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DNA Barcoding and Molecular Phylogeny of the Commercially Valuable Groupers *Epinephelus* (Perciformes: Serranidae) Based on Mitochondrial COI DNA Sequence

Kao-Sung Chen[1], Hernyi J. Hsieh[2], Min-Yu Huang[3], Kayan Ma[4] and Sheng-Tai Hsiao[3*]

[1]Planning and Information Division, Fisheries Research Institute
[2]Penghu Marine Biology Research Center, Fisheries Research Institute
[3]Marine Fisheries Division, Fisheries Research Institute
[4]School of Marine and Tropical Biology, James Cook University, Townville, QLD, Australia

ABSTRACT

The genus *Epinephelus*, which belongs to the family Serranidae, and the subfamily Epinephelinae, comprises about 100 species in all three major oceans. In this study, totally 33 *Epinephelus* species were collected in Taiwan, Australia and the West Pacific during January-December, 2012. The genetic data of the mitochondrial DNA COI gene were applied to investigate the evolutionary divergence (K2P). Furthermore, the phylogenetic tree of the neighbor-joining (NJ) method and the maximum parsimony (MP) method were also constructed. The results of evolutionary divergence revealed that *E. chlorostigma* and *E. areolatus* exhibited the minimum distance (0.004), while *E. spilotoceps* and *E. quoyanus* exhibited the maximum distance (0.210). According to the results of the phylogenetic tree, the genus *Epinephelus* had the following two clades, one contained mostly small- to medium-size "reticulated" groupers; while the other contained medium- to large-size groupers, such as *E. lanceolatus*. These results were similar to the traditional morphometric classification. These results revealed that mitochondrial COI gene is not only an effective "DNA barcoding" marker for species identification, but also a widely accepted marker for investigate the phylogenetic relationship among these commercially valuable groupers.

Keywords: Serranidae, *Epinephelus*, COI, Barcoding, Phylogenetic tree

INTRODUCTION

The genus *Epinephelus* Bloch 1973 was placed in the subfamily Epinephelinae, one of three subfamilies in the family Serranidae, which are commonly known as groupers, rockcods, and seabasses (Heemstra and Randall, 1993).These fishes are important target species for coastal fisheries in tropical and subtropical areas. Due to their increasing market demand and high commercial value, they have been subjected to heavy fishing pressure. However, although they have a very high commercial and ecological value, their interspecific relationships remain poorly understood.

With regard to the phylogeny of the family Serranidae, Jordan and Eigenmann (1890) first attempted to resolve the relationships within family Serranidae by defining the following six subfamilies: Serraninae, Epinephelinae, Anthiinae, Grammistinae, Latinae, and Percichthyinae. Gosline (1966) and Johnson (1983) both porposed that family Serranidae should contain three subfamilies, included

*Correspondence: 199 Hou-Ih Road, Keelung 202, Taiwan. TEL: (02) 2462-2101; FAX: (02) 2463-3110; E-mail: sthsiao@mail.tfrin.gov.tw

Serraninae, Epinephelinae, and Anthiinae, which still commonly recognized today. Furthermore, Johnson (1983) revealed that the subfamily Epinephelinae was monophyletic based on the derived feature of loss of an autogenous distal radial on the first dorsal-fin pterygiophore, Johnson (1983, 1988) thus divided the subfamily into the following five tribes: Niphonini, Epinephelini, Diploprionini, Liopropromini, and Grammistini. Subsequently, Baldwin and Johnson (1993) proposed the relationships among these tribes and demonstrated their monophyly. The tribe Epinephelini comprises more than 150 species in the following 15 genera: *Aethaloperca*, *Alphestes*, *Anyperodon*, *Cephalopholis*, *Cromileptes*, *Dermatolepis*, *Epinephelus*, *Gonioplectrus*, *Gracila*, *Mycteroperca*, *Paranthias*, *Plectropomus*, *Saloptia*, *Triso*, and *Variola* (Johnson, 1983). Among these genera, the genus *Epinephelus* has the highest number of species, comprising more than 98 species (Heemstra and Randall, 1993).

In the past, with the exception for the studies conducted by Johnson (1983, 1988), Baldwin and Johnson (1993), and Heemstra (1991), Heemstra and Randall (1991, 1993), few systematic studies had been conducted to resolve the relationships between the genera belonging to the subfamily Epinephelinae. As a result, this subfamily contains several complexes of sympatric and parapatric species, which are distributed worldwide. These species are primarily distinguished by their color pattern and combinations of overlapping morphomeristic characters. In fact, it is difficult to identify species of groupers based on distinct morphological features, because some species differ only in terms of their color pattern, however, marked different color pattern could exist between distant populations within a single widely distributed species (Heemstra and Randall, 1993), resulting in taxonomic confusion. Thus, the homogeneous nature of the morphology has led to problems in reconstructing evolutionary relationships among the groupers (Smith, 1971).

Mitochondrial DNA sequences have been commonly used in taxonomic and phylogenetic studies of marine fishes in recent years (Miya and Nishida, 1996; Bernardi and Bucciarelli, 1999; ; Tringali *et al*., 1999; Teletchea, 2009) in particular, cytochrome *b* (Maggio *et al*., 2005) and 16S rDNA genes (Pondella *et al*., 2003) are the main genetic markers be utilized. Craig *et al*. (2001) were the first study to use the 16S rDNA sequence as a genetic marker to investigate the phylogenetic relationships of 42 serranid species and propose the monophyly of the family Serranidae (Craig and Hastings, 2007).

