CRYPTIC SPECIES AND EVOLUTIONARY HISTORY OF THE *BOLEOPHTHALMUS PECTINIROSTRIS* COMPLEX, ALONG THE NORTHWESTERN PACIFIC COAST

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Abstract: Two allopatric populations of *Boleophthalmus pectinirostris sensu lato* are found in the western Pacific coast of East Asia and the Strait of Malacca in Malaysia. However, the species status of Malaysian populations remains debated. Mitochondrial *ND*5 gene (718 bp) and nuclear *Rag*1 gene (1395 bp) were used to reconstruct phylogenetic relationships among *Boleophthalmus pectinirostris* fishes by sampling 45 specimens from 11 locations in the northwestern Pacific. The results showed that *Boleophthalmus pectinirostris* fishes can be divided into two monophyletic sister groups: the East Asian and the Malaysian lineages. Species delineation using GMYC and *BEAST species tree analyses supports the inclusion of the East Asian and Malaysian lineages of *Boleophthalmus pectinirostris sensu lato* in two different species. A molecular clock estimated that the divergence time between these two lineages is 2.73 Ma. We concluded that *Boleophthalmus pectinirostris sensu lato* is a complex including two species. The East Asian lineage is *Boleophthalmus pectinirostris sensu stricto,* and the Malaysian lineage is a cryptic species (*Boleophthalmus* sp.). Our findings suggest that the split between *Boleophthalmus pectinirostris sensu stricto* and *Boleophthalmus* sp. can be attributed to geographic isolation due to low sea levels during glacials, and to barriers to gene flow determined by ocean currents during interglacials, in the late Pliocene.

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Introduction

Boleophthalmus Valenciénnes, 1837 is a genus of small fishes which live in coastal intertidal mudflats of the Pacific and Indian Oceans, included in the order Perciformes, suborder Gobioidei, family Gobiidae, subfamily Oxudercinae (Murdy 1989). This genus includes 5 species: *Boleophthalmus birdsongi* (Murdy, 1989), *B. boddarti* (Pallas, 1770), *B. caeruleomaculatus* (McCulloch & Waite, 1918), *B. dussumieri* (Valenciennes，1837) and *B. pectinirostris* (Linnaeus, 1758). According to FishBase (Froese & Pauly 2012) and the Catalog of Fishes (Fricke & Eschmeyer 2012), two *Boleophthalmus* species are recorded from the northwest Pacific Ocean, i.e., *B. boddarti* south of the Beibu Gulf, and the East Asian *Boleophthalmus pectinirostris*, distributed north of the Beibu Gulf and South China Sea, and the Malaysian lineage, distributed in the Strait of Malacca (Cantor 1849; Koumans 1953; Murdy 1989; Takita et al. 1999; Ni 2008; Polgar & Khaironizam 2008; Polgar & Crosa 2009). Normally, the length of the East Asian *Boleophthalmus pectinirostris* is < 135 mm (Ni 2008) while fishes of the Malaysian lineage can reach 175 mm (Polgar & Crosa 2009). In his cladistic revision, Murdy (1989) hypothesised that the Malaysian population could be a cryptic species. Molecular evidence has been widely used in fish classification and phylogeny (Wang et al. 2010; Guo et al. 2011); the mitochondrial *ND*5 (718 bp) and the nuclear *Rag*1 markers (1395 bp) were commonly used in the classification and phylogeny of Gobiidae (Tang et al. 2010; Tornabene et al. 2013). Therefore, these markers were selected in this study to investigate the species status of Malaysian populations of *B. pectinirostris*.

The Northwest Pacific is topographically characterized by a series of interconnected marginal seas: the South China Sea, the East China Sea, the Yellow Sea and the Japan Sea (Wang 1999). The Pliocene and Pleistocene glacial and interglacial cycles caused sea-level fluctuations that dramatically impacted this area and the structure of these marginal seas (Wang 1999). Phylogenetic studies based on molecular evidence revealed that during the late Pliocene and Pleistocene glaciations, sea level fluctuations caused the formation of land bridges that acted as geographic barriers, driving shorefish lineage differentiation and speciation (Liu et al. 2007; Tang et al. 2010); during interglacials, the sea-level rise caused marginal seas to reconnect, determining complex circulation patterns (Zheng et al. 2006), such as the China Sea coastal current, the Kuroshio Current and the South China Sea warm current, that can also be natural barriers, limiting the gene flow between populations, and promoting lineage differentiation and speciation of fishes (Hua et al. 2009; Shen et al. 2011). Therefore, this study assumes that both the land bridges formed by the sea level drop that occurred during glacials in the Malacca Strait, and the complex current system established during the interglacials in the Northwest Pacific played an important role in the genetic differentiation of coastal fishes and mudskippers. The purpose of this study is to describe the evolutionary history of the geographical separation of populations of *Boleophthalmus pectinirostris* along the Northwest Pacific coast of East Asia and Malaysia, through extensive sampling along the Northwest Pacific Coast, based on mitochondrial and nuclear DNA evidence.

