ASED http://e-ASED.org
Animal Systematics, Evolution and Diversity

Anim. Syst. Evol. Divers. Vol. 28, No. 4: 269-278, October 2012
http://dx.doi.org/10.5635/ASED.2012.28.4.269

Exploring the Utility of Partial Cytochrome *c* Oxidase Subunit 1 for DNA Barcoding of Gobies

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ABSTRACT

Gobiids are hyperdiverse compared with other teleost groups, with about 2,000 species occurring in marine, freshwater, and blackish habitats, and they show a remarkable variety of morphologies and ecology. Testing the effectiveness of DNA barcodes on species that have emerged as a result of radiation remains a major challenge in evolutionary biology. Here, we used the cytochrome *c* oxidase subunit 1 (COI) sequences from 144 species of gobies and related species to evaluate the performance of distance-based DNA barcoding and to conduct a phylogenetic analysis. The average intra-genus genetic distance was considerably higher than that obtained in previous studies. Additionally, the interspecific divergence at higher taxonomic levels was not significantly different from that at the intragenus level, suggesting that congeneric gobies possess substantial interspecific sequence divergence in their COI gene. However, levels of intragenus divergence varied greatly among genera, and we do not provide sufficient evidence for using COI for cryptic species delimitation. Significantly more nucleotide changes were observed at the third codon position than that at the first and the second codons, revealing that extensive variation in COI reflects synonymous changes and little protein level variation. Despite clear signatures in several genera, the COI sequences did resolve genealogical relationships in the phylogenetic analysis well. Our results support the validity of COI barcoding for gobiid species identification, but the utilization of more gene regions will assist to offer a more robust gobiid species phylogeny.

Keywords: cytochrome *c* oxidase subunit 1, barcoding, phylogeny, Gobiidae, Gobioidei

INTRODUCTION

Gobies (family Gobiidae; suborder Gobioidei) are incomparable among vertebrates in their capacity to adapt and diversify, which has led to adaptive radiation and rapid speciation (Zander, 2011). Gobiid fishes are hyperdiverse compared with other teleost groups, with approximately 2,000 species in 210 genera occurring in marine, freshwater, and blackish habitats. These fish show remarkable morphological and ecological variety (Nelson, 2006; Zander, 2011). Gobiid fishes are globally distributed (Nelson, 2006) and frequently represent a dominant component of coral reefs and coastal fish communities throughout much of their range, accounting for $>50\%$ of the energy flow in some coral reef habitats (Herler et al., 2011). Despite their evolutionary and ecological importance, the phylogenetic relationships among species within Gobiidae and their location within Gobioidei are still poorly understood (Murdy, 1989; Parenti and Thomas, 1998; Thacker and Schaefer, 2000; Larson, 2001). To date, the classification of gobies still remains largely reliant on external morphology (Pezold, 1993; Akihito et al., 2000; Nelson, 2006), and diagnostic characters separating species are subtle and problematic.

Molecular biology has contributed to addressing taxon identification and phylogenetic relationship questions. Mitochondrial DNA (mtDNA) markers have historically formed the core of most molecular systematic analyses and are still the most widely used for reconstructing phylogeny (Brown et al., 1979; Moore, 1995; Johns and Avise, 1998); this is probably due to their single copy nature and relative ease of sequencing (Moore, 1995). Genetic divergence is also enhanced by the higher rate of sequence evolution in vertebrate mtDNA compared to that of nuclear coding regions (Johns and Avise, 1998). However, the choice of a suitable gene is crucial for identification and phylogenetic reconstruction among closely related species (Brown et al., 1979; Moore,

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pISSN 2234-6953 eISSN 2234-8190

1995; Johns and Avise, 1998), because different parts of the mtDNA genome evolve at different rates (Avise and Ellis, 1986; Roques et al., 2006).

Mitochondrial cytochrome oxidase subunit I (COI) could serve as a rapid and reliable barcoding marker for identifying species and for discovering new species across the entire animal kingdom (Hebert et al., 2003). Although skepticism has frequently been expressed (Ebach and Holdrege, 2005; Will et al., 2005), DNA barcoding based on COI has been successful to identify species across a wide array of taxa over the last decade (e.g., Hebert et al., 2004; Clare et al., 2007; Hubert et al., 2008; Feng et al., 2011). A clear gap should exist between intra- and interspecific COI sequence divergence with about a 20-fold difference for DNA barcoding to be perfectly effective in delimitating species (Hebert et al., 2003). A standard sequence threshold can be projected to outline species boundaries by employing this barcoding gap. However, utilizing such a threshold value may be challenging, particularly when attempts do not include numerous specimens, such as for critically endangered taxa.