About the identification of fishes, Hebert *et al*. (2003) proposed the standardization of the various approaches used in species identification through the establishment of a DNA barcoding system [based on a single sequence: a 648-bp portion of the mitochondrial gene cytochrome *c* oxidase I (COI)]. Thereafter, several studies about groupers have been conducted by using COI as the genetic marker for taxonomic, phylogenetic, and phylogeographic evaluation (Nikula and Vainola, 2003; Hubert *et al*., 2008, 2012; Sachithanandam *et al*., 2012; Domingues *et al*., 2013).

According to The Fish Database of Taiwan (http://fishdb.sinica.edu.tw/), approximately 29 genera with 119 Serranid fishes have been recorded in Taiwan. Owing to this high species richness, the present study aimed to evaluate the status of the existing *Epinephelus* species and to flag the presence of cryptic diversity, classify the phylogenetic relationship of *Epinephelus*, and to investigate the relationship of nine "reticulated groupers," which are all distributed in the Indo-Pacific Ocean and share similar morphology. Through mitochondrial COI DNA barcoding, the difficulty in morphological identification - which was caused by different life history stages, geographical variation, or sexual dimorphism - could be overcome and thus the incidence of misidentification could be reduced.

MATERIALS AND METHODS

1. Sample Sites and Collection

Samples used in the present study were collected by various methods, including the use of hooklines and spear poles, or were purchased from local fishery harbors in Taiwan (New Taipei City, Keelung, Yilan, Penghu, Taitung) during 2010-2012.

Five exotic species of groupers known to distribute in Australia and the Southeast Asia, including *E. spilotoceps*, *E. bilobatus*, *E. faveatus*, *E. macrospilos*, and *E. polyphekadion*, were provided by the School of Marine and Tropical Biology at James Cook University in Australia. Each specimen was examined for species identification, and the total length (TL) or fork length (FL) was also measured. Muscle tissue (approximately 0.5 × 0.5 cm) near the caudal fin was collected and stored in 95% alcohol for analysis.

2. Polymerase Chain Reaction (PCR) Amplification and Sequencing

Total genomic DNA was extracted from the tissues using a commercial DNA isolation kit (Gentra, Minneapolis, MN, USA). The fragment of the COI gene was amplified from total DNA by PCR using the universal oligonucleotide primers FISH-BCL (5′-TCAACYAATCAYAAAGATATYGGCAC-3′) and FISH-BCH (5′-TAAACTTCAGGGTGACC AAAAAATCA-3′) (Baldwin *et al*., 2011). The PCR reactions were performed in 50 μL of reaction mixture containing 1 μL of DNA solution (approximately 100 ng genomic DNA), 35.5 μL of sterile distilled water, 5 μL of 10 × PCR buffer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), 4 μL of dNTP (2.5mM each), 2 μL of each primer (10μM), and 0.5 μL of 1.25 unit *Taq* polymerase (Takara Bio Inc., Shiga, Japan). PCR was performed in a model Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA)with predenaturation at 95°C for 120 s, denaturation at 94°C for 30 s, annealing at 50°C for 45 s, extension at 72°C for 60 s, and a final extension step at 72°C for 10 min. PCR products were examined by 1% agarose gel to confirm the exact size of the obtained fragment and then eluted using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified PCR products were sent to Mission Biotech (Taipei, Taiwan) for DNA sequencing. DNA sequencing was performed using an ABI PRISM dye terminator cycle sequence kit and an ABI 3730 DNA sequencer by private Mission Biotech company.

The sequence data were manually edited and automatically assembled using the BioEdit 7.0 (Hall, 1999) and Clustal X (Thompsom *et al*., 1997) programs. The nucleotide frequencies and transition/transversion ratios were obtained using Mega 5 software (Tamura *et al*., 2011). A saturation test was performed using DAMBE 5.3.48 program (Xia, 2013). *Cephalopholis* species and *Gracila* species were used as outgroups in phylogenetic analyses. The phylogenetic relationships among the samples were inferred from both neighbor-joining (NJ) and maximum parsimony (MP) methods. NJ trees were constructed using MEGA 5 (Tamura *et al*., 2011) under the Kimura2-parameter (K2P) model of base substitution (Kimura, 1980). MP tree topology was estimated using the tree bisection–reconnection (TBR) algorithm.

RESULTS

In total, 33 *Epinephelus* species (Table 1) were collected from Taiwan, the Southeast Asia, and Australia during January–December 2012. The mitochondrial COI gene was partially sequenced for all individuals examined. Among the 614 base pairs (T: 26.5-31.9%; A: 23.1-26.2%; C: 25.6-30.5%; G: 16.6-19.1%), 234 were considerable variable sites, 219 were parsimony-informative sites, and 15 were singleton variable sites. The content of A + T (54.2%) were higher than that of C + G (45.8%). The mean transition/transversion ratio was 4.78 (K2P), which showed that transition was obviously more than transversion and that there was no substitution saturation at these sites (Fig. 1).

With exception for species as outgroup, the mean divergence distance in 33 *Epinephelus* species was 0.147. Pairwise divergence (K2P distances) between species is illustrated in Table 2. The value of evolutionary distance ranged from 0.004 to 0.209. Among these *Epinephelus* groupers, the distance between the brownspotted grouper *E. chlorostigma* and the areolate grouper *E. areolatus* had the lowest value of 0.004, while the distance between the two reticulated groupers (Heemstra and Randall, 1993),

namely the foursaddle grouper *E. spilotoceps* and the longfin grouper *E. quoyanus*, had the highest value of 0.209. Furthermore, compared with the nine reticulated groupers that had a mean divergence distance of 0.137, the distance between the twinspot grouper *E. bilobatus* and the highfin grouper *E. maculatus* had the lowest value of 0.058.

