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Inference of Phylogenetics and Evolution of *Epinephelus septemfasciatus* and 48 Other Species of *Epinephelus* Genus using Mitochondrial CO1 Fragment Sequences

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Abstract.- Fragments of mitochondrial cytochrome c oxidase subunit I (CO1) gene of *Epinephlus septemfasciatus* were amplified and compared with 48 other species of *Epinephelus* genus collected from NCBI to construct the phylogenetic relationships. Nucleotide sequences of 582 base pair of CD1 gene revealed 231 polymorphic sites. Phylogeny tree was constructed based on maximum likelihood and Bayesian methods. *E. septemfasciatus* did not show any distinct status in the phylogenetic trees, and its distribution was found to be closely related with that of Kuroshio Current indicating it as a warm-water species. Some small clades were found corresponding to limited geographic distribution such as South China Sea, South African Sea and West Indian Sea. Genetic distances were small between pair comparisons of several species which may be attributed to hybridizations. According to phylogenetics, present distributions and currents direction, the groupers likely originated from western Pacific tropic areas and dispersed into Atlantic crossing sea area of South Africa.

Keywords: *Epinephelus* genus, *Epinephelus septemfasciatus*, cytochrome C oxidase subunit 1, convict grouper sex fish, mitochondrial DNA.

INTRODUCTION

Groupers is a common name for all species of subfamily Epinephlinae. They are commercially most important marine fish species of world wide distribution extending from tropical to subtropical sea areas. *Epinephelus* genus, which is the biggest amongst 15 genera of Epinephlinae, comprises 98 species (Heemstra and Randall, 1993). Most groupers inhabit rocky reefs. They are midsized predators which vary in size from less than 0.5 m to nearly 2.0 m (Robins *et al*., 1986). Color pattern and geographic locality are most often used to identify grouper species (Craig *et al*., 2001). However, many species within *Epinephelus* lack morphological specializations; even color and morphology can vary as the growth and environment changes. In addition, interspecific hybridizations are found among many grouper species (Ding *et al*., 2006). All of these have led to a great deal of taxonomic confusion within the genus.

With the rapid development of molecular tools, mitochondrial DNA (mtDNA) has been applied widely in the studies of phylogenetics and evolution as well as an effective marker to assist taxonomy because of its uniparental inheritance (in a majority of animal phyla), high evolutionary rate, lack of introns, large copy numbers in every cell, and limited recombination (Radulovici *et al*., 2010). The mitochondrial cytochrome c oxidase subunit 1 (CO1) gene has been proposed as a DNA barcode and frequently used to recognize provisional species in groups with incomplete taxonomy, and morphological, ecological and behavioural differences are regularly detected upon further examination of divergent taxa (Carr *et al*., 2011).

Epinephelus septemfasciatus was thought to be the only species of *Epinephelus* genus distributed in the high latitudes areas in the West Pacific (Cheng and Yang, 1981) and was known as a cold-water grouper. The focus of this study was to see if such difference of habitats can determine a peculiar status of *E. septemfasciatus* in the phylogeny of *Epinephelus* genus. The phylogenetic relationshops among *E. septemfasciatus* and most *Epinephelus* species will be described and discussed based on CO1 sequence variations. Our results will be helpful in taxonomy and artificial breeding of *Epinephelus* species.

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MATERIALS AND METHODS

Samples

Muscle tissues of 21 *E. septemfasciatus* were collected from the stocked population in Baijia Aquaculture Company, which were imported from Korea in June, 2010. Genomic DNA was extracted following the procedures described by Sambrook *et al*. (1989). The segment of the mtDNA CO1 was amplified with the primers CO1-F: 5'- TCA ACC AAC CAC AAA GAC ATT GGC AC -3' (forward) and CO1-R: 5'- ACT TCA GGG TGA CCG AAG AAT CAG AA -3' (reverse) (Ward *et al*., 2005). Polymerase chain reactions were performed according to the protocols of Ward *et al*. (2005) and purified fragments were sequenced using ABI 3100 DNA sequencer (Applied Biosystems). CO1 fragment sequences of other 48 *Epinephelus* species were collected from Genebank (Table I).

