomic unit for this study, and thus in the analysed matrix, some genera are represented by a combination of genes from multiple species. Details of included sequences and GenBank accessions numbers are provided in Appendix S2.

2.2 | Sequence generation and alignment

Sequencing the COI barcoding gene region involved polymerase chain reaction (PCR) amplification, following standard protocols for DNA extraction, PCR and sequencing (Ivanova, deWaard, & Hebert, 2006; de Waard, Ivanova, Hajibabaei, & Hebert, 2008). PCR was performed using the C_LepFolF/C_LepFolR primers. The COI fragment for the taxa investigated was largely length invariant except for a two-codon insertion in the sequence for the ichneumonid *Alomya* (Alomyinae), the position of which was determined by reference to the translated amino acid sequence.

The length variable 28S rDNA fragments were aligned following the braconid secondary structure model of Gillespie, Yoder, and Wharton (2005); the 16S fragments were aligned in accordance with the secondary structure models of Buckley, Simon, Flook, and Misof (2000) and Wu et al. (2014), while the 18S sequences were largely length-conserved. For all three ribosomal genes, length variable non-homologous regions were excluded. The raw sequence files with secondary structure interpretation and the analysed files are provided in Appendices S4 and S5, respectively.

2.3 | Phylogenetic analyses

Sequences were analysed using maximum likelihood (ML) with the programme RAxML (v.8) (Stamatakis, 2014), using a GTR + G rate model. To estimate support for nodes, we conducted a combined ML search and rapid bootstrap using the "-f a" option and 100 runs. The combined data were partitioned in three different ways for separate analyses: (a) eight partitions comprising three codon positions for COI, pairing and unpairing bases for 16S and 28S bases separately, and 18S as a single partition because of the very few informative positions; (b) four partitions with each gene fragment

