

Evolutionary relationships among squids of the family Gonatidae (Mollusca: Cephalopoda) inferred from three mitochondrial loci

A.R. Lindgren^{a,1}, O.N. Katugin^b, E. Amezcuita^a, M.K. Nishiguchi^{a,*}

^a Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001, USA

^b Pacific Research Fisheries Centre (TINRO-Centre), Vladivostok, Russia

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Abstract

The oceanic squid family Gonatidae (Mollusca: Cephalopoda) is widely distributed in subpolar and temperate waters, exhibiting behavioral and physiological specializations associated with reproduction. Females of several species undergo muscular degeneration upon maturation; origins of this complex morphogenic change are unknown, hindering our understanding of ecological and morpho-physiological adaptations within the family. To provide further information regarding the evolutionary relationships within Gonatidae, three mitochondrial loci (12S rRNA, 16S rRNA, and cytochrome *c* oxidase subunit I) were analyzed for 39 individuals representing fourteen gonatid and six outgroup cephalopod species. In addition to elucidating relationships among gonatids, molecular data provided more information than morphological data for problematic specimens. Although some data sets are incongruent or have low nodal support values, combined molecular analysis confirms the presence of gonatid groups previously established by morphological characteristics (i.e., possessing radular teeth in seven longitudinal rows and muscular mantle tissue). These characteristics are basal to taxa possessing radular teeth in five longitudinal rows and less muscular mantle tissue, indicating that the derived forms are those species exhibiting physiological adaptation such as tissue degeneration upon maturation and egg brooding.

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1. Introduction

Gonatidae (Mollusca: Cephalopoda) is a family of oegopsid oceanic squid characterized by quadriserial armature on the arms, with most species possessing hooks on arms I–III (in two central rows) and a rhomboidal or heart-shaped fin (Nesis, 1973, 1987). The family is composed of 17–19 species (Nesis, 1982, 1985; Sweeney and Roper, 1998) with three recognized genera: *Gonatus*, *Gonatopsis*, and *Berryteuthis*, which are distinguished

from each other by tentacle and radular morphology (Nesis, 1973). *Gonatus* possesses complex tentacle fixing apparatus, and a radula consisting of five longitudinal rows of teeth. *Gonatopsis* is distinguished by the absence of tentacles in adults, and presence of either seven or five longitudinal rows of teeth in the radula. Members of *Berryteuthis* have no hooks on the tentacle club, weakly differentiated fixing apparatus on tentacles, and a radula with seven longitudinal rows of teeth. Nesis (1971, 1973) suggested that the ancestral gonatid was an oceanic shallow water squid, with a powerful and muscular mantle, rhomboid or oval fin, radula with seven rows of longitudinal teeth, arms and tentacles equipped with only suckers, and a poorly developed tentacular fixing apparatus. Thus, more derived forms were species that migrated to deeper waters, exhibiting less muscular mantle and arm

* Corresponding author. Fax: +1 505 646 5665.

E-mail address: nish@nmsu.edu (M.K. Nishiguchi).

¹ Present address: The Ohio State University, Department of Evolution, Ecology and Organismal Biology, 318 West 12th Avenue, Columbus, OH 43210, USA.

crown tissues, and possessing five longitudinal rows of radular teeth.

Several species of gonatid exhibit a characteristic previously identified only with octopods: post-spawning egg care or brooding, where the female cares for the eggs until hatching (Hunt and Seibel, 2000; Katugin, 2003; Okutani et al., 1995; Seibel et al., 2000). All other extant decabrachian cephalopods freely spawn with or without egg-masses, or lay their eggs on substratum, but none are known to exhibit parental care. During brooding, gonatid females do not feed; instead, they utilize the digestive gland weight and lipid energy conserved during maturation. Gonatid females known to brood also exhibit morphological change upon maturation, which is believed to be related to post-spawning egg-care (e.g., Hunt and Seibel, 2000; Katugin, 2003; Katugin and Merzlyakov, 2002; Seibel et al., 2000). During brooding, muscle tissue degrades, tentacles are autotomized, and mantle and arms become water logged (Katugin, 2003; Katugin and Merzlyakov, 2002). To date, seven gonatid species have been recorded to exhibit this change upon maturation, which include *Gonatus berryi*, *Gonatus madokai*, *Gonatus onyx*, *Gonatus pyros*, *Gonatus tinro*, *Gonatus fabricii*, and *Gonatopsis octopedatus* (Arkhipkin and Bjorke, 1999; Katugin, 2003; Katugin and Merzlyakov, 2002; Nesis, 1993; Seibel et al., 2000; Young, 1973).

Although the reproductive strategies of all gonatid species have yet to be determined, brooding appears to occur in more derived forms, those that already exhibit fragile arm and mantle tissue and are usually found at greater depths (Nesis, 1973). Although brooding is present in Octopoda, gonatids are distantly related (e.g., Carlini and Graves, 1999; Lindgren et al., 2004) and exhibit different physiology and behavior during brooding (Boletzky, 1992), indicating that brooding has evolved independently in Gonatidae. Brooding octopods glue their eggs together into “long straight or branched strings” (Norman, 2000), which are either attached to substrate or carried within their arm webs, keeping them oxygenated and free of debris (Boletzky, 1992; Cosgrove, 1993; Norman, 2000). In gonatids, females that brood their eggs hold them between their arms, floating midwater until hatching (Katugin and Merzlyakov, 2002; Okutani et al., 1995; Seibel et al., 2000), and evidently do not exhibit such invested care as octopuses. Male and immature female members of brooding species have been recorded as having delicate arm and mantle tissue, implying that at least in some species, such as in *Gonatus madokai* and *Gonatus tinro* (Katugin, 2003), morphogenic change in tissue precedes or coincides with maturation. Therefore, it is possible that muscular degeneration may be a species character influenced by physiology and environment. Whether or not tissue degeneration and associated egg brooding is a synapomorphy of Gonatidae can be tested using an independent non-morphological approach, such as a molecular phylogenetic analysis.

