Molecular phylogeny of the diverse parasitoid wasp genus *Ophion* Fabricius (Hymenoptera: Ichneumonidae: Ophioninae)

**Abstract.** *Ophion* Fabricius is a diverse genus of nocturnal ichneumonid wasps (Insecta: Hymenoptera) that is particularly species-rich in temperate areas, yet has received little taxonomic attention in the Holarctic region, where most species occur. While there have been some attempts to divide *Ophion* into monophyletic species groups, the vast majority of species have been lumped into a single, paraphyletic group, the *O. luteus* species group, which is defined only by the lack of characters specific to the other groups. The challenging morphology of this large catch-all group has limited attempts to subdivide it, and no phylogenetic hypothesis has been proposed for the genus as a whole. In this study, we use DNA sequence data [28S ribosomal RNA (28S), cytochrome oxidase 1 (COI) and internal transcribed spacer 2 (ITS2)] to present the first molecular phylogeny of *Ophion*. We also describe the secondary structure of ITS2 for the first time in Ichneumonidae, and explore its implications for phylogeny estimation. We define 13 species groups, nine of which were previously considered part of the *O. luteus* species group s.l. The included species groups are the *O. minutus*, *O. areolaris*, *O. scutellaris*, *O. flavidus*, *O. parvulus*, *O. slossonae*, *O. nigrovarius*, *O. pteridis*, *O. luteus* s.s., and *O. obscursatus* species groups, along with three groups lacking described species (Species group 1, New Zealand group, Madagascar group). This study provides a framework for future studies of this diverse and morphologically challenging genus.

**Introduction**

The ichneumonid subfamily Ophioninae consists primarily of nocturnal orange or yellowish parasitoid wasps that are frequently observed at lights. Ophionines are koinobiont endoparasitoids of holometabolous insect larvae, almost always Lepidoptera. There are currently 32 recognized genera within Ophioninae (Yu *et al.*, 2012), almost all of which are most diverse in tropical regions (Gauld, 1985). However, the genus *Ophion* Fabricius (Fig. 1) is one of the few exceptions to this pattern. While the genus has a nearly worldwide distribution, there are relatively few tropical species and the majority of these are restricted to cooler climates at high elevation (Gauld & Mitchell, 1981; Gauld, 1988). In contrast, *Ophion* is by far the most abundant and diverse ophionine genus in virtually all habitats across the Holarctic region (Gauld, 1980, 1988).

Despite being a predominately temperate genus, *Ophion* in the Holarctic region has received little taxonomic attention, as most revisions of Ophioninae have been for tropical regions (Gauld, 1977, 1988; Gauld & Mitchell, 1978, 1981; Fernández-Triana, 2005; Kim *et al.*, 2009; Rousse & Van Noort, 2014). In the Nearctic region, 17 species are currently described (Yu *et al.*, 2012; Schwarzfeld & Sperling, 2014), although it has been estimated based on morphology that there are approximately 50 Neartic species (Gauld, 1985) and recent molecular analyses suggest there are many more (Schwarzfeld & Sperling, 2015). In the Palearctic region, *Ophion* is better known, with 79 described species (Yu *et al.*, 2012); however, there have been
no revisions of the genus across the Palearctic. *Ophion* was revised in the Netherlands by Oosterbroek (1978), and was examined most comprehensively in the UK, with a number of revisions over the years (Gauld, 1973, 1976, 1978) culminating in a thorough revision of the 16 known British *Ophion* species by Brock (1982). However, even among the relatively well-known British fauna, molecular evidence suggests a host of undescribed species (Schwarzfeld & Sperling, 2015).

*Ophion* is a morphologically challenging genus, with little morphological divergence between most species and few informative, discrete characters for identification (Gauld, 1980; Brock, 1982). Thus far, all attempts to divide *Ophion* into monophyletic species groups have been based on morphology and have been hampered by the lack of information regarding the eastern Palearctic and Nearctic species (Gauld, 1985). A number of species groups and species complexes have been informally referred to within *Ophion*; however, these have often not been explicitly defined and were generally discussed in isolation, with little reference to the remainder of the genus (e.g. Gauld, 1973, 1977; Gauld & Mitchell, 1981; Brock, 1982). The most comprehensive classification to date was by Gauld (1985), based in part on the results of a previous morphological analysis (Gauld, 1980). Gauld (1985) outlined eight major species groups, although there was no discussion of what constitutes a major versus a minor species group. The majority of these groups were presumed to be monophyletic and contained a small number of morphologically distinctive species, whereas one large group [the *O. luteus* (L) species group] served as a catch-all for the remaining species. *Ophion* as a whole can be recognized by the absence of a posterior mesosternal transverse carina, the impressed clypeal apex, the long membranous flange on the foretibial spur, the presence of a distinct ramellus (nearly always), and the straight, evenly slender forewing vein Rs + 2r (most species), among other characters (Gauld, 1985). *Ophion* has also been hypothesized to be paraphyletic with respect to the other six genera within the *Ophion* genus group (Afrophion Gauld, Agathophiona Westwood, Alophion Cushman, Xylophion

Gauld, Sclerophion Gauld and Rhopalophion Seyrig), although none of these are found within the Holarctic region (Gauld, 1985).

All Nearctic and Palearctic species have been placed within four, or possibly five, of Gauld’s eight species groups (Gauld, 1985). The *O. areolaris* group was previously classified as the genus *Platophion* Hellén (Gauld, 1977; Brock, 1982) and is characterized by the loss of the occipital carina, among other characters. The *O. similis* species group contains three Palearctic and two undescribed Nearctic species that have a stout body shape, short antennae, small eyes and ocelli, and appear to be diurnal. The *O. dentatus* species group is restricted to the eastern Palearctic region and is characterized by long slender mandibles and long claws. The *O. bicarinatus* species group is primarily Indo-Malayan and Australian but possibly contains the Palearctic *O. minus* Kriechbaumer; it is characterized by having forewing vein Rs + 2r broadened and slightly curved before reaching the pterostigma. The *O. luteus* species group (sensu Gauld, 1985) is a large group that contains the vast majority of Palearctic and Neotropical species, and all of the Nearctic species except for the two undescribed species from the *O. similis* group. It was assumed by Gauld (1985) to be paraphyletic with respect to the other species groups and was only defined by the lack of characters that characterize the other species groups.

