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Species identification and genetic relationships in the squid family Gonatidae (Teuthida, Cephalopoda) based on partial sequencing of mitochondrial and nuclear genes

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Research Article

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Abstract

Squids of the family Gonatidae are key components in oceanic communities. However, issues related to correct species identification, number of species, and their genetic relatedness remain. To address these issues, sequences from three mitochondrial (cytochrome *c* oxidase subunit I [CO1], 16S rRNA, and 12S rRNA) and two nuclear (18S and 28S) genes were analysed in the Gonatidae. Four of the five sequences (12S rRNA, 16S rRNA, 28S, and CO1) yielded rather similar patterns of genetic relationships among the species. Molecular evidence suggested intra-familial subdivision into two major groups of species having either five or seven longitudinal rows of teeth in the radula. The former group included all species of the genus *Gonatus* and two sister-species of *Gonatopsis* s. str. suggesting that all gonatid species with five rows of radular teeth represent a single taxonomic unit of a genus or subfamily level. Species with seven rows of radular teeth formed several 'species' clusters. Sequence analysis also addressed species identification issues in the Gonatidae. Two genetically divergent groups were found among squid which conformed to the description of *Gonatus berryi*. Molecular evidence suggested sister-species relationships between 'large' and 'small' forms of *Boreoteuthis borealis* with size-at-maturity as the only reported difference between these two cohorts. Sequence variation was observed within *Gonatus pyros*. Inclusion of gonatid sequences from the GenBank into the analysis suggested probable species misidentification in several cases. Combined use of several mitochondrial and nuclear sequences served as a valuable tool for species identification and provided a solid background for unravelling molecular genetic and taxonomic relationships in the Gonatidae.

Introduction

Squids of the family Gonatidae are widely distributed in subpolar regions in the Northern and Southern Hemispheres (Nesis, 1973, 1997; Okutani *et al.*, 1988; Roper *et al.*, 2010). These squids are known for their particularly high abundance and taxonomic diversity in the boreal North Pacific, where they are key components in pelagic and near-bottom deep-sea marine and oceanic communities and, in certain areas, some species are found in high-density commercial concentrations and are harvested by fisheries (Okutani *et al.*, 1988; Clarke, 1996; Nesis, 1997; Savinykh, 2005; Hoving *et al.*, 2014). According to studies based primarily on morphology, the family Gonatidae comprises up to 19 species, of which 16 are found in the North Pacific, two in the North Atlantic, and one in the Southern Ocean (Nesis, 1973, 1997; Okutani *et al.*, 1988; Roper *et al.*, 2010). Despite the family's high ecological and economic importance, life-history patterns and systematic relations in the Gonatidae remain poorly understood. Studies at the bio-molecular level are incongruent with morphological findings and question the reliability of the currently accepted generic subdivision of the family (Katugin, 2004; Lindgren *et al.*, 2005; Katugin *et al.*, 2017). Mitochondrial DNA (mtDNA) sequencing, in particular, has proved to be a useful tool for species identification in the Gonatidae, especially for individuals at the early (paralarvae and early juveniles) and late (spawning and spent individuals) ontogenetic stages, when it is difficult to identify individuals morphologically at the species level (Seibel *et al.*, 2000; Bower *et al.*, 2012).

With the aim of resolving these difficulties, some of which are associated with previous studies based on cytochrome *c* oxidase subunit I (CO1) (Katugin *et al.*, 2017), two additional mitochondrial genes (16S rRNA and 12S rRNA), and two nuclear genes (28S and 18S) were analysed in comparison with sequences obtained from BLAST searches of the GenBank database to reassess the relationships among gonatid species from the World Ocean and the reliability of the currently available sequence data. The five gene markers used in the present study for better understanding of relationships among the Gonatidae species were previously used in molecular genetic studies, particularly, on molluscs (e.g. Zubakov *et al.*, 1997; Carlini, 1998; Canapa *et al.*, 2003; Fahey, 2003; Meyer *et al.*, 2010, Plazzi and Passamonti, 2010; Tan and Conaco, 2021).

Materials and methods

Sampling

DNA sequences of the Gonatidae for subsequent analysis were obtained from two major sources: the original collection and GenBank. Most sequences were obtained from squid specimens deposited in the A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, and tissue samples of squids captured during TINRO (Pacific Branch of Russian Federal Institute of Fisheries and Oceanography) research surveys in the Okhotsk, Bering, and Japan seas, and northwestern Pacific Ocean using midwater and bottom trawl nets. Squids were identified to species in the field and in the laboratory based on morphology and using published identification keys (Bublitz, 1981; Jefferts, 1983; Nesis, 1987). Muscle tissue (mantle) was taken for further DNA extraction and sequencing (Table 1). Sequences were further compared with those deposited in the GenBank (NCBI) identified as originating from species of the family Gonatidae. For the analyses, two outgroup species were chosen from two Oegopsid squid families: Architeuthidae (*Architeuthis dux*: KC701757.1) and Ommastrephidae (*Todarodes pacificus*: AB158364 and AB240153; Supplementary Table S1).

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

Tissue samples were collected from the recently captured squids and stored in 96% ethanol at −20°C. Genomic DNA was extracted from 20 mg of preserved mantle tissue using a DNAeasy extraction kit ('DNA-Extran-2', SINTOL, Moscow, Russia) according to the protocol of the manufacturer and then stored at −20°C. PCR amplification was carried out in a 25 µl PCR volume consisting of 10.42 µl double-distilled water, 1 µl 0.2 mM deoxynucleoside triphosphate mix, 4 µl 5× Taq Red buffer, 1.6 µl 2 mM magnesium chloride, 1 µl *Taq* polymerase, and 1 µl DNA template for fragments of three mitochondrial loci: CO1 (658 bp), 16S rRNA (558 bp), and 12S rRNA (260 bp); and two nuclear genes: 28S rRNA (1635 bp) and part of 18S rRNA (402 bp). Ribosomal genes will be referred to subsequently as 16S rRNA, 12S rRNA, 28S, and 18S, respectively. Some primers were designed for this study; others were taken from previous studies (Table 2).

Amplification was performed using a programmable thermal cycler GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) according to the following protocol: 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, 44.5°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR amplification products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and then visualized and photographed under ultraviolet transillumination prior to cleanup and sequencing.

Amplified PCR products were used as a template for sequence reactions carried out on ABI PRISM 3500 (Applied Biosystems) according to the BigDye terminator v3.1 Cycle Sequencing Kit Protocol (Applied Biosystems) with the same primers as for PCR. The sequenced fragments were read by an ABI3500 Genetic Analyzer (Applied Biosystems). Sequences were aligned using ClusterW in MEGA10 (Kumar *et al.*, 2018), and then edited by eye using BioEdit (Hall, 1999). New sequences (total 119) were deposited in the GenBank under the following accession numbers: MW940366–MW940378 (CO1); MZ008014–MZ008067 (16S rRNA); OK482928–OK482983 (12S rRNA); OM836136–OM836167 (28S); and MZ536663–MZ536716 (18S).

Sequence analyses

Sequence analyses were conducted separately for CO1, 16S rRNA, 12S rRNA, 28S, and 18S. The *P*-distance method (pairwise distances) was used to analyse intra- and interspecific variability in MEGA10 using nucleotide code for mitochondrial invertebrates for CO1 and 16S rRNA; and standard code for 12S rRNA, 28S, and 18S. For each marker, neighbour-joining (NJ), maximum-likelihood (ML), and Bayesian (BA) trees were constructed as graphic representations of species subdivision using programs MEGA10 (Kumar *et al.*, 2018) and mrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Analyses were conducted for each gene individually and also for the combined data set. ML and BA trees were used to generate consensus trees. NJ and ML trees were generated with bootstrap support of 1000 pseudo-replicates (Felsenstein, 1985) for nodes. Consensus ML trees were built using RAXML online (https://raxml-ng.vital-it.ch/#/). The best-fitting evolution models were calculated in jModelTest (Posada, 2008). Considering the Akaike information criterion, the best evolution models were TRM3uf + I + G (CO1, samples from our collection only), TrN + I + G (16S rRNA, samples from our collection only), TrN + I (12S rRNA, samples from our collection only), GTR + I + G (28S, samples from our collection only), TIM1 + I (18S, samples from our collection only); TRMuf + I + G (CO1, combined data from our collection and GenBank), HKY + I + G (16S rRNA, combined data from our collection and GenBank), HKY + I (12S rRNA, combined data from our collection and GenBank), GTR + I + G (28S, combined data from our collection and GenBank), and TIM1 + I (18S, combined data from our collection and GenBank). Stationarity was considered when the mean standard deviation of the split frequencies was below 0.01 (Ronquist and Huelsenbeck, 2003). The number of repetitions (generations) in simulations was 1,500,000, burn in was 25%, and the sample frequency was 100.

Species delimitation

Species delimitation used tree topologies obtained in RAXML, MEGA10, and mrBayes. Species groups were selected using the Automatic Barcode Gap Discovery (ABGD) method, which is widely used in molluscan studies (Ekimova *et al.*, 2020; Ghanimi *et al.*, 2020). JC69 (Jukes–Cantor), K80 (Kimura), and simple distances of automatic barcoding gap discovery were used online https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html to investigate the 'barcode gap' (Hebert *et al.*, 2003) and sort the sequences into hypothetical species (Puillandre *et al.*, 2012). For 16S rRNA, 12S rRNA, 28S, and 18S, *P*min = 0.001, *P*max = 0.2, and the relative gap width (*X*) = 1; for CO1, *P*min = 0.001, *P*max = 0.15, and the relative gap width (*X*) = 1. The other parameters remain as default.

Results

Sequence analysis

Sequences of CO1, 16S rRNA, 12S rRNA, 28S, and 18S of the Gonatidae species were analysed (Table 3). Parsimony-informative sites were also calculated for each of the five gene markers (Table 3).

Genetic divergence within and between species

Species hypothesis-free ABGD analysis of the five gene markers revealed different numbers of separate species groups for different markers (Table 4). ABGD for CO1 suggested that resultant groups appeared as the well-known nominal gonatid species, except for *Gonatus* cf. *berryi* and *Boreoteuthis borealis*, each of which appeared subdivided into two species groups.

ABGD for 16S rRNA suggested that most groups corresponded to the nominal gonatid species, with two species groups included in *B. borealis*. However, some species appeared poorly

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Table 1. List of specimens of the Gonatidae from our collection used in the molecular genetic analysis

Species name	Voucher number	CO1 (Katugin *et al*., 2017) and new (59 individuals)	16S new (52 individuals)	18S new (54 individuals)	12S new (56 individuals)	28S new (32 individuals)	Origin	Collection time
Boreoteuthis makko	KON201	KT429699	MZ008014	MZ536663	OK482928	OM836136	50° 46′N, 158° 18′E	1 April 2009
Boreoteuthis makko	KON202	KT429700	MZ008015	MZ536664	OK482929	OM836137	51° 24′N, 155° 32′E	3 March 2001
Boreoteuthis borealis large form	KON203	KT429701	MZ008016	MZ536665	OK482930	OM836138	51° 00′N, 156° 01′E	12 March 2001
Boreoteuthis borealis large form	KON204	KT429702	MZ008017	MZ536666	OK482931	OM836139	51° 15′N, 156° 11′E	4 March 2001
Boreoteuthis borealis large form	KON205	KT429703	MZ008018	MZ536667	OK482932	OM836140	43° 28′N, 148° 22′E	6 May 2009
Boreoteuthis borealis small form	KON206	KT429704	MZ008019	MZ536668	OK482933	OM836141	56° 12′N, 154° 04′E	20 March 2001
Boreoteuthis borealis small form	KON207	KT429705	MZ008020	MZ536669	OK482934	–	56° 12′N, 154° 04′E	20 March 2001
Boreoteuthis borealis small form	KON208	KT429706	MZ008021	MZ536670	OK482935	OM836142	54° 19′N, 153° 23′E	23 March 2001
Boreoteuthis borealis small form	KON209	KT429707	MZ008022	MZ536671	OK482936	OM836143	56° 23′N, 154° 03′E	21 March 2001
Boreoteuthis borealis small form	KON210	KT429708	MZ008023	MZ536672	OK482937	OM836144	57° 51′N, 167° 41′E	6 October 2008
Gonatopsis japonicus	KON211	KT429709	MZ008024	MZ536673	OK482938	–	46° 56′N, 144° 57′E	31 October 2006
Gonatopsis japonicus	KON212	KT429710	MZ008025	MZ536674	OK482939	OM836145	43° 21′N, 146° 42′E	7 May 2009
Gonatopsis japonicus	KON213	KT429711	MZ008026	–	OK482940	–	45° 00′N, 137° 24′E	16 April 2002
Gonatopsis japonicus	KON214	KT429712	MZ008027	MZ536675	OK482941	–	45° 00′N, 137° 24′E	16 April 2002
Gonatopsis octopedatus	KON215	KT429713	MZ008029	MZ536676	OK482942	–	54° 22′N, 153° 30′E	6 April 2001
Gonatopsis octopedatus	KON216	KT429714	MZ008030	MZ536677	OK482943	–	47° 55′N, 140° 20′E	12 May 2007
Gonatopsis octopedatus	KON217	KT429715	–	MZ536678	OK482944	–	48° 00′N, 140° 33′E	11 May 2007
Gonatus kamtschaticus	KON218	KT429676	MZ008031	MZ536680	OK482945	–	54° 23′N, 153° 11′E	24 March 2001
Gonatus kamtschaticus	KON219	KT429677	MZ008032	MZ536681	OK482946	OM836146	59° 28′N, 168° 11′E	8 October 2008
Gonatus kamtschaticus	KON220	KT429678	MZ008033	MZ536682	OK482947	OM836147	43° 34′N, 147° 16′E	7 May 2009
Gonatus madokai	KON221	KT429680	MZ008034	MZ536683	OK482948	OM836148	54° 28′N, 154° 29′E	5 April 2001
Gonatus madokai	KON222	KT429679	MZ008035	–	OK482949	OM836149	54° 28′N, 154° 29′E	5 April 2001
Gonatus madokai	KON223	KT429681	MZ008036	MZ536684	OK482950	OM836150	56° 23′N, 154° 03′E	21 March 2001
Gonatus madokai	KON224	KT429682	MZ008037	MZ536685	–	OM836151	58° 37′N, 167° 13′E	6 October 2008
Gonatus cf. *berryi* 1	KON225	MW940366	MZ008042	MZ536686	OK482951	–	53° 30′N, 152° 13′E	29 August 2010
Gonatus cf. *berryi* 2	KON226	KT429696	MZ008044	MZ536687	OK482952	–	41° 34′N, 15′° 11′E	22 November 2001
Gonatus cf. *berryi* 2	KON227	KT429695	MZ008045	MZ536688	OK482953	–	41° 34′N, 15′° 11′E	22 November 2001
Gonatopsis japonicus	KON228	MW940367	MZ008028	MZ536679	OK482954	–	48° 00′ N, 150° 06′E	17 October 2006
Gonatus madokai	KON229	KT429683	MZ008039	MZ536689	OK482955	OM836152	51° 00′N, 156° 01′E	12 March 2001
Gonatus madokai	KON230	KT429684	MZ008040	MZ536690	OK482956	OM836153	53° 25′N, 154° 11′E	15 March 2001
Gonatus onyx	KON231	KT429685	MZ008049	MZ536695	OK482957	–	46° 31′N, 167° 01′W	7 March 2009