Table 1 List of 33 economically valuable *Epinephelus* species included in this study and two *Cephalopholis* species and one *Gracila* specieswere used as outgroups

Genera	FAO Code	Scientific name		Collection site	Voucher specimens
Epinephelus	Epin66	*Epinephelus*	*akaara*	Taiwan	FRIF0115D
	Epin74		*amblycephalus*	Taiwan	FRIF0047D
	Epin4		*areolatus*	Taiwan	FRIF0021D
	Epin5		*awoara*	Taiwan	FRIF0043D
	Epin82		*bilobatus*	Malaysia	
	Epin86		*bontoides*	Taiwan	FRIF0067D
	Epin28		*coeruleopunctatus*	Taiwan	FRIF0091D
	Epin29		*chlorostigma*	Taiwan	FRIF0486D
	Epin67		*coioides*	Taiwan	FRIF0254D
	Epin68		*corallicola*	Taiwan	FRIF0257D
	Epin69		*cyanopodus*	Taiwan	FRIF0031D
	Epin75		*fasciatomaculosus*	Taiwan	FRIF0086D
	Epin8		*fasciatus*	Taiwan	FRIF0005D
	Epin49		*faveatus*	Malaysia	
	Epin34		*hexagonatus*	Taiwan	FRIF0013D
	Epin83		*lanceolatus*	Taiwan	FRIF0209D
	Epin35		*latifasciatus*	Taiwan	FRIF0123D
	Epin32		*macrospilos*	Australia	
	Epin85		*maculatus*	Taiwan	FRIF0012D
	Epin38		*malabaricus*	Taiwan	FRIF0216D
	Epin39		*melanostigma*	Taiwan	FRIF0051D
	Epin40		*merra*	Taiwan	FRIF0009D
			moara	Taiwan	
	Epin46		*ongus*	Taiwan	FRIF0145D
	Epin41		*polyphekadion*	Australia	
	Epin10		*quoyanus*	Taiwan	FRIF0001D
	Epin50		*radiatus*	Taiwan	FRIF0026D
	Epin51		*retouti*	Taiwan	FRIF0142D
	Epin92		*sexfasciatus*	Taiwan	FRIF0127D
	Epin54		*spilotoceps*	Malaysia	
	Epin12		*tauvina*	Australia	
	Epin97		*trimaculatus*	Taiwan	FRIF0174D
	Epin57		*undulosus*	Taiwan	FRIF0262D
Cephalopholis	Cephal11	*Cephalopholis*	*boenak*	Taiwan	FRIF0010D
	Cephal20		*igarashiensis*	Taiwan	FRIF0175D
Gracila	Gracil1	*Gracila*	*albomarginata*	Taiwan	FRIF0141D

Table 2 Pairwise divergence (K2P distances) between 33 species, the values ranged from 0.004 to 0.209

	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	*E. quoyanus*	—											
2	*E. malabaricus*	0.174											
3	*E. fasciatus*	0.156	0.158										
4	*E. merra*	0.158	0.190	0.146									
5	*E. maculatus*	0.166	0.164	0.155	0.158								
6	*E. hexagonatus*	0.156	0.159	0.127	0.115	0.155							
7	*E. chlorostigma*	0.160	0.180	0.145	0.154	0.142	0.142						
8	*E. moara*	0.164	0.094	0.151	0.161	0.155	0.137	0.154					
9	*E. radiatus*	0.180	0.136	0.165	0.172	0.182	0.132	0.142	0.111				
10	*E. cyanopodus*	0.181	0.186	0.160	0.150	0.146	0.160	0.078	0.158	0.187			
11	*E. awoara*	0.152	0.151	0.135	0.155	0.148	0.137	0.131	0.140	0.138	0.149		
12	*E. amblycephalus*	0.163	0.168	0.147	0.164	0.156	0.156	0.135	0.140	0.154	0.148	0.082	
13	*E. melanostigma*	0.159	0.166	0.146	0.146	0.140	0.109	0.143	0.131	0.150	0.147	0.125	0.138
14	*E. areolatus*	0.163	0.178	0.151	0.157	0.147	0.147	0.004	0.156	0.141	0.077	0.134	0.140
15	*E. bontoides*	0.092	0.186	0.145	0.183	0.147	0.155	0.148	0.161	0.193	0.165	0.141	0.154
16	*E. tauvina*	0.161	0.166	0.135	0.134	0.139	0.099	0.154	0.133	0.150	0.158	0.137	0.138
17	*E. fasciatomaculosus*	0.148	0.165	0.170	0.161	0.167	0.161	0.154	0.155	0.150	0.166	0.087	0.108
18	*E. caeruleopunctatus*	0.159	0.123	0.168	0.149	0.144	0.146	0.172	0.097	0.141	0.169	0.137	0.165
19	*E. akaara*	0.151	0.175	0.145	0.155	0.158	0.157	0.143	0.154	0.136	0.140	0.050	0.073
20	*E. latifasciatus*	0.194	0.100	0.178	0.182	0.181	0.164	0.173	0.091	0.139	0.188	0.169	0.181
21	*E. sexfasciatus*	0.142	0.159	0.148	0.148	0.159	0.148	0.130	0.145	0.150	0.144	0.059	0.095
22	*E. retouti*	0.158	0.174	0.082	0.118	0.154	0.099	0.140	0.149	0.159	0.148	0.142	0.139
23	*E. ongus*	0.169	0.135	0.175	0.168	0.184	0.152	0.179	0.091	0.138	0.179	0.153	0.153
24	*E. trimaculatus*	0.076	0.167	0.148	0.155	0.155	0.150	0.160	0.155	0.187	0.173	0.147	0.143
25	*E. lanceolatus*	0.185	0.144	0.168	0.181	0.185	0.146	0.202	0.119	0.166	0.177	0.162	0.169
26	*E. coioides*	0.167	0.040	0.150	0.172	0.159	0.142	0.176	0.091	0.129	0.173	0.146	0.148
27	*E. corallicola*	0.173	0.136	0.158	0.147	0.160	0.154	0.156	0.101	0.143	0.158	0.144	0.157
28	*E. undulosus*	0.179	0.196	0.180	0.181	0.169	0.172	0.096	0.154	0.159	0.094	0.147	0.153
29	*E. bilobatus*	0.151	0.159	0.149	0.151	0.058	0.151	0.152	0.153	0.162	0.141	0.140	0.143
30	*E. faveatus*	0.109	0.183	0.147	0.169	0.157	0.157	0.166	0.172	0.199	0.171	0.147	0.142
31	*E. macrospilos*	0.089	0.175	0.157	0.161	0.167	0.169	0.166	0.153	0.183	0.163	0.151	0.152
32	*E. polyphekadion*	0.169	0.136	0.184	0.164	0.170	0.147	0.194	0.097	0.157	0.191	0.177	0.172
33	*E. spilotoceps*	0.209	0.193	0.153	0.173	0.170	0.163	0.094	0.164	0.178	0.083	0.149	0.160
34	*G. albomarginata*	0.184	0.204	0.187	0.189	0.188	0.192	0.173	0.179	0.168	0.187	0.190	0.174
35	*C. igarashiensis*	0.203	0.197	0.195	0.192	0.172	0.204	0.160	0.157	0.159	0.183	0.185	0.180
36	*C. boenak*	0.210	0.195	0.201	0.206	0.224	0.187	0.208	0.185	0.192	0.197	0.190	0.199