1 Material and methods

1.1 Sampling and molecular markers

Samples of *Boleophthalmus pectinirostris* from the west Pacific Ocean (East Asian population) include 19 specimens from 7 study sites: Dangjiang (Guangxi province); Zhanjiang (Guangdong province); Quanzhou (Fujian province); Wenzhou (Zhejiang province); Chongming (Shanghai); Suncheon (Korea) and Rokkaku River (Japan) (Figure 1). Eleven *Boleophthalmus pectinirostris* (Malaysian population) were collected in 2 study sites in Malaysia: Pulau Kukup (PK) and Tanjung Piai (TP) (Figure 1). Fifteen *Boleophthalmus boddarti* were collected in 4 sites in Malaysia: Pulau kulup; Tanjung Piai; Carey Island and Sungai Pinang. As outgroups, we utilized 2 *Odontamblyopus lacepedii*; 1 *Odontamblyopus rebecca*; 1 *Periophthalmus modestus* and 1 *Periophthalmus magnuspinnatus* (Table 1). Muscle samples of the specimens were preserved in 95% ethanol. The mitochondrial NADH dehydrogenase subunit gene 5 (ND5) and the nuclear recombination activating gene-1 (Rag1) were selected as molecular markers.
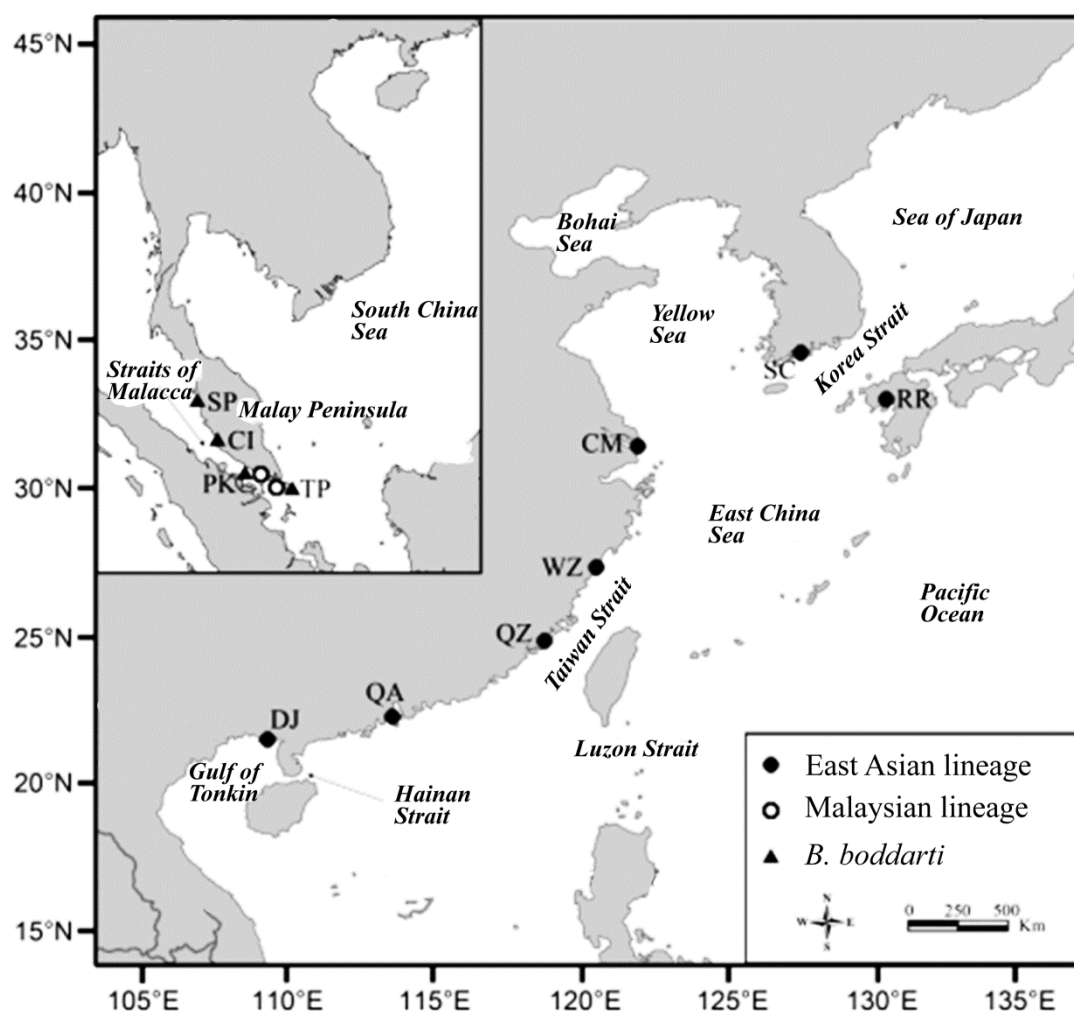

Fig. 1. Sample sites of *Boleophthalmus* fishes along the northwestern Pacific coast. Abbreviations as in Tab. 1.

1.2 DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fish muscle tissue by salting-out. The primers used for ND5 gene amplification are L12321 (5'-GGTCTTAGGAACCAAAAACTCTTGGTGCAA-3') and H13396 (5'-CCTATTTTT CGGATGTCTTG-3') (Mya & Nishida 2000). The PCR reaction (35 cycles) was: pre-denaturation at 94°C for 5min; denaturation at 94°C for 35s; annealing at 55°C for 35s, extension at 72°C for 40s, and final extension at 72°C for 8min. The Rag1 gene marker was amplified with a nested PCR. The primers used for the first round of PCR are RAG1F1 (5'-CTGAGCTGCAGTCAG TACCATAAGATGT-3') and RAG1R1 (5'-CTGAGTCCTTGTGAGCTTCCATRAAYTT-3') (Lopez et al. 2004). The primers used for the second round of PCR are GOBRAG1F1 (5'-GCCAGATCTTCCAGCCTCT-3) and XRAG1R（5'-TACTTGGADGTGTAGAGCC-3'). The PCR reaction was: pre-denaturation at 94°C for 5min; denaturation at 94°C for 35s; annealing at 55°C for 35s, extension at 72°C for 40s, and final extension at 72°C for 8min. PCR products were purified by 2.0% agarose gel-electrophoresis, using a second round of amplification in the ABI 3730 DNA sequencer.

Table 1. Species, sampling localities, codes, haplotypes and GenBank accession numbers.