(*Continued*)

Table 1. (*Continued.*)

Species name	Voucher number	CO1 (Katugin *et al.*, 2017) and new (59 individuals)	16S new (52 individuals)	18S new (54 individuals)	12S new (56 individuals)	28S new (32 individuals)	Origin	Collection time
Gonatus cf. *berryi* 2	KON232	KT429697	MZ008046	MZ536691	OK482958	OM836154	44° 48′N, 149° 16′E	26 April 2009
Gonatus cf. *berryi* 2	KON233	KT429698	MZ008047	MZ536692	OK482959	OM836155	43° 28′N, 148° 22′E	6 May 2009
Gonatus cf. *berryi* 1	KON234	KT429686	MZ008043	MZ536693	OK482960	–	50° 23′N, 157° 26′E	15 April 2009
Gonatus pyros	KON235	MW940368	MZ008057	MZ536694	–	OM836156	55° 38′N, 148° 28′E	20 April 2001
Gonatus onyx	KON236	KT429694	MZ008050	MZ536696	OK482961	OM836157	55° 38′N, 148° 28′E	19 April 2001
Gonatus onyx	KON237	KT429693	MZ008051	MZ536697	OK482962	OM836158	55° 42′N, 148° 04′E	19 April 2001
Gonatus onyx	KON238	KT429674	MZ008052	MZ536698	OK482963	OM836159	57° 51′N, 167° 41′E	6 October 2008
Gonatus onyx	KON239	KT429675	MZ008053	MZ536699	OK482964	OM836160	57° 51′N, 167° 41′E	6 October 2008
Gonatus pyros	KON240	KT429687	MZ008058	MZ536700	OK482965	OM836161	45° 35′N, 151° 18′E	21 April 2009
Gonatus tinro	KON241	KT429688	MZ008060	MZ536701	OK482966	–	43° 37′N, 147° 26′E	6 May 2009
Gonatus pyros	KON242	KT429690	MZ008059	MZ536702	OK482967	OM836162	44° 48′N, 149° 16′E	26 April 2009
Gonatus tinro	KON243	KT429689	MZ008061	MZ536703	OK482968	–	43° 37′N, 147° 26′E	6 May 2009
Gonatus tinro	KON244	KT429692	MZ008062	MZ536704	OK482969	OM836163	54° 33′N, 150° 38′E	2 April 2001
Gonatus tinro	KON245	KT429691	MZ008063	–	OK482970	–	55° 58′N, 154° 01′E	22 March 2001
Berryteuthis magister	KON246	MW940369	–	MZ536705	OK482971	OM836164	46° 11′ N, 140° 59′ E	19 May 2007
Berryteuthis magister	KON247	MW940370	MZ008048	MZ536706	OK482972	–	46° 11′ N, 140° 59′ E	20 May 2007
Berryteuthis magister	KON248	KT429716	MZ008054	MZ536707	OK482973	–	46° 11′ N, 140° 59′ E	14 April 2007
Berryteuthis magister	KON249	MW940371	MZ008055	MZ536708	OK482974	–	46° 11′ N, 140° 59′ E	17 April 2007
Berryteuthis magister	KON250	MW940372	MZ008056	MZ536709	OK482975	OM836165	46° 11′ N, 140° 59′ E	9 May 2007
Gonatus cf. *berryi* 2	KON251	–	–	MZ536710	–	–	54° 23′ N, 152° 06′ E	26 August 2010
Gonatus madokai	KON252	MW940373	MZ008041	MZ536711	–	–	57° 55′ N, 154° 57′ E	19 April 2013
Gonatus cf. *berryi* 2	KON253	MW940374	–	MZ536712	OK482976	–	51° 32′ N, 156° 06′ E	5 April 2013
Okutania anonycha	KON254	MW940375	MZ008064	–	OK482977	–	49° 04′N, 156° 16′E	21 June 2004
Okutania anonycha	KON255	MW940376	MZ008065	–	OK482978	–	49° 04′N, 156° 16′E	21 June 2004
Okutania anonycha	KON256	MW940377	–	MZ536713	OK482979	–	49° 04′N, 156° 16′E	21 June 2004
Okutania anonycha	KON257	KT429671	MZ008066	MZ536714	OK482980	OM836166	49° 04′N, 156° 16′E	21 June 2004
Okutania anonycha	KON258	MW940378	MZ008067	MZ536715	OK482981	–	49° 04′N, 156° 16′E	21 June 2004
Okutania anonycha	KON259	KT429672	–	–	OK482982	–	49° 04′N, 156° 16′E	21 June 2004
Okutania anonycha	KON260	KT429673	–	MZ536716	OK482983	OM836167	49° 04′N, 156° 16′E	21 June 2004

Table 2. Primers used for amplifying and sequencing five genes

Primer	Gene	Direction	5′–3′ sequence	Authors
hdLCO	CO1	Forward	TAATACGACTCACTATAGGGTTTTCWACWAAYCAYAARGATATRGG	Katugin *et al.* (2017)
hdHCO	CO1	Reverse	ATTAACCCTCAC TAAAGTAAACYTCWGGRTGACCAAARAA	Katugin *et al.* (2017)
16sar-L	16S	Forward	CGCCTGTTTAACAAAAACAT	Palumbi *et al.* (1991)
16S R	16S	Reverse	CCGRTYTGAACTCAGCTCACG	Puslednik and Serb (2008)
12S_F_Ceph	12S	Forward	CTTAAAAGGCTTGGCGGTG	This study
12S_R_Ceph	12S	Reverse	CTACCAAGTCCAYCTTC	This study
DigL2	28S	Forward	AAGCATATCACTAAGCGG	Tkach *et al.* (2013)
1500R	28S	Reverse	GCTATCCTGAGGGAAACTTCG	Tkach *et al.* (2013)
#3	18S	Forward	GYG GTG CAT GGC CGT TSK TRG TT	Machida and Knowlton (2015)
#5_RC	18S	Reverse	GTG TGY ACA AAG GBC AGG GAC	Machida and Knowlton (2015)

Table 3. Mean nucleotide frequencies and parsimony-informative sites for nucleotide sequences of five gene markers in the family Gonatidae

Sequence marker	Parsimony-informative sites/mean nucleotide frequencies	A	T/U	C	G
CO1	172	27.0	35.7	21.2	16.1
16S	50	32.7	38.3	10.8	18.2
12S	19	17.6	20.4	28.4	33.5
28S	44	20.9	16.5	33.0	29.6
18S	7	17.6	20.4	28.4	33.5

resolved with very small *P*-distances between them (Supplementary Tables S2 and S3). Four species of the genus *Gonatus* were grouped together, as were two species of genus *Gonatopsis*.

Even though 12S rRNA and 28S revealed fewer groups than 16S rRNA and CO1, these two markers suggested a clear subdivision between 'large' and 'small' forms of *B. borealis* (*P*-distances between them: 12S = 1.6%, 28S = 0.35%) (Supplementary Tables S4 and S5). Even fewer species groups were outputs for 18S (Table 4). Pairwise *P*-distances were calculated within and between 14 taxonomically identifiable groups, or 'species' in the Gonatidae (Supplementary Tables S2–S6).

Phylogenetic reconstructions

Phylogenetic reconstructions using *all three* approaches (BA, ML, and NJ) suggested monophyly in the family Gonatidae for four out of five gene markers (CO1, 28S, 16S rRNA, and 12S rRNA) with 100% bootstrap support and 1.0 posterior probability, whether or not sequences from the GenBank were used. The use of partial CO1, 28S, 16S rRNA, and 12S rRNA sequences yielded generally similar gene trees for the Gonatidae, showing intra-familial subdivision into species. However, there were certain differences between tree topologies constructed for squids from our collection and for the combined array of individuals (squids from our collection plus those from the GenBank).

CO1

Phylogenetic reconstructions for CO1 using NJ (Figure 1) and BA + ML (Figure 2), which were based exclusively on our squid samples, were virtually identical: four clades with almost 100% bootstrap support and high *P*-values, each of the four clades consisting of two sister taxa: (1) *Gonatopsis japonicus* + *Gonatopsis octopedatus* (*P* = 2.7%); (2) *G.* cf. *berryi* 1 + *G.* cf. *berryi* 2 (*P* = 4.8%); (3) *Gonatus kamtschaticus* + *Gonatus madokai* (*P* = 9.6%); and (4) *B. borealis* 'small' form + *B. borealis* 'large' form (*P* = 5.2%). All *Gonatus* spp. and *Gonatopsis* spp. formed a separate clear multi-clade branch with bootstrap values higher than 60% on the NJ and BA + ML trees.

For the combined array of squid samples (ours and from the GenBank), tree topologies for the CO1 using NJ (Figure 3) and BA + ML (Figure 4) were somewhat different from the above-mentioned reconstructions. There were four clades, each consisting of two sister taxa: (1) *G. japonicus* + *G. octopedatus*; (2) *G. kamtschaticus* + *G. madokai*; (3) *B. borealis* 'small' form + *B. borealis* 'large' form; and (4) *Gonatus tinro* + *Gonatus pyros*. Specimens identified morphologically as *G. pyros* split into two sister clades. The clade *G.* cf. *berryi* 1 + *G.* cf. *berryi* 2 was present only in the BA + ML reconstruction. Some GenBank sequences for particular species appeared in clusters with different taxa, e.g. *G. kamtschaticus* clustered either with *G. madokai* or with *G. pyros*; *G. tinro* clustered with *Gonatus onyx* (Supplementary Table S1); *Gonatus fabricii* (AF131873.1, Seibel *et al.*, 2000; AY681065.1, Lindgren *et al.*, 2005; AY557537.1, Lindgren *et al.*, 2004) clustered with *Gonatus steenstrupi* (Taite *et al.*, 2020); *G.* cf. *berryi* 2 (KT429695.1–KT429698.1; MW940374) appeared in the same cluster with *G. berryi* (AB749280.1, Bower *et al.*, 2012) and *Gonatus californiensis* (AF144724.1; GU112108.1; GU112109.1). *G. japonicus* appeared as a separate line close to the *G. madokai* cluster (Supplementary Table S1), most likely erroneously, since all other individuals of *G. japonicus* grouped in a single cluster. One sequence deposited under the name *G. berryi* (AF000040.1, Carlini and Graves, 1999) showed up as a sister taxon to *Gonatus antarcticus* (AY681064.1; AY557536.1) and *Gonatopsis* cf. *okutanii* (EU735401.1).

16S rRNA

Phylogenetic trees constructed for 16S rRNA based on only our array of the Gonatidae samples using NJ (Figure 5) and BA + ML (Figure 6) were similar. Four clades with high bootstrap support on the 16S rRNA NJ and BA + ML trees were composed of the following sister taxa: (1) *G. japonicus* + *G. octopedatus* (*P* = 0.27%); (2) *G. pyros* + *G. tinro* (*P* = 0.14%); (3) *G. kamtschaticus* + *G. madokai* (*P* = 0.43%); and (4) *B. borealis* 'small' form + *B. borealis* 'large' form (*P* = 1.28%). *Boreoteuthis makko* formed an independent branch with 0.86 *pp* and a 99% bootstrap value on the BA + ML reconstruction.

