Graphical Abstract

Filippovia
(1+ species)

Notonykia
(2+ species)

Onychoteuthis
(12+ species)

Ancistroteuthis
(1+ species)

Moroteuthopsis
(3+ species)

Onykia
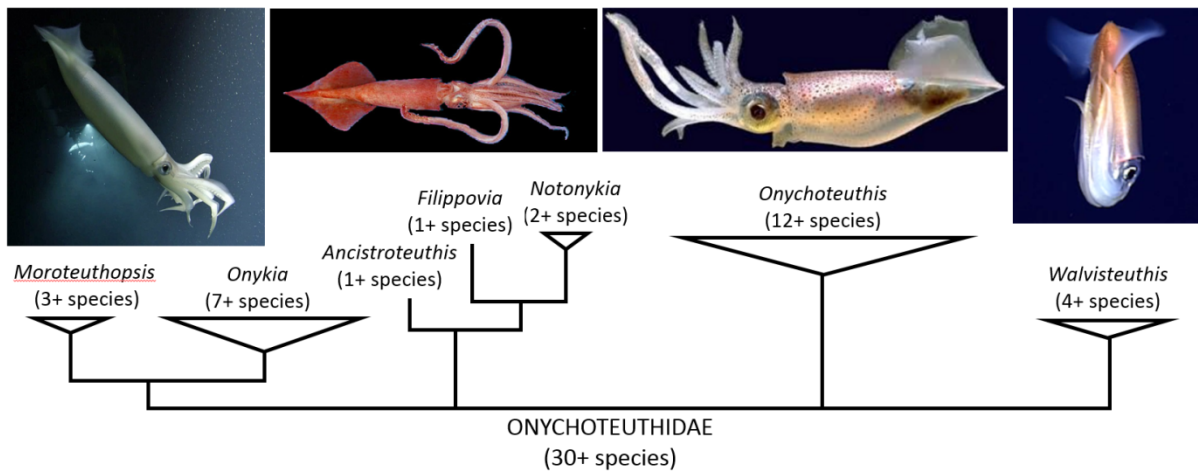(7+ species)

Walvisteuthis
(4+ species)

ONYCHOTEUTHIDAE
(30+ species)

Highlights

- Our results support monophyly of the family Onychoteuthidae and each of its seven genera
- Total onychoteuthid species diversity likely exceeds 34 species, making it the third-most diverse oegopsid (deep-sea squid) family
- 25% (n = 7) of the species recovered in the present study appear to represent undescribed taxa
- The genus name '*Kondakovia*' is recognized as a junior synonym of *Moroteuthopsis*, which presently contains three species including the abundant and ecologically important Southern Hemisphere species *M. ingens*

A mitochondrial phylogeny of the family Onychoteuthidae Gray, 1847 (Cephalopoda: Oegopsida)

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Abstract

The oegopsid squid family Onychoteuthidae was recently revised based on morphology, but sufficient material for a complementary molecular analysis has not been available until now. In the present study, over 250 sequences of cytochrome *c* oxidase subunit I (COI) and 16S rRNA for 222 individuals were analysed to create a combined phylogeny for the family. Results support monophyly for the family and all seven onychoteuthid genera (including *Moroteuthopsis*, established herein as the senior genus name for species formerly attributed to *Kondakovia*); 29 genetically distinct species were recovered, with the BIN analysis for COI showing good congruence overall with morphological species groupings. No sequences were available for five additional known species, making the total family diversity likely to exceed 34 species. Seven of the BINs formed in this study appear to represent undescribed taxa, suggesting that even in this relatively well-studied family, much additional work remains before a comprehensive understanding of the diversity and evolutionary relationships for Onychoteuthidae can be achieved.

Key words

Onychoteuthidae, hooked squids, phylogeny, DNA barcode, cephalopod

1. Introduction

Onychoteuthid ('hooked') squids are known from all oceans, apart from the Arctic (to date), and are characterised by the presence of two hook series on the tentacle clubs, while the arms possess only suckers (Bolstad 2010). Some taxa occur in great abundance, have high ecological importance, and are also well known from fisheries bycatch (e.g., *Onykia ingens* [Smith, 1881]) in southern temperate to sub-Antarctic waters); others remain known from just one or two preserved specimens (e.g., *Onychoteuthis mollis* [Appellöf, 1891, *sensu stricto*]; *Onykia aequatorialis* [Thiele, 1920]). A recent morphology-based review of the family (Bolstad 2010) raised the number of known species from the 12–14 accepted by most previous studies (e.g., Nesis 1987; Kubodera et al. 1998) to 25, making it one of the more diverse oegopsid families. Seven genera were recognised in the review, ranging from monotypic (*Ancistroteuthis* Gray, 1849; *Filippovia* Bolstad, 2010) to quite speciose (seven to ten species in *Onychoteuthis* Lichtenstein, 1818, and *Onykia* Lesueur, 1821) with the remaining genera each containing two or three known species ('*Callimachus*' [= *Walvisteuthis* Nesis and Nikitina, 1986; see Vecchione et al. 2015]; *Kondakovia* Filippova, 1972; *Notonykia* Nesis, Roeleveld and Nikitina,

1998). As most of the examined material had been fixed in formalin, a molecular component was not possible at the time. However, tissue samples from fresh material were collected where possible, in the hope of eventually undertaking a complementary genetic study.

Most of the existing molecular data for onychoteuthids have been gathered as components of wider phylogenetic analyses of cephalopods. Among these studies, there is little concurrence on the position of the Onychoteuthidae within Oegopsida, or on the family's nearest relatives. Carlini and Graves (1999) used mitochondrial cytochrome *c* oxidase subunit 1 [COI] to investigate relationships among Cephalopoda and found the Onychoteuthidae to consistently fall as sister to the Ommastrephidae Steenstrup, 1857, although this relationship was not well supported. Lindgren *et al*. (2004) used a combination of COI, histone H3, 18S rRNA, and 28S rRNA, and found that the Onychoteuthidae aligned most closely with Ancistrocheiridae Pfeffer, 1912, and were very distantly related to the Ommastrephidae. Strugnell (2004) found a well-supported (posterior probability = 1) sister-taxon relationship between the Onychoteuthidae and the Ommastrephidae in a topology resulting from Bayesian phylogenetic analyses from six genes (mitochondrial: COI, 12S rRNA, 16S rRNA; nuclear: octopine dehydrogenase, pax-6, and rhodopsin; topologies resulting from Bayesian analyses of the nuclear genes only did not resolve the phylogenetic relationship of the Onychoteuthidae to that of other oegopsid families). Lindgren (2010) used the same four genes as in her 2004 study, plus 16S rRNA, and included a greater diversity of taxa, and found the Onychoteuthidae sister to the Gonatidae Hoyle, 1886. Most recently, Lindgren *et al*. (2012) constructed a multi-gene cephalopod phylogeny in which onychoteuthids were represented by eight species, with data from four to ten genes each, and fell sister to two of the enoploteuthid families (Enoploteuthidae Pfeffer, 1900, and Pyroteuthidae Pfeffer, 1912).