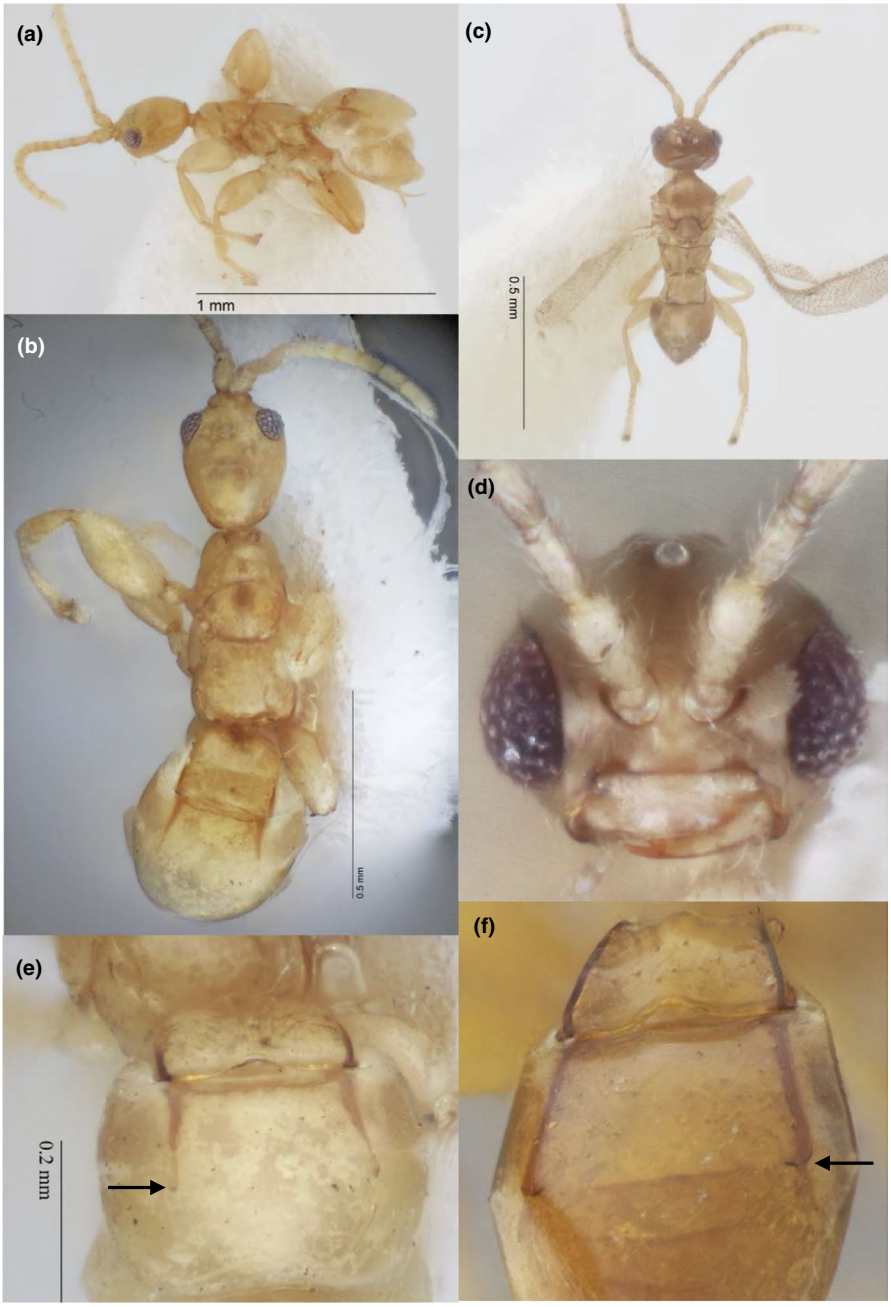

FIGURE 1 *Masona* morphology. (a–e) *Masona similis* van Achterberg, 1995, paratypes from Australia; (f) *Masona popeye* Quicke & Chaul, 2019 from Brazil. (a) Female habitus (near) lateral view. (b) Female habitus, dorsal view. (c) Male habitus, lateral view. (d) Face. (e, f) Metasomal tergites 1–3 showing abrupt end of lateral marginal thickening of tergum of tergite 2 and lateral articulation with tergite 3 [Colour figure can be viewed at wileyonlinelibrary.com]

treated as a single partition; and (c) all data combined as a single partition. Trees were visualized using FigTree (1.4.3) (Rambaut, 2016).

2.4 | Morphology

New data are presented for the fully winged *M. timpaynei* Quicke sp. n. (see Appendix S1) from Western Australia, Millstream National Park, 17 June 2014, collected by Neil Brougham, that was collected as part of the Global Malaise trap Barcode Initiative, and for a new apterous species from Brazil (Quicke et al., 2019). New DNA sequence data are presented for the new Australian species. Phase contrast microscopy of the slide-mounted, Australian winged female *Masona* was carried out using an IX83 microscope (Olympus Company). Images of *M. similis* were generated using a Visionary Digital BK+ imaging system with a Canon EOS 7D 18 megapixel camera, and Zerene Stacker, Zerene Systems LLC, PMAX software and cropped and resized in Adobe Photoshop CS6 (Adobe Systems Inc.).

Terminology follows van Achterberg (1988) except for wing venation nomenclature, which follows Sharkey and Wharton (1997); see also figure 2.2 in Quicke (2015) for comparison of wing venation naming systems.

The body parts of the new species from Australia are mounted in Canada balsam on two cavity microscope slides,