Within the *O. luteus* group, Brock (1982) suggested some sister-group relationships between British species, but these have not been tested with molecular characters and no hypothesis was proposed for the phylogeny of the genus as a whole. Quicke et al. (2009) included nine species of *Ophion* as part of their broad taxonomic sampling to obtain a phylogeny of Ichneumonidae based on 28S rDNA. Recently, Schwarzfeld & Sperling (2014) defined one monophyletic species group (the *O. scutellaris* Thomson species group) from within the larger *O. luteus* group, and revised the included Nearctic species.

Two of the most commonly used molecular markers for hymenopteran phylogenies are the D2–D3 expansion region of 28S ribosomal RNA (28S) and the mitochondrial gene cytochrome oxidase 1 (COI) (e.g. Mardulyn & Whitfield, 1999; Dowton & Austin, 2001; Whitfield et al., 2002; Michel-Salzat & Whitfield, 2004; Klopfstein et al., 2011). COI is often used at the species level, due to its relatively high mutation rate (Monti et al., 2005; Li et al., 2010; Williams et al., 2012); however, it can also be informative at higher phylogenetic levels (Klopfstein et al., 2010; Quicke et al., 2012). In contrast, 28S is a highly conserved gene that has been widely used for higher-level insect phylogenies (Caterino et al., 2000; Heraty et al., 2011; Sharkey et al., 2012), although it has also proved useful in distinguishing species (Monaghan et al., 2005; Derycke et al., 2008; Raupach et al., 2010).

The internal transcribed spacer 2 (ITS2) of nuclear ribosomal RNA is another rapidly evolving locus that has been most often used at the species-level in insects (e.g. Campbell et al., 1993; Gomez-Zurita et al., 2000; Wagenet et al., 2006; Li et al., 2010; Schwarzfeld & Sperling, 2014). However, the highly conserved secondary structure of this gene, with characteristic paired domains and unpaired regions, also makes this an informative
gene at higher phylogenetic levels (Coleman & Vacquier, 2002; Coleman, 2007). Incorporating secondary structure can improve alignment of rRNA, an otherwise problematic task due to the presence of numerous indels in the length variable loop regions (Kjer, 1995; Coleman & Vacquier, 2002). Also, since the nonindependence of paired regions can mislead phylogenetic analyses, incorporating secondary structure can allow the use of rRNA-specific models for phylogeny estimation (Savill et al., 2001; Telford et al., 2005; Wolf et al., 2008; Letsch & Kjer, 2011).

The goal of this study is to obtain a preliminary molecular phylogeny of *Ophion*, with an emphasis on the *O. luteus* species group *sensu* Gauld (1985), based on COI, 28S, and ITS2 DNA. The primary focus is on Nearctic species; however, representatives of most species known from the western Palearctic region are included, as well as a few species from Costa Rica, Madagascar, Taiwan, Australia and New Zealand.

**Methods**

**Specimens**

A total of 493 specimens of Nearctic *Ophion* were newly sequenced for this study. The majority of these specimens are from Canada, with a smaller number from the US. Most were newly collected for this study; however, 25 specimens were sequenced from alcohol-preserved material on loan from the Canadian National Collection of Insects, Arachnids and Nematodes. Specimens were selected for sequencing to represent the range of morphological variation observed in over 4000 specimens from a variety of habitats. Eighty specimens from 15 species of Palearctic *Ophion* were also newly sequenced, all of which are from the UK, except for one specimen from Spain and one from France.

In addition to the newly sequenced specimens, 93 *Ophion* COI sequences were included from the Barcode of Life database (BOLD). Finally, several *Ophion* sequences from GenBank were also included. This resulted in an additional 21 COI sequences (including eight sequences from Costa Rica, two from Madagascar and two from New Zealand) and nine 28S sequences [including one sequence each from *O. zerus* Gauld (Australia) and *O. bicarinatus* Cameron (Taiwan)].

Outgroups were selected from across the range of genus groups within Ophioninae, as defined by Gauld (1985), although some of these genus groups are not well supported (Quicke et al., 2009). From the *Ophion* genus group, we included *Afrophion* Gauld, *Alophophion* Cushman, *Rhopolophilus* Seyrig and *Xylophion* Gauld. The other included genera were: *Sicophilus* Gauld, *Janzophilus* Gauld (*Sicophilus* group); *Eremotylus* Förster (*Eremotylus* group); *Barytatocephalus* Schulz, *Euryphion* Cameron, *Rhychnophion* Enderlein, *Thyreodon* Brullé (*Thyreodon* group); *Dicamptus* Szépligeti, *Enicospilus* Stephens, *Laticoleus* Townes, *Lepiscelis* Townes, *Leptophion* Cameron, *Ophiogastrilla* Brues, *Pamophion* Gauld, *Simophion* Cushman, *Stauroptocotonus* Brauns (*Enicospilus* group). As the phylogeny of Ophioninae in general is poorly known, we also included a nonophionine member of the higher ophioniformes (*Anomalon* Panzer) as a distant outgroup with which to root the phylogeny.

The provenance and repositories of all specimens are listed in Appendix S1. With the exception of sequences obtained from GenBank or BOLD, all Nearctic taxa were identified by M.D.S. and all Palearctic taxa were identified by G.R.B., with the exception of *O. forticornis* Morley, which was identified by M. R. Shaw (National Museums Scotland, Edinburgh).

**Sequencing and alignment**

DNA was extracted from a single hind leg and COI and ITS2 were sequenced using the primers and protocols described in Schwarzfeld & Sperling (2014). For the D2–D3 region of 28S rDNA, the primers were: 5′-GCC AAC AAG TAC CGT GAG GG-3′ (forward) and 5′-TAG TTC ACC ATC TTT CGG GTC-3′ (reverse) (Laurenne et al., 2006), and PCR protocols followed Schwarzfeld & Sperling (2014), except that cycling conditions were 94°C for 2 min, 30 cycles of 96°C for 15 s, 50°C for 30 s, 72°C for 30 s and a final extension of 75°C for 7 min. Sequences are deposited in NCBI GenBank, and Genbank accession numbers are listed in Appendix S1.