The use of both our samples and samples from the GenBank yielded somewhat different reconstructions. In the NJ (Figure 7)

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Table 4. Result of ABGD analyses

Marker	ABGD model	Barcode gap distances	Number of groups	Groups
CO1	K80, JC, simple distance	0.025	14	(1) *G. kamtschaticus*
				(2) *G. madokai*
				(3) *G.* cf. *berryi* 1
				(4) *G.* cf. *berryi* 2
				(5) *G. pyros*
				(6) *G. onyx*
				(7) *G. tinro*
				(8) *G. octopedatus*
				(9) *G. japonicus*
				(10) *B. borealis* large form
				(11) *B. borealis* small form
				(12) *B. makko*
				(13) *B. magister*
				(14) *O. anonycha*
16S	K80, JC, simple distance	0.008	10	(1) *G. kamtschaticus* *G. madokai* *G.* cf. *berryi* 1 *G.* cf. *berryi* 2
				(2) *G. pyros*
				(3) *G. onyx*
				(4) *G. tinro*
				(5) *G. octopedatus* *G. japonicus*
				(6) *B. borealis* large form
				(7) *B. borealis* small form
				(8) *B. makko*
				(9) *B. magister*
				(10) *O. anonycha*
12S	K80, JC	0.001	8	(1) *G. kamtschaticus* *G. octopedatus* *G. madokai* *G.* cf. *berryi* 1 *G.* cf. *berryi* 2 *B. magister* *O. anonycha*
				(2) *G. tinro*
				(3) *G. japonicus*
				(4) *B. borealis* large form
				(5) *B. borealis* small form
				(6) *B. makko*
				(7) *G. onyx*
				(8) *G. pyros*
	Simple distance	0.001	1	(1) *G. kamtschaticus* *G. madokai* *G.* cf. *berryi* 1 *G.* cf. *berryi* 2 *G. pyros* *G. onyx* *G. tinro* *G. octopedatus* *G. japonicus* *B. borealis* large form *B. borealis* small form *B. makko* *B. magister* *O. anonycha*
28S	K80	0.003	9	(1) *G. kamtschaticus*
				(2) *G. madokai* *G.* cf. *berryi* 2 *G. onyx* *G. pyros*
				(3) *G. tinro*
				(4) *G. japonicus*
				(5) *B. borealis* large form
				(6) *B. borealis* small form
				(7) *B. makko*
				(8) *B. magister*
				(9) *O. anonycha*
	JC	0.003	8	(1) *G. kamtschaticus*
				(2) *G. madokai* *G.* cf. *berryi* 2 *G. onyx* *G. pyros* *G. japonicus*
				(3) *G. tinro*
				(4) *B. borealis* large form
				(5) *B. borealis* small form
				(6) *B. makko*
				(7) *B. magister*
				(8) *O. anonycha*
	Simple distance	0.003	1	(1) *G. kamtschaticus* *G. madokai* *G.* cf. *berryi* 1 *G.* cf. *berryi* 2 *G. pyros* *G. onyx* *G. tinro* *G. octopedatus* *G. japonicus* *B. borealis* large form *B. borealis* small form *B. makko* *B. magister* *O. anonycha*
18S	K80, JC	0.001	6	(1) *B. makko* *B. borealis* large form *B. borealis* small form

(*Continued*)

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Table 4. (*Continued.*)

Marker	ABGD model	Barcode gap distances	Number of groups	Groups
				(2) *G. octopedatus* *G. japonicus*
				(3) *G. kamtschaticus* *G. madokai* *G.* cf. *berryi* 1 *G.* cf. *berryi* 2 *G. pyros* *G. tinro*
				(4) *G. onyx*
				(5) *B. magister*
				(6) *O. anonycha*
	Simple distance	0.001	1	(1) *G. kamtschaticus* *G. madokai* *G.* cf. *berryi* 1 *G.* cf. *berryi* 2 *G. pyros* *G. onyx* *G. tinro* *G. octopedatus* *G. japonicus* *B. borealis* large form *B. borealis* small form *B. makko* *B. magister* *O. anonycha*

JC, Jukes–Cantor model for distances; K80, Kimura model for distances.

and BA + ML (Figure 8) topologies, there were four groups of sister taxa: (1) *G. tinro* + *G. pyros*; (2) *G. japonicus* + *G. octopedatus* along with *G. fabricii* (EU735210.1, Lindgren, 2010) as an outgroup; (3) *G. kamtschaticus* + *G. madokai*; and (4) *B. borealis* 'small' form + *B. borealis* 'large' form. Some GenBank sequences for particular species appeared in clusters with different taxa, e.g. four individuals of *G. kamtschaticus* clustered with different species: *G. madokai*, *G. pyros*, and *G. onyx*. *G. fabricii* clustered with *B. borealis* 'small' form; *G. madokai* clustered with *Berryteuthis magister*; *G. tinro* clustered with *G. onyx*; and *Gonatopsis* sp. (EU735235, Lindgren, 2010) clustered with *G. octopedatus* (Supplementary Table S1). *G. antarcticus* (AY681032, Lindgren *et al.*, 2005) and *G.* cf. *okutanii* (EU735265, Lindgren, 2010) formed a separate cluster, which appeared as an outgroup to all other *Gonatus* and *Gonatopsis* groups. All the 16S rRNA trees were pretty much similar in that most species clades were clearly resolved on all topologies. Similar to the CO1 topologies, the following major groups were present on all the 16S rRNA trees: (1) *Gonatus* + *Gonatopsis*; (2) *B. borealis*; (3) *B. makko*; (4) *B. magister*; and (5) *Okutania anonycha*. The first group included the gonatids with five longitudinal rows of teeth on the radula, and the other four groups were represented by the gonatids with seven rows of teeth. On one tree (BA + ML for our data set), *B. makko* appeared as an outgroup to the rest of the Gonatidae (Figure 6); on the other three phylogenetic reconstructions (Figures 5, 7, and 8), *O. anonycha* appeared as an outgroup to all other clusters.

12S rRNA

Phylogenetic trees constructed for the 12S rRNA sequences of our Gonatidae samples using the NJ (Figure 9) and BA + ML (Figure 10) were virtually similar, and produced rather specific relationships between the 'species' groups. In most cases, combinations of clusters yielded low bootstrap values, and tree topologies did not show clear arrangements of 'species' clusters into major groups, which were evident on the CO1 and 16S reconstructions. Within the *Gonatus* + *Gonatopsis* group, relatively high (about 60%) bootstrap values were obtained for only two 'species' pairs when both our original data set and the combined array of our and GenBank data were used in the analysis: (1) *G.* cf. *berryi* 2 + *G. octopedatus* ($P = 0.8\%$); and (2) *G. pyros* + *G. tinro* ($P = 0.14\%$). *B. borealis* 'small' form and *B. borealis* 'large' form ($P = 1.6\%$), appeared in different branches on the BA + ML trees, and showed up as sister clades on NJ reconstruction with a bootstrap value lower than 50%. *O. anonycha* appeared within the low-supported group of *Gonatus* and *Gonatopsis*, and *G. madokai* and *G. kamtschaticus* appeared outside that group on all the trees. Of particular interest, *B. makko* + *B. magister* ($P = 2\%$) formed a group as two sister-species on both the NJ and BA + ML trees. Reconstructions based on the 12S rRNA sequences for a combined array of our and GenBank samples yielded similar topologies on the NJ and BA + ML trees, and are represented here as a single graph (Figure 11). Two sister groups had bootstrap support greater than 50%: (1) *G. pyros* + *G. tinro* and (2) *G.* cf. *berryi* 2 + *G. octopedatus*. Some species sequences from the GenBank clustered with other species, e.g. *G. kamtschaticus* with *G. pyros* and with *G. madokai*; and *G. tinro* with *G. onyx* (Supplementary Table S1). Those specimens from the GenBank were most probably misidentified. *Berryteuthis anonychus* (AY681018.1, Lindgren *et al.*, 2005) (=*O. anonycha*) appeared as an unresolved unit. Finally, the GenBank sequence for *Gonatopsis* sp. (AY681005.1, Lindgren *et al.*, 2005) clustered with *B. makko*, indicating that these sequences belong to one species.

28S

In the GenBank database, there was only one 28S sequence of 1635 bp for one gonatid specimen *G. fabricii* (MW233722.1, Fernandez-Alvarez *et al.*, 2021). The 28S phylogenetic trees using the NJ and BA + ML for 12 originally sequenced gonatid species plus one species *G. fabricii* from the GenBank were identical and differed only in posterior probabilities and bootstrap support values; therefore, one tree was represented and analysed (Figure 12). On that tree, each species formed either a separate cluster or a branch. One pair of sister-species was evident on the graph: (1) *G. madokai* + *G. kamtschaticus* ($P = 0.38\%$). *B. borealis* 'small' form and *B. borealis* 'large' form appeared as different clusters on clearly separated branches. All five-toothed gonatids (*Gonatus* and *Gonatopsis*) formed a large group with a bootstrap support 64%, and all seven-toothed gonatids (*O. anonycha*; *B. makko*; *B. borealis* 'small'; *B. borealis* 'large'; and *B. magister*) clustered as separate species at the base of the graph.

18S

Phylogenetic analysis of the 18S based on the combined array of 54 original sequences and 7 sequences for the Gonatidae from the GenBank did not yield clear patterns and interpretable clustering on the NJ and BA + ML trees.

Combined data analysis

Consensus trees constructed using BA (Figure 13) and ML (Figure 14), and based on all five gene markers for the Gonatidae from our collection and the two outgroup squid species were similar in that they clearly separated all individual squid species ('species' clusters) as well as a number of species groups with high bootstrap support and *pp* values. On both phylogenetic reconstructions (using ML and BA approaches), robust

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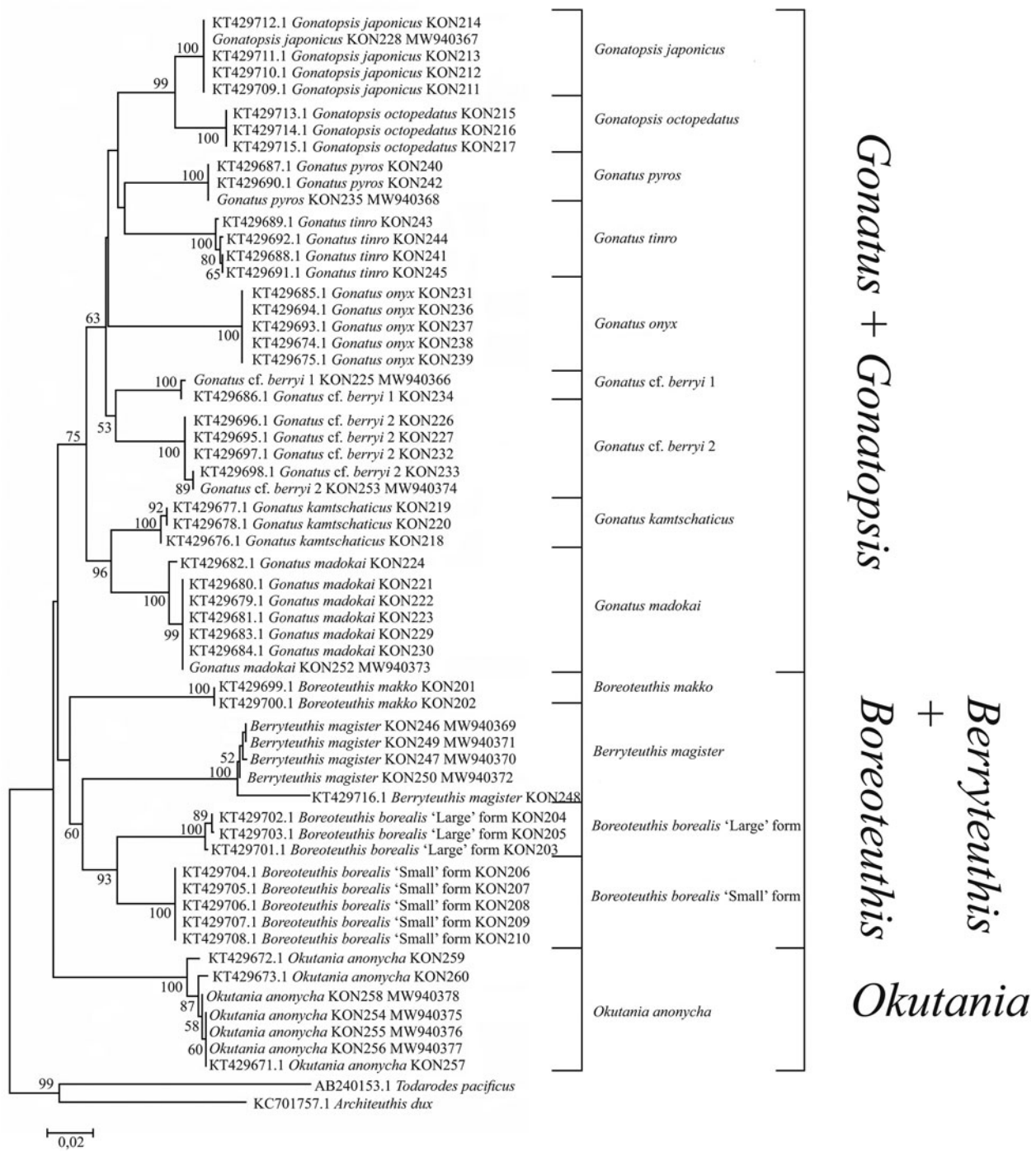

Figure 1. CO1 tree generated by NJ method for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species.

monophyly was revealed which was supported by high bootstrap and *pp* values for the each species within the family Gonatidae.

The main differences between the ML and BA topologies were observed in the branching of squid species with seven rows of teeth on the radula. On the ML reconstruction, there were three outbranchings: (1) two sister-species *B. borealis* 'large' form and *B. borealis* 'small' form; (2) two sister-species *B. makko* and *B. magister*; and finally (3) *O. anonycha*, which was the last to outbranch and appeared close to the group of species with five rows of teeth in the radula. Three basic clusters appear on the BA reconstruction: (1) *B. makko*; (2) 'large' and 'small' sister-forms of *B. borealis* along with *B. magister*, and (3) *O. anonycha* together with the group of species with five rows of teeth on the radula.