No combined sequence-based phylogenetic hypothesis exists for Gonatidae, although an allozyme-based phylogeny has been recently generated (Katugin, 2004). Previous molecular work has been completed using the cytochrome *c* oxidase subunit I locus (COI; Seibel et al., 2000) to identify juvenile gonatids from southern California waters as well as incorporating two species of *Gonatus* in a COI based, higher-level phylogeny to investigate relationships between coleoid cephalopods (Carlini and Graves, 1999). In the present study comparisons between brooding strategies, morphology, and physiology were made to the molecular phylogeny of three mitochondrial loci for 14 gonatid species and analyzed to determine whether these life history characteristics were important in the radiation of this family of squids.

2. Methods

2.1. PCR amplification and sequencing analysis

DNA was extracted from gill or mantle tissues of 39 individuals representing 14 gonatid species (13 nominal species and one undescribed species) as well as six out-group species (Table 1) using Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA). PCR amplification was carried out in 50 μ L reactions using Promega *Taq* Polymerase (see Nishiguchi et al., 2004 for further descriptions of PCR methods) for fragments of three mitochondrial loci: 12S rRNA (404 bp), 16S rRNA (528 bp), and cytochrome *c* oxidase subunit I, COI (658 bp) (see Nishiguchi et al., 2004 for primer information and annealing temperatures). Although nuclear data has proven useful in higher-level analyses (e.g., Lindgren et al., 2004), nuclear loci have been determined to be too conserved for discerning species-level relationships among cephalopods (e.g., Nishiguchi et al., 2004). Sequences were obtained using an ABI PRISM 3100 Genetic Analyzer (Foster City, CA). External primers were excluded from resulting forward and reverse chromatograms which were assembled and edited using either Sequencher v. 4.1 or v. 4.2 (Gene Codes, Ann Arbor, MI). Resulting sequences were compiled and partitioned in Genetic Data Environment (GDE, Smith et al., 1994). For non-coding genes (12S and 16S), sequences were partitioned in GDE using secondary structure models and unambiguous regions; 12S was partitioned into five fragments and 16S was partitioned into eight. The protein-coding gene COI was tested as one entire fragment for the analysis.

2.2. Phylogenetic analysis

Individual and combined sets of molecular data were analyzed using the direct optimization program POY (Wheeler et al., 2002), with parsimony as the optimality

Table 1
Accession numbers for cephalopod taxa used in the present study

	Collection data	12S rRNA	16S rRNA	COI
Nautiloidea (1 sp.)				
Nautilidae	<i>Nautilus pompilius</i>	Voucher at AMNH	AY686585	AY557514
Coleoidea (19 spp.)				
Vampyroteuthidae	<i>Vampyroteuthis infernalis</i>	Hokusei Maru (DC)	AY686586	* AF000071
Sepiolidae	<i>Sepiola affinis</i>	Banyuls sur Mer, France	AY686588	AY557523
Spirulidae	<i>Spirula spirula</i>	DC (voucher at NMNH)	AY686589	* AF000066
Loliginidae	<i>Loligo pealei</i>	Northern Atlantic (WKM)	AY686590	* AF000052
Architeuthidae	<i>Architeuthis dux</i>	DC (voucher at NMNH)	AY686587	* AF000027
Gonatidae (14 spp.)				
	<i>Gonatopsis japonicus 01</i>	Sea of Japan	AY681019	AY681051
	<i>Gonatopsis japonicus 02</i>	Sea of Japan	AY680997	AY681052
	<i>Gonatopsis japonicus 03</i>	Sea of Japan	AY680998	AY681053
	<i>Gonatopsis japonicus 05</i>	Sea of Japan	AY681022	AY681054
	<i>Gonatopsis japonicus 07</i>	Sea of Japan	AY681023	AY681055
	<i>Gonatopsis octopedatus 02</i>	Sea of Okhotsk	AY680999	AY681024
	<i>Gonatopsis borealis (L) 01</i>	Sea of Okhotsk	AY681000	AY681025
	<i>Gonatopsis borealis (L) 02</i>	Sea of Okhotsk	AY681026	AY681058
	<i>Gonatopsis borealis (S) 02</i>	Sea of Okhotsk	AY681001	AY681027
	<i>Gonatopsis borealis (S) 06</i>	Sea of Okhotsk	AY681002	AY681028
	<i>Gonatopsis borealis (S) 07</i>	Sea of Okhotsk	AY681003	AY681029
	<i>Gonatopsis borealis (S) 09</i>	Sea of Okhotsk	AY681004	AY681030
	<i>Gonatopsis sp. 01</i>	Sea of Okhotsk	AY681005	AY681031
	<i>Gonatus antarcticus 01</i>	South Georgia (MC)	AY681032	AY681064
	<i>Gonatus fabricii 01</i>	DE0304 (Sta. 3), 2003 (MV)	AY681006	AY681033
	<i>Gonatus berryi 02</i>	Pacific off Kuril Islands	AY681034	AY681066
	<i>Gonatus tinro 01</i>	Sea of Okhotsk	AY681007	AY681035
	<i>Gonatus tinro 08</i>	Sea of Okhotsk	AY681008	AY681036
	<i>Gonatus tinro 09</i>	Sea of Okhotsk	AY681009	AY681037
	<i>Gonatus kamtschaticus 01</i>	Sea of Okhotsk	AY681010	AY681038
	<i>Gonatus kamtschaticus 04</i>	Sea of Okhotsk	AY681039	AY681070
	<i>Gonatus kamtschaticus sp. 03</i>	Sea of Okhotsk	AY681011	AY681040
	<i>Gonatus kamtschaticus sp. 06</i>	Sea of Okhotsk	AY681012	AY681041
	<i>Gonatus kamtschaticus sp. 07</i>	Sea of Okhotsk	AY681042	AY681073
	<i>Gonatus cf. onyx 01</i>	Sea of Okhotsk	AY681013	AY681043
	<i>Gonatus pyros 02</i>	Pacific off Kuril Islands	AY681044	AY681075
	<i>Gonatus madokai 06</i>	Sea of Okhotsk	AY681014	AY681045
	<i>Gonatus madokai 10</i>	Sea of Okhotsk	AY681015	AY681046
	<i>Gonatus madokai 19</i>	Sea of Okhotsk	AY681016	AY681047
	<i>Berryteuthis magister 02</i>	Sea of Japan (GG)	AY681017	AY681048
	<i>Berryteuthis magister 01</i>	Sea of Okhotsk (GG)	AY681049	AY681079
	<i>Berryteuthis magister 01</i>	Pacific off Vancouver (GG)	AY681050	AY681080
	<i>Berryteuthis anonychus 11</i>	Central North Pacific (SS)	AY681018	AY681081