Only a few studies have addressed gobioid interrelationships based on molecular data (e.g., Akihito et al., 2000; Wang et al., 2001; Thacker, 2003, 2009; Thacker and Hardman, 2005). These studies used different taxon and nucleotide sampling methods. Yet, testing the effectiveness of COI DNA barcodes on species that have emerged as a result of radiation, such as gobies, remains a major challenge in evolutionary biology. Here, we sequenced the COI of 48 species collected from South Korea (Table 1) to evaluate the performance of distance-based DNA barcoding for phylogenetic analyses. We specifically aimed to provide novel data on a comparison of pairwise divergence levels among species in the same genus vs. species in different genera. GenBank sequences were also included in the analyses to use a dataset with large taxonomic coverage (n=144 species) (Table 2).

MATERIALS AND METHODS

Sample collection

Fish were collected using seine and dip nets from January to November 2011 from 21 sites across freshwater systems, coastal areas, and the ocean near South Korea (Table 1). Specimens were identified based on morphological characters. Entire bodies of all individuals were preserved in 95% ethanol, and 44 nominal species were sequenced for COI gene fragments.

DNA isolation, amplification, and sequencing

We used the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) to extract genomic DNA from the right pectoral fin of each fish specimen. The COI was amplified using gobiid-specific primers: GOBYF7558 (forward) 5′-TTT GCW ATT ATG GCW GGA TTT G-3′ and GOBYB 8197 (reverse) 5′-ATT ATT AGG GCG TGG TCG TGG-3′ (Thacker, 2003) and COI fish universal primers, FF2d (forward) 5′-TTC TCC ACC AAC CAC AAR GAY ATY GG-3′ and FF1d (reverse) 5′-CAC CTC AGG GTG TCC GAA RAA YCA RAA-3′ (Ivanova et al., 2007). Each polymerase chain reaction (PCR) amplification was carried out in a 50 μL reaction volume composed of ~75 ng DNA extract, 0.25 mM of each deoxynucleotide, 0.25 mM of each forward and reverse primer, 3 mM $MgCl_2$, 1 × PCR buffer, and 0.25 units of Taq DNA polymerase (Solgent, Daejeon, Korea). GenePro (BIOER) was used to amplify the COI with the following program: 94°C for 10 min, 35 cycles of 30 s at 94°C, 30 s at 54°C (for GOBYF7558−GOBYB8197) and 52°C (for FF2d−FF1d), 30 s at 72°C and final elongation at 72°C for 10 min. PCR products were loaded on 1% agarose gels containing 0.003% ethidium bromide and visualized using the GelDoc-It TM Imaging System (UVP). Amplifications were considered successful when a expected sized band was observed on the agarose gel. PCR products were cleaned using a PCR purification kit (Solgent). The COI was sequenced directly using the BigDye-Terminator V3.1 kit (Applied Biosystems, Foster City, CA, USA) and an ABI3730XL sequencer at Genotech (Daejeon, Korea).

Sequence data analyses

Complementary DNA sequences were assembled using the Bioedit 5.0.9 sequence-editing software (Hall, 1999). Sequences were aligned using Clustal X 2.0 default settings (Larkin et al., 2007). Alignments were translated to amino acids under the vertebrate mitochondrial option using MEGA 5 (Tamura et al., 2011) to detect frameshift mutations and premature stop codons, which may indicate the presence of pseudogenes. The Genbank accession numbers of newly determined sequences were JX679021−JX679066 and are listed in Table 1. Genetic distances were calculated to quantify sequence divergences among species using both *p* distance and Kimura two-parameter (K2P) models (1,000 bootstrapping) (Kimura, 1980), as implemented in MEGA. Rates of synonymous and nonsynonymous substitutions were also calculated with MEGA using both standard and modified (at 1.4 standard errors with 1,000 bootstrapping samples) Nei-Gojobori models (Nei and Gojobori, 1986; Nei and Kumar, 2000). Genetic distances were calculated at intrageneric, intrasubfamilial, intrafamilial, and interfamilial levels. Altogether, 10,296 pairwise distances were compared in this study. The degree of sequence conservation per site, R_{seq}, was defined as $R_{seq}=2-(-\sum p\log_2 p)$ (Ward and Holmes, 2007),

Table 1. Details of gobioid fish species analyzed; data comprised of species name, voucher number, locality, GPS coordinate, collection date, and GenBank accession number; F, E and O in bracket on each location indicates habitat information such as freshwater, estuary and ocean, respectively