Discussion

Apart from two studies (Lindgren *et al.*, 2005; Katugin *et al.*, 2017), very few species of gonatid squids have been analysed using nucleotide sequence analysis, and those studies have provided only a general understanding on the relationships of selected species, either within the class Cephalopoda as a whole (Carlini and Graves, 1999; Takumiya *et al.*, 2005) or among the modern families within the order Teuthida (Lindgren, 2010). DNA barcoding based on the CO1 sequencing has proved to be an effective tool in distinguishing between different species in most of the main animal groups, including the phylum Mollusca (Folmer *et al.*, 1994; Anderson, 2000; Giribet *et al.*, 2006; Chen *et al.*, 2011), in particular, the class Cephalopoda

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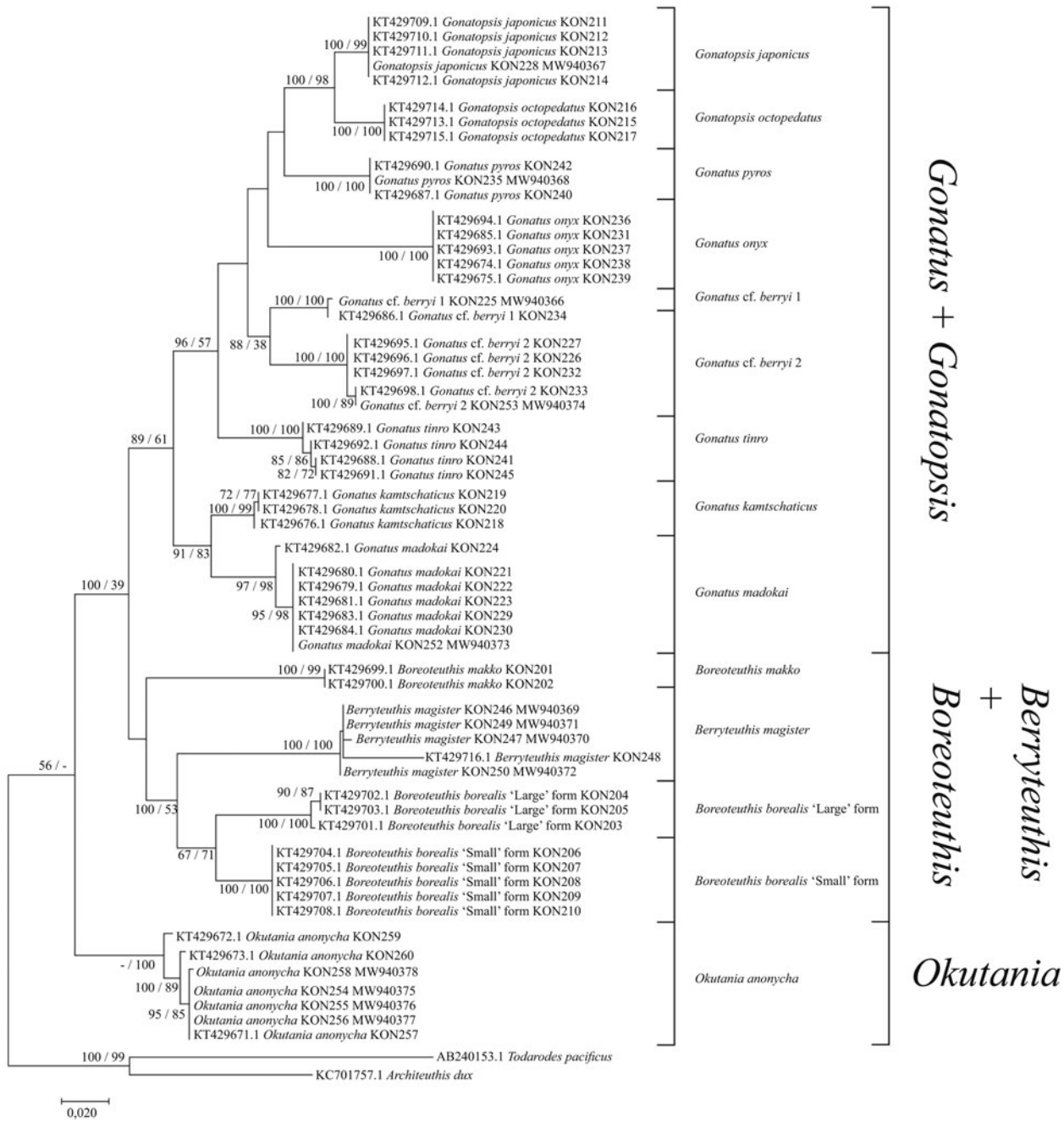

Figure 2. CO1 consensus tree generated by MCMC BA tree reconstruction and ML method for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species. Support levels for branches are shown for three approaches of tree reconstruction (%) in the following order: BA/ML.

(e.g. Carlini and Graves, 1999; Takumiya *et al.*, 2005; Dai *et al.*, 2012; Wen *et al.*, 2017; Maggioni *et al.*, 2020; Afiati *et al.*, 2022). It was shown earlier that partial sequencing of the CO1 gene marker (658 bp) can be successfully used for species identification in the family Gonatidae (Katugin *et al.*, 2017).

However, in some cases, more than one gene is needed to ensure clear species identification, especially among closely related species (Vences *et al.*, 2005; Barr *et al.*, 2009; Lv *et al.*, 2014; Liu *et al.*, 2017; Chan *et al.*, 2022). A number of studies suggest that, in particular, the CO1 marker does not necessarily yield good results in distinguishing between species; known exceptions include the Actinopterygii (Mabragaña *et al.*, 2011), Porifera (Schröder *et al.*, 2003; Neigel *et al.*, 2007), Anthozoa (Shearer *et al.*, 2002), Aranea (Spasojevic *et al.*, 2016), and Aves (Aliabadian *et al.*, 2013).

One of the first molecular markers used in the analysis of phylogenetic relationships among cephalopods was 16S rRNA (Bonnaud *et al.*, 1994). Phylogenetic trees clearly separated species, genera, and families in some teuthoids but included only a few gonatid squids (Bonnaud *et al.*, 1994; Bonnaud and Boucher-Rodoni, 2002). The 16S rRNA gene proved to be a valuable tool for delimitation of different squid genera (Sanchez *et al.*, 2018), and this marker was recommended as a barcode sequence for the class Cephalopoda (Sanchez *et al.*, 2016).

The use of 16S rRNA sequences in a study of the species complex *Sepia pharaonis* (Anderson *et al.*, 2007) was further enhanced by adding CO1 into the analysis (Anderson *et al.*, 2011). The combined use of 16S rRNA and CO1 was also successful in a population genetic study of the short-finned ommastrephid squid *Illex argentinus* (Roldán *et al.*, 2014), as well as for species identification and defining of phylogenetic relationships within the squid family Onychoteuthidae (Bolstad *et al.*, 2018).

Combined use of 12S rRNA and 16S rRNA sequences was used to suggest the paraphyletic nature of the cuttlefish genus

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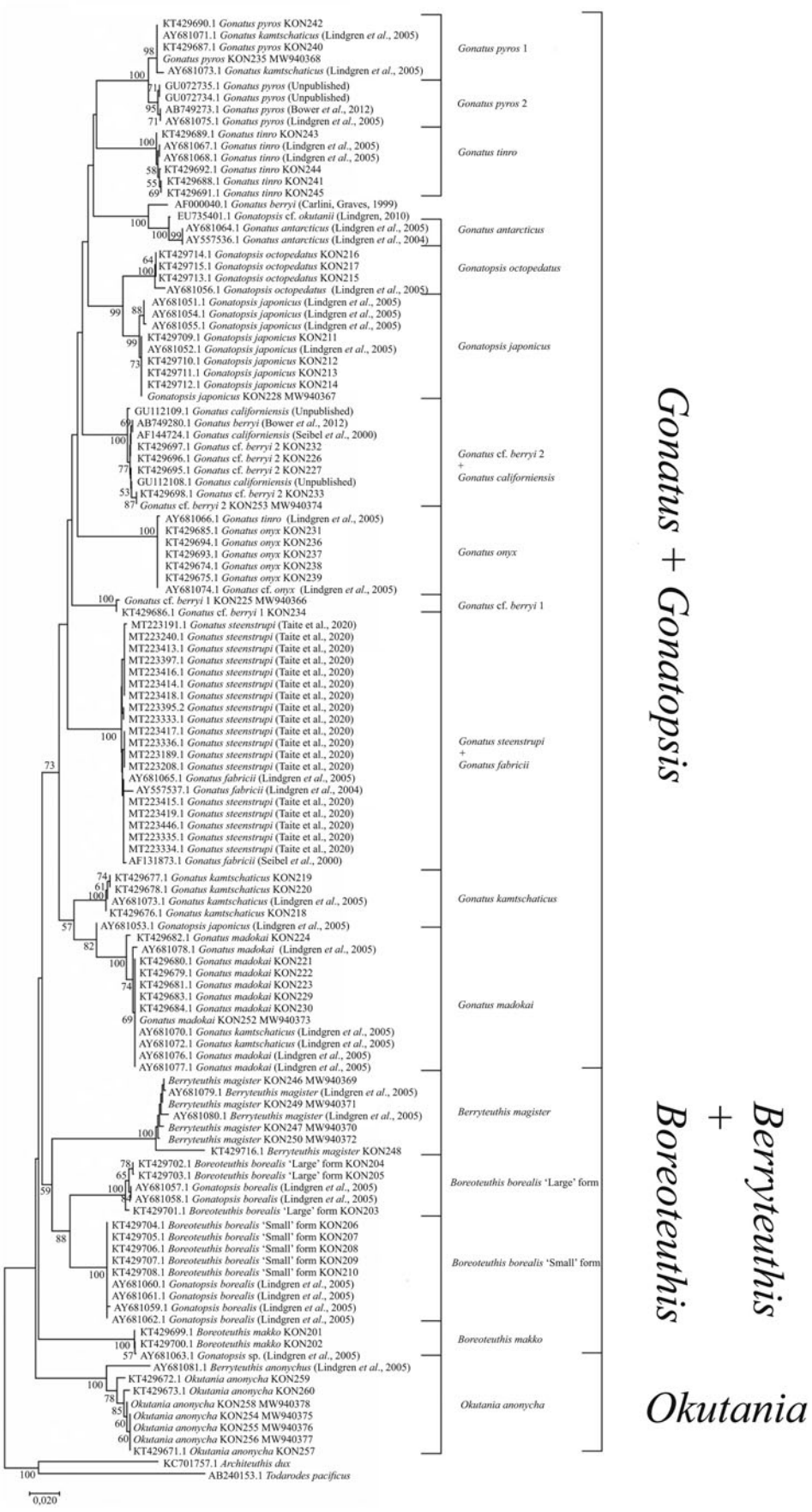

Figure 3. CO1 tree generated by NJ method for the individuals from the family Gonatidae only from our collection (Table 1) and for all analysed specimens including those from the GenBank (Supplementary Table S1).

Sepia and the absence of a direct relationship between geographic distribution and systematics in this genus (Bonnaud *et al.*, 2006). Joint use of 28S and 18S sequences was used to propose phylogenetic relationships among 24 species of coleoid (mainly decapodan) and nautiloid cephalopods (Bonnaud and Boucher-Rodoni, 2002). The research reported here appears to be the first to use an array of all the above-mentioned five gene markers in the analysis of species divergence in the family

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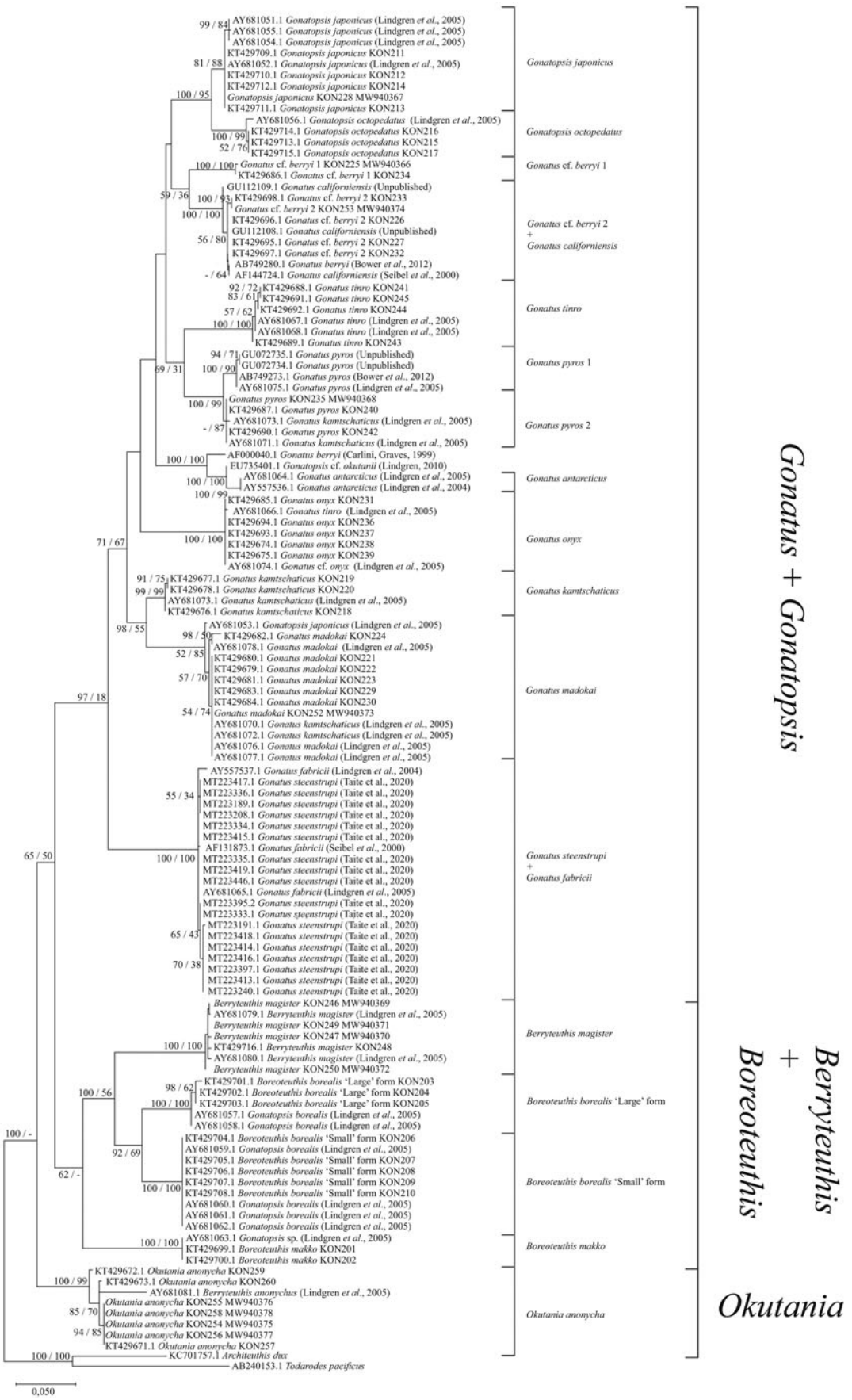

Figure 4. CO1 consensus tree generated by MCMC BA tree reconstruction and ML method for individuals from the family Gonatidae only from our collection (Table 1) and for all analysed specimens including those from the GenBank (Supplementary Table S1). Support levels for branches are shown for three approaches of tree reconstruction (%) in the following order: BA/ML.

