

Molecular phylogeny of obligate fish parasites of the family Cymothoidae (Isopoda, Crustacea): evolution of the attachment mode to host fish and the habitat shift from saline water to freshwater

Hiroki Hata¹  · Atsushi Sogabe² · Shinya Tada¹ · Ryohei Nishimoto³ · Reina Nakano³ · Nobuhiko Kohya³ · Hirohiko Takeshima⁴ · Ryota Kawanishi⁵

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Abstract In host–parasite coevolution, parasite innovations including the acquisition of new habitats and novel traits can trigger evolutionary breakthroughs and enhance parasite diversification via accumulation of new hosts. All species of the family Cymothoidae are obligate fish parasites, attaching to exterior body surfaces of fish, the buccal or opercular cavities, or burrowing into abdominal muscle tissue. In the present study, we constructed a molecular phylogeny of 27 cymothoid species that parasitise 38 fish species, combined with 2 prior cymothoid datasets, based on the sequences of mitochondrial 16S rRNA and COI genes. We explored the evolution of the host attachment mode, and the habitat shift from saline water to freshwater. Our evolutionary trees include two freshwater clades,

an abdominal burrower clade, and cymothoid clades that are closer to the base of Cymothoidae than those initially analysed. We found that the basal clade of Cymothoidae was *Elthusa sacciger*, which is parasitic in the opercular cavity of synphobranchid eels. This result suggests that cymothoids may have originated in deep seas, subsequently expanded to shallow seas, and then to brackish and/or freshwater, by shifting host species. Invasion of freshwater habitats has occurred at least twice; freshwater abdominal muscle burrowers living on armoured catfish constitute a clade allied to *E. sacciger*. The ancestral host attachment site, based on our dataset, was the opercular cavity, followed (sequentially) by buccal colonisation and attachment to the external body.

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✉ Hiroki Hata
hata@sci.ehime-u.ac.jp

¹ Graduate School of Science and Engineering, Ehime University, 2-5 Bunkyo, Matsuyama, Ehime 790-8577, Japan

² Department of Biology, Faculty of Agriculture and Life Science, Hirosaki University, 1-1 Bunkyo, Hirosaki, Aomori 036-8560, Japan

³ Department of Biology, Faculty of Science, Ehime University, 2-5 Bunkyo, Matsuyama, Ehime 790-8577, Japan

⁴ Research Institute for Humanity and Nature, 457-4 Motoyama, Kamigamo, Kita-ku, Kyoto 603-8047, Japan

⁵ Faculty of Environmental Earth Science, Hokkaido University, N10W5 Kita-ku, Sapporo 060-0810, Japan

Introduction

During host–parasite coevolution, parasite innovation (acquisition of new traits and colonisation of new habitats) can enhance parasite diversification via accumulation of new host species (Zietara and Lumme 2002; Ricklefs et al. 2014). The family Cymothoidae is a diverse group of Isopoda containing 366 species of 42 genera to the best of our knowledge (Table S1), all of which are obligate parasites on fish, feeding on blood, mucus, and/or tissue (Adlard and Lester 1995; Horton and Okamura 2003; Bruce and Schotte 2008). Cymothoids have been identified in marine actinopterygian fish (notably Perciformes, Clupeiformes, Beloniformes, and Tetraodontiformes) and certain elasmobranchs (Brusca 1981; Smit et al. 2014). Cymothoids have expanded their distribution to include freshwater fish, being currently parasitic on freshwater actinopterygians including Characiformes, Siluriformes, and Cypriniformes. 310 species (85%) of 32 genera (76%) are marine, 33 species (9%)

of 9 genera (21%) are fresh water inhabitants. At least 27 species in nine genera (including *Riggia*, *Braga*, *Asotana*, and *Paracymothoa*) inhabit Amazonia in South America. Several species of the genus *Ichthyoxenos* live in freshwater habitats of eastern and south-eastern Asia, and central Africa (Brusca 1981; Tsai and Dai 1999).

Cymothoids parasitise the exterior bodies of fish (including the fins), or the buccal or opercular cavities, or burrow into abdominal muscular tissue to create capsules (with small openings) in the abdominal cavity (Brusca 1981; Bruce 1990; Smit et al. 2014). Each cymothoid species exhibits a specific attachment mode to its host fish, and is morphologically specialised in this context (Bruce 1986, 1987, 1990). Seven genera (e.g. *Anilocra*, *Nerocila*, and *Renocila*), 16 genera (e.g. *Ceratothoa* and *Cymothoa*), 17 genera (e.g. *Elthusia* and *Ryukyua*), four genera (e.g. *Ichthyoxenos* and *Artystone*) are known as parasites on fish body surface, in the buccal cavity, opercular cavity, burrowing into abdominal cavity, respectively (Table S1). Host specificity and host range vary among cymothoid species. In general, however, cymothoids that attach to external surfaces of fish exhibit wider host ranges than other species, sometimes straddling fish orders. On the other hand, cymothoids that burrow into the abdominal muscles of fish are highly species-specific, being parasitic on only one or several species within a single family (Tsai and Dai 1999; Thatcher 2006; Yamano et al. 2011). Cymothoids that are parasitic in the buccal and opercular cavities exhibit intermediate width of host range (Bruce 1986; Hadfield et al. 2015).

We used a molecular phylogenetic approach to explore cymothoid evolution in terms of the acquisition of attachment modes, shifts in such modes, habitat expansion (particularly to freshwater), and acquisition of new host fish. Brusca (1981) suggested that external surface attachment was an ancestral trait, citing morphological, zoogeographic, and ecological data. Jones et al. (2008) suggested that buccal- or opercular cavity-dwelling were ancestral in nature, and that external attachment had evolved on several occasions; mitochondrial 16S rRNA sequence data were used to support these conclusions. However, molecular methods have never been used to analyse cymothoids that burrow under the host epidermis to occupy the abdominal cavity, and few data are available on freshwater cymothoids. Thus, it remains unclear whether several cymothoids independently acquired the ability to live in freshwater, or whether a single group acquired that competence and has subsequently diversified. Although there have been several molecular phylogenetic studies on the family Cymothoidae, the reference frameworks are unclear; ambiguous sequences were included in previous phylogenies based on 16S rRNA and COI sequences (Ketmaier et al. 2008), and were stored in GenBank as below. The 16S rRNA

gene sequences differ by a maximum of 5 of the 317 bases among four species in three genera (*Ceratothoa italica* [EF455804–6], *C. collaris* [EF455807], *Nerocila bivittata* [EF455810], and *Anilocra physodes* [EF455808–9]). Such extreme similarity differs from the findings of another study (Jones et al. 2008), which also sequenced 16S rRNA gene, and we found that the sequences in question were those of some members of the genus *Ceratothoa*. It may be that Ketmaier et al. (2008) included these inappropriate sequences in their analyses.