Locality	Code	ND5		Rag1		ND5 + Rag1
		Haplotype	GenBank No.	Haplotype	GenBank No.	Haplotype
Boleophthalmus pectinirostris (*n* = 30)						
Dangjiang, Guangxi DJ	DJDT1	HN01	JX003004	HR01	JX003054	H01
	DJDT2	HN01	JX003005	HR01	JX003055	H01
	DJDT3	HN02	JX003006	HR02	JX003056	H02
Qi Ao Island, Guandong QA	QADT1	HN03	JX003007	HR03	JX003057	H03
				HR04	JX003058	H04
	QADT2	HN04	JX003008	HR05	JX003059	H05
	QADT3	HN05	JX003009	HR06	JX003060	H06
				HR07	JX003061	H07
Fujian, Quanzhou QZ	QZDT1	HN06	JX003010	HR08	JX003062	H08
				HR09	JX003063	H09
Wenzhou, Zhejiang WZ	WZDT1	HN07	JX003011	HR10	JX003064	H10
	WZDT2	HN08	JX003012	HR11	JX003065	H11
	WZDT3	HN09	JX003013	HR10	JX003066	H12
				HR12	JX003067	H13
Chongming, Shanghai CM	CMDT1	HN07	JX003014	HR13	JX003068	H14
	CMDT2	HN01	JX003015	HR01	JX003069	H01
	CMDT3	HN09	JX003016	HR11	JX003070	H15
Suncheon, Korea SC	SCDT1	HN10	JX003017	HR01	JX003071	H16
				HR14	JX003072	H17
	SCDT2	HN10	JX003018	HR14	JX003073	H17
	SCDT3	HN10	JX003019	HR01	JX003074	H16
Rokkaku River, Japan RR	RRDT1	HN11	JX003020	HR01	JX003075	H18
	RRDT2	HN11	JX003021	HR01	JX003076	H18
	RRDT3	HN11	JX003022	HR01	JX003077	H18
Pulau Kukup, Malaysia PK	PKDT1	HN12	JX003023	HR15	JX003078	H19
				HR16	JX003079	H20
	PKDT2	HN12	JX003024	HR17	JX003080	H21
				HR18	JX003081	H22
Tanjung Piai,, Malaysia TP	TPDT1	HN13	JX003025	HR15	JX003082	H23
	TPDT2	HN13	JX003026	HR15	JX003083	H23
				HR16	JX003084	H24
	TPDT3	HN12	JX003027	HR18	JX003085	H22
	TPDT4	HN14	JX003028	HR15	JX003086	H25
	TPDT5	HN15	JX003029	HR19	JX003087	H26
				HR20	JX003088	H27
	TPDT6	HN12	JX003030	HR21	JX003089	H28
	TPDT7	HN16	JX003031	HR22	JX003090	H29
				HR23	JX003091	H30
	TPDT8	HN12	JX003032	HR15	JX003092	H19
	TPDT9	HN16	JX003033	HR18	JX003093	H31

Boleophthalmus boddarti (n = 15)						
Pulau Kukup PK	PKBS1	HN17	JX003034	HR24	JX003094	H32
				HR25	JX003095	H33
	PKBS2	HN17	JX003035	HR26	JX003096	H34
				HR27	JX003097	H35
Tanjung Piai TP	TPBS1	HN17	JX003036	HR24	JX003098	H32
	TPBS2	HN18	JX003037	HR26	JX003099	H36
	TPBS3	HN19	JX003038	HR24	JX003100	H37
	TPBS4	HN20	JX003039	HR25	JX003101	H38
				HR26	JX003102	H39
Carey Island CI	CIBS1	HN21	JX003040	HR26	JX003103	H40
Sungai Pinang SP	SPBS1	HN17	JX003041	HR24	JX003104	H32
	SPBS2	HN22	JX003042	HR26	JX003105	H41
				HR28	JX003106	H42
	SPBS3	HN19	JX003043	HR26	JX003107	H43
				HR27	JX003108	H44
	SPBS4	HN18	JX003044	HR26	JX003109	H36
	SPBS5	HN23	JX003045	HR29	JX003110	H45
	SPBS6	HN17	JX003046	HR24	JX003112	H32
				HR26	JX003113	H34
	SPBS7	HN19	JX003047	HR24	JX003114	H37
	SPBS8	HN17	JX003048	HR24	JX003115	H32
				HR31	JX003116	H47
Odontamblyopus lacepedii						
Jiao Zhou, Shandong JZ	JZLY1	HN24	JX003049	HR32	JX003117	H48
				HR33	JX003118	H49
Wenzhou, Zhejiang WZ	WZLY1	HN25	JX003050	HR34	JX003119	H50
Odontamblyopus rebecca						
Qi Ao Island, Guandong QA	QALB1	HN26	JX003051	HR35	JX003120	H51
				HR36	JX003121	H52
Periophthalmus modestus						
Shankou, Guangxi SK	SKTT1	HN27	JX003052	HR37	JX003122	H53
Periophthalmus magnuspinnatus						
Zhang Zhou, Fujian ZZ	ZZDQ1	HN28	JX003053	HR38	JX003123	H54

1.3 Haplotype network maps and reconstruction of phylogenetic relationships

We used CLUSTAL-X v.1.83 (Thompson et al. 1997), using the default parameters, to align ND5 and Rag1 sequences. We used DnaSP v.4.20 software to find the ND5 gene haplotypes (Rozas et al. 2003). We used Phase v.2.1 software (Stephens et al. 2001; Stephens & Scheet 2005), setting a posterior probability threshold of 0.6, to select the Rag1 gene haplotypes. Some individuals have two Rag1 haplotypes (i.e. they were inferred to be heterozygous; Table 1). We built a Median-Joining haplotype network diagram with Network v.4.6 (Bandelt et al. 1999), and used Maximum Parsimony (Polzin & Daneschmand 2003) to reconstruct the relationship between the haplotypes.