All ingroup taxa (Gonatidae) collected by the authors, except where noted (DC, Dr. David Carlini; GG, Dr. Graham Gillespie; MC, Dr. Martin Collins; MV, Dr. Michael Vecchione; SS, Dr. Satoshi Suyama; WKM, Dr. William Macy; AMNH, American Museum of Natural History, New York; NMNH, National Museum of Natural History, Washington DC).

* Indicates sequences obtained from GenBank.

criterion (for a discussion of POY, see Giribet, 2001). All molecular data were analyzed at the nucleotide level, including the protein-coding gene, COI. In cases where sequences were unavailable for analysis, the data were treated as “missing.” Tree searches in POY were conducted in parallel using a 19 dual processor cluster at Harvard University (darwin.oeb.harvard.edu) with commands described in Lindgren et al. (2004). Each of the four data sets (individual 12S, 16S, COI, and combined molecules) was analyzed under 12 parameter sets with different transition/transversion and indel cost ratios. Gap/transversion ratios of 1 and 2 and transversion/

transition ratios of 1, 2, and 4 were explored. In all cases, gap extension was down-weighted with respect to the first occurrence of an indel event. To determine the optimal parameter set, a sensitivity analysis was conducted (Wheeler, 1995). In sensitivity analysis, character congruence is treated as an extension of parsimony in that the parameter set minimizing overall character incongruence is the “optimal parameter set.” Lastly, nodal support for each analysis under the optimal parameter was assessed using Farris’s parsimony jackknifing procedure (Farris et al., 1995) on 100 replicates (commands -jackboot -replicates 100).

3. Results

Our sensitivity analyses indicated the parameter set that minimized overall character incongruence for the combined molecular analysis was that which weighted the ratio of gap/transversion=2 and transversion/transition=2 (referred to as 221, Table 2). Therefore, 221 is considered the optimal parameter set for both combined and individual data sets to better facilitate comparisons among data sets.

3.1. 12S rRNA

Due to amplification difficulties and specimen availability, outgroup taxa (*Nautilus pompilius*, *Vampyrotheuthis infernalis*), as well as several gonatid taxa (Table 1) were not included in the 12S rRNA individual analysis. Under the optimal parameter set (221), monophyly was shown for Gonatidae (77%, Fig. 1) with *Spirula spirula* as the closest outgroup relative. No genera were recovered as monophyletic. One clade contained representatives of all genera investigated; *Berryteuthis magister* + *Gonatus kamtschaticus* 03, was sister to *Gonatopsis octopedatus*, with *Gonatopsis japonicus* as the outgroup. Within a second clade, *Gonatopsis borealis* was monophyletic (57%), *Gonatus tinro* 01 + *Gonatus cf. onyx* was supported in 100% of jackknife replicates, and all other *Gonatus tinro* individuals fell into a clade with *G. kamtschaticus* 01 and *Gonatus madokai* + *Gonatus kamtschaticus* 06. Although 12S provided additional information regarding genus-level dynamics, many of the nodes remained poorly supported, indicating that 12S alone does not provide enough information to resolve family level relationships.

3.2. COI

Under the optimal parameter set (221), monophyly was supported for Gonatidae (with 100% jackknife support), with a polytomy of other decabrachians sister to the clade (Fig. 2). Within Gonatidae, little generic resolu-

tion was found; however, several interspecies clades were observed. *Gonatopsis octopedatus* formed a clade with specimens of *Gonatopsis japonicus* (80% jackknife support), except for *G. japonicus* 04, which fell within a clade comprised of several *Gonatus kamtschaticus* individuals and two individuals of *Gonatus madokai*. However, this was the only clade that was supported in fewer than 50% of jackknife replicates. Lastly, *Berryteuthis magister* individuals were sister to *Gonatopsis borealis*. Although *G. borealis* includes two forms, one small and one large size, the species is monophyletic (71% jackknife support). Furthermore, the small and large individuals each formed a clade with 100% jackknife support. Thus, COI provided support at the individual and species level, but was not able to resolve genus-level relationships.

3.3. 16S rRNA

The optimal parameter set (221) supported the monophyly of Gonatidae, with *Architeuthis dux* as its sister (54%, Fig. 3). Individuals of *Berryteuthis magister* formed a clade sister to the rest of Gonatidae, indicating monophyly for this species. However, due to amplification difficulties, *B. anonychus* could not be included in the 16S analysis, and therefore the monophyly of *Berryteuthis* could not be tested. Monophyly could not be established for *Gonatopsis* or *Gonatus*, as species from both genera were found throughout several clades. *Gonatopsis borealis* was paraphyletic due to the presence of *Gonatus fabricii* within the clade of small *G. borealis* individuals (86% jackknife support). *Gonatopsis* sp. was sister to all remaining species, although the support values were less than 50%. Individuals from *Gonatus tinro*, *G. pyros*, *G. kamtschaticus*, and *G. cf. onyx* formed a single clade, indicating potential evidence for some intergeneric relationships. *G. kamtschaticus* individuals fell in several different clades, with *G. kamtschaticus* 06 forming the outermost branch of a *Gonatopsis* clade (*Gonatopsis japonicus* and *Gonatopsis octopedatus*). Consequently, the 16S topology provides some support for genus-level groups, however, overall nodal support values remain fairly low.