In the present study, we newly collected two freshwater cymothoid species; these were a burrowing parasite (*Artystone* sp.) from an armoured catfish (*Epactionotus yasi*) living in the Iguazu river, South America, and a buccal parasite (*Ichthyoxenos tanganyikae*) from a cichlid (*Simochromis diagramma*) of Lake Tanganyika. We also collected samples of ten genera of marine cymothoids from the buccal and opercular cavities, and the body surfaces, of their various host fish (ranging from anguilliformes to perciformes) living in various depths (See Table 1). We sought to describe the evolution within the family Cymothoidae. In particular, we explored how the organisms acquired novel host attachment modes, shifted such modes, and expanded into freshwater habitats. We also sought to resolve taxonomic confusion within the family. We constructed phylogenetic trees based on mitochondrial 16S rRNA and COI sequences; we used the families Aegidae, Bopyridae, Cirolanidae, Corallanidae, and Sphaeromatidae as outgroups.

Materials and methods

Sample collection

We collected 29 cymothoid species from 59 species of host fish, as well as 2 species of Aegidae, 1 species of Corallanidae, and 1 species of Sphaeromatidae (Table 1). 27 species of 12 genera cymothoids were newly collected for molecular phylogeny. In total, 15 genera of all the 43 genera of Cymothoidae are included in this study. Host fish were identified by reference to Nakabo (2013). We distinguished four cymothoid attachment modes (Brusca 1981; Smit et al. 2014): (1) buccal-dwelling, where both males and females live inside the buccal cavities of host fish; (2) opercular cavity-dwelling, where females attach to a specific site within the opercular cavity and their body form is distinctly asymmetrical, reflecting the shape of the host operculum; (3) external body-attaching, where the parasites attach to the external body surface (including the fins) of the hosts, being cryptically coloured in contrast to the pale colour of parasites exhibiting other attachment modes; and (4) abdominal-burrowing, where parasites become buried under the host

Table 1 The species studied, with their classification, attachment modes, host species (and the classification thereof), the depth ranges of the host fish, and DDBJ/EMBL/GenBank accession numbers of the sequences studied

Family	Species	Attachment site	Host species	Order of host fish	Family of host fish	Depth (m)	16S No.	COI No.	Sampling locality
Cymothoi- dae	<i>Anilocra apogonae</i>	Body surface	<i>Ostorhinchus fasciatus</i>	Perciformes	Apogonidae	15–30	EF422800	–	Moreton Bay, Qld, AUS (GB)
	<i>Anilocra clupei</i>	Body surface	<i>Etrumeus micropus</i>	Clupeiformes	Clupeidae	–125	–	LC160309	Uwa Sea, Ehime, JPN
	<i>Anilocra clupei</i>	Body surface	<i>Sardinella zunasi</i>	Clupeiformes	Clupeidae	5–	LC159426	LC159540	Moji, Fukuoka, JPN
	<i>Anilocra longicauda</i>	Body surface	<i>Diagramma labiosum</i>	Perciformes	Haemulidae	–	EF422789,97	–	Lizard Is., Qld, AUS (GB)
	<i>Anilocra nemipteri</i>	Body surface	<i>Scolopsis bilineata</i>	Perciformes	Nemipteri- dae	1–25	EF422790,806	–	Lizard Is., Qld, AUS (GB)
	<i>Anilocra physodes</i>	Body surface	<i>Symphodus tinca</i>	Perciformes	Labridae	1–50	–	EF455817	Italy (GB)
	<i>Anilocra pomacentri</i>	Body surface	<i>Chromis nitida</i>	Perciformes	Pomacentri- dae	5–25	EF432778	–	Heron Is., Qld, AUS (GB)
	<i>Anilocra prionuri</i>	Body surface	<i>Prionurus scalprum</i>	Perciformes	Acanthuridae	2–20	LC159427	LC159541	Kushimoto, Wakayama, JPN
	<i>Anilocra</i> sp.	unknown	unknown	–	–	–	EF422795	–	Lizard Is., Qld, AUS (GB)
	<i>Anilocra</i> sp. 1	Body surface	<i>Pterocaesio marri</i>	Perciformes	Caesionidae	–10	LC159428	LC159542	Iou Is., Kagoshima, JPN
	<i>Artystone</i> sp.	Abdominal cavity	<i>Epactionotus yasi</i>	Siluriformes	Loricariidae	–	LC159429	LC159543	Iguazu River
	<i>Ceratothoa arimae</i>	Buccal cavity	<i>Labracoglossa argentiventris</i>	Perciformes	Kyphosidae	–	LC159430	LC159544	Niiijima Is., Tokyo, JPN
	<i>Ceratothoa collaris</i>	Buccal cavity	<i>Dentex gibbosus</i>	Perciformes	Sparidae	20–220	LC159438	LC159551	West Africa
	<i>Ceratothoa collaris</i>	Buccal cavity	<i>Lithognathus mormyrus</i>	Perciformes	Sparidae	10–20	–	EF455816	Tyrrhenian Sea (GB)
	<i>Ceratothoa italica</i>	Buccal cavity	<i>Lithognathus mormyrus</i>	Perciformes	Sparidae	10–20	–	EF455813	Tyrrhenian Sea (GB)
	<i>Ceratothoa oestroides</i>	Buccal cavity	<i>Sparus aurata</i>	Perciformes	Sparidae	1–30	–	GQ240266	Adriatic Sea (GB)
	<i>Ceratothoa oestroides</i>	Buccal cavity	<i>Dicentrarchus labrax</i>	Perciformes	Moronidae	10–100	–	GQ240276	Adriatic Sea (GB)
	<i>Ceratothoa oxyrrhynchaena</i>	Buccal cavity	<i>Rexea prometheoides</i>	Perciformes	Gempylidae	135–540	LC160305	–	Kagoshima Bay, JPN
	<i>Ceratothoa oxyrrhynchaena</i>	Buccal cavity	<i>Dentex hypselosomus</i>	Perciformes	Sparidae	50–200	LC159432	LC159545,9	Yamaguchi, JPN
	<i>Ceratothoa oxyrrhynchaena</i>	Buccal cavity	<i>Dentex abei</i>	Perciformes	Sparidae	50–150	–	LC160310	Amami Is., JPN
	<i>Ceratothoa oxyrrhynchaena</i> ¹	Buccal cavity	<i>Glossanodon semifasciatus</i>	Argentini- formes	Argentinidae	70–1017	LC159433	LC159546	East China Sea
	<i>Ceratothoa oxyrrhynchaena</i>	Buccal cavity	<i>Nippon spinosus</i>	Perciformes	Serranidae	100–200	LC159434	LC159547	Sagami Bay, JPN
	<i>Ceratothoa oxyrrhynchaena</i>	Buccal cavity	<i>Doederleinia berycoides</i>	Perciformes	Acropoma- tidae	100–600	LC159435	LC159548	Ehime, JPN
	<i>Ceratothoa oxyrrhynchaena</i>	Buccal cavity	<i>Chlorophthalmus albatrossis</i>	Aulopi- formes	Chloroph- thalmidae	300–350	LC159437	LC159550	Kagoshima, JPN