Table 2 Continued

	Species	13	14	15	16	17	18	19	20	21	22	23	24
1	*E. quoyanus*												
2	*E. malabaricus*												
3	*E. fasciatus*												
4	*E. merra*												
5	*E. maculatus*												
6	*E. hexagonatus*												
7	*E. chlorostigma*												
8	*E. moara*												
9	*E. radiatus*												
10	*E. cyanopodus*												
11	*E. awoara*												
12	*E. amblycephalus*												
13	*E. melanostigma*												
14	*E. areolatus*	0.142											
15	*E. bontoides*	0.141	0.151										
16	*E. tauvina*	0.039	0.153	0.146									
17	*E. fasciatomaculosus*	0.157	0.155	0.157	0.165								
18	*E. caeruleopunctatus*	0.150	0.174	0.161	0.147	0.152							
19	*E. akaara*	0.125	0.144	0.150	0.131	0.088	0.152						
20	*E. latifasciatus*	0.160	0.175	0.199	0.161	0.179	0.109	0.174					
21	*E. sexfasciatus*	0.145	0.131	0.145	0.147	0.040	0.139	0.062	0.172				
22	*E. retouti*	0.133	0.143	0.157	0.129	0.158	0.148	0.142	0.158	0.141			
23	*E. ongus*	0.140	0.181	0.175	0.143	0.173	0.093	0.154	0.138	0.156	0.172		
24	*E. trimaculatus*	0.158	0.163	0.091	0.165	0.163	0.154	0.158	0.171	0.159	0.150	0.176	
25	*E. lanceolatus*	0.160	0.204	0.197	0.167	0.185	0.152	0.170	0.131	0.180	0.161	0.162	0.162
26	*E. coioides*	0.146	0.174	0.185	0.147	0.158	0.126	0.159	0.101	0.152	0.164	0.134	0.167
27	*E. corallicola*	0.132	0.158	0.171	0.136	0.147	0.059	0.150	0.122	0.131	0.154	0.097	0.177
28	*E. undulosus*	0.142	0.099	0.160	0.166	0.153	0.172	0.136	0.182	0.142	0.162	0.169	0.170
29	*E. bilobatus*	0.149	0.153	0.147	0.155	0.150	0.146	0.144	0.173	0.149	0.156	0.166	0.148
30	*E. faveatus*	0.174	0.167	0.082	0.174	0.153	0.175	0.155	0.206	0.151	0.136	0.181	0.093
31	*E. macrospilos*	0.163	0.169	0.097	0.164	0.159	0.154	0.146	0.188	0.151	0.159	0.170	0.049
32	*E. polyphekadion*	0.161	0.196	0.177	0.155	0.169	0.114	0.180	0.123	0.163	0.165	0.109	0.171
33	*E. spilotoceps*	0.165	0.095	0.182	0.183	0.173	0.158	0.153	0.192	0.153	0.139	0.172	0.197
34	*G. albomarginata*	0.196	0.176	0.183	0.199	0.177	0.170	0.177	0.198	0.177	0.187	0.188	0.170
35	*C. igarashiensis*	0.178	0.162	0.197	0.198	0.175	0.188	0.192	0.181	0.181	0.198	0.188	0.195
36	*C. boenak*	0.181	0.211	0.188	0.187	0.166	0.189	0.201	0.208	0.180	0.210	0.198	0.197