MrBayes v.3.2 (Ronquist et al. 2012) and RAxML v.7.2.6 (Stamatakis 2006), were used to construct a

Bayesian tree and a Maximum-Likelihood tree, partitioning the data by codon positions. We used jModelTest v.0.1.1 software (Posada 2008) to select the best base substitution model for each partition. The MCMC chain for MrBayes was run for $1.2 * 10^7$ generations, sampling topologies every 1000 generations. We discarded the first 3,000 sample trees, built a 50% majority rule consensus tree, and calculated Bayesian Posterior Probabilities (BPP) for each node. Maximum-Likelihood analysis was performed using a Rapid-hill-climbing algorithm, in a GTRGAMMA model with 100 replicates, to find the best-scoring ML tree. 1,000 bootstrap replicates were made to estimate node bootstrap support (BS).

1.4 Definition of species and gene flow

ND5+Rag1-based concatenated gene sequences were used to determine Evolutionary Significant Units (ESU), applying the General Mixed Yule-Coalescent (GMYC) method. GMYC infers the transitional point between intraspecific variations (coalescence of gene trees) and species divergence (the Yule process) to define species boundaries (Pons et al. 2006). At first, we used BEAST v.1.7.2 software (Drummond et al. 2012) to construct an ultrametric tree, setting the population size as constant (Constant-size model), and the average mutation rate with a Lognormal distribution of an average of 1. We run 4 independent runs, each for $5 * 10^7$ generations, sampling every 1000 generations, the first 1/10 of samples were burnins. Then, posterior samples from four independent runs were combined with LogCombiner (BEAST v. 1.7.2) Tracer v.1.5 (Rambaut & Drummond 2007) for the effective sample size (ESS > 200) and we used TreeAnnotator (BEAST v. 1.7.2) to generate a Maximum Clade Credibility Tree. Finally, R software (R Development Core Team 2009) was used with the package SPLITS (Ezard et al. 2013), adopting a Single-threshold GMYC method.

Estimates of gene flow between species were made with IMa2 (Hey 2010a,b), adopting an isolated movement (Isolation with migration, IM) model. Using Hasegawa-Kishino-Yano (HKY) substitution model we ran the MCMC chain for 5×10^7 generations with 5×10^6 generations as burn-ins, and we made sure that ESS > 200, and parameter autocorrelations < 0.05. When the mutation rate (μ) is not provided, IMa2 infers the population mutation rate (θ) and the migration rates per mutation (M). Because effective population size $N = \theta/4\mu$, per-generation migration rate $m = M\mu$, so each generation Effective numbers of gene migrants per generation *2Nm=2θ/4 μ M μ = θ M/2*.
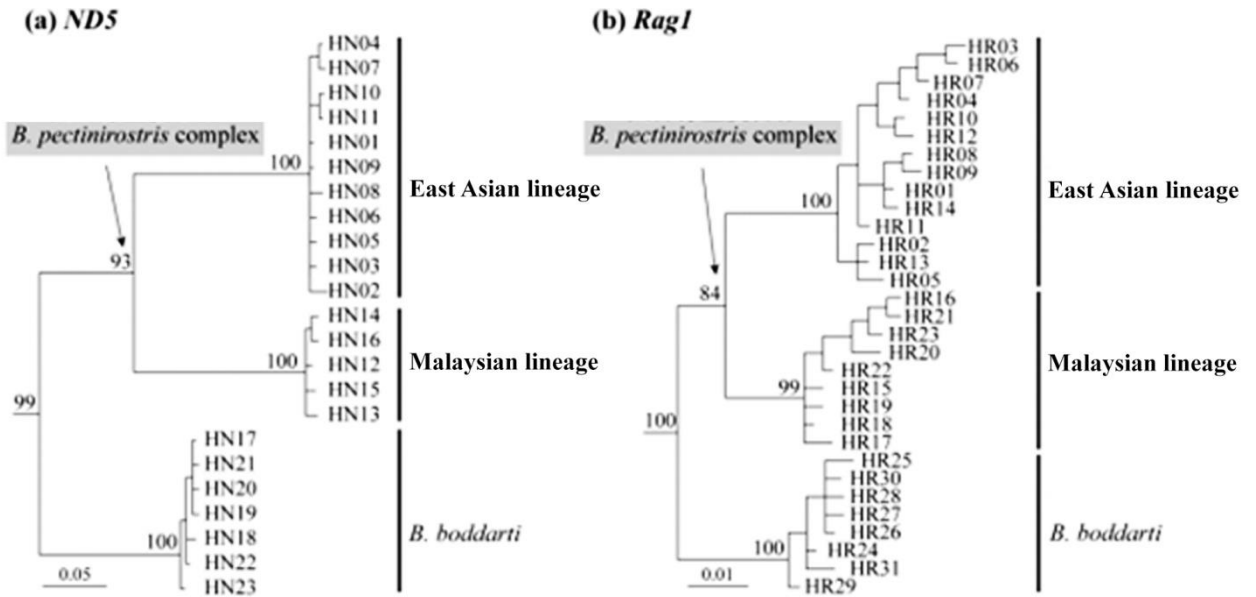
Fig. 2 Bayesian 50% major rule consensus tree based on the (a) ND5 gene and (b) Rag1 gene. Bayesian posterior probabilities are above the nodes; haplotypes as in Tab. 1.