Two molecular studies have also focused specifically on onychoteuthids. Bonnaud et al. (1998) used 16S rRNA to assess relationships among seven onychoteuthid species from four of the presently recognized genera, and found the family to be monophyletic, but noted that the genus '*Moroteuthis*' Verrill, 1882 (=*Onykia*) was not. Wakabayashi et al. (2007) used COI to investigate the identity of *Onykia* paralarvae in the North Pacific, concluding that they represented early life stages of *Onykia robusta* (Verrill, 1876), and providing additional support for the priority of *Onykia* over *Moroteuthis* (as had been suggested by Tsuchiya and Okutani in 1992). They included a total of nine onychoteuthid species, from three of the presently recognized genera. These studies both contributed valuable information to the body of systematic knowledge for the family, although they were each based on a single gene locus. Where possible, multiple genes should be used in combination to gain insight into relationships among squid taxa, especially at higher taxonomic levels—particularly where one of the loci is COI as multiple copies of this gene are known to occur in at least some oegopsids (see, e.g., Strugnell and Lindgren 2007).

A more comprehensive genetic survey of the family is due, especially in light of the recent (2010) morphological review by Bolstad. Sequences from over 200 specimens have been analysed; these include representatives of numerous taxa not previously included in any molecular analysis of the family, such as *Notonykia* and *Ancistroteuthis*, and many of the previously missing taxa from the other five genera. Herein, we use both COI and 16S rRNA to investigate relationships within the family, allowing inclusion and comparison of the data from Bonnaud et al. (1998) and Wakabayashi et al. (2007). Complementing the morphological findings from Bolstad (2010), we aim to provide further understanding of, and systematic stability to, this important family of deep-sea squids.

2. Methods

2.1 Material examined

Specimens were sourced from a variety of institutions (see Appendix 1), including the Geomar Helmholtz Centre for Ocean Research, Kiel, Germany; Monterey Bay Aquarium Research Institute (MBARI), Monterey, CA, USA; National Institute for Water & Atmospheric Research, Ltd (NIWA), Wellington, New Zealand; National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM); National Museum of Nature and Science (NSMT), Tokyo, Japan; and South African Institute for Aquatic Biodiversity (SAIAB), Grahamstown, South Africa. Following morphological examination (in most cases), tissue samples were either frozen, or fixed in 80–100% EtOH until extraction.

Previously published sequences for COI and 16S rRNA were sourced from the Barcode of Life Database (BOLD) and GenBank (Appendix 1). For these sequences, it was not possible to examine parent specimens, but in taxa where morphological identification is straightforward (e.g., in sub-Antarctic and Antarctic waters where few onychoteuthid species occur, and those present have distinctive features), names have been attributed with some confidence. In other cases, likely identifications have been suggested based on zoogeographical probability and the authors' prior knowledge of morphological patterns (see Figs 1 and 2 for known *Onychoteuthis* and *Onykia* species distributions); these attributions are given as (e.g.) *Onychoteuthis* cf. *bergii*. For unique taxa recovered by the molecular analyses that do not appear attributable to any known taxon, and are likely new to science, letters have been used in place of species names, which indicate the material's collector or sequencer (*Onychoteuthis* sp. 'AL4', A. Lischka; *Onychoteuthis* sp. 'W', T. Wakabayashi; *Walvisteuthis* sp. 'C', A. Choy). For clarity, the genera *Onychoteuthis* and *Onykia* are abbreviated *Os.* and *Ok.*, respectively.

2.2 DNA analysis

DNA was extracted following the protocols for the DNeasy Blood & Tissue Kit (QIAGEN), using EconoSpin (Epoch Life Science) columns, with QIAGEN reagents. For specimens that had been stored with formalin-fixed specimens in 80% ethanol at room temperature (some SAIAB material), extraction followed the protocol above, except that specimens were eluted in 100µl, and the eluate was passed through the column a second time.

Sequence data were obtained for two mitochondrial regions (COI and 16S rRNA). The DNA barcode region (648 bp from the 5' end of COI) was chosen because it is the standardized gene region for all metazoans (Hebert et al. 2013). In addition, more onychoteuthid sequences for COI were available from previous studies than from any other gene region. A secondary marker, 16S rRNA, was chosen because it has been previously used for species- and higher-level systematics in other oegopsid families (Braid et al. 2014; Braid et al. 2017) and is often used in dietary studies due to relative ease of amplification (e.g., Braley et al. 2010). Nuclear genes were not included in the present analysis because (1) few sequences were available for comparison from previous studies, and (2) due to the age of many specimens used in the present study, which were collected across a considerable time scale, the DNA produced was not of a high enough quantity or quality to allow the sequencing of nuclear genes.

COI was amplified using modified Folmer et al. (1994) primers from Braid et al. (2014) (LCO1490_CephF/ HCO2198_CephR). For specimens that were difficult to amplify (such as the specimens stored at room temperature in 80% EtOH), internal primers were used to amplify COI in two halves following Braid et al. (2017) (LCO1490_CephF/mCephR; mCephF/HCO2198_CephR). The 16S rRNA region was amplified with 16Sar (Simon et al., 1994) / 16Sb (Xiong and Kocher, 1991). Polymerase chain reaction (PCR) was carried out in 12.5 µl reaction volumes, consisting of 6.25 µl 10% trehalose, 2 µl ddH$_2$O, 1.25 µl 10X buffer, 0.625 µl MgCl$_2$ (50 mM), 0.1 µl forward primer (10

μm), 0.1 μl reverse primer (10 μm), 0.0625 μl 10 mM dNTPs, 0.06 μl Platinum Taq polymerase (5 U/μl), and 2 μl of DNA (~50 ng/μl). PCR products were visualised using 1% agarose gels stained with GenRed (Biotium). The reaction profile for COI was as follows: hot start of 94℃ for 1 min; 5 cycles of 94℃ for 40 s, 45℃ for 40 s, 72℃ for 1 min; 35 cycles of 94℃ for 40 s, 51℃ for 40 s, 72℃ for 1 min; extension at 72℃ for 5 min, hold 4℃ indefinitely. The reaction for 16S rRNA was as follows: hot start of 94℃ for 2 min; 35 cycles of 94℃ for 30 s, 52℃ for 40 s, 72℃ for 1 min; extension of 72℃ for 10 min, hold 4℃ indefinitely. PCR products were sequenced using the same primers that were used for amplification (Macrogen, Korea). Bidirectional sequences were assembled into contigs and edited, in Sequencher v.4.9 (Gene Codes). Sequences were submitted to GenBank (Appendix 1) and checked for contamination through the Basic Local Alignment Search Tool (BLAST) through GenBank. Sequences were uploaded to the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert, 2007) public project titled 'Molecular Phylogeny of the Onychoteuthidae' (project code: MPONY). All BOLD sequences used in the present study were compiled into a public dataset titled 'Genetic assessment of the squid family Onychoteuthidae' (dataset code: DS-ONYCHO17).

Edited contigs were subsequently checked for accuracy and aligned via the "Genious Alignment" algorithm with default parameters (65% similarity 5.0/-4.0) in Geneious R9.1 (Biomatters). As COI is a protein coding gene, the insertion of gaps via alignment is not necessary; however, the starting sequences did not always include the entire 658 bp region of COI, so an alignment was generated without gaps to ensure correct orientation of all sequences. Separate alignments were subsequently concatenated in Geneious and exported for subsequent phylogenetic analyses.

Individual and concatenated (COI+16S rRNA) datasets were analysed using RAxML v. 8 (Stamatakis, 2014) with 1000 rapid bootstrap pseudoreplicates, using the following options: -f a -x <random number seed for rapid bootstrapping; unique for each analysis> -p <random number seed for initial parsimony inferences; unique for each analysis> -# 500 -m GTRGAMMA -s <inputfile> -n <outputfile>. Bootstrap values of 50–59% were deemed very low, 60–69% were deemed low, 70–85% were deemed moderate, and those above 85% were deemed high.