(Sub)family	Species	Voucher	Location	GPS coordinate	Date	GenBank accession no.
Microdesmidae	*Parioglossus dotui*	GB11111	Jeju-si, Jeju-do (O)	N33°28′23″, E126°20′58″	21 Feb 2011	JX679048
Odontobutidae	*Micropercops swinhonis*	GB11112	Seocheon-gun, Chungcheongnam-do (F)	N35°05′57″, E126°45′07″	10 May 2011	JX679042
	Odontobutis interrupta	GB11113	Gyeongju-si, Gyeongsangbuk-do (F)	N36°02′24″, E129°14′41″	7 Dec 2010	JX679045
	Odontobutis obscura	GB11114	Geoje-si, Gyeongsangnam-do (F)	N34°48′51″, E128°38′08″	20 Jun 2011	JX679046
	Odontobutis platycephala	GB11115	Gyeongsan-si, Gyeongsangbuk-do (F)	N35°51′23″, E128°43′41″	23 Dec 2010	JX679047
Amblyopinae	*Odontamblyopus rubicundus*	GB11116	Yeonggwang-gun, Jeollanam-do (O)	N35°25′44″, E126°25′41″	5 Aug 2011	JX679044
	Ctenotrypauchen microcephalus	GB11117	Yeonggwang-gun, Jeollanam-do (O)	N35°25′44″, E126°25′41″	5 Aug 2011	JX679031
Gobiinae	*Istigobius campbelli*	GB11123	Seogwipo-si, Jeju-do (O)	N33°16′15″, E126°35′53″	9 Jun 2011	JX679037
	Leucopsarion petersii	GB11125	Gijang-gun, Busan (O)	N35°19′32″, E129°15′18″	4 Apr 2011	JX679038
	Bathygobius fuscus	GB11127	Seogwipo-si, Jeju-do (O)	N33°28′23″, E126°20′58″	21 Feb 2011	JX679026
	Trimma grammistes	GB11128	Seogwipo-si, Jeju-do (O)	N33°16′15″, E126°35′53″	9 Jun 2011	JX679066
Gobionellinae	*Acanthogobius elongata*	GB11129	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	15 May 2011	JX679021
	Acanthogobius flavimanus	GB11130	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	15 May 2011	JX679022
	Acanthogobius lactipes	GB11131	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	15 May 2011	JX679023
	Acanthogobius luridus	GB11132	Gimpo-si, Gyeonggi-do (O)	N37°42′07″, E126°39′45″	13 Oct 2011	JX679024
	Amblychaeturichthys hexanema	GB11133	Seocheon-gun, Chungcheongnam-do (O)	N36°09′27″, E126°30′01″	9 Jun 2011	JX679025
	Gymnogobius breuntigii	GB11134	Goseong-gun, Gangwon-do (E)	N38°20′08″, E128°30′57″	20 Aug 2011	JX679032
	Gymnogobius mororanus	GB11135	Boryeong-si, Chungcheongnam-do (E)	N36°21′06″, E126°33′36″	20 May 2011	JX679033
	Gymnogobius urotaenia	GB11136	Pohang-si, Gyeongsangbuk-do (E)	N36°08′42″, E129°23′19″	19 Apr 2011	JX679035
	Gymnogobius petschiliensis	GB11137	Seogwipo-si, Jeju-do (E)	N33°14′44″, E126°25′07″	25 Aug 2011	JX679033
	Gymnogobius opperiens	GB11138	Pohang-si, Gyeongsangbuk-do (E)	N36°08′42″, E129°23′19″	19 Apr 2011	JX679036
	Chaeturichthys stigmatias	GB11139	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	14 May 2011	–
	Chaenogobius dolichognathus	GB11140	Jeju-si, Jeju-do (O)	N33°28′23″, E126°20′58″	21 Feb 2011	JX679028
	Chaenogobius gulosus	GB11141	Jeju-si, Jeju-do (O)	N33°28′23″, E126°20′58″	21 Feb 2011	JX679029
	Clariger cosmurus	GB11142	Gijang-gun, Busan (O)	N35°17′30″, E129°15′37″	4 Apr 2011	JX679030
	Lophiogobius ocellicauda	GB11143	Gimpo-si, Gyeonggi-do (O)	N37°42′07″, E126°39′45″	13 Oct 2011	–
	Luciogobius platycephalus	GB11146	Gijang-gun, Busan (O)	N35°17′30″, E129°15′37″	4 Apr 2011	JX679040
	Luciogobius elongatus	GB11148	Gijang-gun, Busan (O)	N35°17′30″, E129°15′37″	4 Apr 2011	JX679039
	Luciogobius saikaensis	GB11149	Gijang-gun, Busan (O)	N35°17′30″, E129°15′37″	4 Apr 2011	JX679041
	Mugilogobius abei	GB11150	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	14 May 2011	JX679043
	Pseudogobius masago	GB11152	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	14 May 2011	JX679051
	Pterogobius zacalles	GB11154	Sinan-gun, Jeollanam-do (O)	N34°03′10″, E125°07′25″	13 Jul 2011	JX679052
	Pterogobius zonoleucus	GB11155	Gijang-gun, Busan (O)	N35°15′51″, E129°14′07″	5 Apr 2011	JX679053
	Rhinogobius brunneus	GB11156	Pohang-si, Gyeongsangbuk-do (F)	N36°07′00″, E129°18′54″	15 Mar 2011	JX679054
	Rhinogobius brunneus CB	GB11157	Goheung-gun, Jeollanam-do (F)	N34°26′40″, E127°30′45″	4 Jun 2011	JX679055
	Rhinogobius brunneus CO	GB11158	Seogwipo-si, Jeju-do (F)	N33°14′38″, E126°20′30″	6 Jul 2011	JX679056
	Rhinogobius giurinus A	GB11159	Seogwipo-si, Jeju-do (F)	N33°14′44″, E126°25′07″	23 Aug 2011	JX679057
	Rhinogobius giurinus B	GB11160	Wanju-gun, Jeollabuk-do (F)	N35°51′15″, E127°10′19″	7 Jul 2011	JX679058
	Synechogobius hasta	GB11161	Boryeong-si, Chungcheongnam-do (E, O)	N36°21′06″, E126°33′36″	14 May 2011	JX679059
	Tridentiger barbatus	GB11162	Gimpo-si, Gyeonggi-do (E, O)	N37°38′15″, E126°32′20″	8 Nov 2011	JX679060
	Tridentiger brevispinis	GB11163	Boryeong-si, Chungcheongnam-do (E, O)	N36°21′06″, E126°33′36″	14 May 2011	JX679062
	Tridentiger bifasciatus	GB11164	Boryeong-si, Chungcheongnam-do (E, O)	N36°21′06″, E126°33′36″	14 May 2011	JX679061
	Tridentiger nudicervicus	GB11165	Boryeong-si, Chungcheongnam-do (E, O)	N36°21′06″, E126°33′36″	14 May 2011	JX679063
	Tridentiger obscurus	GB11166	Tongyeong-si, Gyeongsangnam-do (E, O)	N34°48′35″, E128°14′15″	28 Oct 2011	JX679064
	Tridentiger trigonocephalus	GB11167	Goseong-gun, Gangwon-do (E, O)	N38°28′49″, E128°26′11″	19 Aug 2011	JX679065
Oxudercinae	*Boleophthalmus pectinirostris*	GB11168	Boseong-gun, Jeollanam-do (O)	N34°47′31″, E127°23′46″	15 Aug 2011	JX679027
	Periophthalmus magnuspinnatus	GB11169	Boryeong-si, Chungcheongnam-do (O)	N36°21′06″, E126°33′36″	25 Aug 2011	JX679049
	Periophthalmus modestus	GB11170	Hongseong-gun, Chungcheongnam-do (O)	N36°32′18″, E126°28′15″	9 Oct 2011	JX679050