Data analyses

All the sequences were edited and aligned using CLUSTAL X (Thompson *et al*., 1997). The variable sites, transitions and transversions, base compositions, the genetic distance based on Kimura-2-Parameter between pair sequences, and the ratio of substitution rates at non-synonymous and synonymous sites (dN/dS) based on Nei-Gojobori method were performed in MEGA 5.0 (Tamura *et al*., 2011). The relations between genetic distance and transition and transversion for pairwise sequence comparisons were analyzed by DAMBE (Xia, 2000). The best-fitted nucleotide substitution model was selected in MODELTEST 3.6 (Posada and Crandall, 1998). Phylogenetic relationships were assessed by maximum likelihood (ML) and Bayesian methods. ML tree was constructed in PAUP* 4.0b10 (Swofford, 2002) and Bayesian tree was implemented by MrBayes (Hueslsenbeck and Ronquist, 2001).

RESULTS

Sequence variations

No polymorphism was found in the 21 CO1 sequences of *E. septemfasciatus*. They shared one haplotype. After aligning 49 haplotype sequences, a fragment of 582 bp was used for further analysis. Nucleotide comparison of the segment revealed 231 polymorphic sites, of which 203 were parsimony. The sequence comparisons showed 59 transitions (si) and 19 transversions (sv). The ratio of si/sv is 3.12. Nucleotide compositions of the three codon positions are shown in Table II. Most transitions and transversions were observed at the third codon position.

The pairwise K2P distances between 49 species ranged from 0.002 (*E. fasciatomaculosus* vs. *E. sexfasciatus*) to 0.208 (*E. rivulatus* vs. *E. merra*). The relationship of the genetic distances and the numbers of transitions and transversions are shown in Figure 1. As the genetic distances increase, both transitions and transversions grow linearly, though transitions are always higher than transversions.

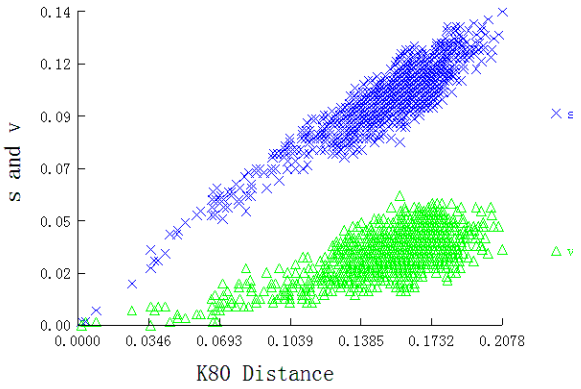

Fig. 1. The relationship of the genetic distances vs. the numbers of transitions and transversions.

The 582 nucleotides coded for 194 amino acids, which defined only 19 variable sites including 7 parsimony informative sites. The information of variable sites for each species is displayed in Figure 2. The ratio of substitution rates at non-synonymous and synonymous sites (dN/dS) under Nei-Gojobori method is about 0.047 indicating a purifying selection.

Phylogenetic relationships

The estimated nucleotide substitution model parameters are shown in Table III. GTR+I+G

Table I.- Genebank data information of the *Epinephelus* species, CO1 fragment sequences of which were used for comparison with *E. septemfasciatus*.