The dataset was also analysed using the GTR+G model within a Bayesian framework using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) implemented within Geneious (Biomatters Ltd, version 7.1.6). Default Monte Carlo Markov Chain (MCMC) setting were used. Random starting trees were used. The analyses were run for 10,000,000 generations, sampling the Markov chain every 10,000 generations. To ensure analyses were not trapped in local optima each analysis was performed twice. Stationarity was considered to be reached when the average standard deviation of split frequencies was less than 0.01 (Ronquist and Huelsenbeck, 2003). In addition the posterior output of the analysis was visualised within Geneious 7.1.6 to check that stationarity had been reached and that the default burnin was adequate. Posterior probabilities of 0.95–0.98 were deemed moderate and those above 0.99 were deemed high.

Intergeneric, and intra- and interspecific distances for COI (Tables 1–3) and 16S rRNA (data not shown) were calculated using MEGA 6.06 (Tamura et al. 2013) using the Tamura–Nei (Tamura and Nei 1993) model with gamma correction. The distances in COI among taxa were used to calculate the 'barcode gap', which is defined as the smallest interspecific distance (Meier et al., 2008). Species boundaries were tested using the Barcode Index Number (BIN) system in BOLD (Ratnasingham and Hebert, 2013).

3. Results

The final dataset was comprised of sequence data, both new and previously published, for 222 specimens (Appendix 1), representing 29 onychoteuthid species, including (to our knowledge) 11 species sequenced herein for the first time: *Moroteuthopsis* aff. *ingens* (see Discussion regarding generic affiliation); *Notonykia nesisi* Bolstad, 2007; *Os. aequimanus* Gabb, 1868; *Onychoteuthis* cf. *banksii* (Leach, 1817, *sensu stricto*); *Os.* cf. *bergii* Lichtenstein, 1818; *Os.* aff. *compacta* (NZ); *Os. meridiopacifica* Rancurel and Okutani, 1990; *Os.* cf. *prolata* Bolstad, Vecchione and Young (in Bolstad, 2008); *Onykia* aff. *robsoni* (NENZ); *Walvisteuthis* cf. *rancureli* (Okutani, 1981); and *Walvisteuthis* sp. 'C'. No sequences/specimens could be located for *Os. horstkottei* Bolstad, 2010 and—although many parent specimens could not be examined for morphological confirmation—none of the sequences sourced online appear to have represented *Os. mollis*, *Ok. indica* Okutani, 1981, *Ok. aequatorialis*, or *W. virilis* Nesis and Nikitina, 1986. It therefore appears that the family contains at least 30, and possibly up to or exceeding 34 species. The maximum-likelihood (ML) tree strongly supported monophyly of the family Onychoteuthidae (bootstrap [BS] = 100]; the Bayesian tree strongly supported monophyly of *Walvisteuthis* (Posterior Probability [PP] = 1.00), and of a clade comprising all other onychoteuthid genera (PP = 0.99). A 'barcode gap' (Meier et al., 2008) was also observed (Table 2), with a maximum intraspecific distance of 2.6%, and a minimum interspecific distance of 3.8%. The BIN analysis recovered 28 clusters (which each appear to represent a species), while one additional species (*W.* cf. *youngorum*) could not be included in this analysis because it was represented only by 16S rRNA.

Within the family, seven monophyletic genera were identified, aligning well overall with the classifications from Bolstad (2010). Among these, *Onychoteuthis* and *Walvisteuthis* (the senior name for *Callimachus* Bolstad, 2010) each formed a monophyletic group (BS = 100; PP = 0.78 and BS = 100; PP = 0.99 respectively), well separated from other genera (minimum intergeneric divergences of 8.9–17.8% and 10.7–18.8%, respectively; see Table 2). The two *Notonykia* species formed a well-supported monophyletic group (BS = 100; PP = 1.00) and were also part of a larger monophyletic group containing the presently monotypic genera *Ancistroteuthis* and *Filippovia* (BS = 100; PP = 0.73). The lowest mean intergeneric divergence values were observed among this group (4.7–11.2%, see Table 2). *Onykia* and *Moroteuthopsis* Pfeffer, 1908 (see Discussion for nomenclatural considerations) also formed a monophyletic group with moderate support resulting from the Bayesian phylogenetic analysis (PP = 0.96).

The Bayesian and ML phylogenies appeared very similar in overall structure and in support values for various groupings: *Walvisteuthis* appears basal to all other genera, and within the remaining genera, *Onykia* and *Moroteuthopsis* form a monophyletic clade, in addition to the *Ancistroteuthis–Filippovia–Notonykia* clade discussed above. Divergence among congeners was variable (see Table 3), with species in some genera, such as *Moroteuthopsis* and *Walvisteuthis*, showing relatively little divergence (3.7–9.4% and 5.6–11.8%, respectively, for COI), while some pairs of congeners in the more speciose genera—*Onychoteuthis* and *Onykia*—were more than 20% divergent.

Within the *Onykia* + *Moroteuthopsis* group, several particularly interesting findings emerged. In *Onykia*, the known species *Ok. robusta* and *Ok. loennbergii* (Ishikawa and Wakiya, 1914) were recovered, as well as a group of four individuals we attribute to *Ok.* cf. *carriboea* Lesueur, 1821—three were small specimens from the Sargasso Sea (type locality for *Ok. carriboea*), with the fourth being a larger individual that was initially attributed to *Ok. robsoni* (Adam, 1962), by its collectors. In the New Zealand region, three morphologically similar, but genetically distinct *Onykia* species were also identified (*Ok.* cf. *robsoni*, *Ok.* aff. *robsoni* [large NZ], and *Ok.* aff. *robsoni* [NENZ]); based on shared morphological characters, individuals of these taxa were previously attributed simply to *Ok. robsoni* (Bolstad 2010).

The species previously known as 'Onykia' *ingens*, however, did not group with the other *Onykia* species. Together with its sister species (aff. *ingens*), *ingens* formed a moderately to well-supported monophyletic group with '*Kondakovia*' *longimana* Filippova, 1972 (BS = 72; PP = 1.00). These three species are therefore considered congeners, under the name *Moroteuthopsis* Pfeffer, 1900 (see Discussion). Individuals identified as '*Kondakovia nigmatullini*' Laptikhovsky, Arkhipkin and Bolstad, 2008, were not genetically distinct from individuals of *K. longimana*.

The closest relationships observed within each genus were usually well supported, but sometimes spanned widely disparate geographic regions. These included the following: *Onychoteuthis aequimanus* and *Os*. cf. *compacta* (New Zealand), and *Os*. cf. *bergii* (Indian Ocean) (BS = 100; PP = 1.00); *Os*. cf. *prolata* (North Pacific + Atlantic) and *Os. compacta* (Pacific) (BS = 100; PP = 1.00); *Notonykia africanae* Nesis, Roeleveld and Nikitina, 1998 (widespread Southern Ocean) and *N. nesisi* (New Zealand) (BS = 100; PP = 1.00); *Onykia robusta* (North Pacific) and *Ok*. cf. *robsoni* 'small NZ' (New Zealand) (BS = 92; PP = 1.00); and *Moroteuthopsis ingens* (widespread Southern Ocean) and *M.* aff. *ingens* (New Zealand) (BS = 96, PP = 1.00). However, several known species in both *Onychoteuthis* and *Onykia* were not available for inclusion in this study, so apparent species relationships within these genera (and within the wider family) could shift if/when these, or other additional, onychoteuthid taxa become available for comparison.