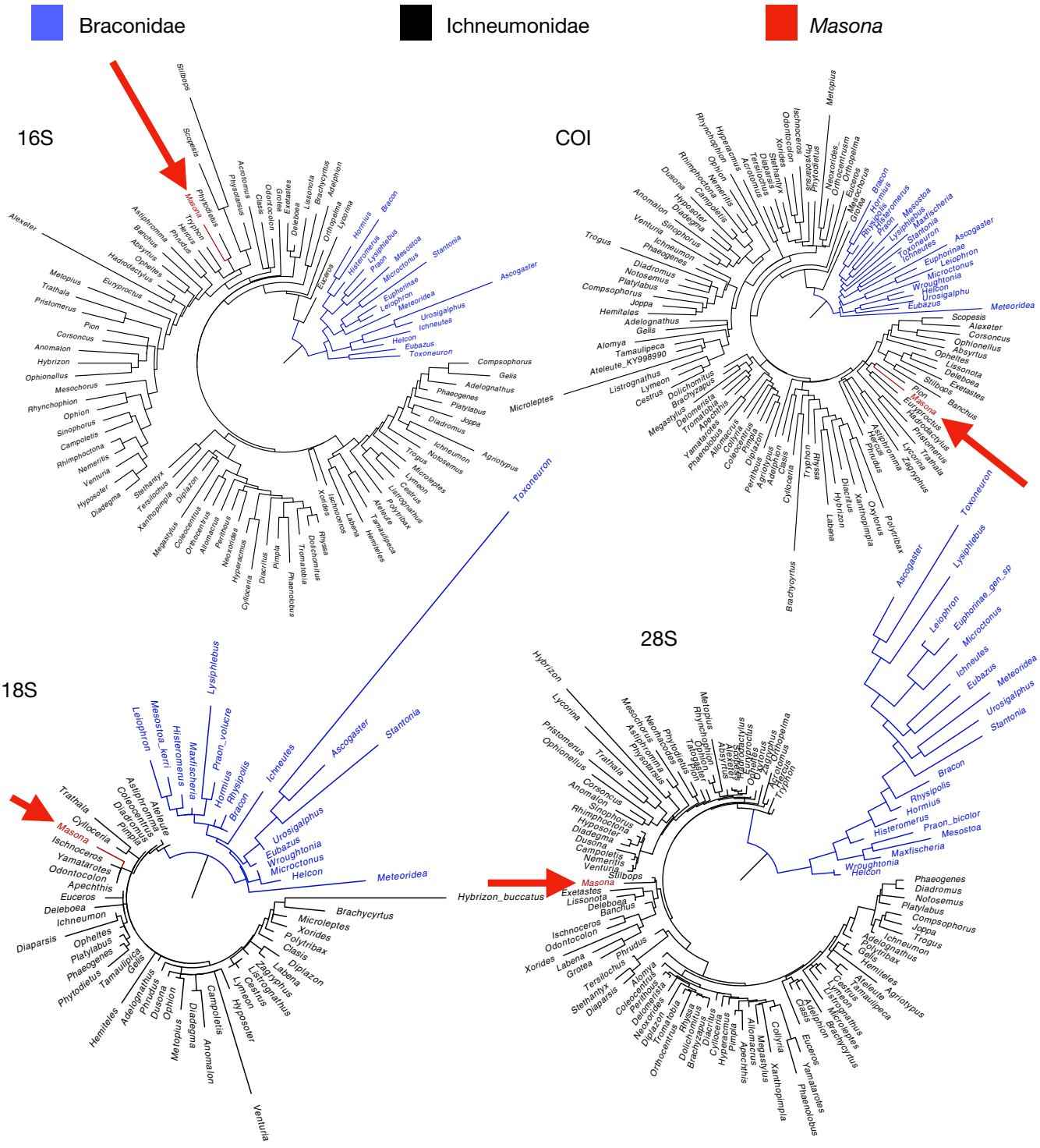

FIGURE 2 Maximum likelihood trees recovered for each of the four gene fragments showing recovered positions of *Masona* with respect to a range of braconids and ichneumonids [Colour figure can be viewed at wileyonlinelibrary.com]

one with the disarticulated head and mesosoma with one fore wing attached, the other with the metasoma and one leg. These are deposited in the Australian National Insect Collection, Canberra.

Masona timpaynei sp. n. Quicke D.L.J.

ZooBank registration: urn:lsid:zoobank.org:pub:832989BC-03E8-43DC-BB31-552F6683F6F3.