Table 2 Continued

	Species	25	26	27	28	29	30	31	32	33	34	35	36
1	*E. quoyanus*												
2	*E. malabaricus*												
3	*E. fasciatus*												
4	*E. merra*												
5	*E. maculatus*												
6	*E. hexagonatus*												
7	*E. chlorostigma*												
8	*E. moara*												
9	*E. radiatus*												
10	*E. cyanopodus*												
11	*E. awoara*												
12	*E. amblycephalus*												
13	*E. melanostigma*												
14	*E. areolatus*												
15	*E. bontoides*												
16	*E. tauvina*												
17	*E. fasciatomaculosus*												
18	*E. caeruleopunctatus*												
19	*E. akaara*												
20	*E. latifasciatus*												
21	*E. sexfasciatus*												
22	*E. retouti*												
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26	*E. coioides*	0.133											
27	*E. corallicola*	0.154	0.136										
28	*E. undulosus*	0.186	0.192	0.156									
29	*E. bilobatus*	0.178	0.161	0.153	0.148								
30	*E. faveatus*	0.193	0.183	0.179	0.167	0.137							
31	*E. macrospilos*	0.180	0.190	0.166	0.172	0.156	0.090						
32	*E. polyphekadion*	0.152	0.119	0.117	0.188	0.161	0.170	0.177					
33	*E. spilotoceps*	0.181	0.179	0.146	0.102	0.165	0.188	0.204	0.183				
34	*G. albomarginata*	0.203	0.203	0.169	0.187	0.195	0.179	0.183	0.203	0.196			
35	*C. igarashiensis*	0.196	0.178	0.181	0.184	0.171	0.216	0.207	0.190	0.207	0.111		
36	*C. boenak*	0.219	0.183	0.188	0.221	0.207	0.195	0.202	0.186	0.225	0.178	0.175	–

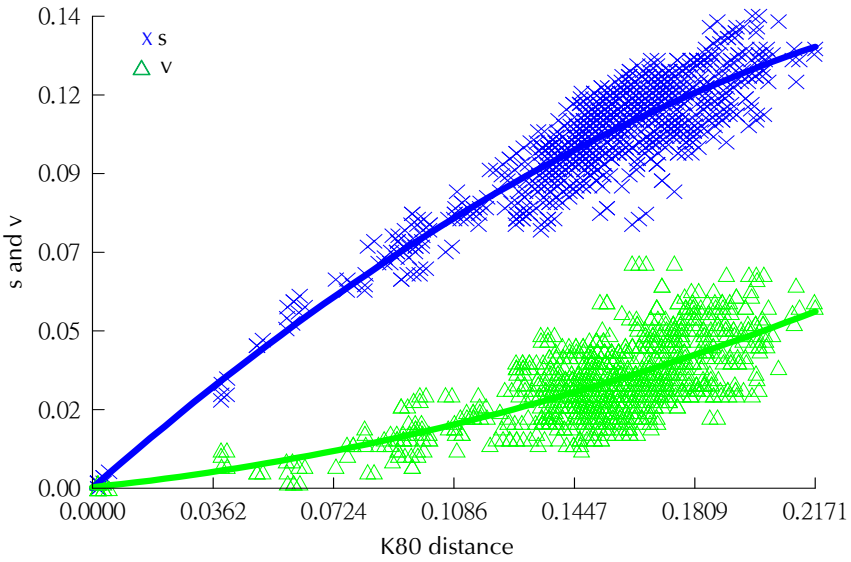

Fig. 1 Transitions (x, s) and transversions (Δ, v) plotted versus Kimura's two-parameter evolutionary distance. Only slight bending and no crossing of the different symbols, representing transitions and transversions, suggest no substitution saturation in this data set.

The NJ tree constructed by the K2P distance is shown in Fig. 2, and the single MP tree is shown in Fig. 3. The MP tree obtained using the TBR algorithm had a tree length of 1246, a consistency index (CI) of 0.2843, and a retention index (RI) of 0.7307. Based on the COI sequences, the tree topologies resolved by NJ and MP methods were not identical; however, the recovered tip clades of both the trees were the same. Different individuals from the same species were all grouped together with a high bootstrap value, respectively. Sequences obtained from the genus *Epinephelus* grouped the species into two clades by taxonomic affinity: one consisted of small- to medium-size groupers and the other consisted of medium- to large-size groupers such as *E. lanceolatus*. All reticulated groupers were found within a monophyletic group.

DISCUSSION

In the present study, a total of 33 *Epinephelus* species were collected, which was very close to the 39 species recorded in the Fish Database of Taiwan. Furthermore, the number of *Epinephelus* species collected in this study was nearly half of the number of species (70) distributed in the Indo-Pacific Ocean (Craig *et al.*, 2011).This reveals that Taiwan has a highly diversity of *Epinephelus* groupers. However, at present, the status of groupers in Taiwan remains poorly understood owing to limited research and the absence of specific fishery focused on them (With the exception of some recreational or commercial hook-and-line activity). Most *Epinephelus* groupers share similar morphology and a lack of species-specific characters. As a result, their fishery statistical data is usually simplified as "groupers" or "seabass" rather than species-specific catch data. This raises fundamental questions regarding fishery resource management and conservation. In particular, many species within genus *Epinephelus* were already listed in different criteria of the IUCN Red List, e.g., the orange-spotted grouper *E. coioides* was listed in "Near Threatened" in 2004, the giant grouper *E. lanceolatus* was listed in "Vulnerable" in 2006 and the Malabar grouper *E. malabaricus* was listed in "Near Threatened" in 2006, although all of them are common species for aquaculture in Taiwan.