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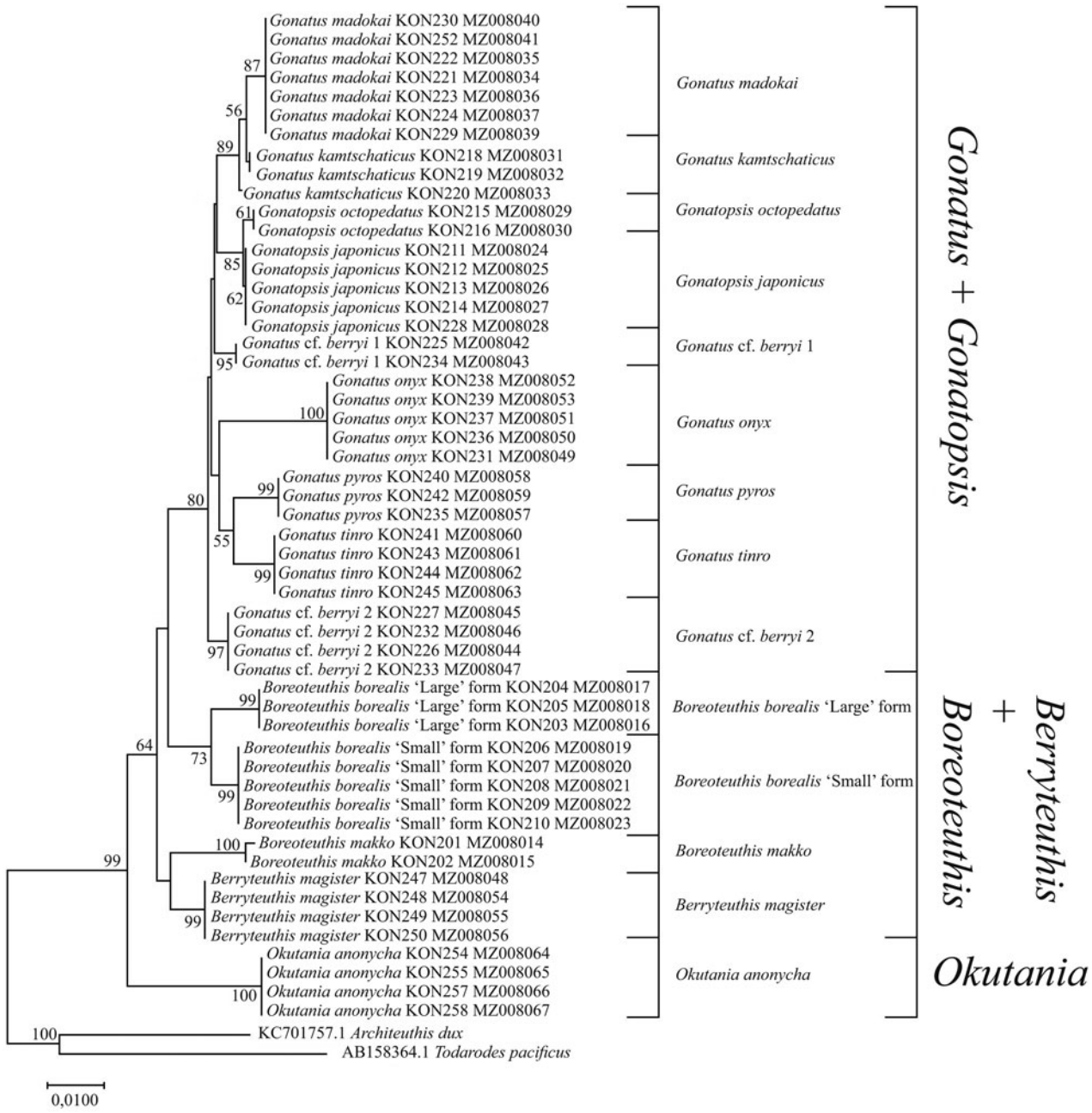

Figure 5. 16S tree generated by NJ method for individuals from family Gonatidae only from our collection (Table 1) and outgroup species.

Gonatidae, using them both separately and combined to generate consensus phylogenies.

Among gene markers of potential use in distinguishing between molluscan species, 18S has been included with 28S (Lindgren *et al.*, 2004) but there were recommendations to use 18S very carefully, particularly for phylogenetic analysis, in other molluscan taxa as well as in the Cephalopoda (Bonnaud and Boucher-Rodoni, 2002). In the present study, a part of 18S (400 bp) was used for the first time to estimate its applicability for distinguishing between the species of the Gonatidae, and it appeared that only one species, *B. magister*, was clearly distinguished while the other 13 gonatid species was a poorly resolved group. The ABGD analyses and phylogenetic reconstructions using 18S were unable to resolve either species (except for *B. magister*) or species groups and genera in the Gonatidae. Despite the existence of an insertion in the *B. magister* 18S sequence and a number of species-specific nucleotide changes (Supplementary Table S7), the resultant low level of between-species differentiation with the 18S marker (with seven parsimony-informative sites) did not support morphologically identifiable species in most cases. Therefore, 18S cannot be considered a suitable molecular marker for the purposes of either barcoding or phylogenetic analysis for the Gonatidae. All the other analysed gene markers were highly informative and revealed separate groups or clusters, which could be considered as different species with relatively high values of support and probabilities. The use of an assemblage of gene markers proved to be effective in species identification and will presumably aid further phylogenetic analyses of the Gonatidae.

The present study found that patterns of differentiation among the gonatid species are similar for the four molecular markers 12S rRNA, 16S rRNA, 28S, and CO1. However, the 12S rRNA, 16S rRNA, and 28S *P*-distances between different species and between individuals within a species were significantly smaller compared to the respective CO1 *P*-distances (Supplementary Tables S2–S5). Subdivision of the Gonatidae into species based on the 12S

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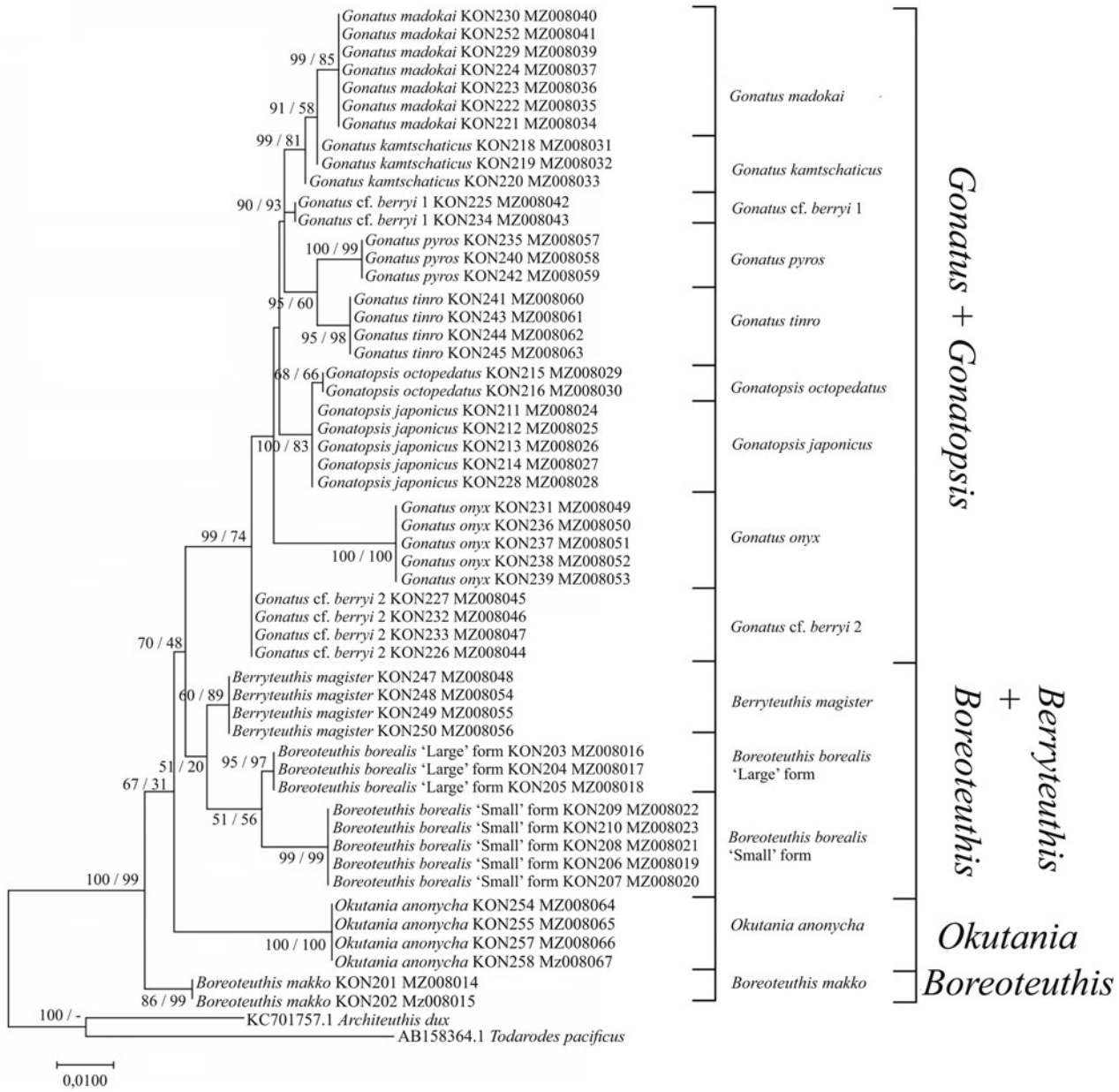

Figure 6. 16S consensus tree generated by MCMC BA tree reconstruction and ML method for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species. Support levels for branches are shown for three approaches of tree reconstruction (%) in the following order: BA/ML.

rRNA, 16S rRNA, and 28S sequence analysis generally followed the reciprocal monophyly criterion (Kizirian and Donnelly, 2004) but it did not fit the '10× rule', according to which the level of divergence between species is at least ten times higher than that within a species (Hickerson *et al.*, 2006). In contrast, CO1 met both criteria. The ABGD approach also suggested that CO1 sequences distinguished among the gonatid species more clearly than 12S rRNA, 16S rRNA, or 28S. However, the latter three gene sequences were more effective than the conventional barcode CO1 in a number of cases, such as in separation between *G.* cf. *berryi* 1 and *G.* cf. *berryi* 2, and between large- and small-sized *B. borealis*, thus corroborating species level divergence between morphologically similar forms.

Analyses of the Gonatidae samples from our collection suggested that, among squid individuals matching the morphological description of *G. berryi*, there were two morphologically similar but genetically different groups. They appeared as independent clusters on gene trees, and can presumably be considered as separate species (Figures 1, 2, 5, 6, 9, and 10). Sequences of 16S rRNA and 12S rRNA were better than CO1 for resolving *G.* cf. *berryi* 1 and *G.* cf. *berryi* 2. On the 16S rRNA and 12S rRNA gene trees, these 'species' appeared in different lineages but as sister clades on the CO1 reconstruction (with 0.88 *pp* and 38% bootstrap support).

When sequences from the GenBank were added to the analyses of the Gonatidae, specimens identified as *G. berryi* (GenBank) and *G.* cf. *berryi* (our data) appeared in three different branches on the CO1 tree, and in two clades on the 12S rRNA and 16S rRNA trees. This observed split of *G. berryi* into separate lineages suggested the existence of hidden taxonomic differences among the examined squid with similar morphological traits. Which of those taxa are actually *G. berryi* is a matter of speculation at present and further research into the taxonomy of this complex of morphologically similar species is needed.

On the 16S rRNA tree, *G.* cf. *berryi* 2 appeared in one group with *G. berryi* AY681034.1 (from Lindgren *et al.*, 2005). However, on the CO1 tree, the same individuals of *G.* cf. *berryi* 2 from the Northwest Pacific clustered together

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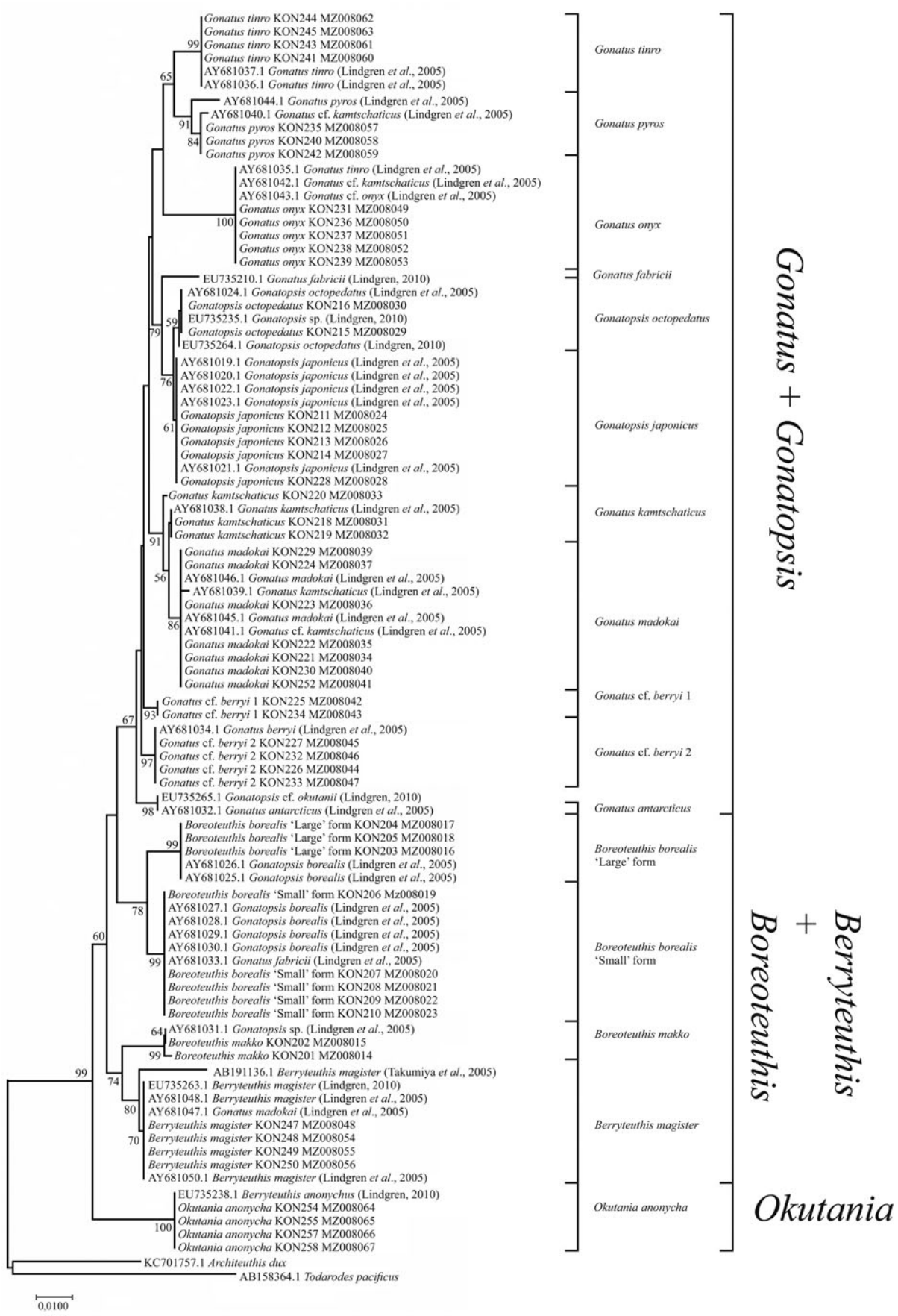

Figure 7. 16S tree generated by NJ method for individuals from family Gonatidae only from our collection (Table 1) and for all analysed specimens including those from the GenBank (Supplementary Table S1).