Table 2. List of reference species used in this study

(Sub)family	Species	Reference	GenBank accession no.
Odontobutidae	*Perccottus glenii*	Thacker and Hardman (2005)	AY722171
Rhyacichthyidae	*Protogobius attiti*	Keith et al. (2011)	HQ639032
	Rhyacichthys guilberti	Keith et al. (2011)	HQ639030
Benthophilinae	*Neogobius melanostomus*	Unpublished	HQ960511
	Proterorhinus marmoratus	Hubert et al. (2012)	EU524305
	Proterorhinus semilunaris	Unpublished	HQ961006
Gobiinae	*Amblyeleotris aurora*	Unpublished	HQ561508
	Amblyeleotris guttata	Steinke et al. (2009)	FJ582710
	Amblyeleotris steinitzi	Steinke et al. (2009)	FJ582712
	Amblyeleotris wheeleri	Unpublished	HQ561506
	Amblygobius decussatus	Steinke et al. (2009)	FJ582722
	Amblygobius phalaena	Hubert et al. (2012)	JQ431409
	Asterropteryx ensifera	Hubert et al. (2012)	JQ431470
	Asterropteryx semipunctata	Hubert et al. (2012)	JQ431471
	Bathygobius coalitus	Hubert et al. (2012)	JQ431479
	Bathygobius cocosensis	Hubert et al. (2012)	JQ431480
	Bathygobius cotticeps	Hubert et al. (2012)	JQ431482
	Bathygobius curacao	Weigt et al. (2012)	JQ839961
	Bathygobius lacertus	Weigt et al. (2012)	JQ839962
	Bathygobius laddi	Unpublished	JF492946
	Bathygobius mystacium	Weigt et al. (2012)	JQ839963
	Bathygobius soporator	Tornabene et al. (2010)	HM748425
	Caffrogobius caffer	Unpublished	HQ945885
	Coryphopterus tortugae	Weigt et al. (2012)	JQ842827
	Croilia mossambica	Unpublished	HQ561481
	Cryptocentrus cryptocentrus	Unpublished	HQ561467
	Cryptocentrus leptocephalus	Steinke et al. (2009)	FJ583296
	Cryptocentrus pavoninoides	Steinke et al. (2009)	FJ583298
	Elacatinus evelynae	Steinke et al. (2009)	FJ583388
	Elacatinus oceanops	Steinke et al. (2009)	FJ583389
	Eviota afelei	Thacker (2003)	AF391391
	Eviota disrupta	Leray et al. (2012)	JN107907
	Eviota distigma	Hubert et al. (2012)	JQ349971
	Eviota indica	Hubert et al. (2012)	JQ349972
	Eviota prasina	Hubert et al. (2012)	JQ349973
	Favonigobius exquisitus	Thacker et al. (2011)	HQ909465
	Fusigobius signipinnis	Steinke et al. (2009)	FJ583414
	Glossogobius aureus	Aquino et al. (2011)	HQ682689
	Glossogobius callidus	Steinke et al. (2009)	JF493535
	Gobiodon ceramensis	Steinke et al. (2009)	FJ583428
	Gobiodon histrio	Steinke et al. (2009)	FJ583450
	Gobiodon okinawae	Steinke et al. (2009)	FJ583454
	Gobiodon quinquestrigatus	Hubert et al. (2012)	JQ431768
	Gobiopterus lacustris	Aquino et al. (2011)	HQ682693
	Gobius bucchichi	Unpublished	JF935258
	Gobius cruentatus	Unpublished	JF935263
	Istigobius decoratus	Steinke et al. (2009)	JF493692
	Istigobius rigilius	Thacker et al. (2011)	HQ536672
	Knipowitschia caucasica	Triantafyllidis et al. (2011)	HQ600736
	Nes longus	Weigt et al. (2012)	JQ841296
	Paragobiodon lacunicolus	Steinke et al. (2009)	FJ583828
	Pomatoschistus tortonesei	Unpublished	FJ751922
	Priolepis cincta	Hubert et al. (2012)	JQ350251
	Priolepis compita	Hubert et al. (2012)	JQ432034
	Priolepis eugenius	Thacker (2003)	AF391329
	Priolepis hipoliti	Thacker et al. (2011)	HQ909484
	Priolepis inhaca	Hubert et al. (2012)	JQ432036
	Priolepis semidoliata	Hubert et al. (2012)	JQ432038
	Priolepis squamogena	Hubert et al. (2012)	JQ432040