Table 1 continued

Family	Species	Attachment site	Host species	Order of host fish	Family of host fish	Depth (m)	16S No.	COI No.	Sampling locality
	<i>Ceratothoa</i> sp.	Buccal cavity	<i>Sphyraena forsteri</i>	Perciformes	Sphyraenidae	6–300	EF422802	–	Cairns, Qld, AUS (GB)
	<i>Ceratothoa</i> sp. 1	Buccal cavity	<i>Trachurus japonicus</i>	Perciformes	Carangidae	50–275	LC159440	LC159553	Imabari, Ehime, JPN
	<i>Ceratothoa</i> sp. 1	Buccal cavity	<i>Decapterus maruadsi</i>	Perciformes	Carangidae	0–20	–	LC160313	Tateyama, Chiba, JPN
	<i>Ceratothoa</i> sp. 1	Buccal cavity	<i>Decapterus muroadsi</i>	Perciformes	Carangidae	1–320	–	LC160314,5	Iburi, Kochi, JPN
	<i>Ceratothoa</i> sp. 2	Buccal cavity	<i>Takifugu flavipterus</i>	Tetraodontiformes	Tetraodontidae	–	LC159439	LC159552	Maizuru, Kyoto, JPN
	<i>Ceratothoa</i> sp. 3	Buccal cavity	<i>Carangoides equula</i>	Perciformes	Carangidae	64–226	LC159441	LC159554	Yawatahama, Ehime, JPN
	<i>Ceratothoa</i> sp. 3	Buccal cavity	<i>Caranx melampygus</i>	Perciformes	Carangidae	0–190	–	LC160316	Iburi, Kochi, JPN
	<i>Ceratothoa</i> sp. 3	Buccal cavity	<i>Pseudocaranx dentex</i>	Perciformes	Carangidae	10–25	LC160306	–	Nichinan, Miyazaki, JPN
	<i>Ceratothoa</i> sp. 3 ¹	Buccal cavity	<i>Scorpaena neglecta</i>	Scorpaeniformes	Scorpaenidae	100–150	LC159443	LC159555	East China Sea
	<i>Ceratothoa verrucosa</i>	Buccal cavity	<i>Evyinnis tumifrons</i>	Perciformes	Sparidae	30–346	–	LC160317	Sagami Bay, JPN
	<i>Ceratothoa verrucosa</i>	Buccal cavity	<i>Evyinnis cardinalis</i>	Perciformes	Sparidae	0–100	–	LC160318	Yawatahama, Ehime, JPN
	<i>Ceratothoa verrucosa</i>	Buccal cavity	<i>Pagrus major</i>	Perciformes	Sparidae	10–200	LC159444	LC159556	Iburi, Kochi, JPN
	<i>Ceratothoa verrucosa</i>	Buccal cavity	<i>Sebastes inermis</i>	Scorpaeniformes	Scorpaenidae	–	LC159445	LC159557	Gogo Is., Ehime, JPN
	<i>Cterissa sakaii</i>	Opercular cavity	<i>Sargocentron rubrum</i>	Beryciformes	Holocentridae	–	–	LC160319	Okinawa, JPN
	<i>Cterissa sakaii</i>	Opercular cavity	<i>Sargocentron praslin</i>	Beryciformes	Holocentridae	1–20	–	LC160320	Ginowan, Okinawa, JPN
	<i>Cterissa sakaii</i>	Opercular cavity	<i>Sargocentron ittodai</i>	Beryciformes	Holocentridae	16–70	LC159446	LC159558	Kunigami, Okinawa, JPN
	<i>Cymothoa eremita</i> ¹	Buccal cavity	<i>Hime japonica</i>	Aulopiiformes	Aulopidae	85–510	LC159447	LC159559	East China Sea
	<i>Cymothoa excisa</i>	Buccal cavity	<i>Cynoscion regalis</i>	Perciformes	Sciaenidae	10–26	–	KP339866	St. Catherines Is., GA, USA (GB)
	<i>Cymothoa indica</i>	Buccal cavity	<i>Sillago ciliata</i>	Perciformes	Sillaginidae	20–22	EF422791,801	–	Moreton Bay, Qld, AUS (GB)
	<i>Cymothoa pulchrum</i>	Buccal cavity	<i>Siganus spinus</i>	Perciformes	Siganidae	1–20	–	LC160321	Yoron Is., Kagoshima, JPN
	<i>Cymothoa pulchrum</i>	Buccal cavity	<i>Chilomycterus reticulatus</i>	Tetraodontiformes	Diodontidae	20–100	LC160307	–	Tosashimizu, Kochi, JPN
	<i>Cymothoa pulchrum</i>	Buccal cavity	<i>Diodon hystrix</i>	Tetraodontiformes	Diodontidae	3–20	–	LC160322	Nago, Okinawa, JPN
	<i>Cymothoa pulchrum</i>	Buccal cavity	<i>Diodon holocanthus</i>	Tetraodontiformes	Diodontidae	2–35	LC159449	LC159560	Amami Is., JPN
	<i>Cymothoa pulchrum</i>	Buccal cavity	<i>Calotomus japonicus</i>	Perciformes	Scaridae	–	LC159450	LC159561	Chiba, JPN
	Cymothoidae gen. sp.	unknown	unknown	–	–	–	EF422792	–	Lizard Is., Qld, AUS (GB)
	Cymothoidae gen. sp.	unknown	unknown	–	–	–	EF422793	–	Lizard Is., Qld, AUS (GB)
	Cymothoidae gen. sp.	unknown	unknown	–	–	–	EF422794	–	Lizard Is., Qld, AUS (GB)
	Cymothoidae gen. sp.	unknown	unknown	–	–	–	EF422796	–	Lizard Is., Qld, AUS (GB)