Morphological and osteological characters have traditionally been used to classify groupers in several studies (Smith, 1971; Randall and Ben-Tuvia, 1983; Heemstra and Randall, 1991, 1993). Some closely related *Epinephelus* species only differ in the color pattern and geographic distribution, however, marked differences in the color pattern could exist between distant populations within a single widely distributed species (Heemstra and Randall, 1993). This increases the difficulty in species identification in the field. Therefore, the mitochondrial COI gene could be applied to set up a DNA barcode database and to evaluate the phylogenetic relationship of the

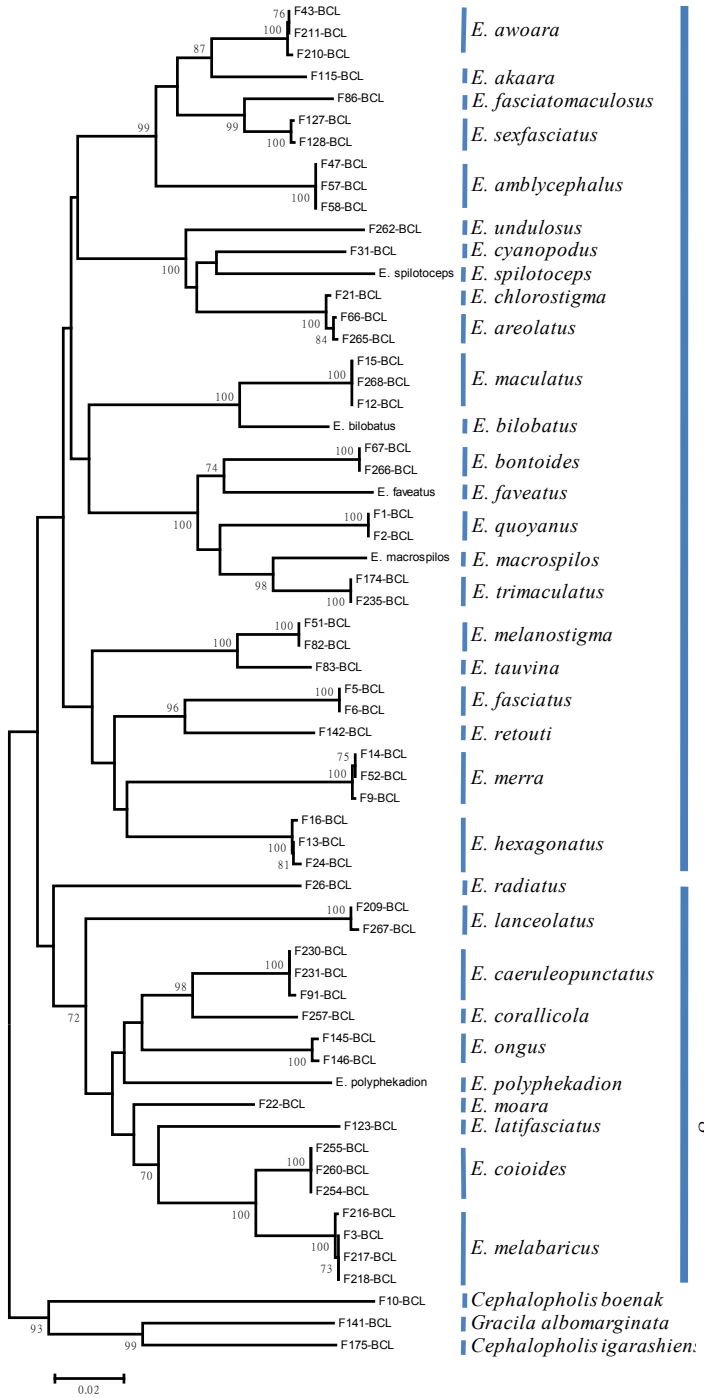

Fig. 2 Neighbor-joining (NJ) tree from mitochondrial COI DNA data of the *Epinephelus* groupers. Bootstrap value are shown as percentages and based on 1000 replicates. Only bootstrap values of > 70% are shown on the branches.

genus *Epinephelus* in the present study. Based on the results of COI gene sequence analysis, two new recorded species were discovered (unpublished): the sixbar grouper *E. sexfasticatus* collected at southern Penghu and a newly valid species: *E. moara* (Liu *et al.*, 2013) collected at Keelung offshore. This suggests that more *Epinephelus* species, including cryptic species, may exist around Taiwan. More researches related to taxonomy, population genetics, reproductive biology, and others are necessary to provide basic information in order to sustain utilization of the *Epinephelus* groupers as a fishery resource.