Table 2. Continued

(Sub)family	Species	Reference	GenBank accession no.
Gobiinae	*Priolepis triops*	Hubert et al. (2012)	JQ432042
	Pycnomma roosevelti	Unpublished	GU224480
	Rhinogobiops nicholsii	Unpublished	JN582118
	Trimma caesiura	Thacker (2009)	EU381039
	Trimma macrophthalma	Hubert et al. (2012)	JQ350401
	Trimma mendelssohni	Hubert et al. (2012)	JQ350407
	Trimma milta	Hubert et al. (2012)	JQ432200
	Valenciennea helsdingenii	Steinke et al. (2009)	FJ584201
	Valenciennea longipinnis	Steinke et al. (2009)	FJ584202
	Valenciennea muralis	Steinke et al. (2009)	FJ584203
	Valenciennea puellaris	Steinke et al. (2009)	FJ584212
	Valenciennea sexguttata	Steinke et al. (2009)	FJ584224
	Valenciennea strigata	Unpublished	HQ945877
	Valenciennea wardii	Steinke et al. (2009)	FJ584240
Gobionellinae	*Awaous aeneofuscus*	Unpublished	HQ945950
	Awaous melanocephalus	Aquilino et al. (2011)	HQ654674
	Ctenogobiops tangaroai	Steinke et al. (2009)	FJ583310
	Ctenogobius saepepallens	Weigt et al. (2012)	JQ840025
	Gnatholepis cauerensis	Unpublished	HQ561511
	Gnatholepis thompsoni	Weigt et al. (2012)	JQ840079
	Gobioides broussonnetii	April et al. (2011)	JN026727
	Gobionellus oceanicus	Weigt et al. (2012)	JQ841902
	Lethops connectens	Unpublished	GU440372
	Oligolepis acutipennis	Aquilino et al. (2011)	HQ654730
	Oligolepis keiensis	Unpublished	HQ945926
	Stenogobius polyzona	Unpublished	HQ945939
	Typhlogobius californiensis	Unpublished	GU440562
Oxudercinae	*Scartelaos histophorus*	Unpublished	FJ238032
	Taenioides sp.	Steinke et al. (2009)	FJ584167
Sicydiinae	*Akihito vanuatu*	Keith et al. (2011)	HQ639065
	Cotylopus rubiripinnis	Keith et al. (2011)	HQ639038
	Lentipes armatus	Keith et al. (2011)	HQ639070
	Sicydium punctatum	Keith et al. (2011)	HQ639050
	Sicyopterus lagocephalus	Hubert et al. (2012)	JQ432152
	Sicyopterus pugnans	Hubert et al. (2012)	JQ432154
	Sicyopus chloe	Keith et al. (2011)	HQ639058
	Stiphodon elegans	Hubert et al. (2012)	JQ432172

where p is the observed frequency of each base at a particular position and the maximal degree of conservation was 2, which was achieved when all nucleotides at a particular site in the 144 species were the same.

A Bayesian inference (BI) tree was established using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with two outgroup species from the Family Rhyacichthydae, *Protogobius attiti* and *Ryacichthys guilberti*. The best-fit model of DNA sequence evolution was chosen using ModelTest 3.8 (Posada and Crandall, 1998) and Akaike information criteria; the chosen model was GTR+I+G. The analysis was run for 10 million generations with sampling of one tree every 500 generations. Two independent Markov Chain Monte Carlo runs were conducted simultaneously. The first 1,000 trees of each run were discarded as burn-in.