Table 1 continued

Family	Species	Attachment site	Host species	Order of host fish	Family of host fish	Depth (m)	16S No.	COI No.	Sampling locality
Cymothoidae	gen. sp.	unknown	unknown	–	–	–	EF422798	–	Lizard Is., Qld, AUS (GB)
Cymothoidae	gen. sp.	unknown	unknown	–	–	–	EF422799	–	Lizard Is., Qld, AUS (GB)
Cymothoidae	gen. sp.	unknown	unknown	–	–	–	EF422804	–	Lizard Is., Qld, AUS (GB)
Cymothoidae	gen. sp.	Opercular cavity	<i>Chrysiptera brownriggii</i>	Perciformes	Pomacentridae	0–12	LC159451	LC159562	Sunabe, Okinawa, JPN
<i>Elthusa moritakii</i>	Opercular cavity	<i>Ereunias grallator</i>	Scorpaeniformes	Ereuniidae	500–	LC159457	LC159568	Kumano, Mie, JPN	
<i>Elthusa sacciger</i> ²	Opercular cavity	<i>Synaphobranchus kaupii</i>	Anguilliformes	Synaphobranchidae	400–2200	–	LC160323	Off the Pacific coast of Tohoku, JPN	
<i>Elthusa sacciger</i>	Opercular cavity	<i>Synaphobranchus affinis</i>	Anguilliformes	Synaphobranchidae	290–2400	LC159452	LC159563	Tokyo Bay, JPN	
<i>Elthusa vulgaris</i>	Buccal cavity	unknown	–	–	–	AF260852 EF455812 AF259546	AF260843	San Diego, CA, USA, (GB)	
<i>Elthusa</i> sp. 1	Opercular cavity	<i>Ventrifossa garmani</i>	Gadiformes	Macrouridae	350–550	LC159453	LC159564	Suruga Bay, JPN	
<i>Elthusa</i> sp. 1	Opercular cavity	<i>Coelorinchus japonicus</i>	Gadiformes	Macrouridae	300–1000	LC159454	LC159565	Suruga Bay, JPN	
<i>Elthusa</i> sp. 2	Opercular cavity	<i>Hexagrammos otakii</i>	Scorpaeniformes	Hexagrammidae	139–155	LC159455	LC159566	Hokkaido, JPN	
<i>Elthusa</i> sp. 2 ³	Opercular cavity	<i>Hemistriperus villosus</i>	Scorpaeniformes	Hemistriperidae	0–550	LC159456	LC159567	Pacific coast of Tohoku, JPN	
<i>Glossobius auritus</i>	Buccal cavity	<i>Cypselurus</i> sp.	Beloniformes	Exocoetidae	–	LC159458	LC159569	Nichinan, Miyazaki, JPN	
<i>Ichthyoxenos tanganyikae</i>	Buccal cavity	<i>Simochromis diagramma</i>	Perciformes	Cichlidae	–	LC159459	LC159570	Mpungu, Zambia	
<i>Joryma hilsae</i>	unknown	unknown	–	–	–	JX413102	KC896399	India (GB)	
<i>Lobothorax</i> sp. 1	Buccal cavity	<i>Trichiurus</i> sp. 2	Perciformes	Trichiuridae	–	LC159460	LC159571	Chinen, Okinawa, JPN	
<i>Mothocya collettei</i>	Opercular cavity	<i>Tylosurus crocodilus</i>	Beloniformes	Belonidae	0–13	LC159461	LC159572	Watarai, Mie, JPN	
<i>Mothocya renardi</i>	Opercular cavity	<i>Tylosurus</i> sp.	Beloniformes	Belonidae	–	EF422803	–	Moreton Bay, Qld, AUS (GB)	
<i>Mothocya parvostis</i>	Opercular cavity	<i>Hyporhamphus sajori</i>	Beloniformes	Hemiramphidae	30–	LC159462	LC159573	Ehime, JPN	
<i>Mothocya</i> sp. 1 ⁴	Opercular cavity	<i>Platybelone argalus platyura</i>	Beloniformes	Belonidae	0–2	–	LC160324	Hachijyo Is., Tokyo, JPN	
<i>Nerocila bivittata</i>	Body surface	<i>Sarpa salpa</i>	Perciformes	Sparidae	5–70	–	EF455819	Tyrrhenian Sea (GB)	
<i>Nerocila japonica</i>	Body surface	<i>Lates japonicus</i>	Perciformes	Latidae	–	LC159463	LC159574	Kochi, JPN	
<i>Nerocila japonica</i>	Body surface	<i>Aluterus monoceros</i>	Tetraodontiformes	Monacanthidae	1–80	–	LC160325	Minamisatsuma, Kagoshima, JPN	
<i>Nerocila japonica</i>	Body surface	<i>Acanthopagrus latus</i>	Perciformes	Sparidae	–50	–	LC160326	Ube, Yamaguchi, JPN	
<i>Nerocila japonica</i>	Body surface	<i>Acanthopagrus schlegelii</i>	Perciformes	Sparidae	15–	LC159464	LC159575	Kanagawa, JPN	
<i>Nerocila japonica</i>	Body surface	<i>Acanthogobius flavimanus</i>	Perciformes	Gobiidae	1–14	–	LC160327,8	Yamaguchi, JPN	