Alignment of COI was unambiguous, and was confirmed by translating nucleotides to amino acids in MESQUITE (Maddison & Maddison, 2011). A total of 681 COI sequences from *Ophion* and 13 from outgroup taxa were included in the study (including sequences from GenBank/BOLD). A few sequences were not successfully sequenced in both directions, or were otherwise partially incomplete; these sequences were included with the gaps coded as missing data. For 28S, alignment was performed by eye in MESQUITE; there were occasional small indels, but generally alignment was unambiguous. The aligned sequences were 725 bp in length. The final dataset included 96 *Ophion* sequences (87 newly sequenced and nine from Genbank), as well as 20 outgroup sequences. We were unable to obtain complete ITS2 sequences for several specimens; as the analysis of ITS2 secondary structure was sensitive to missing data, we only included sequences that were complete and that lacked ambiguous regions. The final dataset included 394 ITS2 sequences. The aligned sequences for each dataset are available on the Dryad Data Repository: doi:10.5061/dryad.49g98.

**ITS2 secondary structure**

The detailed protocol used to obtain the secondary structure of ITS2 is described in Schwarzfeld & Sperling (2015). Briefly, we first folded an arbitrarily chosen sequence using the RNAfold webserver (Hofacker, 2003) and default settings. We then imported this structure into the ITS2 database, and used it as a template for folding the remaining sequences. If any sequences had less than 90% similarity to the existing template, one of the anomalous sequences was directly folded in RNAfold, and then added as an additional template to the database. Using this iterative process, the final dataset consisted of eight
sequences that were folded directly and 386 sequences that were folded using homology. One sequence (from *O. flavidus* Brullé) appeared to be a pseudogene, and was thus excluded from all phylogenetic analyses. The remaining sequences and structures were aligned using 4SALE, a program designed to synchronously align RNA sequences and structures (Seibel *et al.*, 2006). The 28S and 5.8S flanking regions were not included in the phylogenetic analyses; the final alignment of ITS2 was 1705 bp in length.

**Phylogeny estimation**

*Ophion* phylogeny was estimated using maximum likelihood (ML) and maximum parsimony (MP) analyses. Analyses were conducted on each dataset separately (COI, ITS2, and 28S), as well as on two concatenated datasets. The first dataset (‘concatenated_all’) included all specimens and all loci. In order to investigate the effect of taxon sampling and missing data, we also analysed a dataset consisting of only those specimens that were successfully sequenced for all three loci (‘concatenated_common’). As no outgroup sequences were available for ITS2, the ITS2 and concatenated_common trees were rooted with *Ophion minutus* Kriechbaumer, as this was consistently recovered as sister to the remaining ingroup species in the other three analyses, as well as having morphological characters that indicate it is sister to the remaining *Ophion* species.

We conducted MP analyses using PAUP* 4.0 (Swofford, 2002). Analyses were run using heuristic searches with ten replicates of random-addition starting trees (swap = tree–bisection–reconnection, saving no more than 10000 trees per replicate), and a 50% majority-rule tree was constructed. For each analysis, 1000 bootstrap pseudoreplicates were conducted using the same search settings except that simple stepwise addition was used, and a maximum of 100 trees were saved per replicate.

Models for the ML analyses were determined using PARTITIONFINDER version 1.0.1 (Lanfear *et al.*, 2012). This program considers all potential partitions within a dataset, and calculates the best partitioning scheme and best model(s) for each scheme. Only those models able to be implemented in the GARLI web service were included. Models were calculated for COI using the three codon positions as potential partitions, and the ‘greedy’ search algorithm. For the two concatenated analyses, each gene and the codon position for COI were included, with the ‘greedy’ search algorithm. The 28S and ITS2 datasets were not partitioned, and all included models were assessed (‘search = all’). Best models were selected according to the Bayesian information criterion (Schwarz, 1978), as recommended by Lanfear *et al.* (2012).

The ML analyses were conducted using the web service for GARLI 2.1 (Zwickl, 2006), hosted at http://www. molecularrevolution.org (Bazinet *et al.*, 2014). The ITS2 and COI ML analyses used a neighbour-joining (NJ) starting tree constructed in PAUP* (p-distance), while the 28S and concatenated_common analyses used a stepwise addition starting tree. As there were taxa with no overlapping characters in the concatenated_all dataset, a supertree was constructed in CLANN 3.0 (Creevey & McInerney, 2005), using the three individual NJ trees from PAUP* (COI, ITS2, and 28S) and the NJ function in CLANN. This tree was used as the starting tree for the concatenated_all ML analysis. The GARLI web service uses an adaptive approach in which additional search replicates are based on the statistical probability that the best tree has been obtained with 95% confidence. The ITS2, COI and concatenated_all analyses each reached the maximum number of 1000 replicates, while the 28S and concatenated_common analyses were completed after ten replicates. One thousand bootstrap pseudoreplicates were calculated for each dataset, using stepwise addition starting trees.

Finally, in order to incorporate the structural information of the ITS2 sequence data, we calculated 100 NJ bootstrap replicates using an ITS2 structure-specific rate matrix in PROFDISTS (Wolf *et al.*, 2008). The rate matrix was obtained from PROFDISTS, where it was calculated by converting the four bases into a 12-letter alphabet, with each base in three possible states (unpaired, paired-right or paired-left), and then determining a GTR (ML)-corrected substitution model (Seibel *et al.*, 2006; Wolf *et al.*, 2008).

**Results**

The concatenated_all and concatenated_common datasets were each partitioned by locus and codon position (CP), resulting in five partitions. The concatenated_all dataset used CP1: GTR+I+G; CP2: F81+G; CP3: GTR+I+G; ITS2: SYM+G; 28S: K80+I+G. Models for the concatenated_common dataset were the same except that CP1 and CP3 used GTR+G, and CP2 used F81. The individual analyses used the same models as the concatenated_all dataset.