RESULTS

The COI genes of each species were confidently aligned, and the equivocal bases at each end were trimmed to yield a final sequence of 542 bp. No indels were detected. Translation of the sequences did not reveal frame-shift mutations or premature stop codons, confirming that our amplified fragments were functional. Among the 542 nucleotide positions, 245 were polymorphic, and 230 were parsimony informative. The proportion of T, C, A, and G bases for all 144 sequences was 30.5%, 28.1%, 23.1%, and 18.2%, respectively. The GC content was relatively higher at the first codon base (56.3%) than that at the second (43.1%) or third (39.6%). The degree of conservation (R_{seq}) was calculated for each base of the 542 nucleotides; the most common and maximum value was 2, which was achieved when all nucleotides at a particular site

Table 3. Estimated evolutionary parameters (×100) for the nucleotide substitutions in the cytochrome oxidase *c* subunit 1 (COI) barcoding region from 144 gobioid fish species

Genetic distance		All fish		Gobiinae		Gobionellinae	
		Mean	SD	Mean	SD	Mean	SD
Codon nucleotide position							
p distance	First	5.51	1.63	5.45	2.05	5.21	2.06
	Second	0.18	0.15	0.28	0.47	0.02	0.11
	Third	54.31	3.61	55.49	5.19	50.19	7.05
	Overall	19.94	1.66	20.34	2.10	18.42	2.69
K2P distance	First	6.23	2.09	6.14	2.53	5.85	2.52
	Second	0.18	0.15	0.28	0.48	0.02	0.11
	Third	403.10	439.15	499.53	552.80	240.11	145.15
	Overall	28.18	4.06	29.06	4.05	25.34	4.42
Synonymous and nonsynonymous distances							
Nei-Gojobori (N-G)	Synonymous	71.91	6.98	73.41	6.77	66.95	9.32
	Nonsynonymous	1.64	0.94	1.72	0.99	1.29	0.83
	d_N/d_S	0.02	0.00	0.02	0.00	0.02	0.00
Modified N-G	Synonymous	62.78	6.06	64.06	5.88	58.44	8.11
	Nonsynonymous	1.73	1.00	1.81	1.05	1.36	0.88
	d_N/d_S	0.03	0.00	0.03	0.00	0.02	0.00

K2P, Kimura-2-parameter.

of the 144 species were the same. Every third codon base was highly variable with a 0.64 mean R_{seq}. The first (1.72) and the second codon bases (1.98) were nearly monomorphic. Nucleotide genetic distance parameters, *p* and K2P distance, also showed an almost zero rate of substitution for second nucleotide positions, with the first position being an order of magnitude higher and the third position being unparalleled among them (Table 3). The rate of synonymous substitutions was much higher than the rate of nonsynonymous substitutions (Table 3).

Three species could not be separated using the COI sequence analysis, including *Chaenogobius gulosus* (Gobionellinae), *Chaeturichthys stigmatias* (Gobionellinae), and *Lophiogobius ocellicauda* (Gobiinae). The *C. gulosus* sequence was highly similar to those of other congeneric species, as expected, whereas the *C. stigmatias* and *L. ocellicauda* results were very surprising. Multiple specimens of these species should be extensively analyzed in a future study to check the genetic divergence among these species; thus, those sequences were not deposited in GenBank. The average diversity among 142 haplotypes (H_d) was 0.999±0.001 (mean±standard deviation), and the average nucleotide diversity (π) was 0.199 ±0.002. Twenty-six genera were represented by two or more species. Levels of intragenus divergence were generally high (Table 4) but varied greatly among genera. For example, the average within-genus divergence of *Awaous*, *Eviota*, and *Trimma* was 29.85%, 29.13%, and 28.71%, respectively, which was larger than that of overall interspecific divergence. These values were considerably larger than those of *Amblygobius*, *Asterropteryx* and *Rhinogobius* (9.76%, 9.87%, and 10.74%, respectively). Mean interspecific divergences at higher taxonomic levels were slightly larger than that at the intragenus level, resulting in large overlaps among levels (Table 4). As roughly 90% of all gobiid fishes are either in Gobiinae or Gobionellinae, these two subfamilies were compared for nucleotide substitution rate (Table 3). Species from Gobiinae were consistently higher than Gobionelline fishes in every parameter estimated (Table 3).

Table 4. Mean genetic divergences for the cytochrome oxidase *c* subunit 1 (COI) nucleotide sequences (*p* and Kimura-2-parameter [K2P] distances) among 144 gobioid species

Taxonomic level	*p* distance (×100)			K2P distance (×100)		
	Mean	Min	Max	Mean	Min	Max
Within-genus	15.63	0.18	23.80	20.87	0.19	36.13
Within-subfamily	19.69	9.20	26.20	27.87	0.19	41.44
Within-family	20.05	6.83	26.94	28.35	0.94	44.53
Among-family	20.18	10.89	25.83	28.56	13.27	41.06

Four taxonomic levels are represented such as within-genus, within-subfamily, within-family, and among-family.