Table 1 continued

Family	Species	Attachment site	Host species	Order of host fish	Family of host fish	Depth (m)	16S No.	COI No.	Sampling locality
	<i>Nerocila japonica</i>	Body surface	<i>Lepidotrigla hime</i>	Scorpaeniformes	Triglidae	43–357	–	LC160329,30	Uwa Sea, Ehime, JPN
	<i>Nerocila japonica</i>	Body surface	<i>Mola mola</i>	Tetraodontiformes	Molidae	30–70	–	LC160331	Iburi, Kochi, JPN
	<i>Nerocila longispina</i>	Body surface	<i>Terapon puta</i>	Perciformes	Terapontidae	–30	KJ855322	–	India (GB)
	<i>Nerocila longispina</i>	Body surface	<i>Otolithes ruber</i>	Perciformes	Sciaenidae	10–40	KJ855322	–	India (GB)
	<i>Nerocila longispina</i>	unknown	unknown	–	–	–	–	KC896398	India (GB)
	<i>Nerocila monodi</i>	Body surface	<i>Acanthopagrus australis</i>	Perciformes	Sparidae	–	EF422805	–	Brisbane, Qld, AUS (GB)
	<i>Nerocila phaioleura</i>	Body surface	<i>Scomberomorus nipponius</i>	Perciformes	Scombridae	0–200	–	LC160332	Seto Inland Sea, JPN
	<i>Nerocila phaioleura</i>	Body surface	<i>Sardinops sagax melanostictus</i>	Clupeiformes	Clupeidae	0–200	LC159465	LC159576	Sagami Bay, JPN
	<i>Nerocila poruvae</i>	Body surface	unknown	–	–	–	JX413101	–	India (GB)
	<i>Nerocila poruvae</i>	unknown	unknown	–	–	–	–	KJ855321	India (GB)
	<i>Nerocila</i> sp. 1	Body surface	<i>Pennahia argentata</i>	Perciformes	Sciaenidae	40–140	LC159466	LC159577	Tokushima, JPN
	<i>Olencira praegustator</i>	Buccal cavity	unknown	–	–	–	AF259547	AF255791	Charleston, SC, USA (GB)
	<i>Renocila ovata</i>	Body surface	<i>Blenniella chrysospilos</i>	Perciformes	Blenniidae	0–6	EF422788	–	Heron Is., Qld, AUS (GB)
	<i>Ryukyua globosa</i>	Opercular cavity	<i>Amblygaster sirm</i>	Clupeiformes	Clupeidae	10–75	LC159467	LC159578	Henza Is., Okinawa, JPN
Cirolanidae	<i>Cirolana rugicauda</i>	Free-living	none	–	–	–	AF259544	AF255788	South of Lüderitz, Namibia (GB)
Bopyridae	<i>Athelges paguri</i>	Body surface	unknown	–	–	–	–	KT208746	North Sea (GB)
	<i>Bopyroides hippolytes</i>	Branchial chamber	unknown	–	–	–	–	DQ889082	unknown (GB)
	<i>Orthione griffenis</i>	Branchial chamber	<i>Upogebia major</i>	–	–	–	–	KP412462	South Korea (GB)
Corallanidae	<i>Tachaea chinensis</i>	Body surface	<i>Palaemon paucidens</i>	–	–	–	LC160308	–	Osaka, JPN
Aegidae	<i>Aega psora</i>	Free-living	none	–	–	–	–	FJ581463	St. Lawrence Gulf, Canada (GB)
	Aegidae gen. sp.	Body surface	<i>Hyperoglyphe japonica</i>	Perciformes	Centrolophidae	150–1537	LC159469	LC159579	Amami Is., JPN
	Aegidae gen. sp.	Opercular cavity	<i>Fistularia petimba</i>	Gasterosteiformes	Fistulariidae	18–57	LC159470	LC159580	Kimotsuki, Kagoshima, JPN
	<i>Alitropus typus</i>	unknown	<i>Channa striata</i>	Perciformes	Channidae	–	–	KT445864	India (GB)
	<i>Rocinela angustata</i>	Free-living	none	–	–	–	–	EF432739	unknown (GB)
	<i>Syscenus infelix</i>	Free-living	none	–	–	–	–	FJ581911	St. Lawrence Gulf, Canada (GB)

Table 1 continued

Family	Species	Attachment site	Host species	Order of host fish	Family of host fish	Depth (m)	16S No.	COI No.	Sampling locality
Sphaeromatidae	<i>Cymodoce</i> sp.	Body surface	<i>Neoscombrops pacificus</i>	Perciformes	Acropomatidae	60–500	LC159471	LC159581	Uwa Sea, Ehime, JPN

The accession numbers of novel sequences are shown in bold

GB GenBank

¹ Collected by R/V Yoko-Maru in June 2015, ² collected by R/V Wakataka-Maru in November 2013, ³ collected by R/V Wakataka-Maru in July 2014, ⁴ the Kanagawa Prefectural Museum of Natural History (KPM-NH 376)

epidermis, forming a capsule with a small opening within myomeric tissue to occupy the abdominal cavity.

DNA extraction

Samples of dorsal musculature (ca. $2 \times 2 \text{ mm}^3$) were excised from ethanol-preserved specimens. Total genomic DNA was extracted with Genomic DNA Purification Kit (Promega, Wisconsin, USA) following the manufacturer's protocol.

PCR and sequencing

The 16S rRNA gene region of mitochondrial DNA (mtDNA) was amplified using the forward primer (16Sar) 5'-CGC-CTGTTTAACAAAACAT-3' and the reverse primer (16Sbr) 5'-CCGGTCTGAACTCAGATCATGT-3' (Simon et al. 1994) via polymerase chain reaction (PCR). Each PCR was performed in a 10 μL reaction volume containing 3.35 μL sterile distilled H_2O , 5.0 μL Ampdirect Plus (Shimadzu, Kyoto, Japan), 0.3 μL each primer (10 μM solutions), 0.05 μL *Taq* DNA polymerase (BIOTAQ HS DNA Polymerase, Bioline, London, UK), and 1 μL template. The thermal cycle profile was as follows: initial denaturation at 94 $^\circ\text{C}$ for 10 min; 30 cycles of 94 $^\circ\text{C}$ for 30 s, 48 $^\circ\text{C}$ for 1 min, and 72 $^\circ\text{C}$ for 1 min; and a final extension at 72 $^\circ\text{C}$ for 10 min. The COI regions of mtDNA were amplified using the forward primer LCO1490 (5'-GGTCAACAAATCATAAA-GATATTGG-3') and the reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer et al. 1994) using the volumes described above. The thermal cycle profile was as follows: initial denaturation at 94 $^\circ\text{C}$ for 10 min; 35 cycles of 94 $^\circ\text{C}$ for 30 s, 40 $^\circ\text{C}$ for 1 min, and 72 $^\circ\text{C}$ for 1.5 min; and a final extension at 72 $^\circ\text{C}$ for 10 min. PCR products were purified using polyethylene glycol following a published protocol (Rosenthal et al. 1993) and subjected to direct cycle sequencing employing BigDye Terminator version 3.1 technology (Applied Biosystems [ABI], Foster City, CA, USA) using the PCR primers. The sequencing protocol used was that recommended by ABI. Labelled fragments were sequenced on an ABI 3130 platform. All pseudogenes (nuclear copies of mtDNA) evident

on electropherograms were excluded by checking for double peaks and mismatches in overlapping sequences of any given taxon (Mindell et al. 1999); we used Geneious version 7.1.7 software to this end (Kearse et al. 2012).

Sequence analysis

Forward and reverse sequences were assembled using Geneious version 7.1.7. For the 16S rRNA genes, assembled sequences were aligned using MAFFT 7.273 software running the Q-INS-i option that aligns sequences by considering RNA secondary structure (Katoh and Standley 2013). Next, ambiguities were removed by trimAl version 1.2 software (Capella-Gutiérrez et al. 2009). For the COI genes, the assembled sequences were translated into proteins and aligned to identify pseudogenes containing unexpected insertions, deletions, frameshifts, and stop codons (Mindell et al. 1999). The protein sequences were then reverse-translated prior to phylogenetic analysis using MEGA 6.06 software (Tamura et al. 2013).

We ran χ^2 homogeneity tests to explore the compositional homogeneity of nucleotides within 16S rRNA genes, and those of all codons of COI genes. These tests showed that the third codon positions of the COI genes exhibited significant heterogeneity in terms of base frequency. Most phylogenetic reconstruction algorithms assume homogeneity in nucleotide base compositions and can give rise to phylogenetic inaccuracies when this assumption is violated (e.g. Tarrío et al. 2001; Hassanin 2006). Therefore, the first, second, and third codon positions of the COI genes were separately analysed. The third codon position was subjected to RY coding. Thus, the pyrimidines C and T formed a single character, as did the purines A and G (Phillips and Penny 2003).