Species	Distribution[a]	Genebank no.	References*
E. acanthistius	E Pacific	HQ010051	**Gleason *et al*., 2010[b]**
E. adscensionis	W, C & E Atlantic	FJ583396	Steinke *et al*., 2009
E. aeneus	S Mediterranean, E Atlantic	JQ623937	**Keskin *et al*., 2012[b]**
E. akaara	S China Sea, W Pacific	JQ013801	**Wong *et al*., 2011[b]**
E. albomarginatus	S Africa, W Indian	JF493430	**Steinke *et al*., 2011[b]**
E. amblycephalus	S China Sea, W Pacific	JN242614	Zhang and Hanner, 2012
E. andersoni	S Africa, W Indian	JF493433	Steinke *et al*., 2011[b]
E. areolatus	W, E Indian & W Pacific	DQ107870	Ward *et al*, 2005
E. awoara	S China Sea, W Pacific	JQ013798	Wong *et al*., 2011[b]
E. bleekeri	W, E Indian & W Pacific	JQ013804	Wong *et al*., 2011[b]
E. bruneus	S China Sea, W Pacific	JN603834	**Ahn *et al*., 2011[b]**
E. chabaudi	S Africa, W Indian	JF493435	Steinke *et al*., 2011[b]
E. chlorostigma	W, E Indian & W Pacific	JQ412501	Schoelinck *et al*., 2012
E. coeruleopunctatus	W, E Indian & W Pacific	JQ349961	Hubert *et al*., 2012
E. coioides	W, E Indian & W Pacific	JN021213	**Quilang *et al*., 2011[b]**
E. cyanopodus	W Pacific	JQ412503	Schoelinck *et al*., 2012
E. diacanthus	W, E Indian & W Pacific	EF609519	Lakra *et al*., 2011
E. epistictus	W, E Indian & W Pacific	FJ237768	**Zhang and Hanner, 2008[b]**
E. ergastularius	E Australia, W Pacific	DQ107884	Ward *et al*, 2005
E. fario	S China Sea, W Pacific	EU600142	Wang *et al*., 2008[b]
E. fasciatomaculosus	S China Sea, W Pacific	JQ013799	Wong *et al*., 2011[b]
E. fasciatus	W, E Pacific, Indian & Atlantic	JQ431717	Hubert *et al*., 2012
E. flavocaeruleus	W, E Indian	JQ349963	Hubert *et al*., 2012
E. fuscoguttatus	W, E Indian & W Pacific	JQ013803	Wong *et al*., 2011[b]
E. hexagonatus	W, E Indian & W, C Pacific	JQ431719	Hubert *et al*., 2012
E. lanceolatus	W, E Indian & W, C Pacific	HQ174836	**Meng *et al*., 2010[b]**
E. latifasciatus	E Indian & W Pacific	EU014219	Lakra *et al*, 2011
E. longispinis	W, E Indian & W Pacific	JF493443	**Steinke *et al*., 2011[b]**
E. macrospilos	W, E Indian & W, C Pacific	JF493445	Steinke *et al*., 2011[b]
E. maculatus	E Indian & W, C Pacific	JN242490	Zhang and Hanner, 2012
E. malabaricus	W, E Indian & W Pacific	DQ107871	Ward *et al*, 2005
E. marginatus	E Atlantic & S Africa, W Indian	JQ623938	**Keskin *et al*., 2012[b]**
E. melanostigma	W, E Indian & W, C Pacific	JQ349966	Hubert *et al*., 2012
E. merra	W, E Indian & W, C Pacific	JQ431723	Hubert *et al*., 2012
E. morrhua	W, E Indian & W, C Pacific	DQ107896	Ward *et al*, 2005
E. multinotatus	W, E Indian & W, C Pacific	DQ107888	Ward *et al*, 2005
E. ongus	W, E Indian & W, C Pacific	FJ583399	Steinke *et al*., 2009
E. poecilonotus	W, E Indian & W, C Pacific	JF493454	Steinke *et al*., 2011[b]
E. posteli	S Africa, W Indian	JF493455	Steinke *et al*., 2011[b]
E. quoyanus	E Indian & W Pacific	DQ107864	Ward *et al*, 2005
E. retouti	W Indian & W Pacific	JQ431724	Hubert *et al*., 2012
E. rivulatus	W Indian & W Pacific	DQ107860	Ward *et al*, 2005
E. septemfasciatus	W & WN Pacific	this study	
E. sexfasciatus	S China Sea, W Pacific	EU595122	Zhang and Hanner, 2008[b]
E. spilotoceps	W Indian & W, C Pacific	FJ237775	Zhang and Hanner, 2008[b]
E. tauvina	W Indian & W, C Pacific	JQ623939	Keskin *et al*., 2012[b]
E. trimaculatus	W Pacific	JQ013805	Wong *et al*., 2011[b]
E. tukula	W, E Indian & W, C Pacific	JF493460	Steinke *et al*., 2011[b]
E. undulosus	W, E Indian & W Pacific	EF609352	Ward and Holmes, 2007