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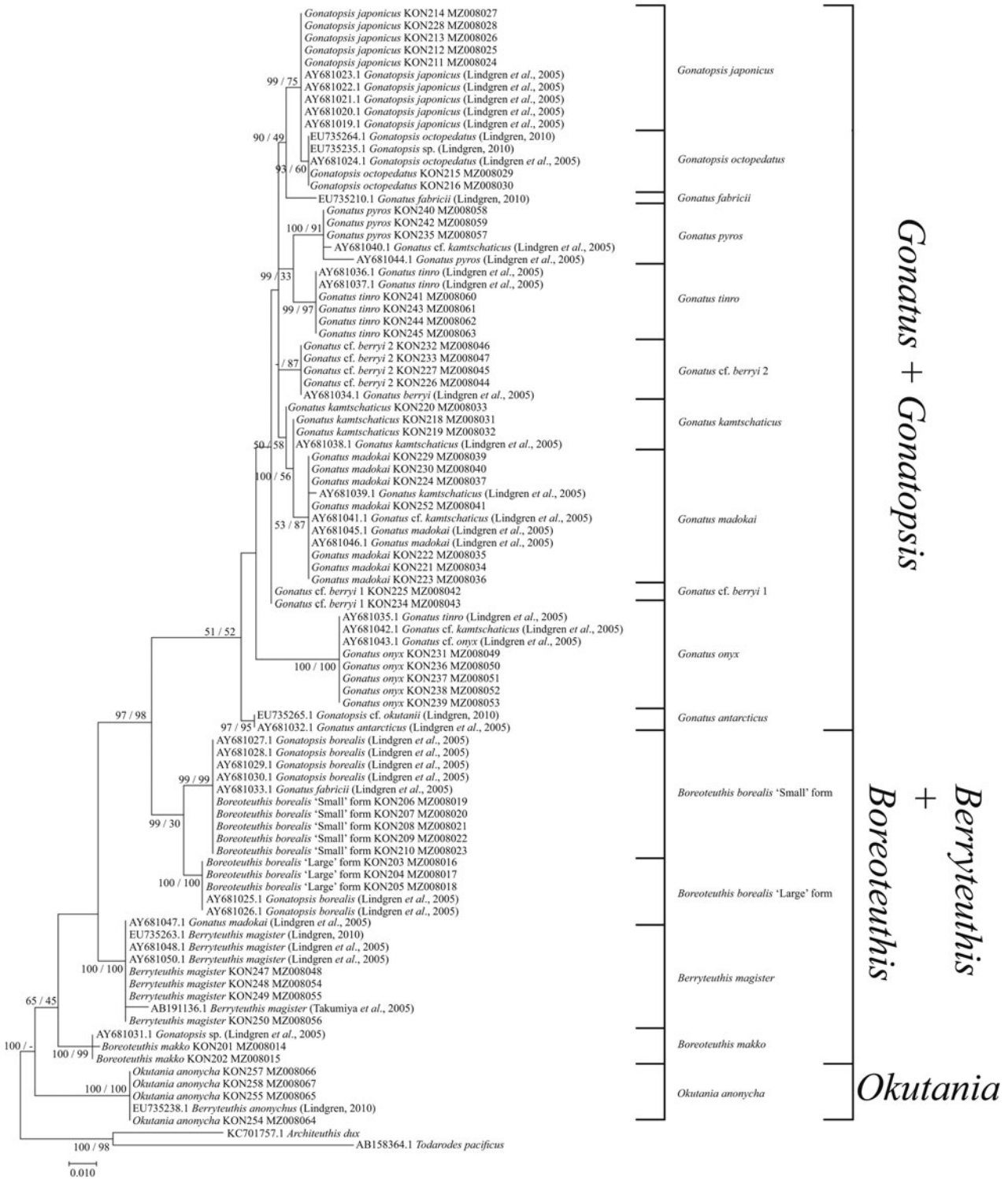

Figure 8. 16S consensus tree generated by MCMC BA tree reconstruction and ML method for individuals from the family Gonatidae only from our collection (Table 1) and for all analysed specimens including those from the GenBank (Supplementary Table S1). Support levels for branches are shown for three approaches of tree reconstruction (%) in the following order: BA/ML.

with *G. berryi* AB749280.1 (from Bower *et al.*, 2012) and with *G. californiensis* (GU112109, GU112108, AF144724) from the Northeast Pacific. Such a pattern of clustering suggests either erroneous identification of specimens of *G. californiensis* from which sequences were deposited in the GenBank, or a much wider geographic distribution for this gonatid species, which was considered to be endemic to the eastern North Pacific and to represent the southernmost species of the genus *Gonatus* there (Young, 1972).

Since correct identification of these species is based primarily on tentacle club morphology, identification becomes difficult in individuals with broken tentacles; and other features, such as fin size and proportion, may provide important distinctions. Berry (1912) first described young individuals of *G. fabricii* with very large fins, and that morphological feature was the basis for establishing the new species *G. berryi* (Naef, 1923). Later, the species was re-described and individuals with extremely large hooks, strongly differentiated tentacle clubs and large

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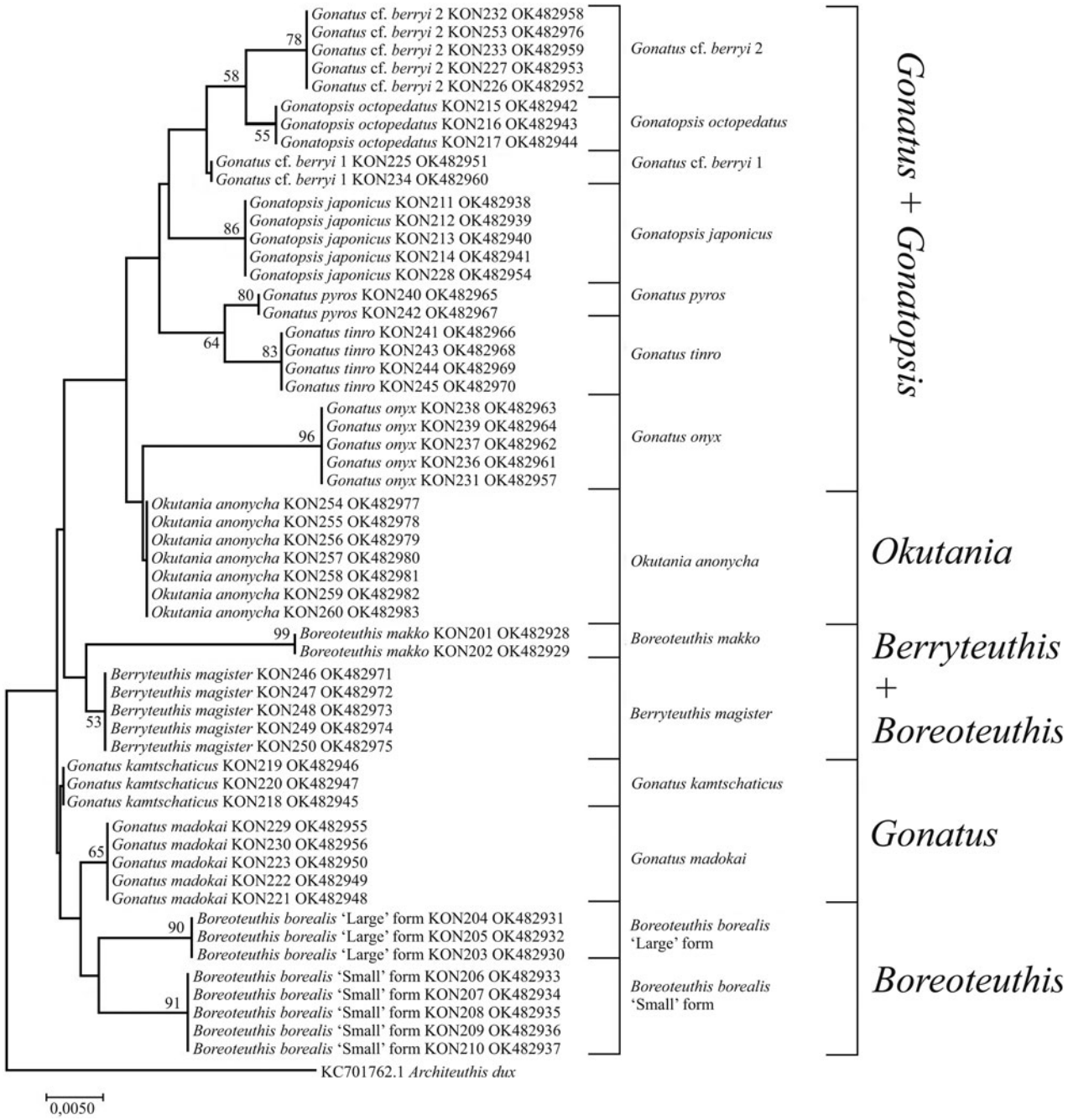

Figure 9. 12S tree generated by NJ method for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species.

fins were considered to represent *G. berryi* (Young, 1972). In contrast, individuals with smaller hooks, a somewhat different arrangement of hooks and suckers on the tentacle clubs, and much smaller fins were placed by Young (1972) into a new species, *G. californiensis*.

Our data on gonatid squid measurements (Katugin, unpublished) suggest that there exists individual variability in the above-mentioned morphological features and, in some cases, the so-called 'species characters' may overlap, which hampers correct identification, especially in individuals with missing or broken tentacles. Individuals that belong to *G.* cf. *berryi* 1 and *G.* cf. *berryi* 2 are characterized by a large, *G. berryi*-like central hook on the club manus but they present a wide variety of features such as the relative size of the fins, from 'large' fins characteristic of *G. berryi* to the (much) 'smaller' fins peculiar to *G. californiensis*. Undoubtedly, further research into the morphology of the genetically differentiated groups, provisionally named *G.* cf. *berryi* 1 and *G.* cf. *berryi* 2, is needed to determine their taxonomic status.

Although, in the Gonatidae, 12S rRNA, 16S rRNA, and 28S exhibited lower variability levels than CO1, the tree-free ABGD approach applied to all of four gene markers showed clear separation between large- and small-sized *B. borealis*, and *P*-distances indicated high levels of genetic divergence between these two groups (CO1: $P = 5.2\%$; 12S: $P = 1.6\%$; 16S: $P = 1.3\%$; and 28S: $P = 0.4\%$). Previously, tree-free ABGD using the CO1 sequence revealed significant differentiation between these two groups (Katugin *et al.*, 2017). The molecular differences between these size cohorts may therefore indicate the presence of two different species within the *B. borealis* species complex. Representatives of the 'large' and 'small' cohorts in *B. borealis* differ from each other in a number of biological traits, such as size-at-maturity

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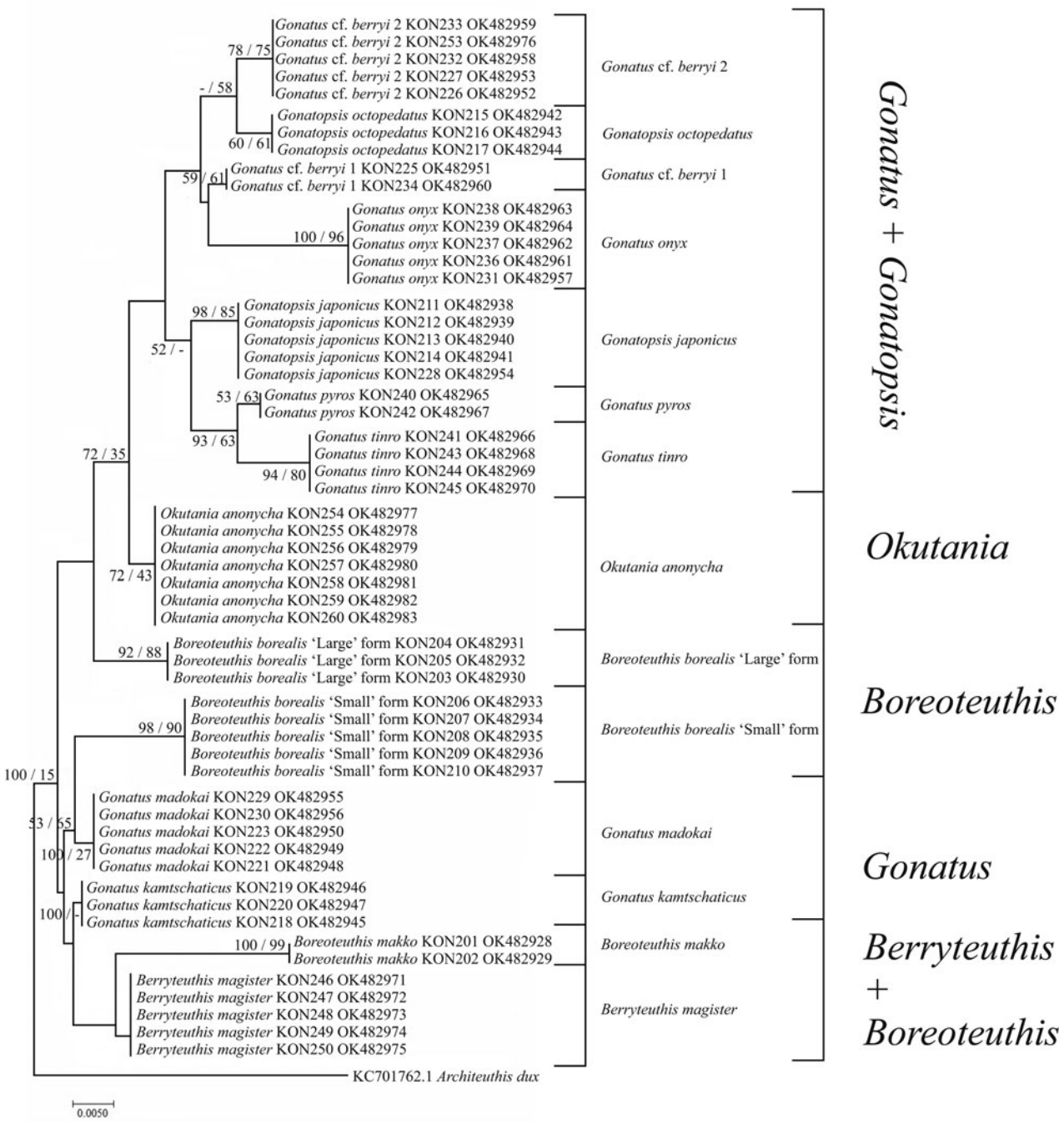

Figure 10. 12S consensus tree generated by MCMC BA tree reconstruction and ML method for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species. Support levels for branches are shown for three methods of tree reconstruction (%) in the following order: BA/ML.

and (presumably) growth rates, and also in patterns of geographic distribution (Nesis, 1989; Nesis and Nezlin, 1993; Zuev *et al.*, 2007), which agrees with molecular evidence indicating that these two size cohorts are different taxonomic units presumably at the species rank.