Phylogenetic analysis

Phylogenetic trees were constructed based on the individual 16S rRNA and COI datasets, and their combination. We used both the maximum likelihood (ML) and Bayesian inference (BI) methods. Individual substitution models were used to analyse the 16S rRNA data, and the first,

second, and third codon positions of COI. We calculated Akaike Information Criteria and selected the best substitution model using Kakusan 4 software (Tanabe 2011). Following this model selections, we used the GTR + G model when applying both the ML and BI methods to the 16S rRNA data. For COI analysis, we employed the GTR + G model when evaluating all three codon positions by the ML method, and the SYM + G, GTR + G, and F81 + G models to evaluate the first, second, and third codon positions, respectively, when applying the BI method. For the combined dataset, we used the GTR + G model to evaluate all markers by the ML method, and the GTR + G, SYM + G, GTR + G, and F81 + G models to evaluate the 16S rRNA data, and the first, second, and third codon positions of COI, respectively, when applying the BI method. ML and BI analyses were performed using RAxML version 8 (Stamatakis 2014) and MrBayes version 3.2.6 (Ronquist et al. 2012) software, respectively. In the BI method, two independent Markov-chain Monte Carlo runs, each of 1,000,000 generations, were performed; the trees were sampled every 100 generations. The first 4000 trees were discarded as burn-in. A majority-rule consensus tree was constructed from the remaining 6000 trees. We confirmed that all analyses attained the stationary condition well before the end of the burn-in period. To this end, we plotted the natural logarithms of the likelihoods of all sampled trees against the generation time, and confirmed that average standard deviation of split frequencies and the potential scale reduction factor for all parameters reached 0.006 and 1.00, respectively. We also confirmed that evaluating effective sample size values for all parameters were more than 200.

Evolution of the attachment mode

To explore how attachment modes were acquired by, and shifted within, Cymothoidae, we identified the ancestral mode using the Trace Character History function of Mesquite 3.04 (Maddison and Maddison 2015) software running an ML reconstruction algorithm.

Results and discussion

Phylogeny of the Cymothoidae

Three phylogenetic trees were constructed based on the 16S rRNA, COI, and combined datasets. These trees included two freshwater clades, an abdominal burrower, and the clade closer to the base of Cymothoidae (which were analysed for the first time). The topologies were nearly consistent among the trees, although the bootstrap values/Bayesian posterior probabilities were low in both the 16S rRNA and COI trees (Figs. 1, 2, 3, S1). We base

most of our results on the tree constructed using the combined dataset (Figs. 1, S1).

Phylogenetic analysis revealed that the basal branches within the family Cymothoidae were *Elthusa sacciger*; parasitic in the opercular cavity of synphobranchid eels living in the deep sea (between 400 and 3000 m) of the Western Pacific, and the freshwater abdomen-burrowing *Artystone* sp., parasitic on an armoured catfish of the Iguassu River, South America. The opercular cavity parasite clade, including the genera *Elthusa* and *Cterissa*, arose next (Figs. 1, 2, 3, and S2). These parasites colonise both deep-water fish (Ereuniidae, Hemitripterae, Hexagrammidae, and Macrouridae) and shallow water coastal fish belonging to Holocentridae. The mouth-dwelling *Ceratothoa* and *Glossobius* are next; most of their host fish species are coastal in nature. The next clade contains only *Ichthyoxenos tanganyikae*, which parasitises the mouth of a Tanganyikan cichlid (*Simochromis diagramma*); this species seems to form a monophyletic group with the *Mothocya* species parasitic in the opercular cavities of marine Belontiidae and Hemiramphidae. Subsequently, two clades of parasites living in the buccal and opercular cavities arose; one clade includes *Elthusa vulgaris* and the other includes *Lobothorax* and *Ryukyua*. Finally, *Cymothoa* (parasitic in the mouth) and *Anilocra* and *Nerocila* (parasitic on the external body surfaces of marine fish) arose and became diversified.

The abdominal muscle burrower *Artystone* sp. parasitises a freshwater loricariid fish, *Epactionotus yasi*, and forms a highly supported clade with *Alitropus typus*, a species of Aegidae that is parasitic in the opercular cavities or on the body surfaces of fish that live in brackish or freshwater of southern Asia to eastern Australia (Pillai 1967; Bruce 1983; Nair and Nair 1983; Bruce 2009). This was apparent in the phylogenetic tree based on COI sequences (Fig. 3).

Habitat expansion and shift to a new host fish

One of the basal taxa of Cymothoidae is *Elthusa sacciger*, which is parasitic on deep-sea Synphobranchidae. Smit et al. (2014) indicated that Cymothoidae may have originated in the Jurassic era (199–145 Ma) because fossils of bopyrid isopods, a family closely related to Cymothoidae (Boyko et al. 2013), have been dated to that time. In addition, the fossil record suggests that cirrolanid isopods scavenged a marine fish, *Pachyrhizodus marathonensis*, of the extinct order Crossozognathiformes, in the Albian, Early Cretaceous (113–100 Ma) (Wilson et al. 2011). In terms of marine fish, most existing orders that marine cymothoids parasitise were absent in the Jurassic era (Inoue et al. 2015), the exception being the order Anguilliformes. Deep-sea Synphobranchidae originated in the Triassic era (Inoue et al. 2010; Johnson et al. 2012), and it is thus possible

that the isopods parasitic in the opercular cavity of these deep-sea eels constitute the origin of Cymothoidae, which later diversified and expanded their habitat to include shallow seas and freshwater by evolving to live with newly derived fish. Intensive collection of deep-sea cymothoids is required to rigorously define the phylogenetic position of parasites of synphobranchid eels and to evaluate the hypothesis we advance above. On the other hand, phylogeny of cymothoids and that of host fishes are not concordant because of dynamic host shift by cymothoids (Fig. 1). Especially, cymothoids living in the buccal cavity and on external body surfaces of fishes have wide ranges of host fish species (Fig. 1; Table 1) and suggesting frequent host shift in the coevolution between cymothoids and host fishes.

In the present study, we analysed two freshwater species, one of which was a flesh-burrowing *Artystone* sp. parasitic on an armoured catfish, *Epactionotus yasi*, of the Iguassu River basin, South America; the other was the freshwater buccal parasite, *Ichthyoxenos tanganyikae*, which is parasitic on a Tanganyikan cichlid, *Simochromis diagramma*. *Artystone* sp. forms a highly supported clade with *Alitropus typus*, a species of Aegidae that is parasitic in the opercular cavity or body surface of brackish or freshwater fish of southern Asia to eastern Australia (Pillai 1967; Bruce 1983; Nair and Nair 1983; Bruce 2009). This suggests that *Alitropus* (which contains only *A. typus*) may belong to Cymothoidae rather than Aegidae. *Alitropus typus* has seven pairs of prehensile legs and short coxae on pereopods 5–7, and is unique among aegid species (Chilton 1926; Bruce 1983; note that *Rocinela simplex* of Chilton [1926] is a junior synonym of *A. typus*). However, this pereopod morphology is common in Cymothoidae (Brusca 1981). Further, this clade diverges near the base of the cymothoid phylogenetic tree, suggesting that the lineage may have immigrated to freshwater in Gondwanaland, and diversified following the break-up of that continent. *Alitropus typus* and *Artystone* sp. colonise siluriform fish that also originated in Gondwanaland (106.1 Ma; Near et al. 2012), partly supporting this hypothesis.