[a], According to FishBase, www.fishbase.org; [b], sequences have been released in NCBI but unpublished for articles
The bold references are not published and the sequences have been directly submitted.

Table II.- Variations in nucleotide composition of CO1 gene sequence of *Epinephelus* species.

	N	T	C	A	G	*si*	*sv*	*si/sv*
Total	582	29.6	27.7	24.8	17.9	59.0	19.0	3.12
1st	194	16.2	25.6	28.0	30.2	6.0	0.0	21.3
2nd	194	42.2	29.5	13.9	14.4	0.0	0.0	1.28
3rd	194	30.4	28.2	32.5	8.9	58.0	18.0	2.87

si, transitions; *sv*, transversions.

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                                  111111
                      1112224567 779234589
                      1580791932 345215282
E. acanthistius       LASPDIMIIR MNETVIIDN
E. adscensionis       .......... .........
E. aeneus             .......... ....I....
E. akaara             .......... ....I.T..
E. albomarginatus     .......... ....I....
E. amblycephalus      .......... ....I.T..
E. andersoni          .......... .........
E. areolatus          .......VV. .........
E. awoara             .........Q ..K.I.TN.
E. bleekeri           .......... ....I....
E. bruneus            .......... ....I....
E. chabaudi           .......... .........
E. chlorostigma       .......VV. ....I....
E. coeruleopunctatus  ..IS.Y.... ....I....
E. coioides           .......... ....I.T..
E. cyanopodus         .......VV. .........
E. diacanthus         .......V.. ....I....
E. epistictus         MV........ ....I....
E. ergastularius      .......... ........I
E. fario              .......... .........
E. fasciatomaculosus  .......... ....I.T..
E. fasciatus          .......... ....I....
E. flavocaeruleus     ....HY.VV. .........
E. fuscoguttatus      .......... ....I....
E. hexagonatus        .......... .........
E. lanceolatus        .......... ....I....
E. latifasciatus      .......... ...A.....
E. longispinis        .......... ....I....
E. macrospilos        .......... ....IV...
E. maculatus          .......... ....I....
E. malabaricus        .......... ....I.T..
E. marginatus         .......... ....LT.....
E. melanostigma       ....HYK... ....IV...
E. merra              .......... .........
E. morrhua            .......... .........
E. multinotatus       .......VV. .....V...
E. ongus              .......... ....I....
E. poecilonotus       .......... ....I....
E. posteli            .......... ....I....
E. quoyanus           .......... ....I....
E. retouti            .......... ....I....
E. rivulatus          .......... ....I....
E. septemfasciatusse  .......... .........
E. sexfasciatus       .......... ....I.T..
E. spilotoceps        .......VV. .........
E. tauvina            .......... ....I.T..
E. trimaculatus       .......... .........
E. tukula             .......... .........
E. undulosus          .......V.. ....I....
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Fig. 2. Variable sites of cytochrome C oxidase subunit 1 amino acid sequences.

model with I = 0.587 and G = 1.360 was selected as the best-fitted model. Both ML tree and Bayesian tree showed a consensus for the phylogenetic relationships of 49 species (Figs. 3, 4). They were divided into several obvious groups although some lacked high statistical support. Six species were present only in South China and were distinctly separated from others. These six species had relatively close relationship in the ML the tree. Only three species were clustered and were distant with the other three species. Excluding the six species of South China Sea, the remaining 43 species comprised two main groups. One of the two groups can be subdivided into three clades, one of which has 100% support in both ML and Bayesian trees. The five species of this clade seemed to have a