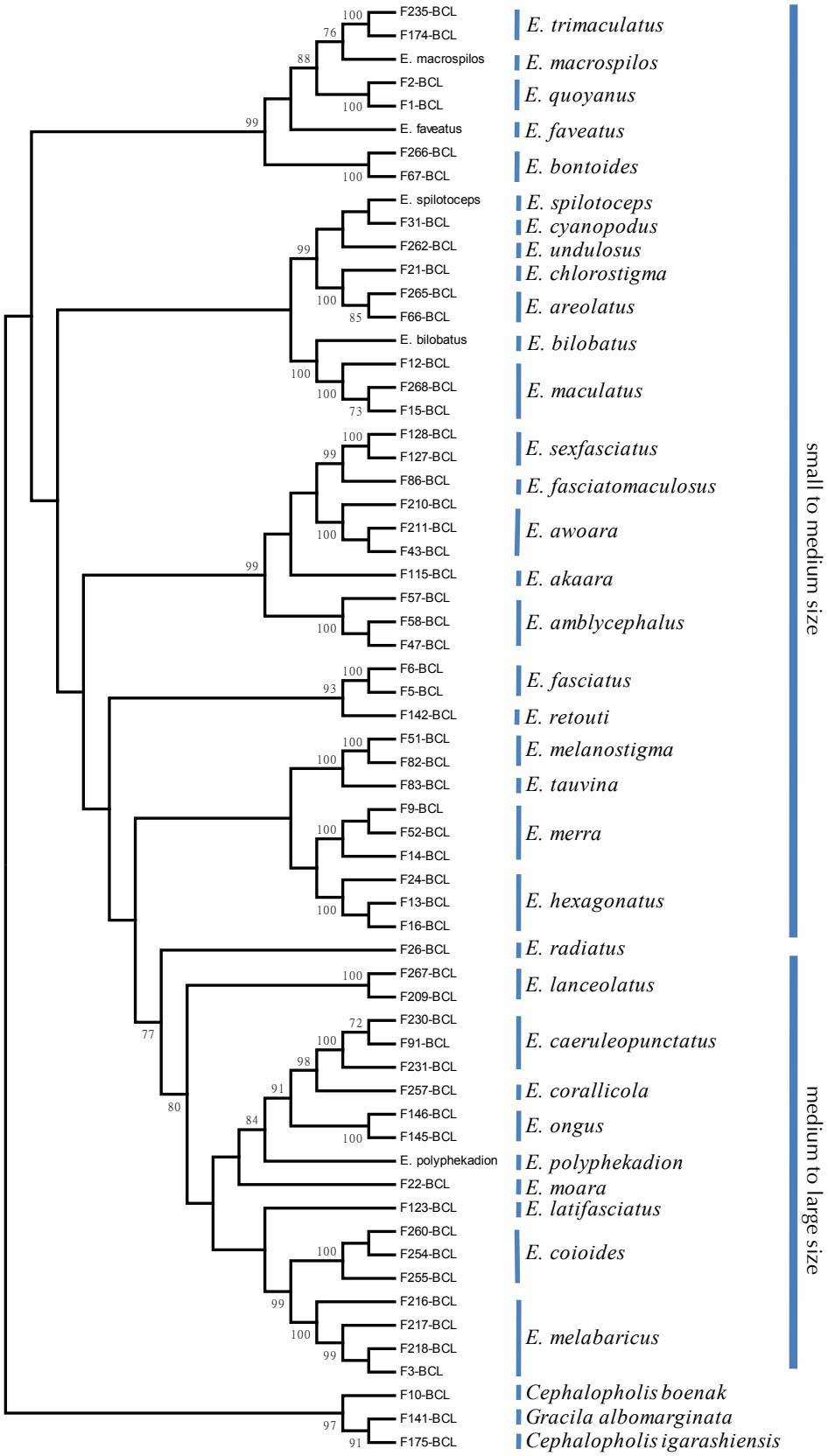

Fig. 3 Maximum parsimony (MP) tree from mitochondrial COI DNA data of the *Epinephelus* groupers. Bootstrap value are shown as percentages and based on 1000 replicates. Only bootstrap values of > 70% are shown on the branches.

With regard to systematic, lower percoid fishes whose affinities are unclear, have been traditionally assigned the family Serranidae. Therefore several studies, including cladistics (Bladwin and Johnson, 1993; Gosline, 1966; Johnson, 1983; Jordan and Eigenmann, 1890; Kendall, 1979) and phylogenetic analysis (Craig *et al.*, 2001, 2007; Zhuang, 2013), proposed different systematic hypotheses to classify the relationship of subfamilies and genera within this family; however, it still remains unclear due to the enormous number of species, worldwide distribution and uninformative morphometric characters. Until recently, it was recognized that *Epinephelus* spp. should be belong to the family Epinephelidae (a new proposed family, including Diploprioninae, Epinephelinae, Liopropominae, and Grammistinae) (Craig and Hasting, 2007). On the other hand, there are very few studies regarding interspecies relationships within the family Serranidae, such as genera *Epinephelus* (Kang and Song, 2004; Sachithanandam *et al*., 2012), *Paralabrax* (Pondella *et al*., 2003) and *Plectropomus* (Chakkaravathy *et al*., 2011; Saad *et al*., 2012). Due to the accumulation of large amounts of molecular data, many novel relationships among groupers have been figured out (Craig and Hasting, 2007; Smith and Craig, 2007).

The results of both the NJ and MP phylogenetic trees were similar to the results of several previous studies which used different genes as genetic markers, including, for example, genes encoding mitochondrial 12S, 16S, and Cyt *b*, and nuclear markers *Tmo-4C4* and Histone H3 (Craig *et al*., 2007). It supported that the COI gene also could be a suitable genetic marker for either DNA barcoding or phylogenetic relationship of groupers. Both phylogenetic trees had two major clades: one clade contained medium- to large-size groupers (approximately 70-100 cm TL or more), except for the white streaked grouper *E. ongus* (maximum size: 40 cm), and the other clade contained small- to medium-size groupers (approximately 30-60 cm TL), including the blacktip grouper *E. fasciatus* and star-spotted grouper *E. hexagonatus*. In particular, all nine "reticulated groupers" were found within this clade. This result was consist with Craig *et al.* (2001, 2007), although the bootstrap value in present study was low, and supported that *Epinephelus* groupers of Indo-Pacific Ocean could be separated into two main groups which display a range of different body size and color patterns. Because of more diverse color patterns and wider body size range within medium- to large-size groupers, it revealed that more complex phylogenetic relationship could be exist within these species. However, more researches are needed to verify such relationships.