1.5 Genetic distance, tree species and differentiation time

MEGA v. 5.05 (Tamura et al. 2010) was used to calculate the average genetic distance between the species with the Kimura two-parameter (K2P) substitution model. The species tree was built using *BEAST v. 1.7.2 (Heled et al. 2010; Drummond et al. 2012). We used a relaxed LogNormal clock model, a Tree prior as the Yule process. We ran 4 independent runs, and each for 5×10^7 generations, sampling every 1000 generations, burn-in was the first one-tenth. Posterior samples from 4 runs were combined with LogCombiner, Tracer was used to inspect ESS. TreeAnnotator was used to generate a maximum clade incredible tree. Due to the lack of gobiid fossils, this study used Mukai et al. (2005)'s estimate of *Rhinogobius* ND5 gene mutation rate, $(1.95 \pm 0.17)\%$ per million years per lineage per site to estimate divergence time.
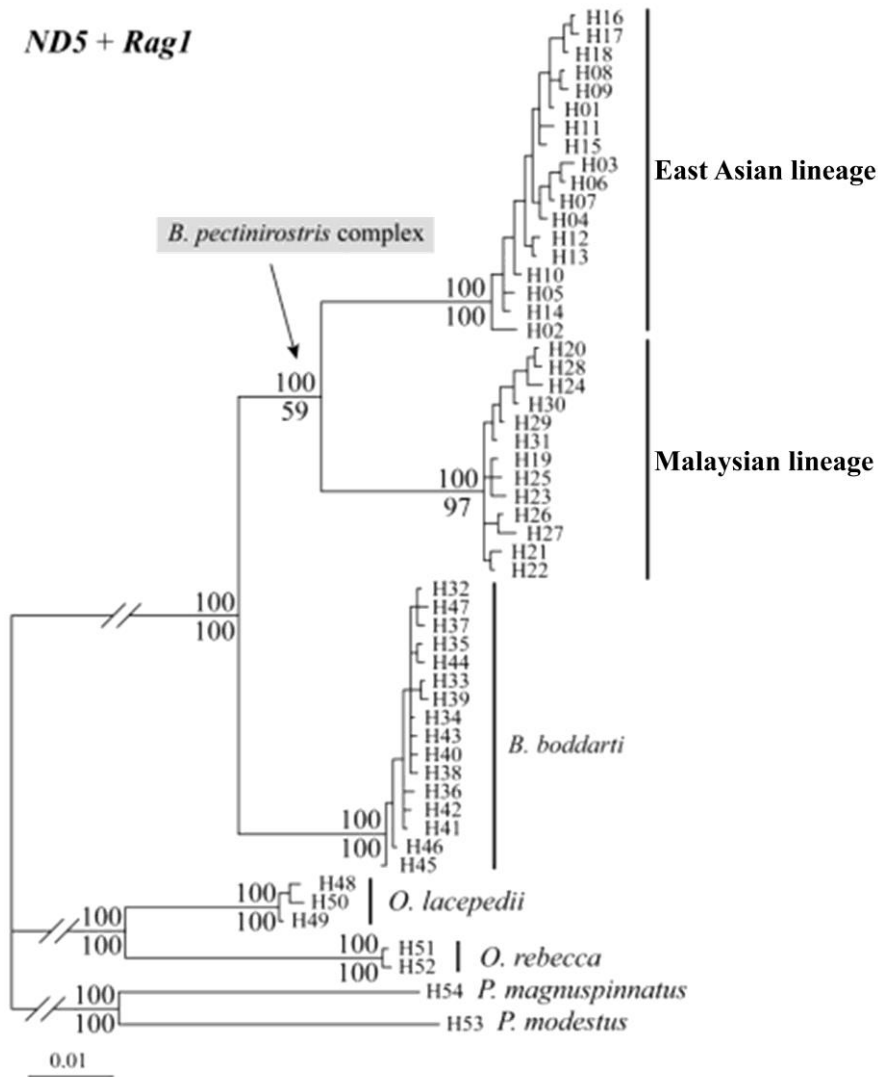
Fig. 3. Bayesian consensus tree based on combined data of ND5 and Rag1 genes. Bayesian posterior probabilities are above the nodes and are bootstrap support values are below the nodes; haplotypes as in Tab. 1.

2. RESULTS

2.1 Sequence analysis

The gene sequences of the ND5 marker are 718 bp in length, 117 variable loci, there are 109 parsimony-informative sites; the sequences of the Rag1 marker are1395 bp in length, 51 variable loci, 40 parsimony-informative sites. The 45 specimens of *Boleophthalmus pectinirostris* included 23 ND5 haplotypes and 31 Rag1 haplotypes. GenBank accession numbers are shown in Table 1.

2.2 Phylogenetic relationships and the haplotype network

Bayesian analysis of ND5 and Rag1 markers (Figure 2) and the combined Bayesian and maximum likelihood analyses (Figure 3) have a consistent topology. These analyses show that *Boleophthalmus pectinirostris* and *B. boddarti* form a monophyletic group (posterior probability BPP = 84%-100%;