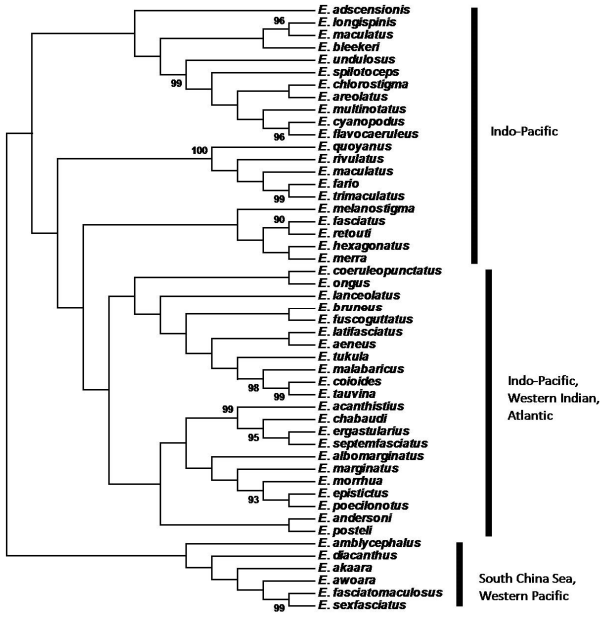

Fig. 3. The consensus tree of 49 species based on ML analyses. >90% bootstrap support was shown.

Table III.- Nucleotide substitution model parameter estimates for Model test analyses.

Model selected	Base frequencies	Rate matrix
GTR + I + G		R(a) [A-C] = 1.7542
- lnL = 6639.4458	freqA = 0.2647	R(b) [A-G] = 37.1796
K = 10	freqC = 0.2895	R(c) [A-T] = 3.4675
AIC = 13298.8916	freqG = 0.1402	R(d) [C-G] = 0.6745
(I) = 0.5870	freqT = 0.3056	R(e) [C-T] = 23.8452
(G) = 1.3597		R(f) [G-T] = 1.0000

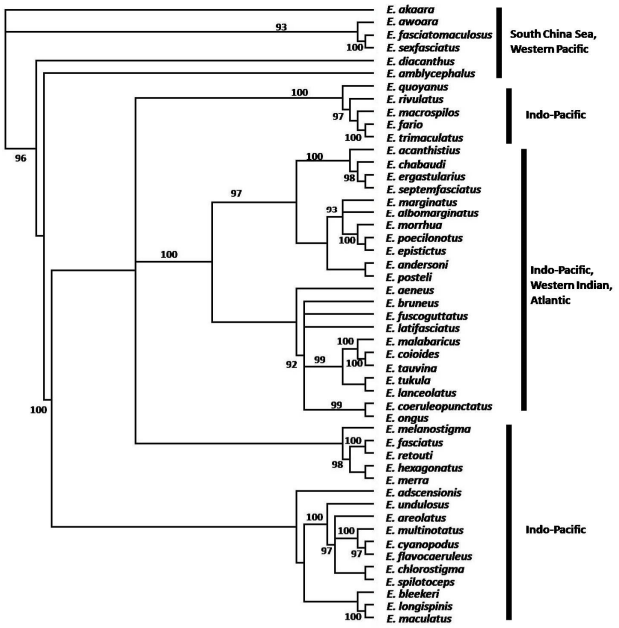

Fig. 4. The consensus tree of 49 species based on Bayesian analyses. >90% bootstrap support was shown.

common distribution with limits of Eastern Indian–Western Pacific. No obvious geographic association was found between clusters and the remaining species. *E. septemfasciatus* were observed as a sibling species of *E. ergastularius*. They are distant geographically with *E. acanthistius* and *E. chabaudi* but clustered with them interestingly. The species from Atlantic didn't diverge with those in Indian and Pacific.