The nine reticulated species, including the twinspot grouper *E. bilobatus*, the barred-chest grouper *E. faveatus*, the star-spotted grouper *E. hexagonatus,* the highfin grouper *E. maculatus*, the snubnose grouper *E. macrospilos*, the one blotch grouper *E. melanostigma*, the honeycomb grouper *E. merra*, the longfin grouper *E. quoyanus*, and the foursaddle grouper *E. spilotoceps*, are usually found in shallow-water coral reef habitats and have a rounded caudal fin and close-set dark brown spots with the pale interspaces forming a network on the body (Heemstra and Randall, 1993). However, they are often confused and subsequently misidentified. The results of the present study suggested that these nine species could have close phylogenetic relationships. All of these nine species are only distributed in the Indo-Pacific Ocean. Some species are widespread, while some have narrow distribution to a specific area. Therefore, an interesting evolutionary issue arises: how did the evolutionary process result in the current status of either, divergence or convergence? Unfortunately, the evolutionary process has still not been determined after analysis of their geographical distribution and phylogenetic relationships.

The results of evolutionary distance revealed that the distance between the twinspot grouper *E. bilobatus* and the highfin grouper *E. maculatus* had the lowest value of 0.058 among these nine species, which consists with morphological key (Heemstra and Randall, 1993). These results suggest that *E. bilobatus* and *E. maculates* could be sister species despite having different geographical distributions that do not overlap. The brownspotted grouper *E. chlorostigma* and the areolate grouper *E. areolatus*

had the lowest value of 0.004 among all the species in the present study, which consists with morphological key. This finding suggested that these two species are very close to one another. These two species have often been confused because of their overlapping geographical distribution and their diagnostic features that vary but overlap in terms of the number of gill rakers on the lower limb (15-18 vs. 14-16) and dorsal fin rays (16-18 vs. 15-17).Their maximum size (70 cm vs. 40 cm), color pattern, and shape of the caudal fin also differ.

The orangespotted grouper *E. coioides* and the Malabar grouper *E. malabaricus*, both are two most common aquaculture species, are very close species based on evolutionary distance (value: 0.04) and also consist with morphological key. This corresponds to the status of aquaculture of groupers in which two species are usually bred in one pond, resulting in hybridization. However, several species pairs could not consistent with morphological classification, including the one blotch grouper *E. melanostigma* and the foursaddle grouper *E. spilotoceps* (evolutionary distance: 0.165), the palemargin grouper *E. bontoides* and the camouflage grouper *E. polyphekadion* (evolutionary distance: 0.177), and the blacktip grouper *E. fasciatus* and the redtipped grouper *E. retouti* (evolutionary distance: 0.082). Since Zhuang (2013) suggested that mitochondrial protein-coding gene ND2 could be a better genetic marker for DNA barcoding in groupers due to the higher percentage of variable sites (52.2% > 36.9% of COI). This indicates that the most suitable genetic markers are still need to be investigated and the validity of the current systematic of genus *Epinephelus* requires to be re-examination.

There were 33 *Epiephelus* grouper species collected and identified, including 2 new recorded species: *E. sexfasticatus* and *E. moara*. The molecular species identification and phylogenetic relationship among *Epinephelus* groupers were investigated by mitochondrial COI gene and the morphological characters were checked with the morphological key (Heemstra and Randall, 1993). Molecular and morphometrical results are roughly similar, though there were some differences in certain species pairs. This study not only census the status of commercially valuable *Epinephelus* groupers in Taiwan, but also provides important molecular resources for the species identification, population genetic structure, fishery management and conservation biology of groupers. Since several species of these 33 grouper species have already been assessed as "endangered," "vulnerable," or "near threatened" on the IUCN Red List (http://www.iucnredlist.org/) and many other species also exposed to overfishing, thus it's important to pay more attention on these commercially valuable wild grouper for sustainable resource utilization.

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台灣產經濟性石斑魚屬生命條碼及分子親緣演化關係分析

陳高松[1]・謝恆毅[2]・黃閔裕[3]・馬嘉欣[4]・蕭聖代[3*]

[1]行政院農業委員會水產試驗所企劃資訊組
[2]行政院農業委員會水產試驗所澎湖海洋生物研究中心
[3]行政院農業委員會水產試驗所海洋漁業組
[4]澳洲詹姆士庫克大學海洋及熱帶生物研究所

摘　要

石斑魚屬在分類上屬於鮨科、石斑魚亞科，全世界約有 100 餘種分布於三個主要洋區。本研究於 2012 年間於台灣及澳洲共計採集 33 種石斑魚屬 (Genus *Epinephelus*) 的石斑魚，利用粒線體 COI 基因序列資料進行魚種鑑定、遺傳距離計算，及利用 neighbor-joining (NJ) 方法與 maximum parsimony (MP) 方法建構分子親緣關係樹，藉以探討本屬內各魚種之關係。研究結果顯示以密點石斑 (*E. chlorostigma*) 與寶石石斑 (*E. areolatus*) 的演化距離最近 (0.004)，吻斑石斑 (*E. spilotoceps*) 與玳瑁石斑 (*E. quoyanus*) 的演化距離最遠 (0.209)。分子親緣關係樹主要分成兩群，具網狀斑紋 (Reticulated grouper) 的中小體型種類為一群，龍膽石斑等中大型體型種類為一群。這些結果與傳統型態分類結果相似，顯示粒線體 COI 基因除了作為生命條碼的遺傳標誌外，亦能應用於探討這些高經濟性石斑魚種的親緣演化關係。

關鍵詞：鮨科、COI 基因、生命條碼、分子親緣演化樹

*通訊作者 / 基隆市和一路 199 號，TEL: (02) 2462-2101; FAX: (02) 2463-3110; E-mail: stshiao@mail.tfrin.gov