4. Discussion

This study represents the most complete molecular analysis of an oegopsid family to date, with at least 85% of known species included. The morphological study by Bolstad (2010) and the results of the present molecular study together allow us to present an integrated view of the alpha taxonomy of the Onychoteuthidae. The results of these studies are largely complimentary, although the genetic data suggest the presence of several additional taxa (some morphologically cryptic), and the need for taxonomic reorganisation of at least two genera: '*Kondakovia*'/*Moroteuthopsis* and *Onykia*. Specific remarks on each genus follow.

The distances calculated among onychoteuthid taxa (Tables 1–3) were comparable to what little is known for other oegopsid families to date. Within the DNA barcode region, Braid et al. (2014) reported intra-species evolutionary distances of 0–*0.2*–1.0% (min–*mean*–max) among eight species in the Mastigoteuthidae, and in an ongoing global revision of the Octopoteuthidae (J Kelly, pers. comm.) intra-specific distances of 0–*0.2*–0.4% have been observed. These values are comparable overall to the 0–*0.23*–2.6% found herein. The much higher maximum divergence found in onychoteuthids was due to a single species (*Onychoteuthis compacta* (Berry, 1913), where a single specimen [OnychoNSMT264/MPONY049-17] was quite divergent from the others, while still grouping together in the BIN analysis, indicating that it likely represents variation within a single species). The next highest maximum intraspecific divergence was 1.3% in *Onykia robusta*, which is similar to that found for the Mastigoteuthidae. Within the combined phylogeny, superficially higher intraspecific divergences are also observed in taxa where some individuals were only represented by a single locus (either COI or 16S rRNA) rather than both (e.g., *F. knipovitchi* [Filippova, 1972], *Os. compacta*, and *Os. prolata*).

Within the Onychoteuthidae, interspecific distances in the barcode region among any pair of species ranged from 3.8% to 32.9% (mean 20.0%), which is also comparable to the other two families (Mastigoteuthidae 10.5–*25.6*–35.3%, Braid et al. 2014; Octopoteuthidae 3.0–*16.9*–25.1%, J Kelly, pers. comm.), although the higher minimum interspecific value observed in the mastigoteuthids could suggest that this family is older, and has been diverging for a longer period of time, than either the onychoteuthids or the octopoteuthids. The observed mean intergeneric differences in these

three families could also support this conjecture, being greater in the Mastigoteuthidae (18.9–34.1%, Braid et al. 2014) than in either the Onychoteuthidae (4.7–18.8%) or the Octopoteuthidae (12.3–18.6%, J Kelly, pers. comm.).

4.1 Ancistroteuthis, Filippovia, Notonykia

The three least speciose genera formed a monophyletic group: *Ancistroteuthis* (one known species, although no material known to physically match *Ancistroteuthis lichtensteinii* (Férussac, 1835) 'Type B', *sensu* Vecchione et al. 2010, was available), *Filippovia* (one known species), and *Notonykia* (two known species). The lowest minimum intergeneric distances within the family were observed among these four species (4.7–10.8%; Table 2). These genera share certain morphological characteristics (see Fig. 3), including muscular tissues (as opposed to ammoniacal, seen in *Moroteuthopsis* and *Onykia*), smooth skin on the mantle, large ventral tentacular hooks with asymmetrical bases, and the absence of photophores. However, other substantial morphological differences observed among them (Fig. 3; particularly in their hard parts, such as the gladius—see Bolstad, 2010) support their current taxonomic status as separate genera. Herein, this clade received similar support levels (BS = 100; PP = 0.73) to those for some genus-level clades (*Moroteuthopsis*, *Onychoteuthis*), suggesting that these taxa are closely related and could be united into a single genus (under the senior name *Ancistroteuthis*). Yet the level of morphological variation among these taxa makes such a grouping counterintuitive. Genetic and morphological data in combination make a powerful taxonomic tool, but may also yield conflicting results, as in this case. Following much deliberation, we presently follow the more conservative route of retaining the separate genera, with the knowledge that additional work is needed in this group. For example, inclusion of *Ancistroteuthis lichtensteinii* 'Type B' sequences in future analyses could help clarify these relationships. The recognition of additional characters (not necessarily morphological) could also provide additional insight. If the present tree does accurately reflect the positions of the known taxa within the clade, they may each have evolved from some common wide-ranging or Atlantic temperate ancestor, with ongoing evolution in the northern Atlantic leading to the Mediterranean/Eastern Atlantic *Ancistroteuthis*, and evolution in more southern regions giving rise to the sub-Antarctic *Filippovia* and southern temperate *Notonykia*.

4.2 Onychoteuthis

Onychoteuthis appears to be the most speciose genus in the family. Bolstad (2010) reported ten species, eight of which were included here (with five sequenced for the first time) and at least one of which remains unavailable for sequencing to date (*Os. horstkottei*). The combined phylogeny shows 12 distinct groupings of sequences, which were each supported by the BIN analysis. Because BINs have a high observed congruence with species (Ratnasingham and Hebert, 2013; Braid et al., 2014; Braid et al., 2017), it appears that the total number of species worldwide is likely to be at least 13—probably higher, as many *Onychoteuthis* species presently appear to have limited geographic ranges, and some areas remain poorly sampled (see Fig. 1).

The present analyses reveal several sister relationships within *Onychoteuthis*. One group includes two species from the New Zealand region (aff. *compacta* [NZ] and *aequimanus*) and one from the Indian Ocean (cf. *bergii*), with very high Bayesian and ML support (Fig. 3). Another strongly supported group comprises the Pacific *Os. compacta* and the more cosmopolitan *Os.* cf. *prolata*. The latter species was sequenced mostly from the Atlantic, with one individual from the North Pacific (AY616980.1) that was 1.7–1.9% divergent from the Atlantic individuals, for 16S rRNA (COI was not available). This divergence may indicate population-level variation, or the existence of two different species; resolving the status and distribution of *Os. prolata* will require a morphological examination of individuals from both locations, along with additional COI sequences from the North Pacific

individuals. A third (rather poorly supported) clade included the North Pacific sister taxa *Os. borealijaponica* Okada, 1927, and *Os. lacrima* Bolstad and Seki (in Bolstad, 2008), which are the only two *Onychoteuthis* species with tear-drop-shaped posterior visceral photophores (as opposed to circular).

The remaining five *Onychoteuthis* species form a clade with high Bayesian support (but low maximum-likelihood support) with the previously mentioned NZ–Indian *Onychoteuthis* group, but no obvious geographic pattern is apparent: three Atlantic species (*Os.* cf. *banksii*, *Os.* aff. *compacta*, *Os.* sp. 'AL4'), the southwestern Pacific and Indian *Os. meridiopacifica*, and one quite unique Pacific individual (*Os.* sp. 'W', BOLD:ADH0337/SSONY054-16), which is ~11% divergent from its apparently closest (Atlantic) congener. Although specimens representing most of these species were examined, morphological comparisons are difficult because the majority of sequences came from (1) juvenile specimens, (2) poorly conserved specimens lacking salient morphological features, or in a few cases (3) other studies where the parent specimen could not be examined. As a result, several of the species IDs are, somewhat riskily, suggested herein (indicated with 'cf.') according to our current understanding of the known *Onychoteuthis* species' geographic ranges (see Fig. 1).