DISCUSSION

Effect of mtDNA CO1 marker

Recently, mitochondrial CO1 gene fragments have been widely used as DNA bar coding to identify the species effectively. CO1 gene has been documented to be relatively conservative comparing with mitochondrial control region and Cytb gene in most fish species. Thus it can reflect large variations between species rather than within species. We failed to detect intraspecific polymorphism within *E. septemfasciatus* CO1 sequences in this study. However, quite high polymorphism was found among *Epinephelus* species. Simon *et al.* (1994) have reported AT rich nucleotide base compositions in CO1 gene of many organisms, but it is not obvious for groupers. Almost transitions and transversions occurred on the third codon positions and transitions are always higher than transversions indicating the nucleotide substitutions are yet unsaturated. This data suggest that CO1 markers are effective parameters for phylogenetic analyses of groupers.

Status of E. septemfasciatus

E. septemfasciatus did not show a special status in both ML and Bayes tree as previous exception. Strangely it was clustered with *E. ergastularius* (Eastern Australia), *E. chabaudi* (South Africa and Eastern Indian) and *E. acanthistius* (Eastern Pacific), which do not have common distribution and are distant apart from each other (Table I). If the Western Pacific tropic area is thought to be the centre of the groupers' distribution, one of the possible interpretations would be that these four species might have been derived from a long distant colonization. Specially, *E. septemfasciatus* was very close to *E. ergastularius* with a of 0.035 genetic distance. They are also highly similar in appearance with grey and white bands on the body. It appears that the two species have common ancestors with trans-equatorial dispersal. Trans-equatorial dispersal was widely proposed as a mechanism for anti-tropical distribution in marine organisms (Lindberg, 1991), such as *Sardinops* (Bowen and Grant, 1997), *Merluccius* (Quinterio *et al*., 2000) and *Mytilus* spp. (Gérard *et al*., 2008).

Although *E. septemfasciatus* can survive in the cold water under 10°C, its gonads can not develop in water at <20°C according to our artificial breeding trial. The distribution of high latitude