3 | RESULTS

3.1 | Molecular phylogenetics

DNA extracted from the Australian *M. timpaynei* sp. n. DNA extract was successfully sequenced for the barcode gene (564 BPs), 28S D2 + D3 (674 BPs), 16S (397 BPs) and 18S

TABLE 1 Results of BLAST searching *Masona* sequences on GenBank and accessions numbers of best matching sequences (GenBank accessed 9.ii.2019; full details and accessions numbers are given in Appendix S6)

	CO1	16S	28S	18S
Winged female *Masona*	88% to various unidentified and a diplazontine	84% to cryptine and claseine genera	93% to tersilochine, stilbopine and ctenopelmatine genera	98% to various cryptine genera
Previously published *Masona* spp (COI: JF963525; 28S: AJ302914)	84% to various Aphidiinae	—	91% to a predicted ant (Formicidae) sequence, and to a rhyssaline braconid	—

(155 BPs). The GC contents of these were 26.1%, 60.5%, 20.9% and 53.5%, respectively. DNA sequences for the current and previously published *Masona* species were BLAST searched (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) on 8 February 2019. The top matches for each of the four gene fragments are summarized in Table 1 (see Appendix S5 for full details). The highest similarity matches (98%) were obtained for the highly conserved 18S rDNA gene. All other sequences gave far lower scores ranging from 84% to 93%. For the newly generated barcode sequence, the top 100 nearest matches were all members of the Ichneumonidae, ranging in sequence identity from 87% to 88%; the top 100 nearest matches for the 28S sequence were also all members of the Ichneumonidae, ranging in sequence identity from 90% to 93%, and the best matches for 16S and 18S similarly were members of the Ichneumonidae. However, the closest matching ichneumonids represented a diversity of subfamilies including members of the informal brachycyrtiformes, ichneumoniformes and basal ophioniformes.

Individual ML analyses of the four genes all recovered the Ichneumonidae separated from the Braconidae with 100% bootstrap support (Figure 2). The 16S and COI sequences both recover *Masona* within a monophyletic ophioniformes clade, in the former case rather close to the base. The 28S tree recovered the ophioniformes as a grade taxon from within which were derived all other ichneumonid groupings, but *Masona* associated most closely to the basal ophioniformes subfamilies Banchinae and Stilbopinae. The 18S tree recovered *Masona* within the Ichneumonidae together with Xoridinae and an Acaenitinae.

In combined analyses of the four genes (Figure 3), the ophioniformes were recovered as a basal ichneumonid grade rendered paraphyletic by the Xoridinae (eight