One interesting insight provided by the molecular results is the apparently independent evolution, on several occasions, of the distinctive morphological features previously believed to characterise *Os. compacta*. Previously, in addition to *Os. compacta* material from the type locality (Hawaii), a substantial number of Atlantic specimens were examined and, according to morphology (hook shape, body proportions, chromatophore patterns) were ultimately also attributed—with some trepidation—to *Os. compacta* (Bolstad 2010, pp. 36–40). Now it appears that some of these 'specific' characters can be found in multiple taxa. For example, chromatophores are absent from a large distal portion of the ventral mantle surface, creating a 'bare patch' (see Bolstad 2010, fig. 16C), in north Pacific *Os. compacta*, New Zealand *Os.* aff. *compacta*, and Atlantic *Os.* aff. *compacta*, which are not only genetically distinct species, but also fall into different clades within *Onychoteuthis*. This is an excellent example of the value of integrative taxonomy, with molecular information providing additional insight into relationships that were simply not apparent based on morphology alone. However, caution is also needed when additional taxa are known to exist within a group but are not available for inclusion, since their later addition can alter the positions of related taxa within a tree. Should sequences become available for the known *Onychoteuthis* species not available for sequencing in this study (*horstkottei* and potentially *mollis*), and/or other undescribed species, positions and apparent affiliations of these taxa may well change.

4.3 Onykia

Six species of *Onykia* were monophyletic in ML and Bayesian topologies, not counting *Moroteuthopsis* (formerly *Onykia*) *ingens* and its sister species, *M.* aff. *ingens*. In addition to the known species *Ok. robusta* and *Ok. loennbergii*, the BIN analysis revealed four genetically distinct species all initially identified as *Ok.* '*robsoni*' based on their morphology: one from the western central Atlantic (Sargasso Sea and Gulf of Mexico), which we attribute to *Ok.* cf. *carriboea*, and three from New Zealand (Fig. 2). Of these, *Ok.* aff. *robsoni* (large NZ)—which is an undescribed New Zealand species known in local fisheries codes as 'Big MRQ'—appears basal to all other species (with high support), and quite divergent, with a difference of about 15% to the next closest congener. This species possesses the identifying characters previously believed unique to *Onykia robsoni*—sagittate fins, a long gladius rostrum (25–30% total gladius length), and round, well-separated, blister-like warts on the mantle. These features are shared by *Ok.* aff. *robsoni* (small NZ), and as a result these taxa have been considered a single species (*Ok. robsoni*) in most previous publications (e.g,. Bolstad 2007, 2010), although *Onykia* aff. *robsoni* [large NZ] was sequenced as '*M. robusta*' by Bonnaud et

al. (1998), and was included here (sequence AJ223489.) The third '*robsoni*'-like species, which we herein attribute to *Ok*. aff. *robsoni* (NENZ), was represented by a single individual (NIWA 95573/BOLD:ADH2128/MPONY059-17); it also shares these morphological characters and—to our knowledge—has not previously been encountered (or recognized), having been collected in north-eastern New Zealand, a region that remains quite poorly represented in local collections. It is clear that further examinations of *Onykia* '*robsoni*' material (large and small) from New Zealand, and elsewhere, are needed, and that at least one of these taxa (probably two, and possibly even three) may be recognised as new species as a result.

Further insight into these four different '*robsoni*' species, and their relationship with *Onykia carriboea* (the type species of the genus, originally described from the Sargasso Sea), could be gained by examining and sequencing ontogenetic series of small *Onykia* individuals from the regions in question. *Onykia carriboea* was recently reported to have a circumglobal southern temperate range (Bolstad 2010). However, with at least four morphologically similar species (at adulthood) now recognised from different regions, it is likely that small '*Ok. carriboea*' specimens from different regions will eventually be linked to adults of some of these '*robsoni*' species. This would necessitate a careful review of the names, specific characters, and zoogeography of these taxa. Some possible outcomes could include: (1) *Onykia carriboea* (*sensu stricto*) being present in the Gulf of Mexico (GoM), Sargasso Sea, and southward in the Atlantic, eventually proving a senior synonym for *Ok. aequatorialis* (presently known only from spent individuals from the GoM/equatorial Atlantic), or for *Ok. robsoni* (*sensu stricto*, type locality Angola), leaving all three New Zealand species as presently undescribed taxa; or (2) *Ok. carriboea* being restricted to the equatorial Atlantic, with *Ok. robsoni sensu stricto* distributed throughout notalian temperate waters including the south Atlantic and South Pacific (including New Zealand, probably our *Ok*. cf. *robsoni* [small NZ]), leaving *Ok*. aff. *robsoni* (large NZ) and *Ok*. aff. *robsoni* (NENZ) as undescribed taxa.

4.4 '*Kondakovia*'/*Moroteuthopsis*

A monophyletic genus comprising '*Kondakovia*' *longimana*, '*Onykia*' *ingens*, and its undescribed sister species '*Onykia*' aff. *ingens*, is recognized herein for the first time. '*Onykia*' *ingens* is the type species for Pfeffer's (1900) genus *Moroteuthopsis* (most recently considered a subgenus within *Onykia*, acknowledging morphological differences between '*Onykia*' *ingens* and all other *Onykia* species, which were at that time attributed to *Moroteuthis*—see Bolstad 2010). Thus *Moroteuthopsis* takes priority as the genus name (over *Kondakovia* Filippova, 1972) for the species group comprising the species *ingens*, aff. *ingens* and *longimana*, and will be used throughout the remainder of this study. *Moroteuthopsis* shares many character states with *Onykia* (which retains all species attributed to the subgenus *Onykia* [*Onykia*] in Bolstad 2010), including warty or longitudinally ridged skin, large body size, ammoniacal tissues, and the absence of photophores (see Fig. 3), but can be separated as follows. *Onykia* species possess a Y-shaped ridge in the funnel, and symmetrical grooves on the claw portion of the tentacular hooks (see Bolstad 2010, p. 62), both of which are absent in *Moroteuthopsis*. For all species where adult gladius morphology is known, the proportion of gladius length comprised by the rostrum is also longer in *Onykia* (≥20%) than in *Moroteuthopsis* (10–15%). Some morphological similarities previously noted among *M. ingens* and *M. longimana*, including sexual dimorphism in the beaks (Bolstad 2010), which may indicate similar head-to-head mating strategies (see Bolstad 2006), may also prove consistent within the genus *Moroteuthopsis*, but cannot be confirmed until more (and larger) specimens of *M*. aff. *ingens* become available.

The '*Kondakovia nigmatullini*' sequences included in this study were largely taken from specimens identified by this species' primary author (V Laptikhovsky) and are thus considered to be good

representatives of the *K. nigmatullini* morphotype. Since the maximum observed difference in COI among the *M. longimana* and '*K. nigmatullini*' sequences obtained in this study was 0.3%, and the maximum divergence found within *M. longimana* samples was 0.6%, and all individuals of these two species were assigned the same BIN, molecular data do not support the status of '*K. nigmatullini*' as a distinct species from *M. longimana*. The morphological differences that are consistent among specimens attributed to '*K. nigmatullini*,' and its apparent prevalence in sub-Antarctic waters (compared with true Antarctic specimens of *M. longimana*), could indicate ongoing modification which has not (yet) resulted in noticeable reproductive isolation of these groups, as has been reported in *Uroteuthis edulis* (Hoyle, 1885) (Takemoto & Yamashita, 2012) and *Alloteuthis subulata* Lamarck, 1878 (? = *A. media* [Linnaeus, 1858]; see Gebhart & Knebelsberger, 2015).