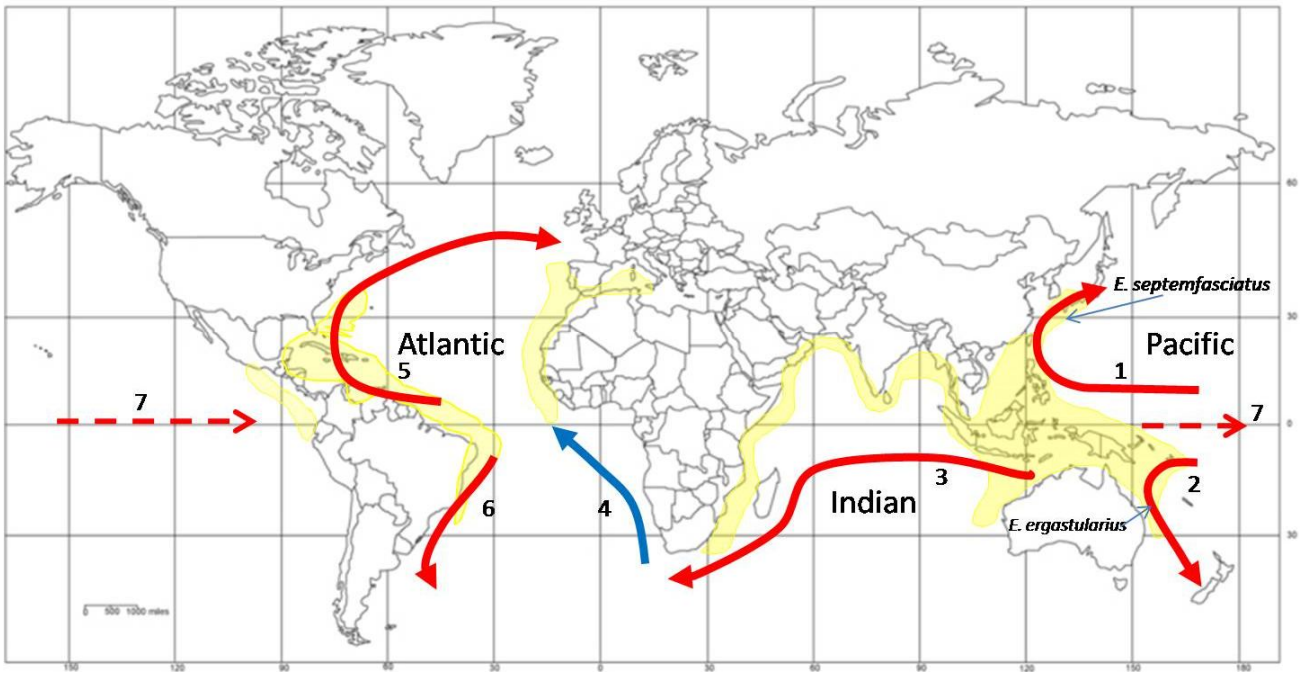

Fig. 5. General distributions (yellow shadow) and potential dispersal routes associated with several major ocean currents. 1. Kuroshio Current; 2. Eastern Australia Current; 3. South Equatorial Current; 4. Benguela Current; 5. North Atlantic Current; 6. Brazil Current; 7. Equatorial Counter.

likely benefits from the strong warm current, Kuroshio, which is consistent with the distribution of Kuroshio Currents. Likewise, Eastern Australia Currents may also contribute a lot to the southern dispersal of *E. ergastularius* (Fig. 5).

Geographic association

Most groupers have common and wide distribution in Indo-Pacific tropic sea areas. Thus it is difficult to find clear associations between phylogenetic clades and large geographic scales in this study. However, some clades with high bootstrap supports were still found corresponding to geographic limits, indicating that selection may have occurred for these clades. Six species mostly distributed in Western Pacific tropic area were first separated from phylogenetic trees, most of them are found in South China Sea. It was also noted that a small clade containing *E. andersoni* and other six species have a common distribution in South Africa and Western Indian sea. The clades including *E. fario* and *E. trimaculatus* are only distributed in Western Pacific, whereas *E. coioides* and *E. tauvina* belong to Western Indian waters (Figs. 3, 4).

Hybrids?

The genetic distances between *E. fasciatomaculosus* and *E. sexfasciatus*, *E. fario* and *E. trimaculatus*, and *E. coioides* and *E. tauvina* are very small (0.002, 0.003 and 0.009, respectively). It is quite uncommon between different relative species. These three pairs have similar appearance. *E. fasciatomaculosus* and *E. sexfasciatus* have bands in the body, *E. fario* and *E. trimaculatus* are full of spots, and *E. coioides* and *E. tauvina* have both bands and spots. In addition, these three pairs have same and limited distribution. As the maternal inheritance of mtDNA, we postulated these pair fish may originate in from hybrids.

Origin and evolution

According to Heemstra and Randall (1993), 111 species of 159 groupers are concentrated in the Central Indo-Pacific tropic area. This area is full of islands and reefs which is thought to be an ideal environment for the groupers. However, many

grouper species can also reach both sides of Atlantic such as Caribbean Sea, Mediterranean. Since both Atlantic and Indian oceans are younger than Pacific, the grouper are likely to have originated from Pacific, dispersed to west after crossing South Africa and finally colonizing into Atlantic. Such evolutionary routes were also consistent with the directions of ocean currents which could assist the dispersal of groupers from Indian to Atlantic ocean (Fig. 5). A few species such as *E. acanthistius*, were also found in eastern Pacific, which might have migrated from Atlantic rather than western Pacific. In the phylogenetic tree, *E. acanthistius* was close to *E. chabaudi* which is distributed in western Indian waters (Figs. 3, 4). In addition, large depth, far distance, and lack of reefs and rocks are disadvantageous to a trans-Pacific dispersal for the groupers of western Pacific in spite of an equatorial counter current from west to east.

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