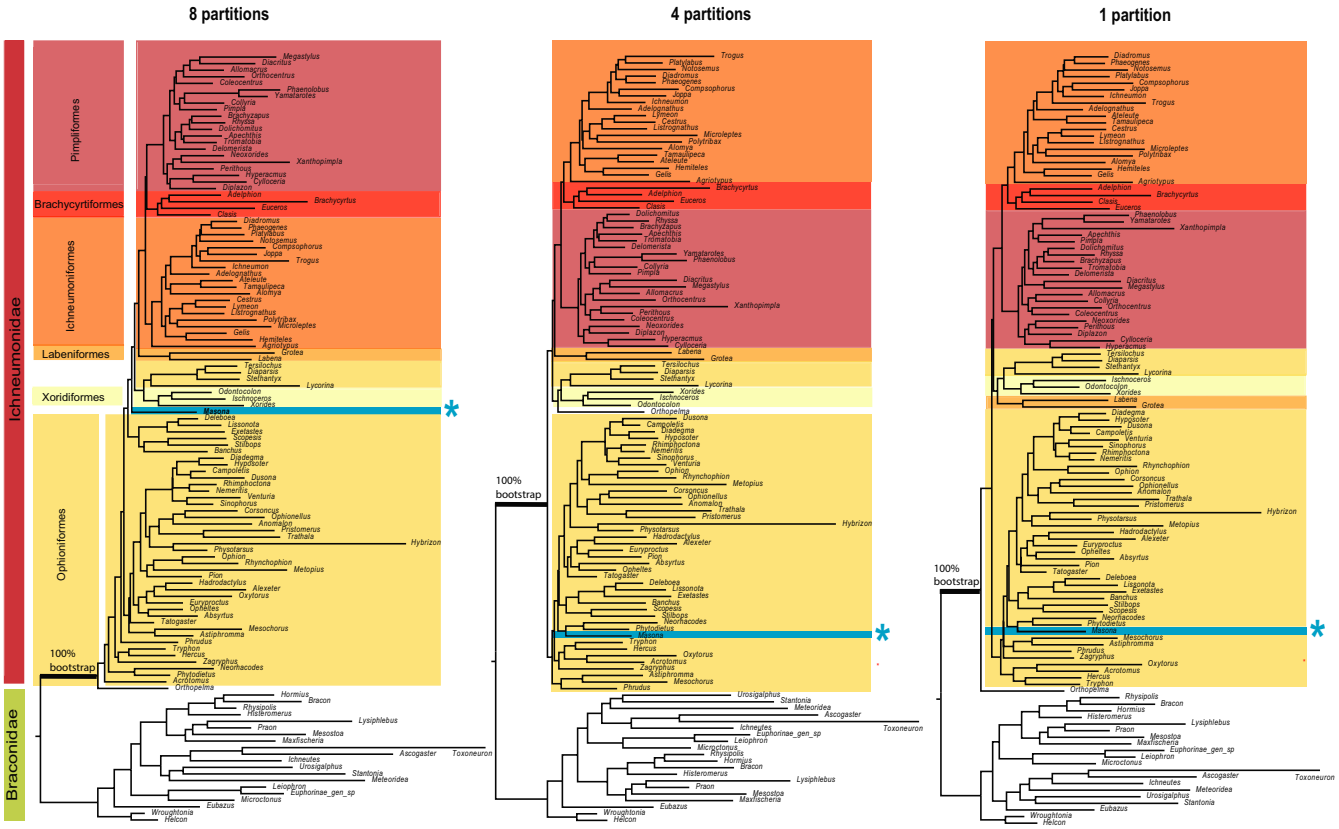

FIGURE 3 Maximum likelihood trees based on four concatenated gene fragments showing recovered positions of *Masona* (red arrow and asterisk) with three different treatments of DNA partitions [Colour figure can be viewed at wileyonlinelibrary.com]

partition analysis) with *Orthopelma* basal, by Xoridinae and *Orthopelma* (four partition analysis) or by Xoridinae plus Labeninae (with *Orthopelma* basal). In all analyses, *Masona* was recovered within this ophioniformes grade.

3.2 | Morphological characters

Previously described *Masona* species are strongly sexually dimorphic with apterous prognathous females and fully winged, more orthognathous males (Figure 1a,b cf 1c). Females lack ocelli (Figure 1b), but ocelli are present in males (Figure 1d). In both sexes, the face is short and rather featureless and the mandibles strongly twisted and sickle-shaped. No groove separates metasomal tergites 2 and 3 dorsally, and, in most specimens, these tergites form a more-or-less continuous curve in profile. However, close examination of *M. similis* (Figure 1e) and *M. popeye* (Figure 1f) shows that in both, the two tergites are flexibly connected, that is a narrow lateral margin of the second tergite is more strongly sclerotized than the third, and posteriorly is modified forming points of articulation with the third tergite.