Bootstrap support BS = 100%); the East Asian and Malaysian populations of *Boleophthalmus pectinirostris* form monophyletic groups and are in a sister group relationship (BPP=97%-100%; Bootstrap support BS=100%), and will be referred here as the East Asian and Malaysian lineages, respectively (Figs. 2, 3). The haplotype network diagrams also show that the East Asian and Malaysian lineages of *Boleophthalmus pectinirostris* are clearly genetically differentiated from *B. boddarti* (Figure 4). The ND5 haplotype network shows that the East Asian and Malaysian lineages of *B. pectinirostris* are separated by 64 mutational steps, and that these are separated by 73 mutational steps from *B. boddarti*; within these lineages, there are never more than 5 mutational steps between connected haplotypes (Figure 4A). The Rag1 gene haplotype network shows that the East Asian and Malaysian lineages of *B. pectinirostris* are separated by 16 mutational steps, and that these are separated by 24 mutational steps from *B. boddarti*; within these lineages, there are never more than 2 mutational steps between connected haplotypes (Figure 4B).
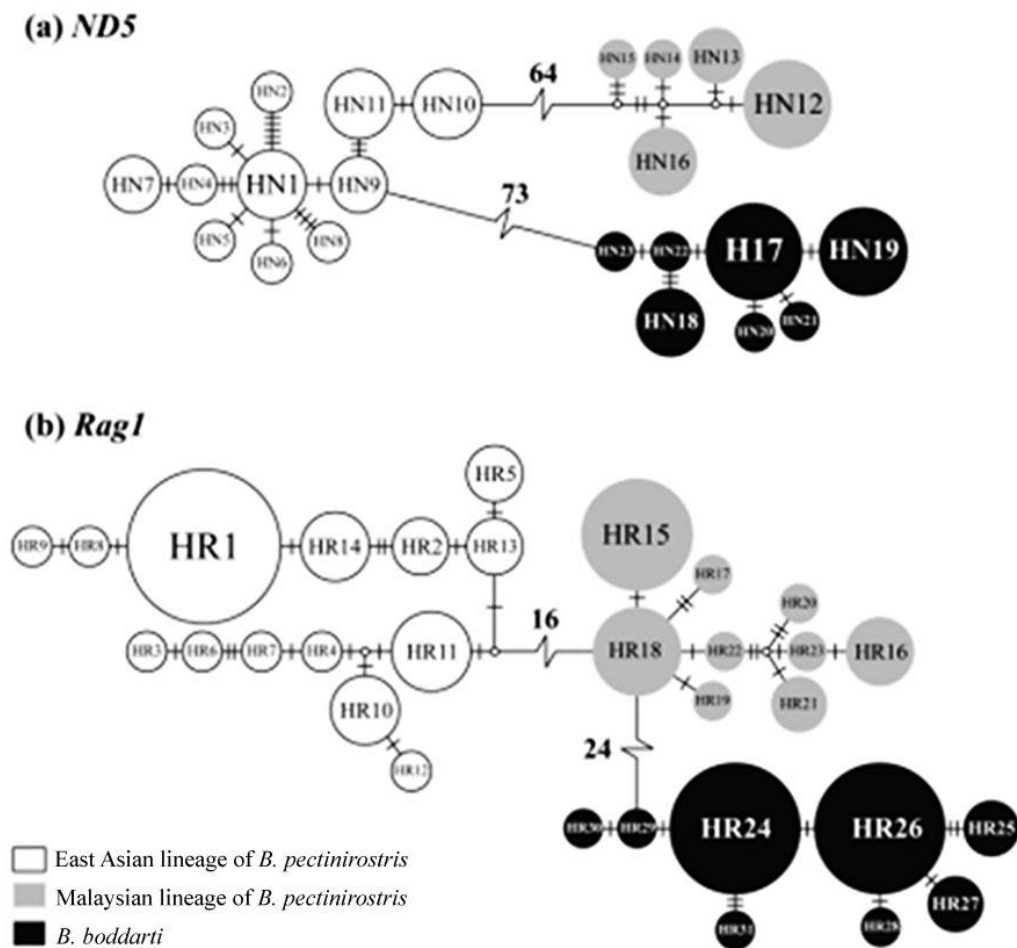

Fig. 4 Haplotype Median-Joining network of *Boleophthalmus* fishes in the northwestern Pacific. The area of circles is proportional to the haplotype frequencies, and empty circles are missing haplotypes. Lines linking haplotypes indicate the evolutionary paths among haplotypes, vertical bars or numbers on the linking lines represent mutation steps between haplotypes.
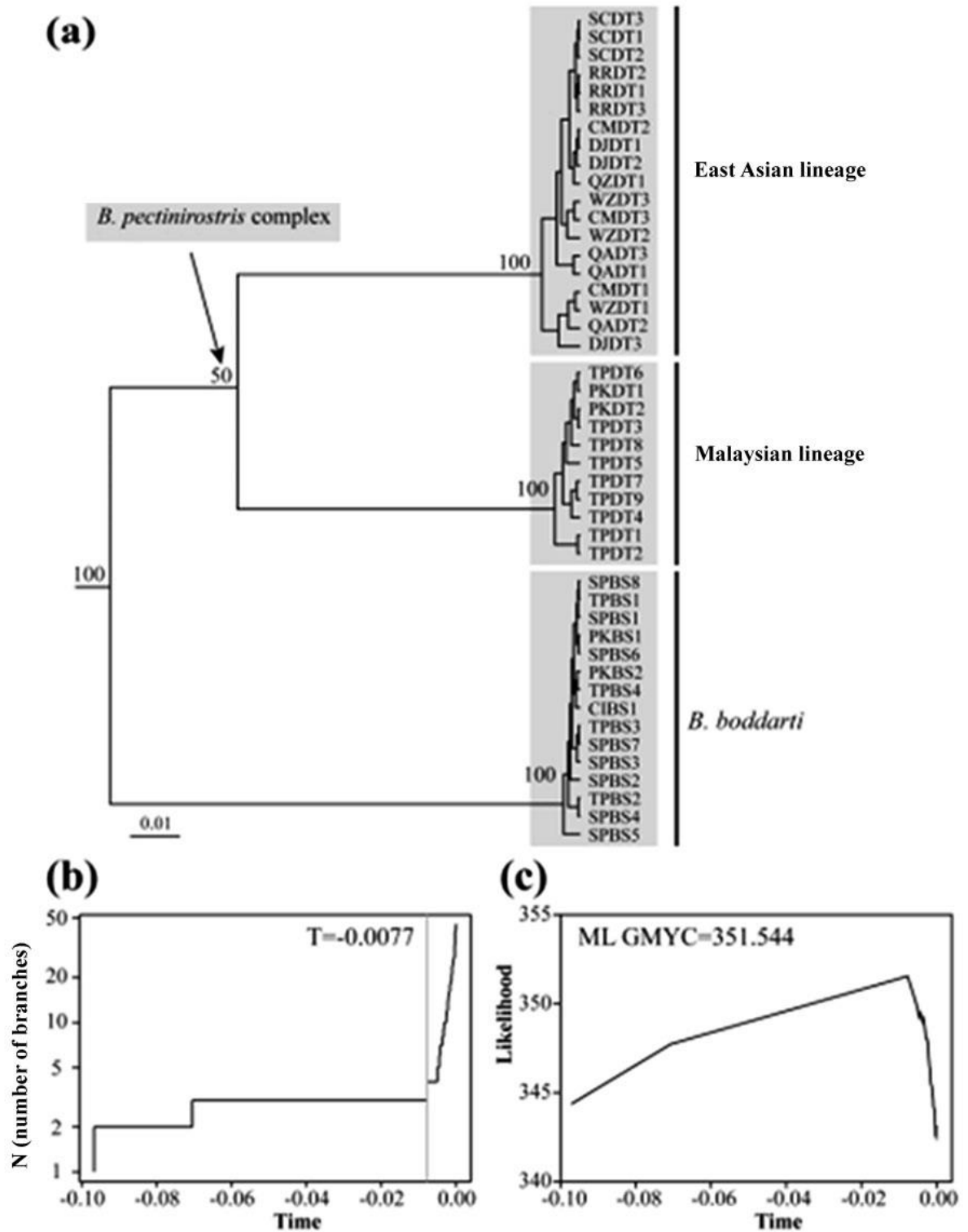
Fig. 5 The ultrametric tree implemented with BEAST (a), relationship between time and lineage (b), and relationship between time and likelihood (c) based on combined data of ND5 and Rag1 genes. Above nodes are posterior probabilities and the gray areas indicate three ESUs divided by GMYC method (a), gray line indicates the transition point suggested by GMYC (b).