Table 2

Weighted tree lengths for the individual and combined analyses at different gap/tv and tv/ts cost ratios and ILD values

Gap/tv	tv/ts	COI	12S	16S	Combined	ILD values
1	∞	338	109	230	693	0.0231
1	1	1026	227	438	1736	0.0259
1	2	1383	342	675	2455	0.0224
1	4	2061	561	1141	3851	0.0229
2	∞	338	163	321	845	0.0272
2	1	1026	290	539	1904	0.0257
2	2	1383	460	866	2768	0.0213
2	4	2061	788	1508	4476	0.0266
4	∞	338	268	478	1117	0.0295
4	1	1026	399	696	2191	0.0319
4	2	1383	668	1172	3321	0.0295
4	4	2061	1204	2121	5551	0.0297

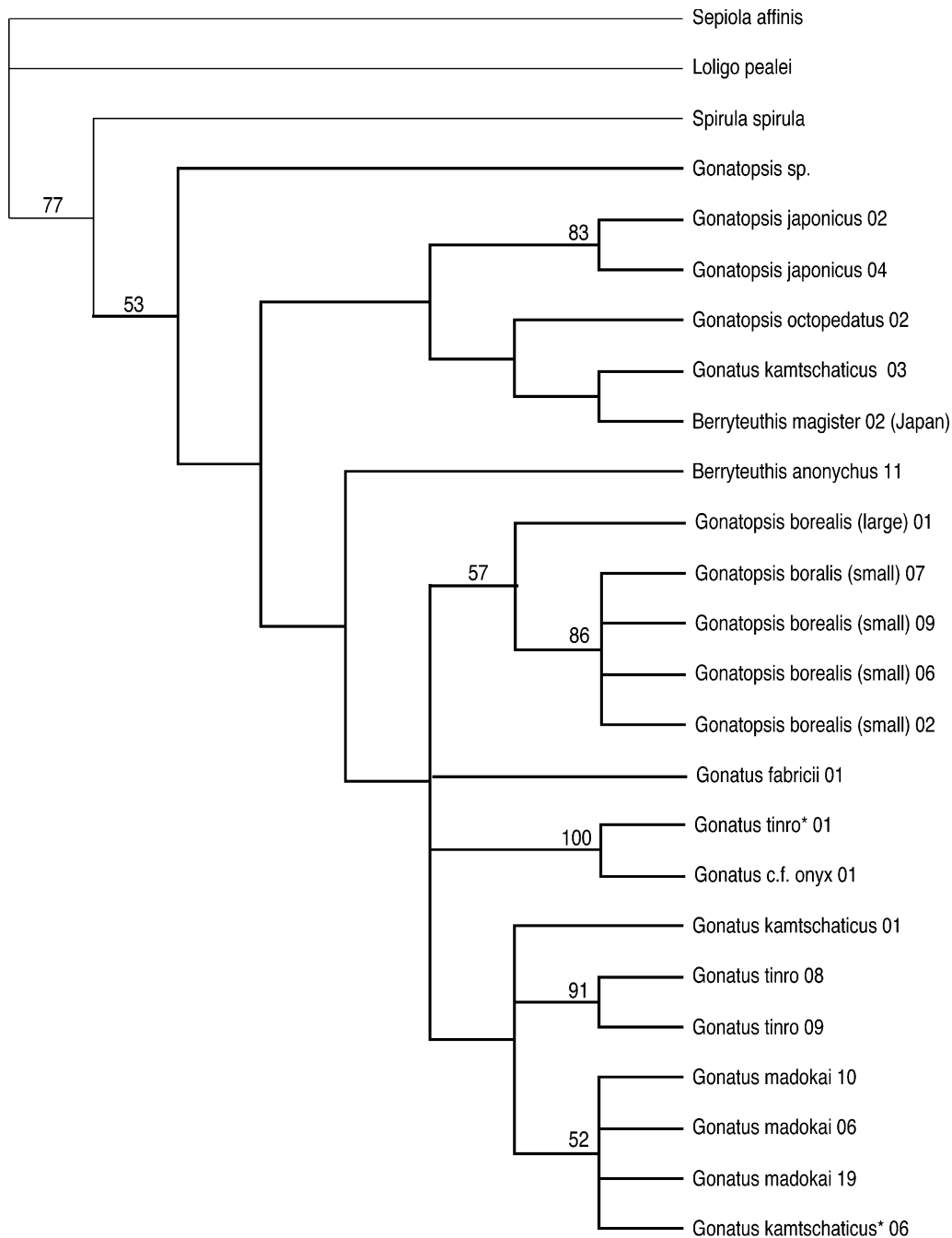


Fig. 1. 12S rRNA cladogram generated from a eight equally parsimonious trees, each of 460 equally weighted steps under the optimal parameter set (221). Lines in bold indicate individuals from the family Gonatidae. Asterisk (*) refers to specimens that may have been misidentified. Jackknife support values greater than 50% are noted above corresponding nodes.