Thirteen major ingroup clades (= species groups) were recovered in this study, although not all clades were represented in all analyses (Figs 2, 3; Figures S1–S4; Table S1). In the COI and concatenated_all analyses, bootstrap support was much stronger in the MP analyses, compared with the ML analyses; however, for the other analyses, support values were similar between the two methods (Table S1). The ITS2-specific NJ bootstrap values from PROFDISTS were almost identical to the MP bootstrap values for ITS2, and the analysis did not produce any divergent results. Bootstrap values are only shown for the major nodes in Fig. 2; however, all bootstrap values are included in Figure S1. The trees from the individual analyses can be seen in Figures S2–S4, with bootstrap values shown for the major nodes.

The two concatenated datasets recovered the same major clades; however, bootstrap support was generally higher in the concatenated_common analyses, with most major nodes having high bootstrap values (Fig. 3, Table S1). The same clades were also recovered by the analyses of ITS2 and COI. However, the pattern of divergence differed between these two loci, with ITS2 showing high sequence divergence between species groups and very low sequence divergence within groups (Figure S3), while COI had a less distinct separation of within-group and between-group divergence (Figure S2). The 28S tree was the
Fig. 2. (A–E) Maximum likelihood tree from the concatenated_all dataset, including 704 Ophion and 21 outgroup specimens and three loci [cytochrome oxidase 1 (COI), internal transcribed spacer 2 (ITS2) and 28S ribosomal RNA (28S)]. Maximum likelihood and maximum parsimony bootstrap values > 50% are given for the major nodes (MP/ML).

© 2015 The Royal Entomological Society, Systematic Entomology, 41, 191–206
least resolved of any of the analyses, with few clades having strong bootstrap support (Figure S4). Summary statistics for all analyses are shown in Table S2.

At the root of the tree, a polytomy was recovered including *Alophophion*, a clade containing *Afrophion*, *Xylophion*, *O. zerus*, *O. ventricosus* Gravenhorst and *O. minutus* (labelled I in Fig. 2), and a clade containing all remaining *Ophion* as well as *Rhapalophion* (labelled II in Fig. 2). However there was no bootstrap support for either of these clades. *O. minutus* and *O. ventricosus* formed a monophyletic clade, though with weak bootstrap support (Fig. 2; Table S1). With the exception of the highly divergent *O. flavidus* sequence, *O. minutus* had
the most atypically shaped ITS2, compared with other species (Fig. 4H). We were not able to successfully sequence ITS2 for \( O. \) ventricosus.

Within clade II, there was little bootstrap support for the deeper nodes in the concatenated_all tree; however, the concatenated_common tree was quite well resolved, with most nodes having strong bootstrap support (Fig. 3). The \( O. \) scutellaris group was recovered as monophyletic, with weak support according to the concatenated_all dataset, but very strong support in the concatenated_common tree (Table S1). In the concatenated_all tree, it also included the genus Rhopalophion, although only 28S was available for this genus. The \( O. \) areolaris group was represented by a single species in this study, and was recovered as sister to the \( O. \) scutellaris group, with strong support according to concatenated_common analysis (MP: 98, ML: 100), but no support according to concatenated_all.

The \( O. \) slossonae Davis and \( O. \) parvulus Kriechbaum species groups were also recovered as sister clades, with strong support in the concatenated_common analysis (Figs 2, 3). The \( O. \) parvulus group was strongly supported by all analyses.

© 2015 The Royal Entomological Society, Systematic Entomology, 41, 191–206
Within this group, there was a deep divergence within *O. parvulus*, indicating that two species are present (*O. parvulus* A and *O. parvulus* B, Fig. 2). The Nearctic specimens in this group were also separated into two strongly supported clades (Fig. 2; Figure S1). In one of these clades, however, sequencing appears to have amplified a nuclear mitochondrial pseudogene (numt) (Gellissen et al., 1983; Lopez et al., 1994), as there is a 2-bp deletion at the same location in each sequence that would result in a frameshift in the coding mitochondrial gene. The *O. slossonae* group was strongly supported in the concatenated_common analysis (MP: 100, ML: 96) and in the concatenated_all parsimony analysis (MP: 90). However, it was recovered with less than 50% support in the concatenated_all ML analysis. This group includes *O. slossonae*, as well as morphospecies A from Schwarzfeld & Sperling (2015).

In the concatenated_all analysis, the *O. flavidus* group was recovered with strong support (MP: 96, ML: 87). It was the sister group to (*O. slossonae* group + *O. parvulus* group), although with no bootstrap support. It was not included in the concatenated_common dataset, as we were only able to successfully sequence ITS2 for one specimen within this group (*O. flavidus*).

This sequence was highly divergent from all other species, indicating it may be a pseudogene, as it greatly exceeds the amount of expected variation in ITS2 (Kita & Ito, 2000; Alvarez & Wendel, 2003). The secondary structure was equally distinct; although the conserved regions of the structure were maintained, the unusually branched Helix III provides further evidence that this sequence may be a pseudogene (Fig. 4G). However, another indication of ITS2 pseudogenes (lower GC content) was not observed in this sequence (Buckler et al., 1997; Kita & Ito, 2000). Attempts to sequence two other specimens in this species group resulted in very small amounts of clean sequence that were clearly similar to that obtained for *O. flavidus*.

Two COI sequences from New Zealand *Ophion* were included from GenBank. These two specimens formed a strongly supported monophyletic group (MP: 99, ML: 88), which was sister to (*O. flavidus* group + (*O. parvulus* group + *O. slossonae* group)), although without bootstrap support.