Our phylogenetic data provide little evidence to support the previous claims at the generic and higher taxonomic levels, based on phenetic analyses. Several species did not cluster into their respective groups, and the BI tree failed to correctly identify some genera or subfamilies (Fig. 1). For example, *Tridentiger barbatus* (Gobiinellinae) clustered into the *Asterropteryx* clade (Gobiinae) with high nodal support rather than with other species of *Tridentiger*. *Pterogobius zacalles* clustered with *Acanthogobius*, whereas *P. zonoleucus* formed a monophyletic group with the *Rhinogobius brun-*

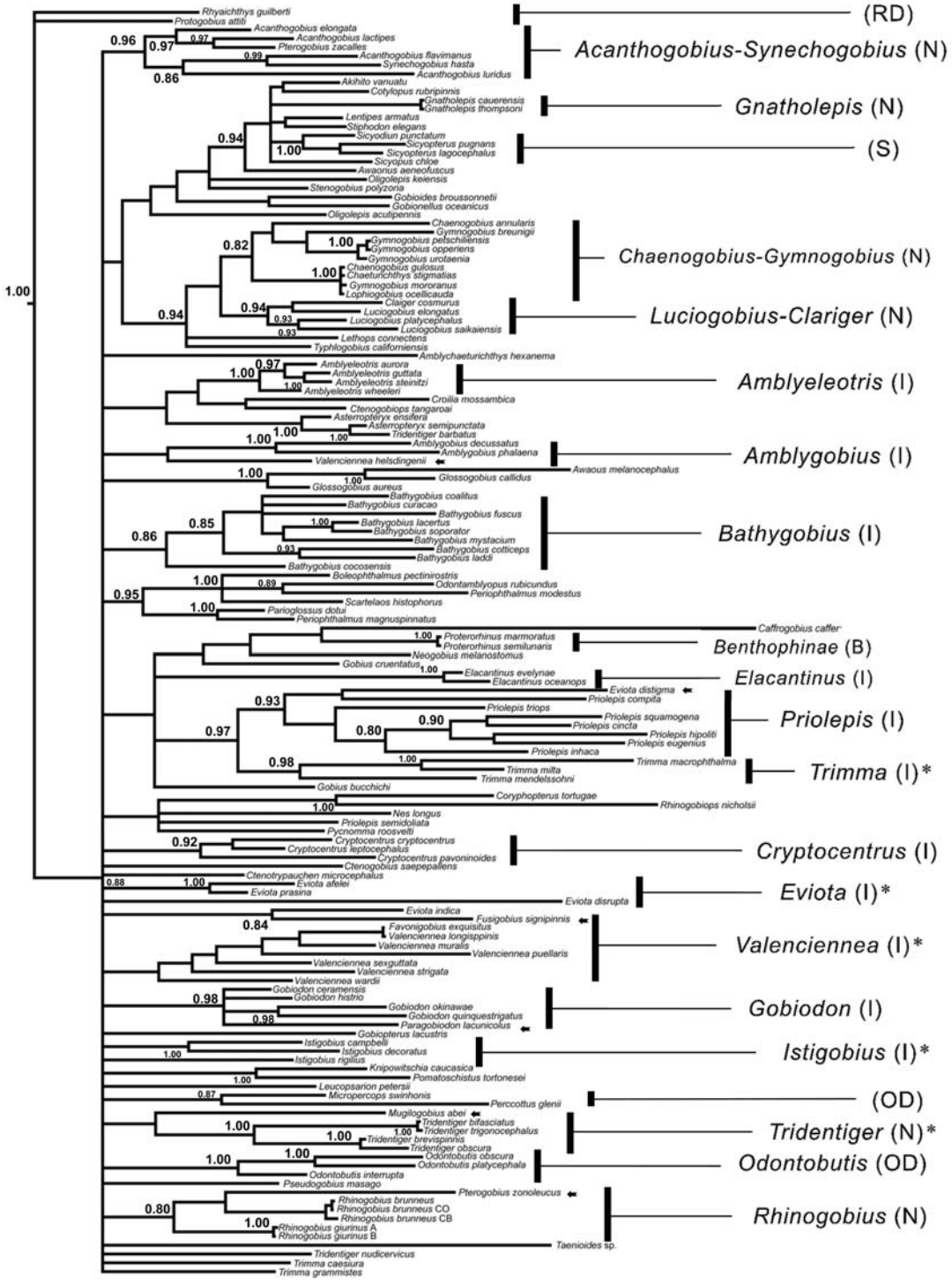

Fig. 1. Molecular phylogeny of Gobioidei based on Bayesian inference from 542 bp of the mitochondrial cytochrome oxidase *c* subunit 1 (COI) gene with two outgroup species from the Family Rhyacichthyidae: *Protogobius attiti* and *Rhyacichthys guilberti*. Under the chosen model, GTR+I+G, analysis was run for 10 million generations with sampling of one tree every 500 generations. Numbers above branches indicate posterior probabilities (>0.8). Abbreviations in brackets on the right side indicate higher taxonomic names (subfamily and family) in current usage, including Amblyopinae (A), Benthophilinae (B), Gobiinae (I), Gobionellinae (N), Oxudercinae (O), Sicydiinae (S), Microdesmidae (MD), Odontobutidae (OD), and Rhyacichthyidae (RD). Asterisks immediately after the higher taxonomic names and arrows on nodes indicate taxa failing to resolve monophyletic assemblages, and species clustered into unrelated groups, respectively.