Inclusion of sequence data for gonatid species available in the GenBank not only added valuable new information on the genetic subdivisions among the family members, but also revealed several inconsistencies, which should be considered in molecular phylogenetic studies of that group of squid. Sequence analyses from both our collection and GenBank suggested that, based on the CO1, 12S rRNA, 16S rRNA, and 28S tree topologies, such inconsistencies were revealed for the pair *G. berryi*–*G. californiensis*, and in the following taxa: *G. pyros* from the North Pacific, and morphologically very close *G. fabricii* and *G. steenstrupi* from the North Atlantic (Figure 3).

Species identified as *G. pyros* formed two sister clades on the CO1 and 16S rRNA trees. Those clusters had high bootstrap support of >90%. However, *P*-distances between them were small (1.3% for CO1; 0.9% for 16S rRNA). One group of *G. pyros* specimens consisted of individuals from our collection, which were captured in the northwestern Pacific Ocean, and of individuals from the GenBank. Another group was composed of animals from the GenBank alone. Though *G. pyros* is clearly separable from all other gonatids in being the only species with a large photophore on the ventral surface of each eye (Young, 1972), in some cases, shreds of eye tissue can be mistaken for a photophore, which may lead to a species misidentification. Further investigation of the '*G. pyros*' group is needed to exclude possible erroneous species identification.

On the CO1 trees constructed for the combined data set (Figures 3 and 4) and using BA, ML, and NJ approaches, two

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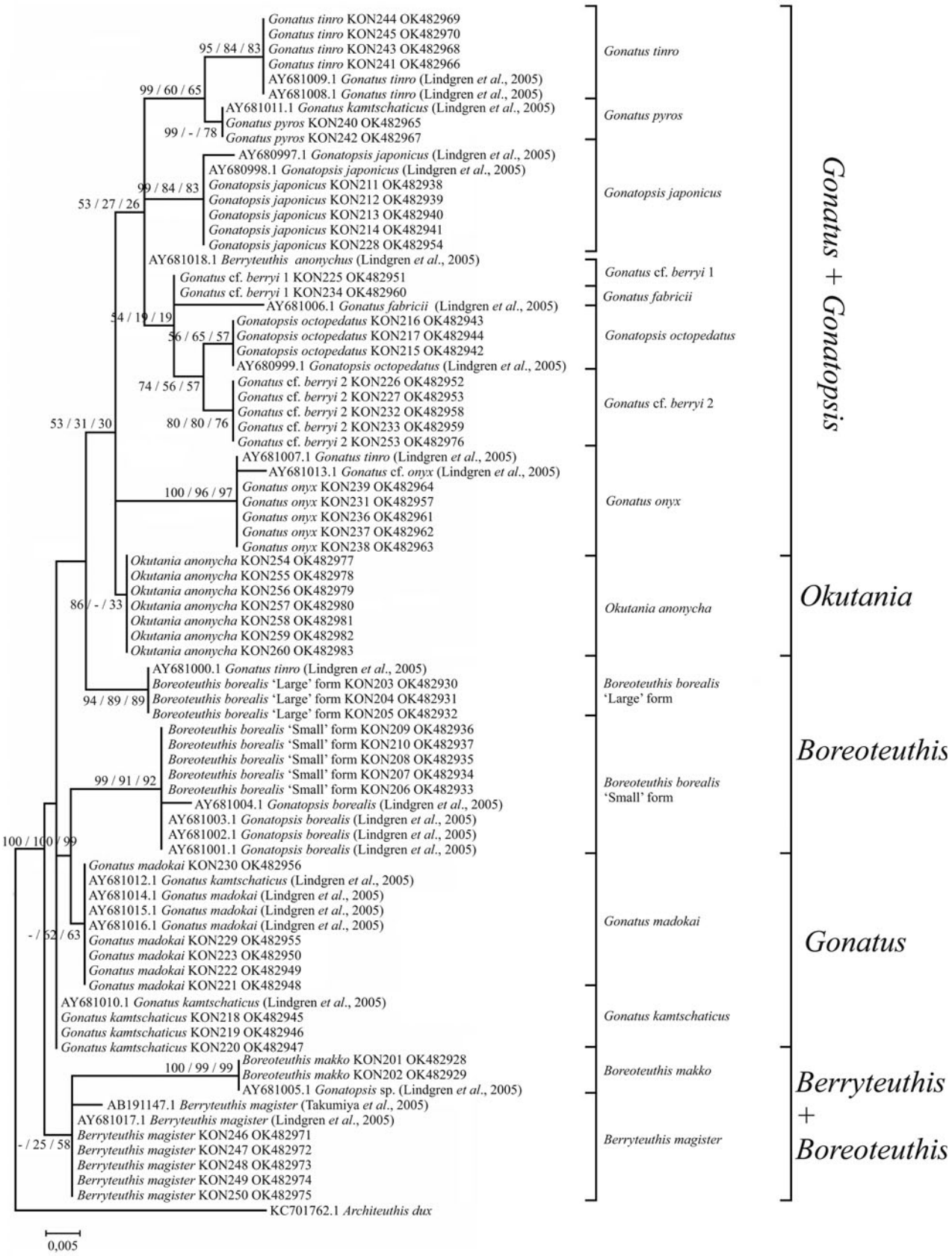

Figure 11. 12S consensus tree generated by MCMC BA tree reconstruction, ML, and NJ methods for individuals from the family Gonatidae only from our collection (Table 1) and for all analysed specimens including those from the GenBank (Supplementary Table S1). Support levels of branches are shown for three methods of tree reconstruction (%) in the following order: BA/ML/NJ.

nominal species, *G. fabricii* and *G. steenstrupi*, showed no genetic differentiation between each other, which concurred with the recently published study on identification of the North Atlantic cephalopods using morphology and DNA barcoding (Taite *et al.*, 2020). Sequences deposited in the GenBank under the name *G. fabricii* (Lindgren *et al.*, 2005; Lindgren, 2010) were

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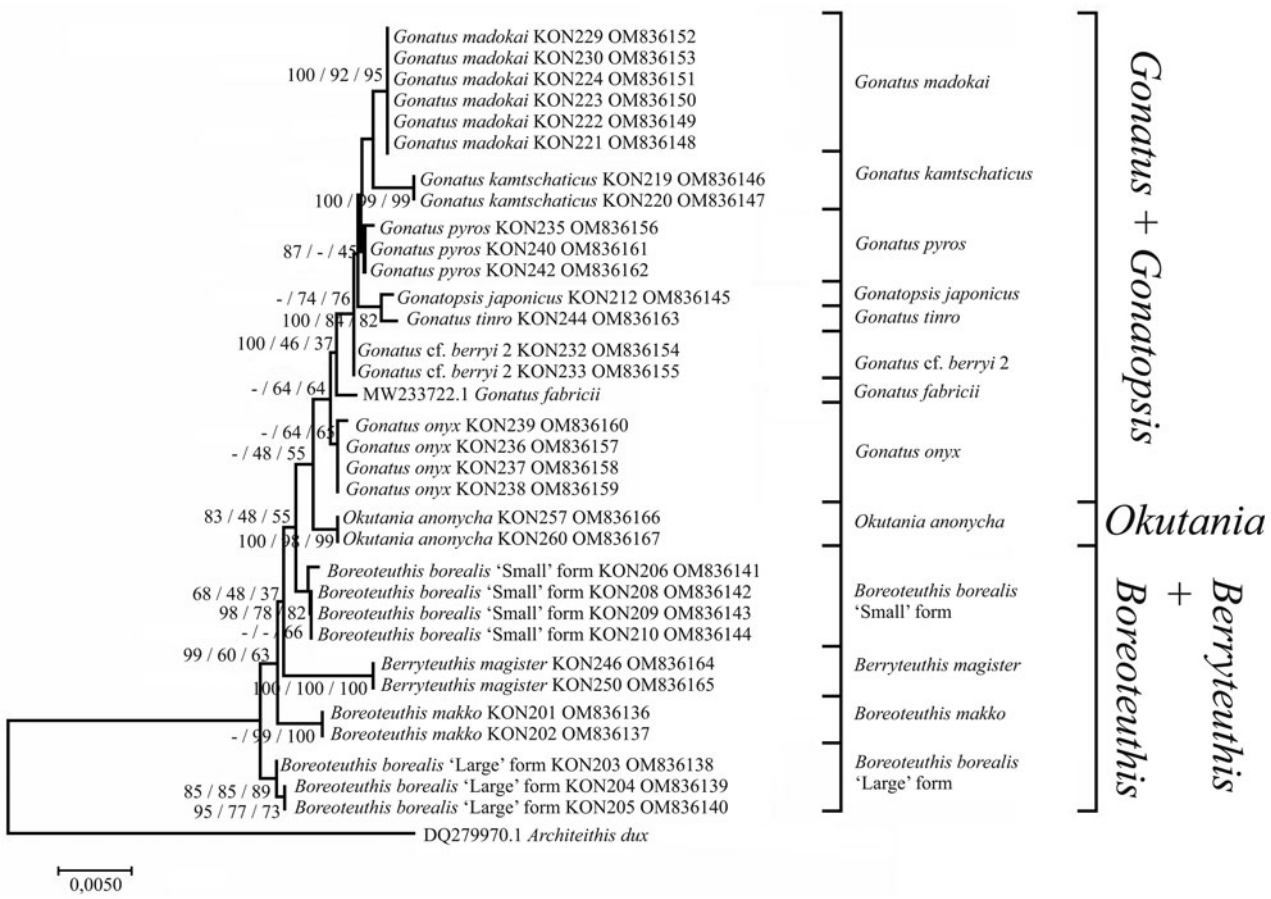

Figure 12. 28S consensus tree generated by MCMC BA tree reconstruction, ML, and NJ methods for individuals from the family Gonatidae only from our collection (Table 1) and for all analysed specimens including those from the GenBank (Supplementary Table S1). Support levels for branches are shown for three methods of tree reconstruction (%) in the following order: BA/ML/NJ.

used in our study. Specimens analysed by Taite *et al.* (2020), along with those from Vecchione *et al.* (2010), formed a single haplotype network, suggesting that they comprise one species. However, some individuals were morphologically identified as *G. fabricii* and some as *G. steenstrupi*. All of them differed genetically from *G. fabricii* collected from the Bear Seamount and in the Arctic (Lindgren, unpublished), which may indicate that they represent *G. steenstrupi*. Therefore, GenBank sequences of *G. fabricii* (Lindgren *et al.*, 2005; Lindgren, 2010) may in fact belong to *G. steenstrupi*. Possible misidentification could be due to significant overlap in morphological traits between these two closely related species, as pointed out earlier (e.g. Vecchione *et al.*, 2010). This example, along with the above-mentioned issues for some other gonatid species, highlights the importance of correct initial morphologically based species identification for further interpretation of the observed genetic divergence at the species level.

Three molecular markers (CO1, 16S rRNA, and 28S) have been beneficial in understanding the systematic relationships among the higher level taxonomic groups in the cephalopods (e.g. Lindgren, 2010; Allcock *et al.*, 2015; Sanchez *et al.*, 2016). In our study of the family Gonatidae, individual phylogenies constructed using sequences of four gene markers (i.e. with the addition of 12S rRNA to the other three) along with consensus trees for the combined sequences, have provided further insight into the taxonomy of the Gonatidae above the species level. At the genus level, conventional morphology-based taxonomy of the Gonatidae earlier suggested that the family is composed of either three genera: *Gonatus*, *Gonatopsis*, and *Berryteuthis* (e.g. Okutani, 1968; Nesis, 1973, 1987, 1997; Bublitz, 1981; Okutani and Clarke, 1992), or four genera: *Gonatus*, *Gonatopsis*, *Berryteuthis*, and *Eogonatus* (e.g. Okutani *et al.*, 1988; Roper *et al.*, 2010). The present study corroborates earlier studies using multi-locus biochemical genetics (allozymes) approaches (Katugin, 2004), and molecular genetic (mtDNA) approaches (Lindgren *et al.*, 2005; Katugin *et al.*, 2017). They agree that conventional views on generic subdivision of the family Gonatidae do not reflect phylogenetic relationships among the gonatid species, and therefore, some of the genera may be invalid. In particular, gonatid species with eight arms and without tentacles in juveniles and adults were considered to belong to the widely accepted nominal genus *Gonatopsis* Sasaki, 1920. However, some of the species with eight appendages in the arm crown in that genus, e.g. *Gonatopsis borealis* (recently identified as *B. borealis*) possess a radula with seven longitudinal rows of teeth, and others (e.g. *G. octopedatus* and *G. japonicus*) have only five rows of teeth on the radula. Genetic evidence suggested that those two groups of the eight-armed species are paraphyletic, and therefore should be placed in different genera (Katugin, 2004; Lindgren *et al.*, 2005).