The *O. pteridis* Kriechbaumer and *O. nigrovarius* Provancher species groups were recovered as sister groups, although with no support in the concatenated_all analyses, and only weak support in the concatenated_common analyses. Together, these two groups were sister to the *O. luteus* group, with strong support in the concatenated_common analyses (MP: 92, ML: 86), although again support was lacking in the concatenated_all analyses. The *O. pteridis* group was strongly supported in most analyses, although the ML analysis of the concatenated_all dataset had a bootstrap of <50. The *O. luteus* s.s. group was strongly supported by both datasets, with bootstrap support ranging from 82 (concatenated_all ML) to 100 (concatenated_common MP, ML). This group includes the Palearctic *O. luteus* (L) and the Nearctic species *O. elongatus* Hooker. The *O. nigrovarius* group includes two species: the Palearctic *O. perkinsi* Brock and the Nearctic *O. nigrovarius*. It was strongly supported in the concatenated_all dataset (MP: 95, ML: 71); however, only *O. nigrovarius* was available for the concatenated_common dataset.

Two COI sequences of *Ophion* from Madagascar were included from GenBank, forming a strongly supported monophyletic group (MP: 100, ML: 87). Species group 1 was recovered with moderate bootstrap support in the concatenated_all dataset (MP: 86, ML: 71). Support was very strong in the concatenated_common dataset (MP and ML: 100), although this dataset only included two specimens from this group. *O. longigena* Thomson was recovered as sister to this group in the concatenated_all analyses, although with very low bootstrap support; only COI was available for this species. The placement of the Madagascar group and species group 1 varied between analyses. The concatenated_all MP analysis recovered [Madagascar group + (Species group 1 + *O. longigena*)] as sister to the *O. obscuratus* group, as did the MP and ML analyses of the concatenated_common dataset (although *O. longigena* and the Madagascar group were not included in this dataset). In comparison, the ML analysis of concatenated_all recovered the Madagascar group as sister to (*luteus group + (pteridis group + nigrovarius group)), while (species group 1 + *O. longigena*) was sister to this combined clade.

© 2015 The Royal Entomological Society, Systematic Entomology, 41, 191–206
The *O. obscuratus* Fabricius species group was recovered as monophyletic by most analyses. According to the concatenated_all ML analysis, two specimens (one sequence each of *O. mocsaryi* and *O. costatus*) were not included in this clade; however, these were each only represented by 28S. Bootstrap support for this group was very low, with the strongest support for this group coming from the concatenated_common dataset (Figs 2, 3; Table S1). The *O. obscuratus* group includes morphospecies B from Schwarzfeld & Sperling (2015), as well as a monophyletic clade consisting of several Palearctic species (*O. crassicornis* Brock, *O. costatus* Ratzburg, *O. mocsaryi* Brauns, *O. brevicornis* Morley, *O. forticornis* Morley, and apparently two species within ‘*O. obscuratus’*) (Fig. 2). Within this Palearctic group, specimens identified as *O. costatus* and *O. mocsaryi* were found in three clades: one of *O. costatus*, one of *O. mocsaryi*, and a third clade with two specimens identified as *mocsaryi* and two as *costatus*. An additional specimen of *O. mocsaryi* was found separately from the above three clades, but as it was only represented by ITS2, this placement probably reflects the locus, rather than indicating an additional species. Two specimens identified as *O. obscuratus* (labelled *O. obscuratus C*; Fig. 2) were not recovered as part of this Palearctic clade, although they were part of the *obscuratus* group (Fig. 2).

The single representative of *O. bicarinatus* from GenBank, and two Nearctic specimens (MS10835 and UASM341138) were recovered as sister to the clade including species group 1 and the Madagascar, *obscuratus*, *luteus*, *pteridis*, and *nigrovarius* groups in the concatenated_all analysis, although without support. *O. bicarinatus* was only represented by 28S, which was quite uninformative in this portion of the tree (Figure S4). The other two specimens were also represented by COI and ITS2; however, their placement on the tree varied depending on which locus was examined (Figures S2 and S3). In the concatenated_common tree, they were recovered as sister to the *obscuratus* group, with weak support, possibly indicating they should be included in this species group (Fig. 3).

**ITS2 secondary structure**

The ITS2 of rDNA varied from 717 to 1056 bp in length, with all except two species having greater than 850 bp. The secondary structure was congruent with the conserved features of ITS2 observed across a wide range of taxa (Schultz *et al.*, 2005; Coleman, 2007). In particular, all species have recognizable helices II and III, the hallmark helices of almost all known ITS2 sequences (Coleman, 2007). Helix II is relatively short, almost always unbranched, and has a pyrimidine-pyrimidine mismatch bulge near its base. Helix III is long, often branched and more highly variable, but with a conserved sequence motif near the apex on the 5′ side. In all Ichneumonidae examined (M.D Schwarzfeld, unpublished data), this motif is CGGTCGATCGAGTCC. In *Ophion*, there are an additional two to four helices between helix II and helix III. The first of these (tentatively labelled helix IIA) is present in all species and is almost always very long (Fig. 4).

Within *Ophion*, the secondary structure of ITS2 was quite conserved among many taxa, and highly divergent between others. The vast majority of specimens were successfully modelled from a single template, with a minimum of 90% of the base pairs assigned to the template structure. This includes all specimens from the *obscuratus*, *luteus*, *pteridis* and *nigrovarius* groups and species group 1 (Fig. 4A). The remaining species groups each required a separate template. In most cases, these differed only slightly in shape, particularly in the number of short helices between helix IIa and helix III, and in the branching pattern of helix III. The structures for *O. minutus* and *O. flavidas* were the most distinct (Fig. 4G, H) The *O. flavidas* structure was particularly unusual as helix IIa was branched, a pattern rarely observed across Metazoa (Coleman, 2007). The *slossonae* group was the only species group to have two distinct secondary structures (Fig. 4E, F). One of these structures (Fig. 4F) was found in a single undescribed species (three specimens) and differed from all other sequences by having a large deletion in the centre of the sequence, thus lacking most of helix IIa.

**Discussion**

This study presents the first phylogenetic hypothesis for the genus *Ophion*. Gauld (1985) suggested that the genus *Ophion* was paraphyletic with respect to the six other genera within the *Ophion* genus group. Four of these genera were included in this study (Afrophion, Alopophion, Rhopalophion and Xylophion), and our results support a close relationship between these species and *Ophion*. While more sampling is needed, these results suggest that by erecting a new genus for clade I (the *O. minutus* group, *O. zerus*, *Xylophion*, and *Afrophion*) the remaining *Ophion* (possibly including *Rhopalophion*) would be a monophyletic genus (= clade II in this study).