The first winged female *Masona* species is described and illustrated as new in online supporting materials (Appendix S1) despite being fragmented and slide-mounted because of its systematic importance. It is not only winged but was collected in a Malaise trap rather than in a ground-layer trap, suggesting it is well capable of flying and possibly attacks hosts in a different niche than its apterous congeners. It is superficially similar to the males of the two other species of the genus for which males are known (*M. similis* and *M. prognatha* van Achterberg, 1995) (Figure 1c). Its head is only moderately elongate and not very prognathous. Wing venation was hardly visible in the intact specimen with normal transmitted or incident light (Appendix S1), but after slide-mounting, phase contrast microscopy revealed a more extensive pattern of fore wing venation (Appendices S1 Figure D and S6 Figure A) comprising M + CU, 1M, 1CU, 2CU, r-rs, m-cu and a combined longitudinal RS + M that divides. Veins (RS + M) a and 2m-cu are absent. The arrangement is almost identical to that of the ichneumonid genus *Neorhacodes* Hedicke, 1922 (Neorhacodinae; Appendix S6 Figure B). No venation was observable in the hind wing. In addition, the pedicellus of the new Australian species is very large, approximately 0.8 × the length of the scapus, a condition also shown by hormiine braconids and neorhacodine ichneumonids.

The lower part of the mesopleuron of most species possesses a longitudinal groove that we interpret as a sternaulus rather than a precoxal sulcus because of its more ventral location (Appendix S1 Figures A,E).

The Australian specimen is assigned here to *Masona* despite a few differences from the females of all the other known species. Notably, it is fully winged, with a head shape typical of that of males (known only for *M. similis* and *M. prognatha*). Sexually dimorphic wing development is a common trait in smaller-bodied ichneumonoids (Quicke, 2015). In common with females of the other species of *Masona*, it lacks ocelli. However, the pedicellus is small relative to the scapus in other females but large (up to 80% length) in males (Figure 1d; also figure 753 in van Achterberg, 1995). Thus, our female *M. timpaynei* sp. n. is male-like in this respect too. A similarly large pedicellus occurs in some ophioniform Ichneumonidae such as in Neorhacodinae (see figure 113c in Broad, Shaw, & Fitton, 2018) and many Tersilochinae (see figure 152 in Broad et al., 2018).

Robust legs (Appendix S1 Figure A) are probably adaptive to the wasps having to push bodily through some substrate to reach hosts, being found in the Ichneumonidae, notably in many Metopiinae, Orthocentrinae and *Hyperacmus* Holmgren, 1858. The shape of the ovipositor dorsal valve, narrowed, apically almost needle-like and with an elongate notch (Appendix S6 Figure G), strongly suggests that *Masona* are endoparasitoids (Belshaw, Grafen, & Quicke, 2003), and is reminiscent of the ovipositor of some Ctenopelmatinae and Metopiinae. Given the morphological assessment above, a revised diagnosis of Masoninae is provided in Appendix S1.

4 | DISCUSSION

The new molecular and morphological data presented here show that Masoninae are members of the Ichneumonidae rather than the Braconidae. In the original description of the subfamily, van Achterberg (1995) wrote 'I include the genus [*Masona*] in the Braconidae because it shares its synapomorphies such as the united second and third tergites and the reduction of veins of fore wing'. Many braconids have far more reduced venation than most ichneumonids (Matthews, 1974), a trend that is probably associated with their generally smaller body size, but the bulk of Braconidae has as complete a wing venation as ichneumonids. However, the more important feature would seem to be the connection between the second and third metasomal tergites which in Braconidae (excepting Aphidiinae) are immovably fused, whereas in the Ichneumonidae, they are flexibly joined (Sharkey & Wahl, 1992). In *Masona*, there is no groove separating them, and in many specimens, these tergites appear superficially to be connected in a braconid-like way. However, they are often angled with respect to one another and closer inspection reveals that the third tergite is articulated laterally with the second (Figure 1e,f). The second and third tergites are also convergently fused in various derived lineages of Ichneumonidae (Sharkey & Wahl, 1992). The other three morphological apomorphies of the Braconidae mentioned by Sharkey & Wahl are all characters of the hind wing which are not discernible in any known Masoninae.