Another example of an artificial combination of distantly related species in one genus concerns *B. magister* (initially described as *Gonatus magister* Berry, 1913) and *B. anonychus* (initially described as *Gonatus anonychus* Pearcy and Voss, 1963), which were placed in the same genus based on two character states: the absence of hooks on the tentacle club and a radula with seven teeth (Okutani, 1968; Nesis, 1973; Jefferts, 1983). Allozyme and mtDNA studies suggest strong genetic divergence between these two species, arguably sufficient evidence that they may not belong to the same genus (Katugin, 2004; Katugin

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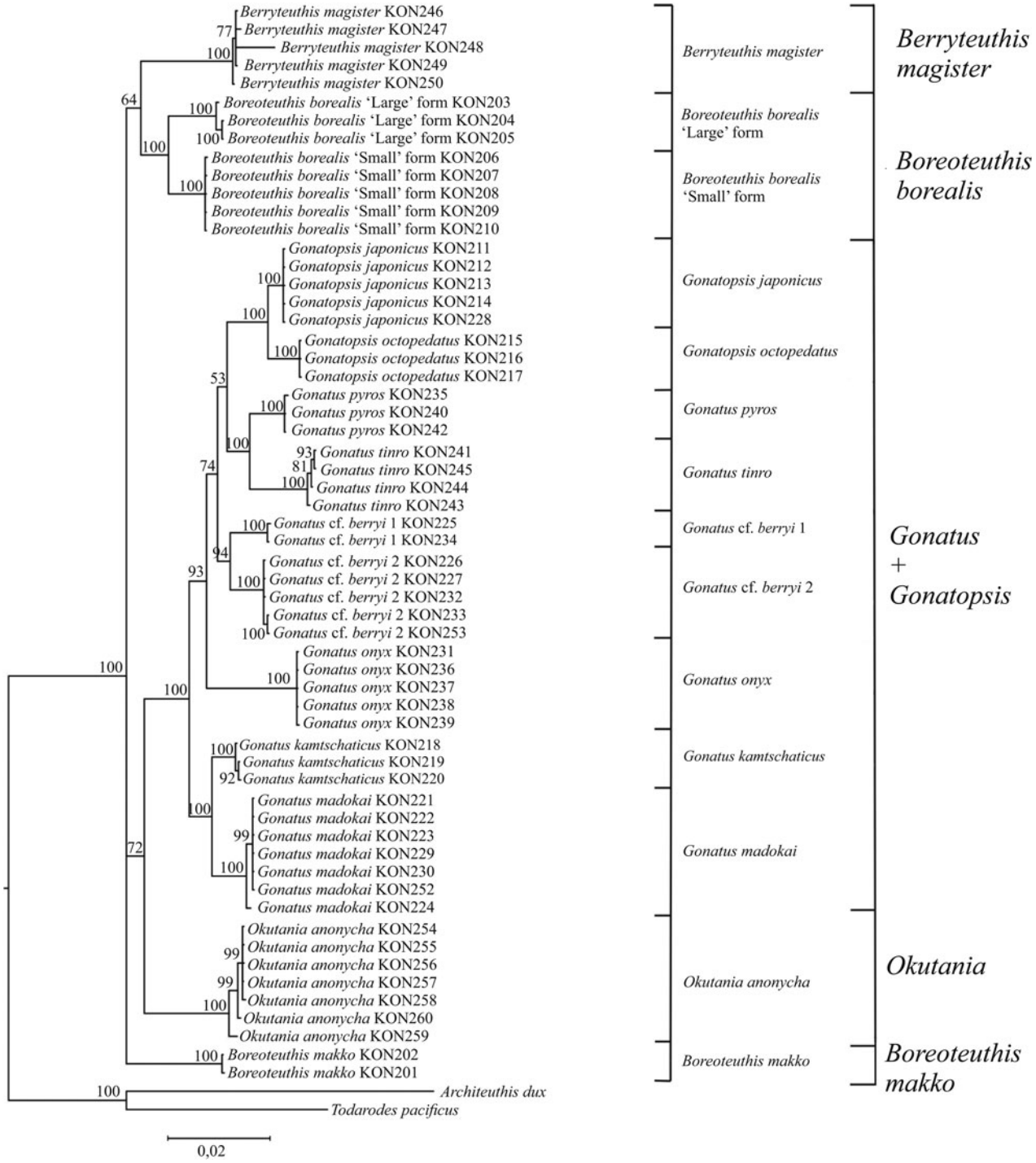

Figure 13. Consensus tree generated for the combined CO1, 16S, 18S, 12S, and 28S data by ML method for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species.

et al., 2017). To address the inconsistencies between morphologic evidence, genetic divergence patterns and taxonomy at the genus level within the Gonatidae, two new genera were introduced: *Boreoteuthis* Nesis, 1971, which was first proposed as a separate subgenus in the genus *Gonatopsis*; and *Okutania* Katugin, 2004. As for all gonatid species known to date, morphological evidence and patterns of molecular divergence suggest taxonomic subdivision of the family into two subfamilies: Gonatinae and Berryteuthinae (Katugin, 2004). The former includes species with five rows of teeth on the radula, and the latter those with seven rows. The Gonatinae comprise all species of the genus *Gonatus* Gray, 1849, and two species of the genus *Gonatopsis* s. str. Sasaki, 1920, and together they constitute a monophyletic group on the consensus gene trees (Figures 12 and 13) with high bootstrap support (100%) and *pp*-values (1.00). From all the obtained individual gene and consensus phylogenies, *G. octopedatus* and *G. japonicus* form a monophyletic group (*pp* 1.00 and bootstrap 100%) and appear as sister-species within the large monophyletic group of *Gonatus* spp. (*pp* 1.00, bootstrap 100%), along with a monophyletic group of two sister-species, *G. kamtschaticus* and *G. madokai* (*pp* 1.00, bootstrap 95%), and other species for which combination into groups is not highly supported by bootstrap values.

Our findings suggest that evolutionary loss of tentacles in juveniles and adults in *Gonatopsis* s. str. happened independently from the loss of tentacles in the *Boreoteuthis* lineage, and was

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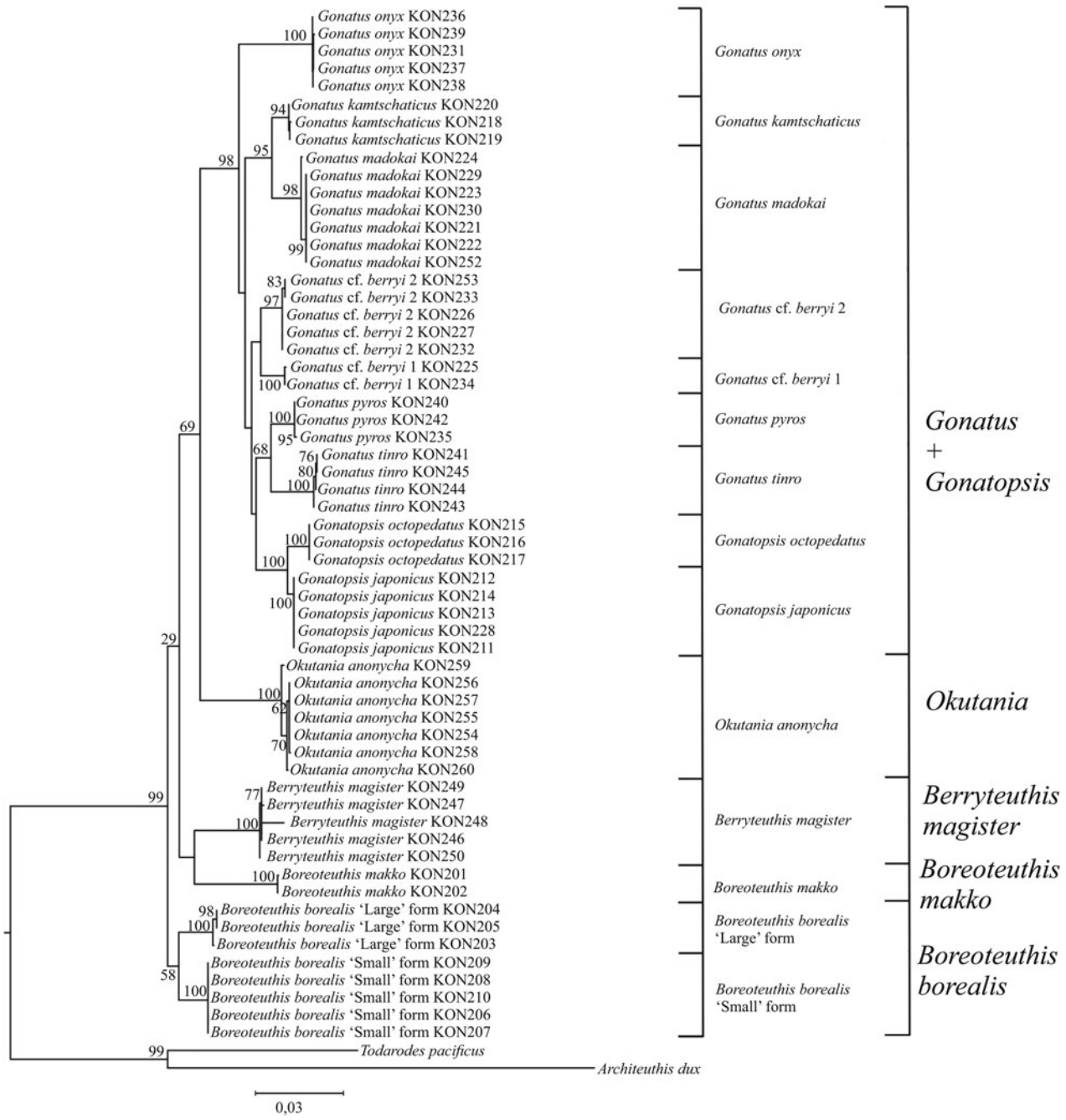

Figure 14. Consensus tree generated for the combined CO1, 16S, 18S, 12S, and 28S data by MCMC BA tree reconstruction for individuals from the family Gonatidae only from our collection (Table 1) and outgroup species.

not accompanied by strong genetic divergence of two *Gonatopsis* species from the other five radular-toothed gonatids of the *Gonatus* lineage, which possess tentacles in the adult stage. Therefore, from a molecular genetic standpoint, species that comprise *Gonatopsis* s. str., in fact, belong to the genus *Gonatus*. Taking these findings into account, *Gonatopsis* should be downranked to the subgenus level within genus *Gonatus*. With respect to the Berryteuthinae, which comprises species with seven rows of teeth on the radula, the analysis of tree topologies constructed for four genes, both individually and when combined, revealed specific patterns of their molecular divergence. Contrary to an evident evolutionary commonality (=monophyly) of all Gonatinae species with radular teeth in five rows, the modern seven-toothed gonatids are split into several different lineages, which stem from the basis of the family gene trees. Genetic interrelations between five 'species' lineages, or clusters (*B. magister*, *O. anonycha*, *B. makko*, *B. borealis* 'large', and *B. borealis* 'small') are not always consistent among the 12S rRNA, 16S rRNA, 28S, and CO1 gene trees, and the resultant tree topologies could be interpreted in different ways depending on the gene used for reconstruction, or a clustering approach. The order of clustering for species with a seven-rowed radula differs between the individual gene trees, as well as between the combined molecules phylogenies constructed using BA and ML approaches. For example, the position of *O. anonycha*, which possesses a number of 'primitive' character states, such as minute almost equal-sized suckers all over the tentacle club manus, and hookless arms in males (Pearcy and Voss, 1963; Bublitz, 1981) that may vary, so the seven-toothed gonatids, this species appears as the most proximal to the stem of five-toothed species on the BA and ML consensus trees, and the most distal to all other confamilial species and groups of species on the CO1 trees. Irrespective of variable tree topologies, there

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exist five species (in, supposedly, three genera) of gonatids with a seven-rowed radula, which are clearly identifiable morphologically and genetically, and which are closer to the base of the family clade than an evidently derived monophyletic group of species with a five-rowed radula. Differences in the Gonatidae phylogeny versions, based on different data sources, are discussed elsewhere (e.g. Nesis, 1973; Bublitz, 1981; Jefferts, 1983; Katugin, 2004; Lindgren *et al*., 2005).

Conclusion

The use of partially sequenced mitochondrial (CO1, 16S rRNA, and 12S rRNA) and nuclear (18S and 28S) genes provided deeper insight into the species identification, delimitation, and subdivisions within the family Gonatidae. The 12S rRNA, 16S rRNA, 28S, and CO1 sequences proved to be of high value and 18S of low value for the study of genetic relatedness among the gonatid squids. Molecular evidence based on the analysis of four valuable gene markers suggested the existence of a general subdivision of the family members into species with radular teeth in rows of five and those with rows of seven. The species with a five-rowed radula form a unique monophyletic group with a taxonomic status of a single-polymorphic genus or subfamily. The species with a seven-rowed radula represent several independent lineages, and their relations with each other and a derived lineage of species with a five-rowed radula are yet to be interpreted phylogenetically. The molecular genetic relationships among the Gonatidae revealed in this study provide a solid basis for further taxonomic decisions and studies on phylogeny of this squid family.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315423000759

Author's contribution. Oleg N. Katugin: data collection, research planning, and writing.

Anna O. Zolotova: sequencing, data analysis using software, and writing.

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Competing interests. None.

Ethical standards. The authors declare no violation of ethical standards.

Data availability. Data used in this study are publicly available at the NCBI GenBank (https://www.ncbi.nlm.nih.gov/); new DNA sequences that have been obtained for squid species via sequencing procedures have been posted to NCBI GenBank and will be publicly available at NCBI GenBank after publication of the article.

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