This study could be regarded as a preliminary analysis due to limited geographical sampling; only three of the eight species groups outlined by Gauld (1985) are included in the analysis (the *O. bicarinatus*, *O. areolarius* and *O. luteus* groups), and the sampling is heavily biased towards Canadian and western Palearctic material. Delimitation of taxa above the species level is necessarily subjective. We delimited species groups primarily based on distinct sequencing gaps between well-supported clades. However, some species groups were less supported and further sampling may fill in some of the sequencing gaps, thus requiring a reanalysis of these species groups. Despite these limitations, this is the first attempt at resolving the ‘*luteus anathema’* (Gauld, 1985, p. 125), and as such provides a valuable framework for understanding the relationships between the majority of *Ophion* species. While the vast majority of *Ophion* in this study are undescribed (Schwarzfeld & Sperling, 2015), there is at least one described species in all except species group 1 and the Madagascar and New Zealand groups.

Thirteen species groups are designated in this study, most of which are newly coined here. With the exception of *O. minutus*, *O. zerus*, *O. bicarinatus* (*O. bicarinatus* group), *O. ocellaris*
Fig. 5. Characters of selected Ophion species groups. Forewing: (A) O. minutus group (O. minutus), (B) O. parvulus group (undescribed sp.), (C) O. obscuratus group (undescribed sp.). Mid-tibial spurs: (D) O. scutellaris group (O. idoneus). Scutellum: (E) O. scutellaris group (O. keala), (F) O. pteridis group (undescribed sp.), (G) O. obscuratus group (undescribed sp.). First tergite: (H) O. parvulus group (undescribed sp.), (I) O. pteridis group (undescribed sp.). Hind trochantellus (Tr): (J) O. luteus group (O. luteus), (K) O. parvulus group (undescribed sp.). Mandibles: (L) O. luteus group (O. luteus), (M) O. pteridis group (undescribed sp.), IA, internal angle; face: (N) O. nigrovarius, (O) O. slossonae group (undescribed sp.). (Photographs: (A–C), (E), (H–M), Rylee Isitt.)

Ulbright (O. areolaris group), and the two species groups from Madagascar and New Zealand, the remaining species were all presumably included within the catch-all O. luteus species group (Gauld, 1985).

**Ophion minutus Kriechbaumer species group**

According to Gauld (1985), the O. bicarinatus species group includes O. bicarinatus, all of the Australian species (including O. zerus), and possibly O. minutus; it is characterized by having forewing vein Rs+2r thickened and slightly curved before reaching the pterostigma (Fig. 5A). *Ophion ventricosus* Gravenhorst also has a slightly thickened Rs+2r (Brock, 1982) but was not mentioned by Gauld (1985). Similarly thickened Rs+2r veins are found in other ophionine genera, such as *Eremotylus* Förster, which lends support to these species being sister to the remaining *Ophion*. In contrast, the O. luteus species group sensu Gauld (1985) has Rs+2r straight and of equal thickness along its length (Fig. 5B, C).

While only weakly supported by the sequence data, the O. minutus + *ventricosus* clade is further supported by biology, as both
species have been reared from Geometridae, unlike the majority of *Ophion*, which are parasitoids of Noctuoidea (Brock, 1982). In their analysis of 28S across Ichneumonidae, Quicke *et al.* (2009) recovered *O. zerus* and *O. minutus* as a clade, but did not recover *O. ventricosus* within *Ophion*. The 28S sequence from Genbank identified as *Ophion bicarinatus* was not recovered near *O. minutus*, but rather was near the *O. obscuratus* group and related species groups; it was similarly recovered by Quicke *et al.* (2009). This may indicate a misidentification of this species by Quicke *et al.* (2009), as it would be surprising to find a species with a thickened Rs + 2r within a group with otherwise typical venation. In this study, we have included *O. minutus* and *O. ventricosus* in the *O. minutus* species group but left *O. zerus* unassociated, pending further study of *O. bicarinatus* and of other members of the *O. bicarinatus* species group sensu Gauld (1985). *O. minutus* had the most atypical-shaped ITS2 compared with other species, providing additional evidence that *O. minutus* is distinct from all *Ophion* species in clade II. Determining the ITS2 sequence and structure of other species in clade I would be informative for understanding the root of the *Ophion* phylogeny. In most cases, we named each species group after the earliest-named species within it; however, as the term ‘*O. minutus* species group’ has previously been used in the literature (e.g. Gauld, 1977), we are retaining it for this group.

*O. areolaris* Brauns species group

Due to the lack of the occipital carina, the *O. areolaris* species group (Gauld, 1985) has been treated as a separate genus, *Platophion* Hellén, by some authors (Hellén, 1926; Brock, 1982); however, it was synonymized with *Ophion* by Gauld (1980). This study supports Gauld’s conclusion that the species group should be included within *Ophion*, probably as sister to the *O. scutellaris* species group. This group is also unusual within *Ophion*, as it has been reared from Thyatirinae (Drepanidae) (Brock, 1982), unlike the usual Noctuoidea hosts. As well as lacking the occipital carina, species of the *O. areolaris* group are unusual in their rather square dorsum of the mesoscutellum, black inter-ocellar area and, at least in some species, pronounced sexually dimorphic colour patterns (males can be considerably darker).

*O. scutellaris* Thomson species group

The *O. scutellaris* group is treated in detail by Schwarzfeld & Sperling (2014). The members of this group are mostly dark reddish, early-season species and can be recognized by a suite of morphological characters [e.g. subequal mid-tibial spurs (Fig. 5D), carinate scutellum (Fig. 5E)]. This group includes a minimum of seven Nearctic species and two Palearctic species. The recovery of *Rhopalophion* within this clade suggests that it should be transferred to *Ophion*. Quicke *et al.* (2009) also recovered it as sister to *O. scutellaris*, using the same 28S sequence as in the current study. However, as only a single 28S sequence was included in this study, additional taxon and gene sampling is needed before a formal name change is proposed.