3.4. Combined molecules

The optimal parameter set (221) for the combined molecular data analysis (Fig. 4) recovers monophyly of Gonatidae (100% jackknife support), as well as monophyly of Decabrachia (90%). All gonatid species, with the exception of *Berryteuthis magister*, formed a single clade (58% jackknife support). Within this primary gonatid clade, *Gonatopsis sp.* forms the outermost branch, with a *Gonatopsis borealis* clade (89% jackknife support) basal to

all other gonatids. Within the *Gonatopsis borealis* clade, small and large individuals formed two clades, each with 100% jackknife support. *Berryteuthis anonychus* is embedded within *Gonatopsis* and *Gonatus*, recovering non-monophyly of all gonatid genera. However, several *Gonatus* and *Gonatopsis* clades are present. *Gonatus tinro*, *Gonatus cf. onyx*, *Gonatus pyros*, and several individuals from *G. kamtschaticus* form a single clade, with *G. tinro* + *G. cf. onyx* (100%), and *G. kamtschaticus* 07 sister to *G. kamtschaticus* 03 + *G. pyros* (95%). All *Gonatopsis japonicus* individuals

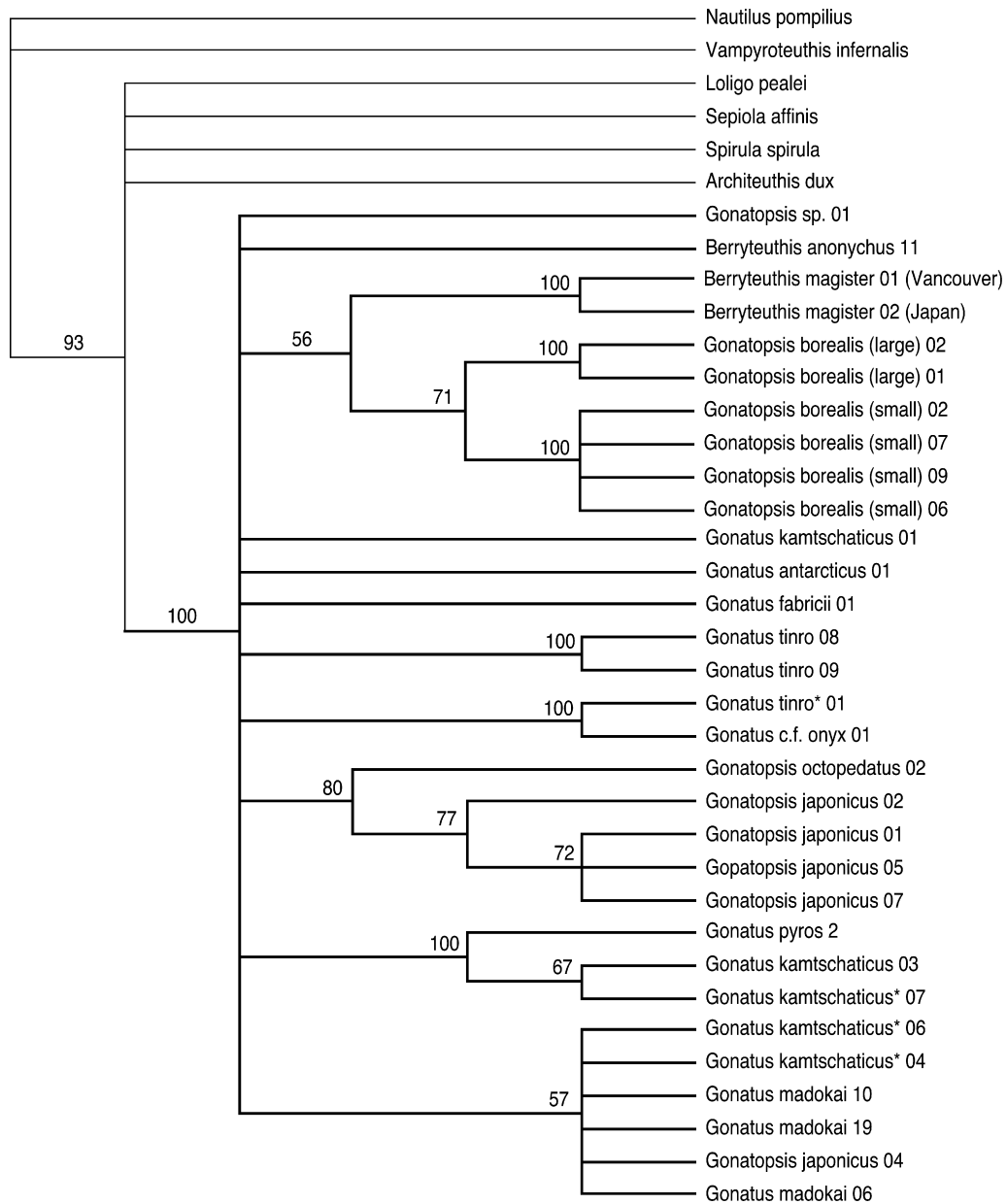


Fig. 2. COI consensus tree generated from 20 equally parsimonious trees of 1383 equally weighted steps under the optimal parameter set (221). Individuals from Gonatidae are indicated in bold. Asterisk (*) refers to specimens that may have been misidentified. Jackknife support values greater than 50% are listed.

form a clade with *Gonatopsis octopedatus* (96%); this clade is sister to a crown *Gonatus* clade (supported in 54% of replicates) consisting of *G. berryi*, *G. antarcticus*, *G. kamtschaticus* (01, 04), *G. kamtschaticus* (06), and *G. madokai*. Within the crown clade, *Gonatus berryi* and *Gonatus antarcticus* are sister to *G. kamtschaticus* 01 + (*G. madokai* + *G. kamtschaticus* 06 + *G. kamtschaticus* 04).

4. Discussion

Although individual data sets disagreed to some extent, the combined molecular analyses provided the

highest degree of resolution and nodal support (Fig. 4). The topology of the combined analysis is congruent with morphological characteristics such as the number of teeth in a transverse row of a radula, tissue degeneration upon sexual maturation (Fig. 4), and allozyme data (Katugin, 2004).