In contrast, *Ichthyoxenos tanganyikae* is closely related to the opercular cavity-parasitic genus *Mothocya* that lives in shallow coastal regions, suggesting that this species also arose by invasion of the lacustrine habitat, perhaps via the Congo River where a close relative, *I. expansus*, has been described from the opercular cavity of the characiform fish *Eugnathichthys eetveldii* (Fryer 1968; Lincoln 1972). *Ichthyoxenos tanganyikae* is apparently asymmetrical, as are cymothoids that parasitise the opercular cavity, suggesting that this mouth-parasitic group originated from a parasite of the opercular cavity (Fig. 4). These two freshwater clades arose independently from different marine ancestors; the *Artystone*

clade evolved much earlier than did the *I. tanganyikae* clade.

Evolution of the attachment mode to host fish

Our results suggest that the ancestor of the family Cymothoidae was most likely an opercular parasite (Fig. 4), although more taxon sampling and molecular analyses are necessary to resolve the basal part of cymothoid phylogeny. Subsequently, at a very early stage, certain lineages seem to have invaded freshwater habitats and some acquired the ability to burrow into the abdominal cavity. All abdominal cavity-burrowing species (the genera *Ichthyoxenos*, *Artystone*, and *Riggia*) are strictly confined to brackish or freshwater, with the exception of only one species, *Ourozeuktes bopyroides*, that is marine and parasitic on a leatherjacket (*Acanthaluteres spilomelanurus*) of one of the most derived fish orders, Tetraodontiformes (Brusca 1981; Saunders 2012; Trilles and Hipeau-Jacquotte 2012). Further analyses on these species are required to define the timing and frequency of evolution of abdominal cavity burrowers. Mouth-dwelling capacity evolved only once and then became diversified. Three clades of mouth parasites have reverted to opercular cavity parasitism. The final evolutionary step was attachment to the external surface of fish. Based on the 16S rRNA data, parasites of the buccal or opercular cavity may be the ancestors of organisms exhibiting external attachment today (Jones et al. 2008). Our 16S rRNA and COI dataset supports this suggestion. Furthermore, we have defined three clades near the base of the tree, suggesting that parasites of the opercular cavity are the most ancient. For the first time, we used molecular data to reveal the ancient origin of freshwater abdominal burrowers, and the independent expansion into a freshwater habitat by a Tanganyikan mouth-dwelling cymothoid.

Phylogenetic relationships among species of Cymothoidae and fish parasites of Aegidae

Taxonomic studies of the family Cymothoidae are challenging when only morphological data are available; intra-specific variation is sufficiently great to sometimes overwhelm inter-specific variation (Smit et al. 2014). This is attributable to the morphological convergence of cymothoid species that share morphologically similar host fish, and divergence among cymothoid populations that utilise different species of host fish but nonetheless belong to a single species. In such cases, molecular markers are important in taxonomic terms, as has also been found for bivalves parasitic on various host species (Goto et al. 2012). Taxonomy and phylogeny based on