Masona species are variable with regard to the development of a lateral groove on the mesopleuron. Having taken it that *Masona* is a braconid, van Achterberg (1995, 2001) referred to this structure as the precoxal sulcus (see Appendix S6 Figure D) whereas had it been described in the Ichneumonidae the groove would have been referred to as a sternaulus. For a long time, it was broadly assumed that these were homologous although the groove in the Ichneumonidae is located more ventrally. However, the occurrence of both grooves in the opiine braconid *Sternaulopius* Fischer (Wharton, 2006) (Appendix S6E) shows that they are different structures. Importantly, the groove in *Masona* is situated far ventrally (Appendices S1E and S6C), suggesting it is probably a sternaulus rather than a precoxal sulcus.

4.1 | Molecular data

The first published molecular data for a masonine were a 28S rDNA gene fragment used by Belshaw and Quicke (2002) in a study of ancestral biological state reconstructions in the Ichneumonoidea. The aberrant nature of the sequence was highlighted in that paper with the statement 'The only other taxa for which placement was uncertain with respect to these rootings were the Xoridinae, Labeninae, and *Hybrizon* Fallén, 1813 in the Ichneumonidae and *Masona* in the Braconidae'. Depending upon tree reconstruction method and parameters, the *Masona* sequence associated with either the braconid sigalphoid subfamily complex OR amongst the outgroups used comprising the Apidae, Bethylidae and Dryinidae, which, being aculeate hymenopterans were generally thought to be the likeliest sister group of the Ichneumonoidea at that time. Quicke et al. (2012) published a barcode for another *Masona* species from Australia (GenBank: JF963525, sample ID BCLDQ1128, BOLD BIN BOLD: AAI1711) and in the published tree it was recovered as sister group to the Aphidiinae though on a long branch.

Our molecular trees (Figures 2 and 3) failed to recover several groups as monophyletic which have usually been recovered in other investigations and make morphological sense. Notably, in several cases, the Ichneumonidae were rooted among the ophioniformes, whereas the latter were recovered monophyletic by Quicke et al. (2009) who analysed an elided 28S rDNA data set combined with morphology. Indeed, Quicke et al.'s (2019) analyses of 28S sequence data alone from 1,001 ichneumonid species often recovered some or all Xoridinae among the ophioniformes contrary to generally accepted views. We believe our current trees to be misleading in this respect and generally due to the rather small number of exemplars included, as well as the relatively anomalous sequences of Xoridinae (28S) and Tersilochinae (CO1, 28S) and the overall low level of variation in the 18S gene fragment.

This study highlights the need to generate robust molecular data sets for highly modified, morphologically reduced Hymenoptera, to test hypotheses on their phylogenetic placement. In many cases, these taxa are rarely collected and so such studies are invariably opportunistic.

ACKNOWLEDGEMENTS

We are very grateful to Somrudee Attachit (Mahidol University) for her assistance and expertise in phase contrast microscopy. Júlio Cezar Mário Chaul (Universidade Federal de Viçosa, Brazil) kindly allowed us to use a photograph of *Masona popeye* from Brazil. Andrew Bennett kindly allowed us to use DNA extracts from various specimens to generate additional sequence data. We also wish to thank Gavin R. Broad and an anonymous referee for their helpful comments, and Mike Sharkey for discussion of wing venation nomenclature. This work was supported by the Senior Postdoctoral Fellowship under Ratchadaphiseksomphot Fund, Graduate School, Chulalongkorn University to DLJQ and Ratchadaphiseksomphot Fund, Chulalongkorn University (CU-GR_62_06_23_03) to BAB. Involvement in this project by ADA and EFJ was supported by the Australian Biological Resources Study grants RF214-16 and CT215-08, and PDNH was funded by a NSERC Discovery grant.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Quicke DLJ, Austin AD, Fagan-Jeffries EP, Hebert PDN, Butcher BA. Molecular phylogeny places the enigmatic subfamily Masoninae within the Ichneumonidae, not the Braconidae. *Zool Scr*. 2020;49:64–71. https://doi.org/10.1111/zsc.12390