*O. parvulus* Kriechbaumer species group

This group was very strongly supported by the sequence data. Morphologically, this group can be recognized by the evenly arched forewing vein Rs (i.e. not sinuous) (Fig. 5B; cf. Fig. 5C) and by the unusually short first tergite, with the spiracles in line with the ventral membrane (Fig. 5H). Brock (1982) referred to *O. parvulus* as forming a ‘close species pair’ with *O. pteridis*, but our results show they are not in fact closely related. Thus far all Nearctic specimens in this group have been collected in late summer (August or September), although one of the two Palearctic species was collected in May.

*O. slossonae* Davis species group

This species group is highly morphologically variable and specimens have been collected from early June to mid-September (with one specimen from Florida in March). This is also the only species group to have two distinct ITS2 structures in different species (Fig. 4E, F). Additional morphological study of this group may reveal characters that are consistent within the group or, conversely, might suggest it should be further subdivided. The nominate species, *O. slossonae* Davis, is by far the largest Nearctic species we have examined; however, other species in this group are of a more typical size.

*O. pteridis* Kriechbaumer species group

This is a late-flying species group, with almost all collection records from July to September. Species in this group have strong scutellar carinae (Fig. 5F; cf. Fig. 5G) and a distinctly sinuous first tergite (Fig. 5I). Again, there is no evidence that *O. pteridis* is closely related to *O. parvulus*, as was suggested by Brock (1982).

*O. luteus* (L.) species group

Species from this group have been collected from May to September, with one species from Arizona in March or April (Appendix S1). While morphologically variable, most species can be recognized by the long hind trochantellus (Fig. 5J; cf. Fig. 5K), relatively transverse face, and blunt-tipped mandibles with weak or absent internal angles (Fig. 5L; cf. Fig. 5M). *O. elongatus* is a distinctly elongated Nearctic species which was recovered within this group, based on two COI sequences obtained from BOLD; we have not examined these specimens to determine whether this species also has the typical *luteus* group characters.

O. nigrovarius Provancher species group

Ophion nigrovarius and O. perkinsi are both buccate-headed (i.e. have expanded genae; Fig. 5N; cf. Fig. 5O), which supports the molecular data linking them. Based on morphology, Brock (1982) believed O. perkinsi was closely related to O. brevicornis; however, in our study O. brevicornis was part of the Palearctic clade within the O. obscuratus group. Ophion nigrovarius is a particularly interesting species as it has been recorded from Phyllophaga (Coleoptera: Scarabaeidae), the only known record of a coleopteran host for Ophion (Townes, 1971). No hosts have been recorded for O. perkinsi (Yu et al., 2012).

Species group 1

This is a morphologically diverse clade; while COI had a maximum sequence divergence of only 2.3% within the 12 included specimens, we predict based on morphology that the group contains a minimum of four species, none of which are apparently described (M.D. Schwarzfeld, unpublished data). Specimens have been collected from May to September. Some of these species are buccate-headed, though not as dramatically as in the O. nigrovarius group, while others are more typical. It is possible that this group should be expanded to include O. longigena, another buccate-headed species, however, additional specimens and genes need to be examined, as only a single COI sequence was included in this study and its inclusion was not supported by bootstrap analyses.

New Zealand and Madagascar groups

Gauld (1985) stated that all described New Zealand Ophion species were found in the O. peregrinus species group, based on a number of morphological characters. It is therefore possible that the two New Zealand sequences included in this study belong to this group. However, as the sequences from GenBank were not identified to the species level, and we have not examined the specimens, we refrain from assuming that they are part of this named species group. Similarly, the two sequences from Madagascar are clearly distinct from all other Ophion in this study; however, with such limited sampling from the area, we are unable to draw any general conclusions regarding Ophion of Madagascar.

O. obscuratus Fabricius species group

While this study provides much resolution of the species groups within Ophion, the O. obscuratus group remains an unresolved challenge. This species complex consists of many apparently closely related species, some of which are morphologically distinct, while others are less so (M.D. Schwarzfeld, unpublished data). While most evidence points to this group being monophyletic, bootstrap support is very low. This group is nonetheless a useful categorization pending further study. It is possible that three unplaced specimens (O. bicarinatus, UASM341138 and MS10835) also belong to this group; however, more evidence is needed before they can be conclusively placed.

Brock (1982) states that O. mocsaryi and O. costatus are more closely related than any other pair of British Ophion, and suggests they should be synonymized. Our study supports this close relationship, but suggests that part of the difficulty in distinguishing these species may be because they form part of a species complex of more than two species. Brock’s other hypothesis, that the mocsaryi–costatus species pair is closely related to the pteridis–parvalis pair, is not supported by our study, as the latter species are not closely related to each other or to the members of the obscuratus group. In the Nearctic region, this group includes an early-season, yellow-marked Ophion species that is by far the most abundant species in all Canadian localities that have been well sampled; however, the precise boundaries of this species are still undetermined (M.D. Schwarzfeld, unpublished data). In total, the O. obscuratus group contained 56% of all COI sequences and 67% of ITS2 sequences in this study.

ITS2 secondary structure

The highly variable unpaired regions of ITS2, combined with the nonindependence of pairing regions, can potentially mislead phylogenetic inferences (Tillier & Collins, 1995; Telford et al., 2005; Wolf et al., 2008; Letsch & Kjer, 2011). However, using an ITS2-specific model that incorporates secondary structure had very little impact on the results of this study. This may be because the observed variation in ITS2, namely large differences between species groups and low divergence within them, provided a strong enough signal that the choice of model did not significantly affect the results.