The single known specimen of *M.* aff. *ingens*, NIWA 96264 (BOLD:ADG8616/MPONY044-17), was a small individual (ML ~50mm) initially attributed to *M. ingens* but with some morphological differences noted—shorter, broader fins than are generally found in *M. ingens*, and with Arms IV shorter and more slender than the remaining arms, in contrast to *M. ingens*, where Arms I are noticeably shortest. This specimen was collected on New Zealand's Chatham Rise and was identified as a single unique individual among 50-odd small specimens of *M. ingens* (*sensu stricto*); thus it appears sympatric, and a review of *M.* '*ingens*' material from the region may reveal additional specimens in collections, and permit reliable morphological differentiation of these species, and formal description of *M.* aff. *ingens*.

4.5 Walvisteuthis

Four species were recovered within *Walvisteuthis*: *W.* cf. *rancureli* (Indian Ocean; condition of material did not permit detailed analysis of morphological features), and a group of three more closely related species, comprising the Hawaiian *W.* cf. *youngorum* Bolstad, 2010, and W. sp. 'C' (a single ex-stomach-content specimen, BOLD:ADH2906/MPONY082-17), and *W. jeremiahi* (from the Gulf of Mexico and tropical Atlantic). The original description of *W. virilis* (Nesis, Roeleveld and Nikitina, 1986) reported the holotype (a mature male) to have pointed teeth around the inner circumference of the sucker rings as well as markedly enlarged suckers on the distal portions of Arms III; to date, no confirmed individual of *W. virilis* with these features has been available for examination by the present authors, nor has any individual of other *Walvisteuthis* species examined by us possessed these features. It remains unknown whether these are specific characters of *W. virilis*, and if so, whether they develop only at maturity, and possibly only in males. No specimen with the distinctive features of *W. virilis* was available for inclusion in this study, nor was any material from the type locality (Walvis Ridge, west of southern Africa) available. Two genetically distinct species with potentially adjacent distributions to *W. virilis* were included, however, and it is possible that one of these may eventually prove synonymous with *W. virilis*. If the distribution of *W. rancureli* (type locality: Indian Ocean) should prove widespread throughout sub-tropical and temperate waters of the Southern Hemisphere, then *W. rancureli* could take priority over *W. virilis*. Alternatively, if the range of *W. virilis* should prove to extend northward into the tropical Atlantic, Sargasso Sea, and Gulf of Mexico, then *W. virilis* could prove senior to *W. jeremiahi*. Additional morphological and molecular work is certainly needed on members of this genus, throughout its many ontogenetic stages and from as many geographic locations as possible.

In both the Bayesian and maximum-likelihood analyses, *Walvisteuthis* was recovered as a sister taxon to the other onychoteuthid genera. Members of this genus are morphologically anomalous within the family (e.g., fin and gladius shape, arm sucker size and dentition; see detailed discussion of '*Callimachus*' in Bolstad 2010); recent footage of *Walvisteuthis* cf. *youngorum in situ* (National Oceanic and Atmospheric Administration, 2015) shows a vertical body posture, utilizing the terminal,

ovate fins in a vigorous flapping motion (also unique among the—admittedly limited—live observations of other onychoteuthids to date). Similar positions/gaits have been observed in some other decapodiforms that share the relatively small, stout mantle shape and rounded, terminal fins, including members of the Histioteuthidae (KB, pers. obs., and see Thomas et al. 2017), Bathyteuthidae (Bush et al. 2012), and *Spirula spirula*. Vertical positioning could be a neotenous and/or primitive trait; certainly, many ancestral cephalopods maintained an obligate head-down posture, necessitated by their shells. Interestingly, the short phylogenetic branch lengths observed within *Walvisteuthis* indicate that its species have diverged less than the species within many other genera: the maximum interspecific distance observed for COI within *Walvisteuthis* was 11.8%, only slightly higher than that minimum interspecific distance observed in some other genera (e.g., *Onykia*, see Table 3). On the combined maximum likelihood phylogeny, some *Walvisteuthis* species appear to have diverged as little as 3%. However, pairwise distances for *W. youngorum* may be artificially low (and its branch artificially short) since it was represented by a single 16S rRNA sequence; COI was not available for this individual.

4.6 Zoogeography of the Onychoteuthidae

Onychoteuthid genera demonstrate a variety of distribution patterns ranging from very localized (e.g., *Ancistroteuthis*, known only from the Mediterranean and nearby eastern Atlantic [e.g., *Ancistroteuthis* 'type B' from 21°S]; *Filippovia*, restricted to the sub-Antarctic) to cosmopolitan in tropical to temperate waters (*Onychoteuthis* and *Walvisteuthis*). Within widespread genera, some species also appear wide-ranging, such as *Os. prolata*, while others presently appear localized, such as *Os. lacrima* (western North Pacific, Hawaii to Japan) and *Os. horstkottei* (eastern central Pacific, Central America to California). The highest diversity is presently known from the Pacific Ocean (at least nine species around Hawaii and at least ten around New Zealand) and the lowest (apart from Arctic waters, where no onychoteuthids have been reported) in the sub-Antarctic to Antarctic (only *Filippovia knipovitchi* and *Moroteuthopsis longimana*).

The zoogeography of the hypothetical *Ancistroteuthis/Filippovia/Notonykia* clade is particularly interesting: *Ancistroteuthis lichtensteinii* (one of the most localized onychoteuthid taxa) appears not only restricted to waters far from the other two genera, but also appears more closely related to the sub-Antarctic *Filippovia* than to *Notonykia*, which occupies circumglobal southern notalian waters (i.e., a wide band separating the known distributions of *Filippovia* and *Ancistroteuthis*). Disjunct distributions of closely related genera, particularly with a Northern–Southern Hemisphere split, have also been observed in other families (e.g., *Gonatopsis* Sasaki, 1920, and *Gonatus* Gray, 1849, in the Gonatidae). Of course, many onychoteuthid species distributions remain poorly understood, and physical absence from a region cannot be concluded from the absence of local records.

5. Conclusions

This study represents the most complete molecular analysis of an oegopsid family to date, with at least 85% of known species included (29 in total, including 11 sequenced for the first time). The present results have improved our knowledge of the family Onychoteuthidae substantially, clarifying the relationships among many taxa and providing additional information on a number of recently described species. A truly integrative taxonomic approach to alpha taxonomy involving simultaneous and coordinated studies of molecular and morphological characters (rather than sequential studies as done here) would save considerable effort in recognizing species differences and in obtaining specimens for study (see Braid et al. 2014, 2017; Braid and Bolstad 2015). While combining molecular and morphological approaches is not always practical, the clear value of this approach indicates that the obstacles need to be overcome.

Following this study of the Onychoteuthidae, much still remains to be resolved. Some species whose taxonomy remains uncertain (e.g., *Walvisteuthis virilis*) were not available for inclusion; a number of genetically distinct taxa included in this study could not be confidently attributed to known species, as parent specimens were not available for morphological examination; and a number of additional (sometimes sympatric) taxa await description, in order to resolve species complexes, such as *Onychoteuthis compacta* and *Onykia* 'robsoni'. Wherever possible, tissue samples should be collected and parent specimens saved (and preserved with care, especially those in good condition), especially from the regions where new or (or yet unresolved) taxa are thought to exist, including in particular the Indian Ocean, New Zealand, and the southern Atlantic.

6. Acknowledgments

7. References [citations solely for taxon authorities are in blue]

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Table 1. Pairwise intra- and inter-specific (percent) evolutionary distances for cytochrome *c* oxidase subunit I (COI) across 29 species of the Onychoteuthidae.