There is a clear separation between two major evolutionary lineages: rather primitive, muscular gonatids with seven longitudinal rows of radular teeth and more advanced, less muscular (at least in late ontogenetic stages) gonatids with five rows of radular teeth. The radula of *Nautilus* contains thirteen elements (nine longitudinal rows of teeth and four rows of plates) whereas

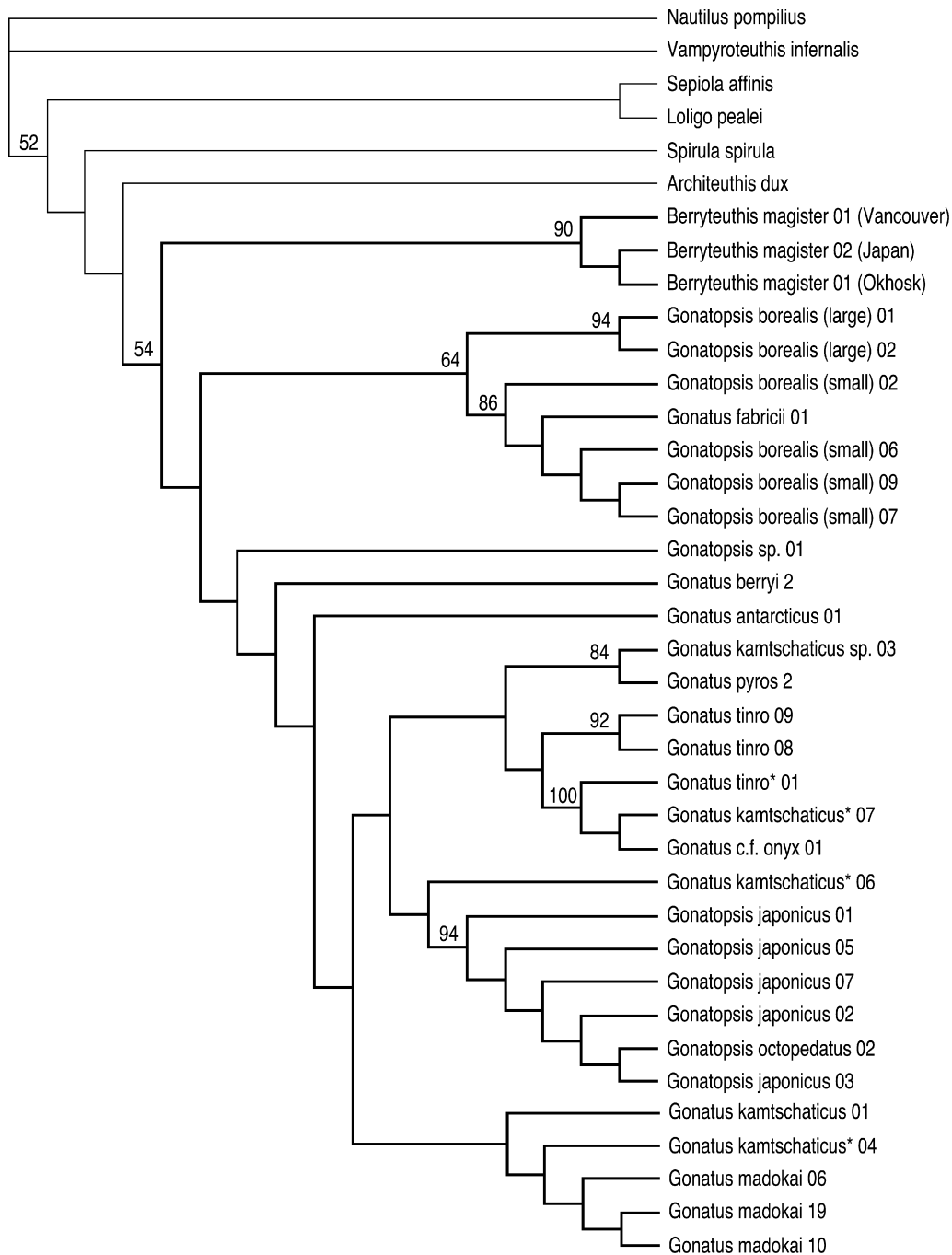


Fig. 3. 16S rRNA cladogram generated from a single tree of 866 equally weighted steps under the optimal parameter set (221). Gonatid taxa are illustrated in bold. Asterisk (*) refers to specimens that may have been misidentified. Jackknife support values greater than 50% are noted above corresponding nodes.

most decabrachians contain nine (seven rows of longitudinal teeth and two rows of plates; Nixon, 1998). Our findings suggest the more basal gonatid group is comprised of the 7-toothed recent taxa: two paraphyletic *Berryteuthis* species (*B. magister* and *B. anonychus*), and several representatives of *Gonatopsis* (large and small sized *G. borealis* and *Gonatopsis* sp., partly conforming to the description of *Gonatopsis makko* (Okutani and Nemoto, 1964)). The more derived 5-toothed recent

species are monophyletic with 7-toothed *B. anonychus* as sister.

The combined analysis establishes several other relationships that can be compared to earlier morphological investigations. For example, Nesis (1973) believed that *Berryteuthis anonychus* was the more ancestral form within *Berryteuthis*, and hence the closest exemplar of the family prototype. However, our findings suggest that *B. anonychus* may be the derived form (sister to all taxa