As the ITS2 sequences were folded in silico, further studies are needed to confirm that these structures are accurate (Eddy, 2004; Marinho et al., 2013). Overall, the ITS2 structures were consistent with the conserved patterns observed by Coleman (2007), loosely corresponding to the four-domain model predicted for ITS2 (Schultz et al., 2005). They did, however, differ in significant ways. Most dramatically, ITS2 in Ophion is considerably longer than in most other species that have been studied (Schließlich et al., 1994; Gomez-Zurita et al., 2000; Young & Coleman, 2004; Coleman, 2007; Marinho et al., 2011), including many other Ichneumonidae (Ashfaq et al., 2005; Wagener et al., 2006). This length contributed to difficulties in sequencing the gene, making it less optimal for phylogenetic analyses in Ophion compared with other taxa. Much of the additional length was found in the third helix between helix II and helix III. We have called this domain helix IIA, assuming it is equivalent to the IIA of other insect species (Coleman, 2007). However, the lack of a variable helix IV in most Ophion species indicates that perhaps this domain is equivalent to helix IV in other taxa. It is biologically interesting that one species within the

© 2015 The Royal Entomological Society, Systematic Entomology, 41, 191–206
O. slossonae group lacked this long helix, even though all other specimens within the species group had a more typical-shaped ITS2. The nearly identical structure found in species group 1 and the pteridis, luteus, nigrovius and obscuratus groups further support the monophyly of this clade.

Species delimitation and pseudogenes

The vast majority of Nearctic species in this study are undescribed. However, the presence of several previously unrecognized putative new species within the British material shows that even in well-studied areas there is much work to be done at the species level within Ophion (Schwarzfeld & Sperling, 2015). This also suggests that using a single exemplar per species for phylogenetic inference can potentially mislead the analysis if there is any doubt as to the identity of the species involved. Until Ophion are better resolved at the species level, we are limited in our understanding of their overall phylogeny.

The amplification of nuclear mitochondrial pseudogenes (nonfunctional copies of mitochondrial genes that have been incorporated into the nuclear genome, or numts) can mislead phylogenetic analyses based on mitochondrial DNA (Song et al., 2008; Buhay, 2009; Moulton et al., 2010). In this study, the amplification of a potential numt in some members of the parvulus group means the results of the analyses should be viewed cautiously. As ITS2 and 28S also support the monophyly of this group, it appears that, at least at the species group level, the numts remain phylogenetically informative. Their presence, however, limits the utility of COI for species delimitation. These numts were recognized due to a presumably homologous 2-bp deletion in several otherwise clean sequences. However, not all numts contain indels or stop codons, and may be difficult to recognize (Song et al., 2008; Buhay, 2009; Moulton et al., 2010). Similarly, the amplification of a possible ITS2 pseudogene in the O. flavidus species group could also potentially mislead phylogenetic analyses. Both of these examples emphasize the importance of not relying on a single molecular marker for phylogenetic inference (Dupuis et al., 2012).

Conclusion

This study provides a framework for future studies of the diverse and morphologically challenging genus Ophion. For the first time, the ‘luteus anathema’ (Gauld, 1985, page 125) is divided into more manageable, discrete units that can be examined for morphological characters and biological information. Many species are morphologically very similar between species groups, and there are also highly divergent species within groups. With this phylogeny as a guide, it will now be possible to conduct more targeted morphological analyses, with reanalysis of known morphological characters and discovery of new characters, to better characterize the species groups and to facilitate the delimitation and description of species within each group.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12152

Figure S1. Maximum likelihood tree from the concatenated_all dataset, including 704 Ophion and 21 outgroup specimens and three loci (COI, ITS2, 28S). Maximum likelihood and maximum parsimony bootstrap values > 50% are presented (MP/ML). For clarity, occasional weak bootstrap values for short branches that conflicted between analyses were excluded from the figure.

Figure S2. Maximum likelihood tree of 681 Ophion and 12 outgroup COI sequences. Maximum likelihood and maximum parsimony bootstrap values > 50% are given for major nodes (MP/ML).

Figure S3. Maximum likelihood tree of 393 Ophion ITS2 sequences. Maximum likelihood and maximum parsimony bootstrap values > 50% are given for major nodes (MP/ML).

Figure S4. Maximum likelihood tree of 96 Ophion and 20 outgroup 28S D2-D3 sequences. Maximum likelihood and maximum parsimony bootstrap values > 50% are given for major nodes (MP/ML).

Table S1. Summary of bootstrap support values for 12 species groups of Nearctic and western Palearctic Ophion (Only a single species of the O. areolaris species-group was included in the analyses, and therefore this group is not included in the table.). nm, non-monophyletic; all, all taxa and all loci included; common, only taxa with all three loci present included.

Table S2. Summary of maximum likelihood (ML) and maximum parsimony (MP) analyses of three genes of Ophion.

Appendix S1. List of all specimens included in this study.

Acknowledgements

Thank you to the collectors who provided specimens to M.D.S., especially John Acorn, John Adams, Gary Anweiler, Charley Bird, Heather Bird, Brett Bodeux, Bryan Brunet, Jason Dombroskie, Julian Dupuis, Jessica Edwards, Dave Holden, Wes Hunting, Dave Langor, Nina Lany, Dave Lawrie, Lisa Lumley, Doug Macaulay, Boyd Mori, Ted Pike, Greg Pohl and Chris Schmidt. Thank you also to the numerous light-trappers who have sent specimens to G.R.B. for the Nocturnal Ichneumonoidea Recording Scheme. We are grateful to Jeremy deWaard for providing many specimens as well as nearly 100 COI sequences through BOLD. Thanks also to Andrew Bennett (Canadian National Collection) for the loan of pinned and alcohol-preserved specimens, and to Mark Shaw for sending a leg from O. forticornis. Matthias Wolf kindly provided assistance with 4Sale and ProfDistS. Thank you to Rylee Isitt for help with photographing specimens and to

© 2015 The Royal Entomological Society, Systematic Entomology, 41, 191–206
Andrea Jackson for a live shot of *Ophion*. Many thanks to Pascal Rousse and two anonymous reviewers for their comments on an earlier version of this manuscript. This work was supported by the Sustainable Forest Management Network; Alberta Conservation Association Grants in Biodiversity; Natural Sciences and Engineering Research Council (NSERC) and Alberta Ingenuity (Alberta Innovates) student scholarships to M.D.S.; and by an NSERC Discovery grant to F.A.H.S.

References


Accepted 4 September 2015
First published online 30 October 2015