	Percent divergence		
	Min	Mean	Max
Intraspecific	0	0.24	2.60
Interspecific	3.80	17.39	32.94

Table 2. Minimum intergeneric (percent) pairwise divergences for cytochrome *c* oxidase subunit I (COI) across seven genera of the Onychoteuthidae.

	Ancistroteuthis	*Filippovia*	*Moroteuthopsis*	*Notonykia*	*Onychoteuthis*	*Onykia*
Filippovia	10.8	–	–	–	–	–
Moroteuthopsis	12.9	11.2	–	–	–	–
Notonykia	6.8	4.7	8.0	–	–	–
Onychoteuthis	15.9	16.0	14.4	8.9	–	–
Onykia	15.6	18.4	13.1	10.8	17.8	–
Walvisteuthis	18.4	18.8	14.8	10.7	17.6	15.9

Table 3. Inter-specific (percent) distances for cytochrome *c* oxidase subunit I (COI) within each of the five non-monospecific genera in the Onychoteuthidae.

	Percent divergence		
	Min	Mean	Max
Moroteuthopsis	3.76	7.91	9.43
Notonykia	-	8.54	-
Onychoteuthis	7.47	15.20	21.31
Onykia	10.57	16.24	21.18
Walvisteuthis	5.55	8.50	11.77

Figure captions

Figure 1. Known distributions of named species in the genus *Onychoteuthis* (asterisk indicates type locality), and locations of provisionally identified material sequenced in this study (symbols). Shading indicates regions with little material known to date. Species codes: aeq = *aequimanus*, ban = *banksii*, ber = *bergii*, bor = *borealijaponica*, com = *compacta*, hor = *horstkottei*, lac = *lacrima*, mer = *meridiopacifica*, mol = *mollis*, pro = *prolata*.

Figure 2. Known distributions of named species in the genera *Moroteuthopsis* and *Onykia* (asterisk indicates type locality), and locations of provisionally identified material sequenced in this study (symbols). Shading indicates regions with little material known to date. Species codes: aeq = *aequatorialis*, car = *carriboea*, ing = *ingens*, loe = *loennbergii*, lon = *longimana*, rba = *robusta*, rbi = *robsoni*.

Figure 3. Combined phylogeny of all onychoteuthid specimens sequenced for this study and from previously published sequences for COI and 16S rRNA (see Appendix 1). Upper hemisphere at nodes indicates Bayesian posterior probabilities (blue > 0.95, red ≤ 0.95); lower hemisphere indicates bootstrap support from the maximum-likelihood analysis, based on 1000 bootstrap replicates (blue > 70; red ≤ 70). Nodes with bootstrap support below 50% have been collapsed. Barcode Index Numbers (BINs) for COI are indicated. Clades are identified to the highest taxonomic level. Morphological character states are represented by symbols, which are defined in the legend; grey symbols indicate variable presence of a character within the group.