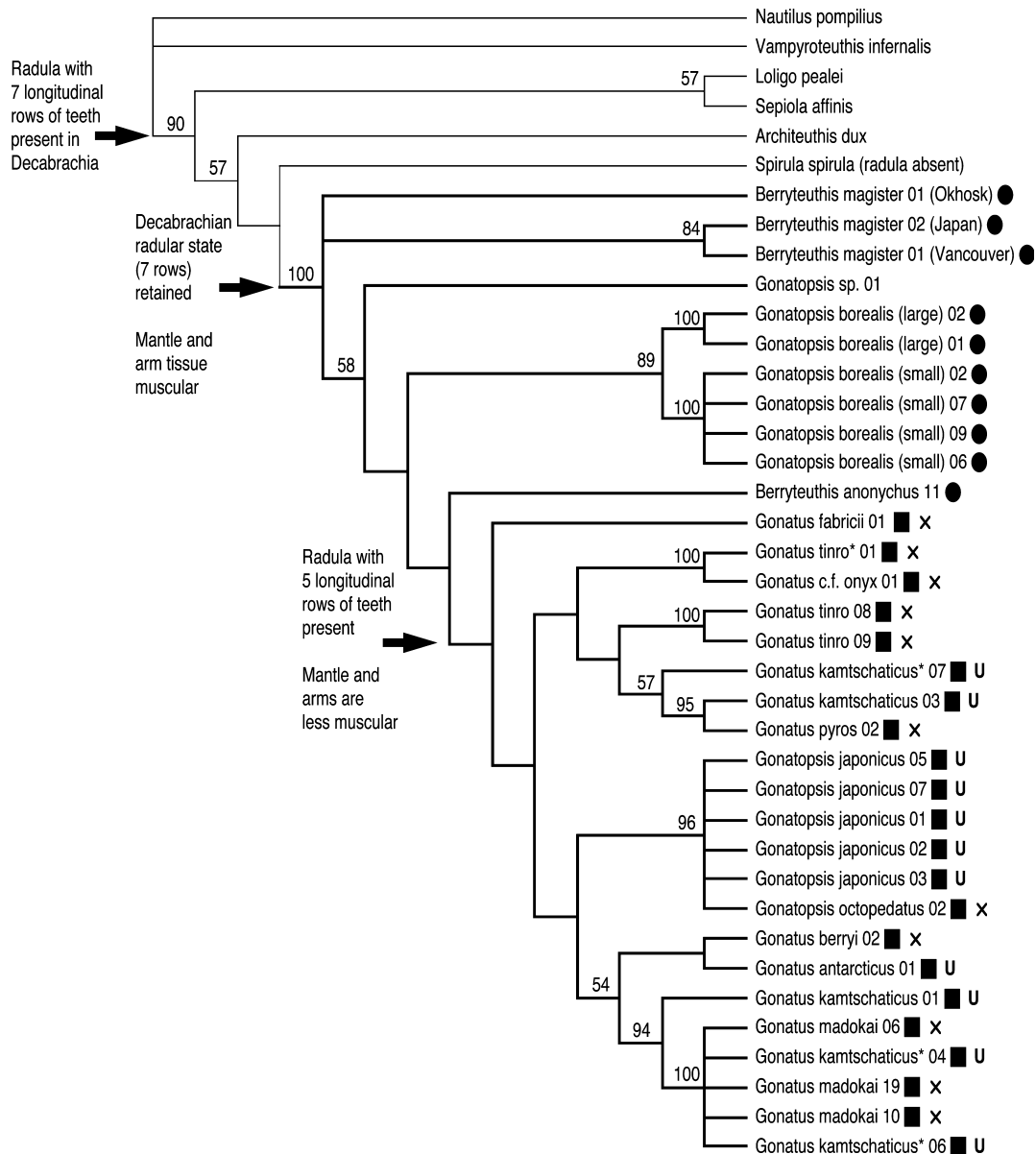


Fig. 4. Combined molecules phylogeny generated from five equally parsimonious trees, each of 2768 steps under the optimal parameter set (221). Bold lines indicate species within the family Gonatidae. Jackknife values greater than 50% are listed above corresponding nodes. Asterisk (*) indicates specimens that may have been misidentified. Circles indicate gonatid species known to have muscular mantle and arm tissues (no degeneration). Black squares indicate species that may have some mantle and arm tissue with a higher water content level (noted from Nesis, 1973). Females recorded to have watery arm and mantle tissue and brood their eggs are indicated with an X. Species with no information on degeneration are indicated with a U. Although *G. japonicus* may undergo degeneration, further analysis is warranted and was therefore coded as "U."

with 5 radular rows of teeth), while *Berryteuthis magister* is most basal of all gonatid species sampled. This outcome is in concert with Jefferts (1983), who considered *B. magister* to be the most primitive type within the family based on morphology alone. Our findings, as well as previous allozyme analysis (Katugin, 2004) confirm this result, suggesting that *B. magister* is more basal, whereas *B. anonychus* is more derived. Furthermore, monophyly of *B. magister* did not receive high nodal support, and is congruent with the fact that the species is polymorphic with geographical (Katugin, 2000) and presumably ecological (Okutani et al., 1987) subspecies. Further

studies into the population dynamics of *B. magister* are needed to better understand the evolutionary dynamics within this species.

One of the most geographically distinct species, *Gonatus fabricii*, which inhabits Arctic and North Atlantic waters, was found as the sister to all other *Gonatus* spp., *Gonatopsis japonicus*, and *Gonatopsis octopedatus*, demonstrating that it is the most basal of the taxa with five longitudinal rows of radular teeth. The position of *Gonatus fabricii* suggests that morphological change to a 5-rowed state may have occurred prior to geographic radiation.

Gonatus antarcticus is also geographically distinct from other gonatids, and is distributed sub-polar in notal waters close to Antarctica, migrating as far north as South Africa, Cook Strait and north Peru (Nesis, 1982). However, *G. antarcticus* nested within a *Gonatus* clade, sister to *Gonatus berryi*, which is native to the North Pacific. This may be indirect evidence that gonatid squids migrated to the Southern Ocean not from North Atlantic, but rather via a Pacific route, as was suggested by Nesis (1985, 1997).