2.3 GMYC analysis, species tree and gene flow

According to the ND5+Rag1 combined tree (Figure 5), the genus *Boleophthalmus* includes three evolutionary distinct clades distributed in the northwest Pacific Ocean, which correspond to the *Boleophthalmus pectinirostris* East Asian and Malaysian lineages, and *Boleophthalmus boddarti*. The relationships between the above lineages or species are likely species divergence (Yule process); within each of these three clades, gene tree coalescence explained the gene tree pattern; therefore, the East Asian, Malaysian lineages and *B. boddarti* should be considered as three distinct species (Figure 5). The *BEAST tree species (Figure 6) further supports the result of the GMYC analysis (species discrimination), with strong nodes' support values (BPP = 100%). Isolation with Migration model also inferred that (pairwise) gene flow between East Asian, Malaysian lineages of *B. pectinirostris* and *B. boddarti* is very small: the effective number of gene migrations per generation is close to zero (Figure 7).
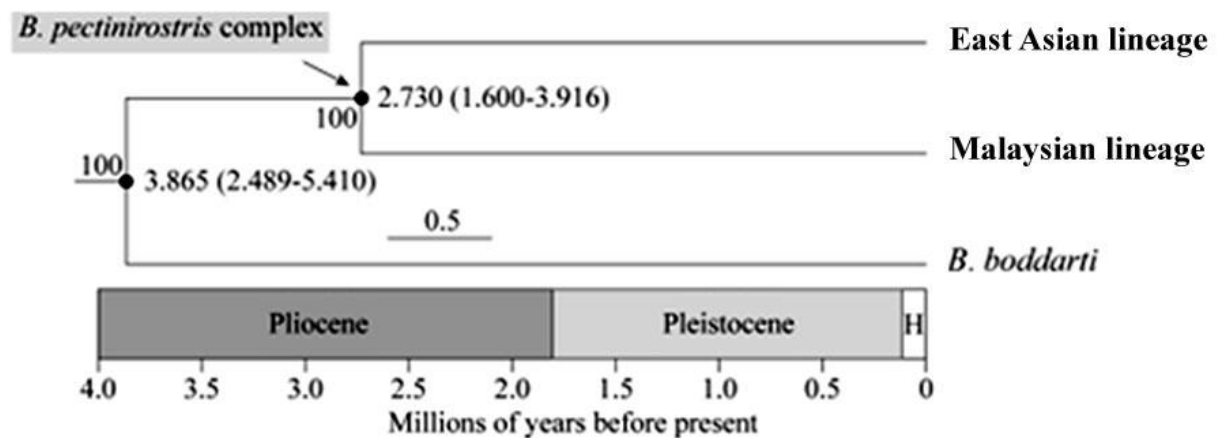

Fig. 6 *BEAST species tree and chronogram based on combined ND5 and Rag1 genes. Below nodes, posterior probabilities; to the right side of nodes, divergence time with 95% confident intervals (Ma); on the time axis H = Holocene.

2.4 Genetic distance and time of divergence

Calculations based on the ND5 marker show that the interspecific K2P genetic distances between East Asian and Malaysia lineages of *Boleophthalmus pectinirostris*, and both these lineages and *B. boddarti* are 10.41-11.87%, while the intraspecific or intralineages K2P genetic distances within *Boleophthalmus pectinirostris* are 00.32-0.57% (Table 2). Calculations based on the Rag1 marker show that the interspecific K2P genetic distances between East Asian and Malaysia lineages of *Boleophthalmus pectinirostris*, and both these lineages and *B. boddarti* are 1.5%-1.8%, while the intraspecific or intralineages K2P genetic distances within *Boleophthalmus pectinirostris* are 0.18%-0.30%. The molecular clock showed that the divergence time between the *Boleophthalmus pectinirostris* East Asian and Malaysian lineages is of 2.73 million years, whereas the divergence time between both of these

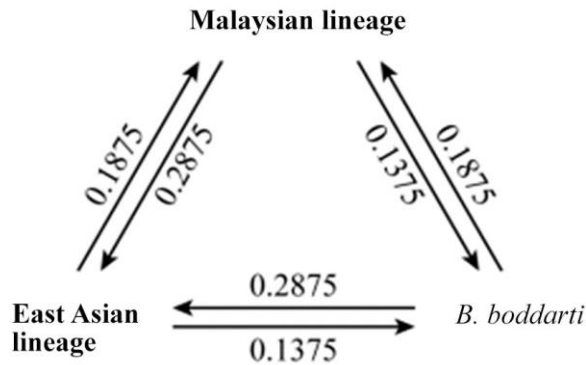lineages and *B. boddarti* is 3.86 million years (Figure 6).