Appendix caption

Appendix 1. Source material for molecular phylogenetic analysis of the Onychoteuthidae, using cytochrome *c* oxidase subunit I (COI) and 16S rRNA. *[see separate Excel file]*
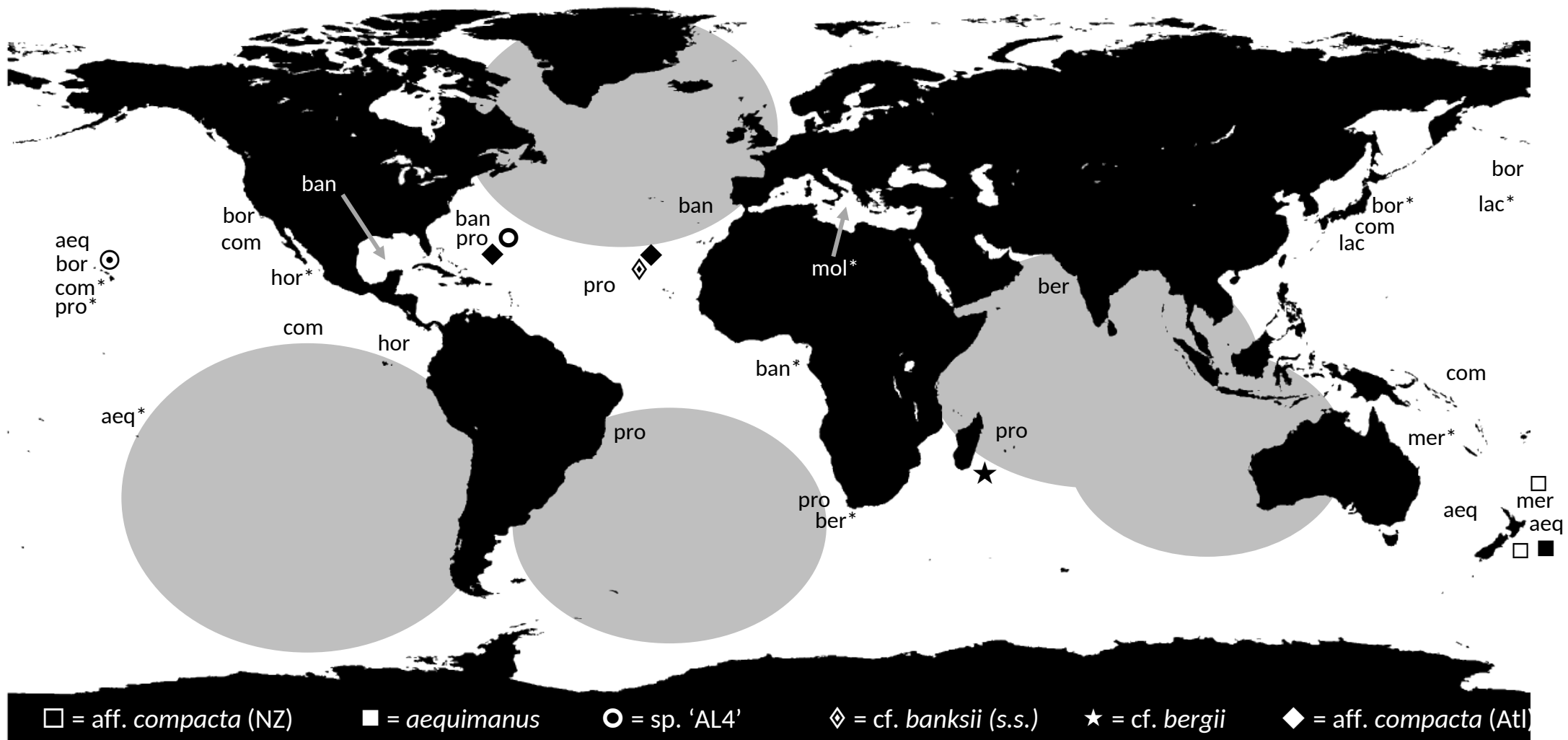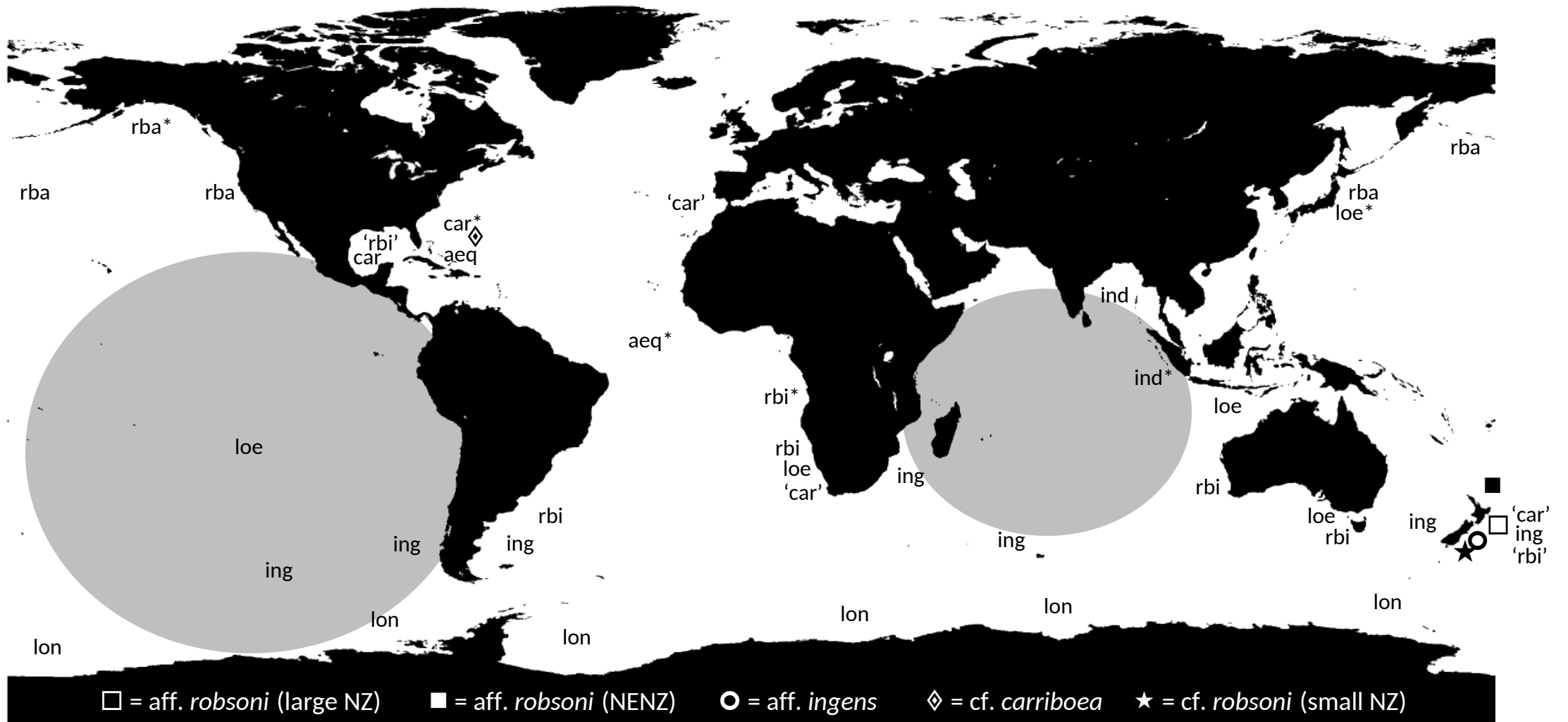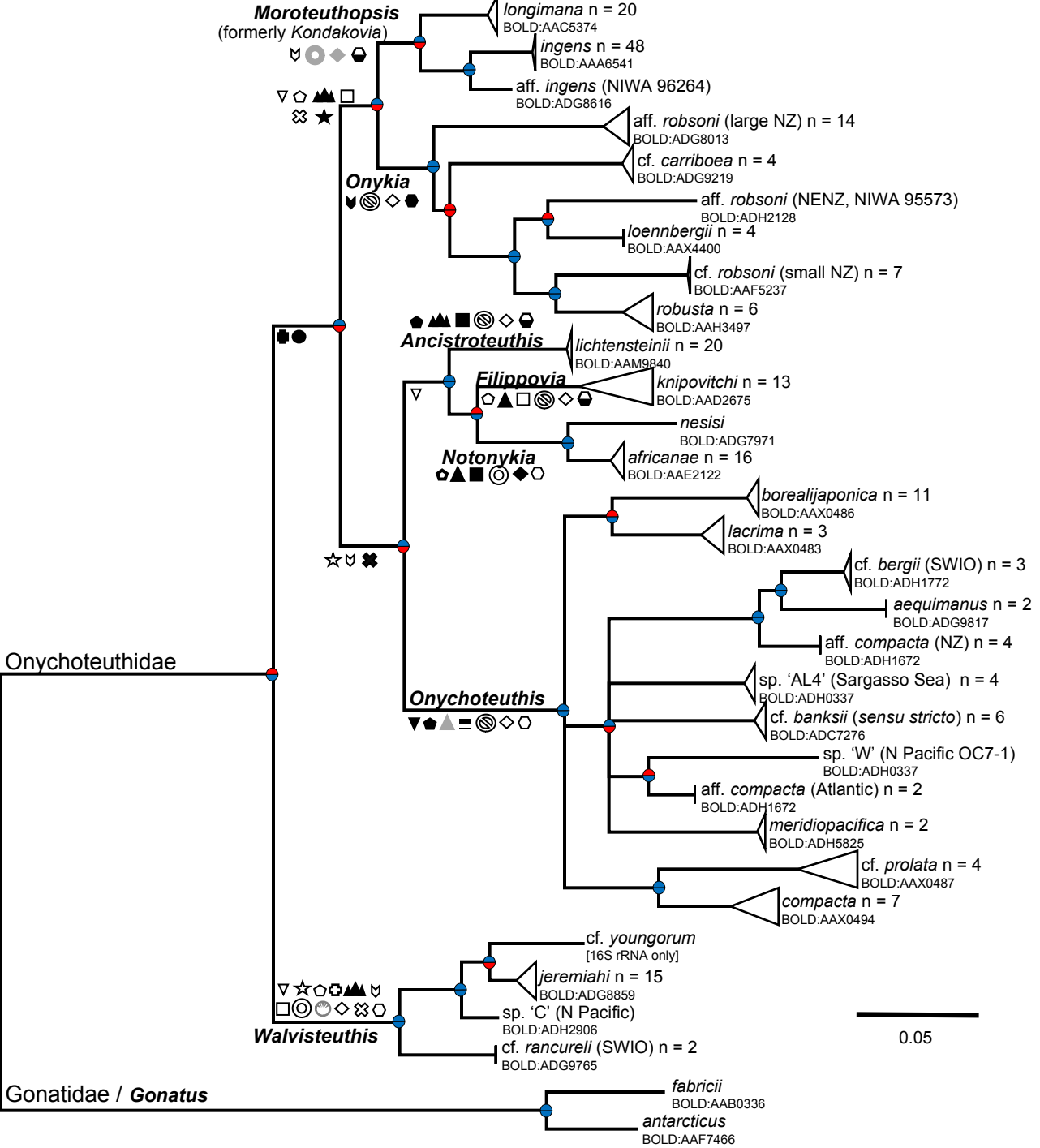
Figure 1

Legend:
□ = aff. *compacta* (NZ) ■ = *aequimanus* ⊙ = sp. 'AL4' ◊ = cf. *banksii (s.s.)* ★ = cf. *bergii* ◆ = aff. *compacta* (Atl)

Figure 2

Legend: □ = aff. *robsoni* (large NZ)   ■ = aff. *robsoni* (NENZ)   ○ = aff. *ingens*   ◇ = cf. *carriboea*   ★ = cf. *robsoni* (small NZ)

Figure 3