We have also demonstrated that individuals conforming to the description of *Gonatus kamtschaticus* represent a heterogeneous group, comprised of at least two paraphyletic taxa. This species was originally described by Middendorff (1849) from a specimen caught in the oceanic waters off the Kuril Islands; later, the same species was re-described under the new name *Gonatus middendorffi* (Kubodera and Okutani, 1981), with holotype and paratype specimens also obtained from the oceanic area off the Kuril Islands. Our study contains both large- and medium-sized squid that conformed to the species description. The most readily identifiable specimen was *G. kamtschaticus* 01 (= *G. middendorffi*), a fairly large, still maturing female with dorsal mantle length (ML) of 403 mm, which fell within a *Gonatus madokai* group with jackknife support of 94% (Fig. 4). A much smaller individual, listed as *G. kamtschaticus* 07, a maturing female with ML of only 132 mm, was recovered within a separate clade, closely related to the *Gonatus* group represented by *G. tinro*, *G. pyros*, *G. cf. onyx*, and *G. kamtschaticus* 03. Additionally, in the 16S analysis *G. kamtschaticus* 07 formed a clade with *G. tinro* 01 and *G. cf. onyx* 01. It is possible that either *G. kamtschaticus* 07 was misidentified or that there was sequence contamination. To further resolve this issue, additional data needs to be collected. From our analyses, we can draw several conclusions regarding *Gonatus kamtschaticus*: *G. kamtschaticus* (04 and 06) may be *G. madokai*. The 04 specimen was an immature female of ML 313 mm with both *G. madokai* and *G. kamtschaticus* features, with its mantle consistency similar to *G. madokai*. The specimen had a rather slim body (mantle width index of 0.22, more typical of *G. kamtschaticus*), and fin proportions similar to those of *G. kamtschaticus* (the fin was as wide as long, with fin length index of 0.44, more typical of *G. kamtschaticus*). However, it lacked the tentacles for valid identification. The *G. kamtschaticus* 06 specimen also lacked tentacles, and was initially questionable whether it represented *G. madokai* or *G. kamtschaticus*. *Gonatus madokai* and *G. kamtschaticus* can be hard to distinguish morphologically when specimens are damaged, such as when tentacles or fins are missing, and *G. kamtschaticus* is as yet an unresolved group of closely related species without evident differences in morphology (= sibling species). This situation indicates that morphologically based identification can sometimes be erroneous and lead to

misinterpretation of the results, resulting in an incorrect classification, providing additional support that molecular data is useful not only for phylogenetics, but also for species identification in the Gonatidae.

Gonatus cf. onyx and *Gonatus tinro* 01 were recovered as sister taxa, with all other *G. tinro* individuals forming a clade with *G. kamtschaticus* individuals and *G. pyros*. Further investigation of the *G. tinro* 01 specimen indicated that it was most likely misidentified. This specimen, identified as a maturing female with ML of approximately 160 mm (onset of sexual maturation in *G. tinro* females occurs at around 210 mm ML), was collected from a trawl net and was in extremely bad condition, with arms significantly damaged, tentacles torn and missing, and fins absent. Therefore, we infer that *G. tinro* 01 was actually a specimen of *G. onyx*; however, further sampling of *G. onyx* would provide more support of this finding.

Within *Gonatopsis*, a sister relationship between *Gonatopsis japonicus* and *Gonatopsis octopedatus* is further supported by the presence of a 5-toothed radula, as well as their location in deep waters of the Japan Sea (this species being much rarer in the Okhotsk Sea). Furthermore, there is indirect evidence that in *G. japonicus*, musculature degenerates upon maturation; parts of the arm crown (only eight arms, no trace of tentacles), presumably belonging to *G. japonicus*, have been occasionally observed floating on the surface in the Japan Sea (Katugin and Mokrin, personal observation).

The possibility of a recent speciation event may be evident in *Gonatopsis borealis*, which was divided into two subgroups (large and small), with 100% nodal support for each clade. Large-sized squids mature at mantle length over 200 mm in males and over 250 mm in females, while mature small-sized are 130–150 and 150–170 mm, respectively (Nesis, 1989). Distribution ranges of these two groups are different, with the small group distributed predominantly in northern areas, while the large group inhabits mostly southerly offshore oceanic areas, although they are found in sympatry along the Kuril Islands and in south Okhotsk Sea (Nesis, 1997; Nesis and Nezhlin, 1993). Our findings also support preliminary results from allozyme electrophoretic comparisons of *G. borealis* groups (Katugin, unpublished), where fixed differences at a number of protein loci have been observed between the two groups, suggesting their reproductive isolation from each other. Although no morphological traits other than size allows discrimination between small- and large-sized groups of *G. borealis*, two independent sources of genetic evidence (allozyme and DNA) along with geographical patterns suggest that they are taxonomically distinct. Whether they represent two subspecies within a polymorphic species, or they have already diverged at a specific level within a super-species remains unclear, and further research into the problem is warranted.

Physiological transitions within this family raise several pertinent questions. If weaker musculature exists in males and immature females, is muscular degeneration in brooding females the result of physiological or hormonal cues? Can female change be considered a synapomorphy supporting the monophyly of five-toothed gonatids? Was morphological change and egg brooding a cause for speciation and radiation, or vice versa? At the present time, it is difficult to fully answer these questions since the reproductive characteristics of several key taxa remain unknown. No mature females have been recorded for *G. kamtschaticus*, *G. antarcticus*, *G. californiensis*, or *G. steenstrupi*, and although *G. japonicus* may undergo muscular degeneration (Nesis, 1997; Katugin and Mokrin, personal observation), further investigation is needed. Nesis (1971) believed that all deep-water taxa were also those with water-rich tissue and the ability to degenerate during maturation, but he was not able to substantiate his beliefs due to the lack of specimen availability. Okutani et al. (1995) refuted Nesis “deep water rule” in observing an unidentified species of *Gonatopsis* in the Okhotsk Sea near the Hokkaido coast (later it was suggested by Tsuchiya et al. (2002) that the species in fact belongs to the genus *Gonatus*), which was found to release juveniles from an egg-mass between the arms near in-shore shallower waters. Observations on spent females of *Gonatus madokai* also suggest that prevailing currents largely influence their distribution patterns in the Okhotsk Sea, as they are frequently found in shallow depths (Katugin and Merzlyakov, 2002; Katugin et al., 2004).

Gonatids remain an elusive group, due in part to the lack of available mature specimens, but also because considerable ontogenetic change is seen in at least several species. While this study was able to provide further insight into the evolutionary dynamics within Gonatidae and support previous morphological hypotheses, future studies of this complex group of decabrachians will provide additional information as to how reproductive strategies, ecological factors, biogeography, and physiological modifications may affect speciation in pelagic marine invertebrates.

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