Fig. 7 Gene flows among East Asia lineage and Malaysia lineage of *Boleophthalmus pectinirostris* and *B. boddarti* estimated by the IM model. Numbers are the effective numbers of gene migrants per generation.

Gene	Species/Lineage	Mean Kimura 2-parameter genetic distance		
		BPE	BPM	BB
ND5	*B. pectinirostris*, **East Asian lineage BPE**	0.57%		
	B. pectinirostris, **Malaysian lineage BPM**	10.41%	0.53%	
	B. boddarti **BB**	11.52%	11.87%	0.32%
*Rag*1	*B. pectinirostris*, **East Asian lineage BPE**	0.30%		
	B. pectinirostris, **Malaysian lineage BPM**	1.50%	0.27%	
	B. boddarti **BB**	1.80%	1.70%	0.18%

Table 2. Intra-specific and inter-specific Kimura 2-parameter genetic distance (K2P) of *Boleophthalmus* fishes.

3. Discussion

3.1 Species boundaries and cryptic species

In recent years, molecular evidence revealed that cryptic species are common among gobies (Mukai et al. 2005; Lima et al. 2005; Sota et al. 2005; Kon et al. 2007; Neilson & Stepien 2009; Tang et al. 2010). Based on molecular phylogenetic reconstructions using mitochondrial and nuclear markers, this study shows that East Asian and Malaysian populations of *Boleophthalmus pectinirostris* are monophyletic and are sister groups. The phylogenetic species concept considers species as monophyletic sets of populations resulting from phylogenetic analyses (de Queiroz 2007; Wiens 2007). According to this concept, *Boleophthalmus pectinirostris* East Asian and Malaysian lineages can be defined as two species. GMYC analysis and *BEAST species tree reconstructions, as well as gene flow estimates also

show that the East Asian and Malaysian lineages of *Boleophthalmus pectinirostris* are distinct. In terms of genetic distance, interspecific divergence levels of mitochondrial DNA are generally 10 times larger than intraspecific divergence levels (Herbert et al. 2004; Hickerson et al. 2006). Divergence between *Boleophthalmus pectinirostris* East Asian and Malaysian lineages measured by the ND5 marker is 18-19 times the divergence observed within these lineages, thus also supporting the boundary between these two species. Since the type locality of this fish is China (Murdy 1989; Ni 2008), it can be concluded that the East Asian lineage is *Boleophthalmus pectinirostris sensu stricto*, while the Malaysian lineage is a cryptic species (*Boleophthalmus* sp.). This discovery furthers the knowledge of the western Pacific genus *Boleophthalmus*, limiting the distribution of this species to East Asia (North of Beibu Gulf in the South China Sea), and showing that the cryptic species *Boleophthalmus* sp. is currently limited to the Straits of Malacca.

3.2 Evolutionary history

During the Late Pliocene and Pleistocene largest glaciation, the sea level was 120-140 m lower than today (Lambeck et al. 2002); at that time, the Yellow Sea, Bohai Sea, South China and the whole region of Haidong in the China Sea rose above water level (Wang 1999). Previous research showed that during the Late Pliocene sea-level minimum, the Taiwan and Tsushima Straits emerged, thus isolating the East China Sea, the South China Sea, and the Japan Sea, resulting in the diversification of the northwest Pacific shorefish genera *Odontamblyopus* and *Mugil* (Tang et al. 2010; Shen et al. 2011). This study estimated the divergence time between *Boleophthalmus pectinirostris* (East Asian lineage) and the cryptic species (Malaysian lineage) at 2.73 million years. Accordingly, the Late Pliocene sea-level minimum also caused the Straits of Malacca to emerge, thus isolating the South China Sea and the Indian Ocean, possibly resulting in the differentiation between the cryptic species and the sister *Boleophthalmus pectinirostris*.

On the other hand, the complex hydrological characteristics of South China Sea and adjacent waters also have a significant impact on species' dispersal and distribution (Gordon & Fine 1996; Guan & Fang 2006). During the life history of *Boleophthalmus pectinirostris*, only the larval planktonic stage is capable of long-distance dispersal, and this stage duration is of about 35 days (Takegaki 2008). The mating season of mudskippers is approximately from April to September; in this period, a warm sea current flows along coast in the South China Sea, from central Viet Nam to Hainan Island to Guangdong, and then in a northeast direction; at the same time, the warm current circulation of the southern part of the South China Sea flows in a south-east direction, under the influence of the summer monsoon (Li & Sun 2005). The two opposite currents may have transported the planktonic larvae of the East Asian lineage and of the cryptic species in opposite directions, enforcing the genetic isolation, and facilitating speciation. Recent studies also hypothesised that interglacial currents created biogeographical barriers that were one of the factors facilitating the differentiation of several marine species of the Pacific northwest region (Kojima et al. 2003; 2004; Liu et al. 2008; Tsang et al. 2008; Yin et al. 2009; Shen et al. 2011).

In conclusion, *Boleophthalmus pectinirostris* of the Pacific northwest region is a species complex,

which includes two species: the East Asian population is *Boleophthalmus pectinirostris* (*Boleophthalmus pectinirostris sensu stricto*), and the Malaysian population is a cryptic species (*Boleophthalmus* sp.). Species differentiation between the mudskipper *Boleophthalmus pectinirostris* and the cryptic species may have been caused by geographic isolation during the Late Pliocene, due to glacial sea-level minima and interglacial sea currents patterns, which induced genetic isolation. In order to describe the mechanisms of differentiation between these two species, further studies are needed.

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