neus complex, albeit without good nodal support. The BI tree also failed to resolve monophyletic assemblages of some taxa, such as *Awaous*, *Eviota*, *Trimma*, and Ondontobutidae. Those exceptions aside, the tree largely assigned species to identical major groups.

DISCUSSION

The COI sequences did resolve genealogical relationships well at the level of genera and family in Gobioidei. As previously noted in Che et al. (2012), this was possibly due to at least two factors. Most likely, the 542 nucleotides do not provide sufficient phylogenetically informative characters to recover the true phylogeny when examining hundreds of taxa with enormous diversity. In addition, the fast mutation rate and saturation in the third codon position can be a disadvantage at deeper phylogenetic levels, and the subsequent long terminal branches may impede resolution of ancient speciation due to the chance accumulation of shared character states (Huelsenbeck, 1997). Despite the poor monophyletic resolution in several taxa, some clear phylogenetic signatures were observed in the COI sequence data. For example, several major congeneric species, *Rhinogobius* and *Bathygobius*, tended to cluster together with no exception and, in most cases, so did consubfamilial species. It is believed that the utilization of more gene regions including nuclear DNA will assist in offering a more reliable phylogeny within Gobiidae and their placement within Gobioidei. Several nuclear genes such as recombination activating genes 1 and 2 (Rag 1 and 2) and ryandine receptor 3 (Ryr3) may have slower rate of sequence evolution in gobies compared to that of mtDNA genes (Yamada et al., 2009).

Our results support the validity of COI barcoding for species identification in gobiid species, although no attempt was made to include numerous specimens for any one species. One fundamental barcoding criterion is that congeneric divergence should be significantly higher than that of conspecific divergence (Hubert et al., 2008). The average intragenus distance (K2P) for 28 genera with multiple species in the present study was 21.09%, which was considerably higher than the values obtained among fish species in previous studies (9.93% from Ward et al., 2005; 9.54% from Ward and Holmes, 2007). In addition, the interspecific divergence at higher taxonomic levels was not significantly larger than that at the intragenus level, suggesting that congeneric gobies possess substantial interspecific sequence divergence in their COI genes. Significantly more nucleotide changes were observed at the third codon position than those at the first and the second, revealing that the extensive variation shown among the COI sequences typically reflects synonymous changes and little variation at the protein level. Consequently, the proportion of nonsynonymous to synonymous changes was far less than one (Table 2). As previously noted in Ward and Holmes (2007), this result must be due to exceptionally strong purifying selection of the COI gene and confirms that the ability of COI to identify species in Gobiidae is dependent on the degenerate nature of the genetic code.

We did not provide sufficient evidence for the utility of the COI towards cryptic species identification in several species complexes. Gobiidae taxonomy has been studied extensively for the last several decades, but confusion still exists. One typical case is the taxonomic status of the *Gymnogobius* species complex (*G. urotaenia*, *G. opperiens*, and *G. petschiliensis*) (e.g., Harada et al., 2002). In our results, overall K2P divergence within *Gymnogobius* was 12.74%, whereas the average value among the *Gymnogobius* species complex was only 1.81%, probably reflecting a short history of reproductive isolation. The *Rhinogobius brunneus* complex (in our analysis, *Rhinogobius brunneus*, *R. brunneus* CB, and *R. brunneus* CO) is also a representative example of a cryptic species complex in Gobiidae (Kawanabe and Mizuno, 1989; Kim, 1995). Overall, K2P divergence within *Rhinogobius* was $>10\%$, whereas the average value among the *R. brunneus* complex was just 2%. Although more work needs to be done with multiple specimens, the COI sequence may not be a reliable tool to delineate cryptic and complex species boundaries in the family Gobiidae.

ACKNOWLEDGMENTS

We thank Mr. Seong Jang Jo and Jin Young Choo for their assistance collecting fish. We also thank Seongjun Park for logistical phylogenetic analyses support. Su Youn Baek provided assistance with the laboratory studies. This study was supported by the grant "Korean Tree of Life, 4th Year: Gobioidei Fishes (No. 211C000068)" funded by the National Institute of Biological Resources, Korean Government and awarded to H. Y. Suk.

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Received September 23, 2012
Revised October 18, 2012
Accepted October 20, 2012