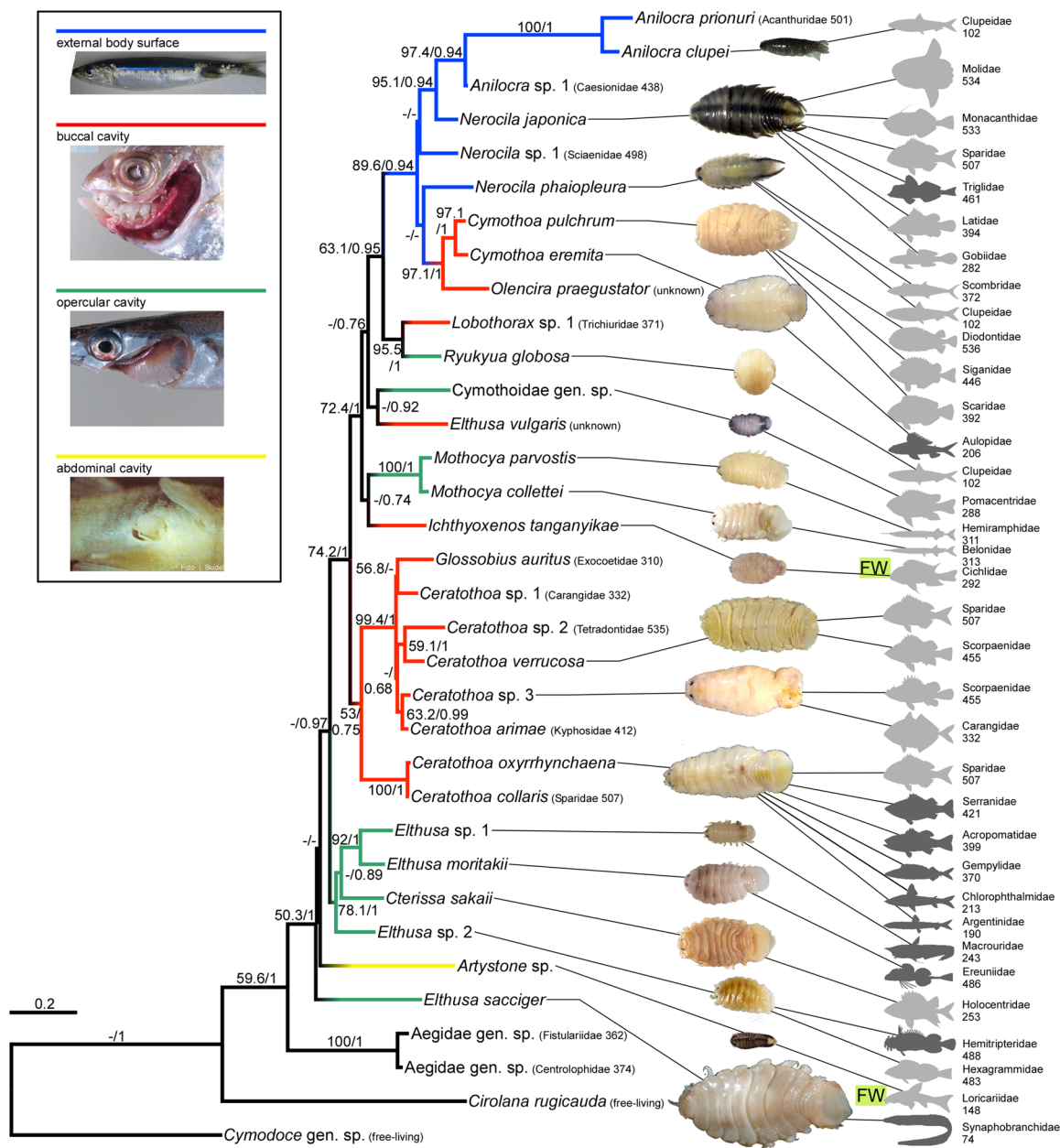


Fig. 1 A maximum likelihood tree of the family Cymothoidae based on combined data on mitochondrial 16S rRNA and COI genes. The numbers adjacent to branches are the maximum likelihood bootstrap values followed by the Bayesian posterior probabilities. Values >50 and 0.5 (bootstrap values and probabilities, respectively) are shown. The host fish families and the numbers of families (informative in terms of phylogenetic position; 1 to 536 indicate Myxinidae to Dio-

dontidae, respectively; Nelson et al. 2016) are shown in parentheses or following silhouettes of the families. Dark- and light-grey fish inhabit deep (>200 m) and shallow water, respectively; 'FW' identifies freshwater fish. See Table 1 for details of host fish species. The photograph of *Artystone* sp. is reproduced with the kind permission of I. Seidel

both morphology and molecular markers have been conducted on Cymothoidae (Hadfield 2012; Martin 2015), and most genera are well established. Our results also confirmed the monophyly of most genera. However, the genus *Elthusa* is not a monophyletic group, but rather contains at least three distinct clades: *Elthusa sacciger*;

Elthusa spp. parasitic in the opercular cavity of Ereuniidae, Hemitripterae, Hexagrammidae, Macrouridae; and *E. vulgaris*. In addition, our data suggest that *Alitropus typus* belongs to Cymothoidae, not Aegidae; both molecular phylogeny and morphology support this suggestion.

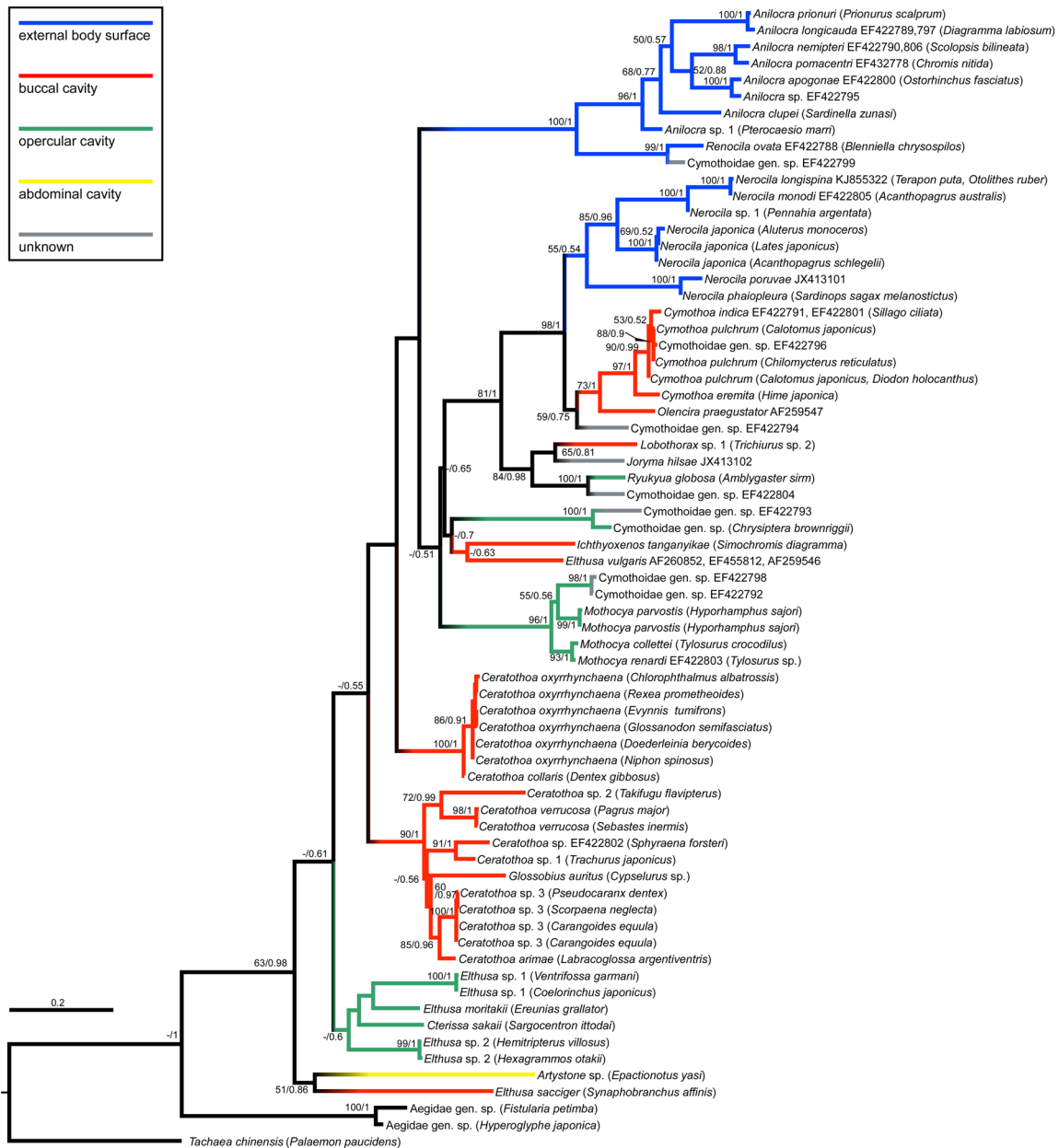


Fig. 2 Maximum likelihood tree of the family Cymothoidae based on mitochondrial 16S rRNA sequence data. The *numbers* adjacent to the branches are the maximum likelihood bootstrap values followed

by the Bayesian posterior probabilities. Values >50 and 0.5 (bootstrap values and probabilities, respectively) are shown. The host fish species are shown in *parentheses*

Conclusions

We constructed a molecular phylogeny of the family Cymothoidae based on mitochondrial 16S rRNA and COI sequences. The phylogenetic tree suggests that

cymothoid isopods may have originated in the deep sea, and then expanded their habitats to include shallow seas and brackish or freshwater by shifting their host species. Invasion of freshwater has occurred (independently) at least twice. The ancestral attachment mode is most likely

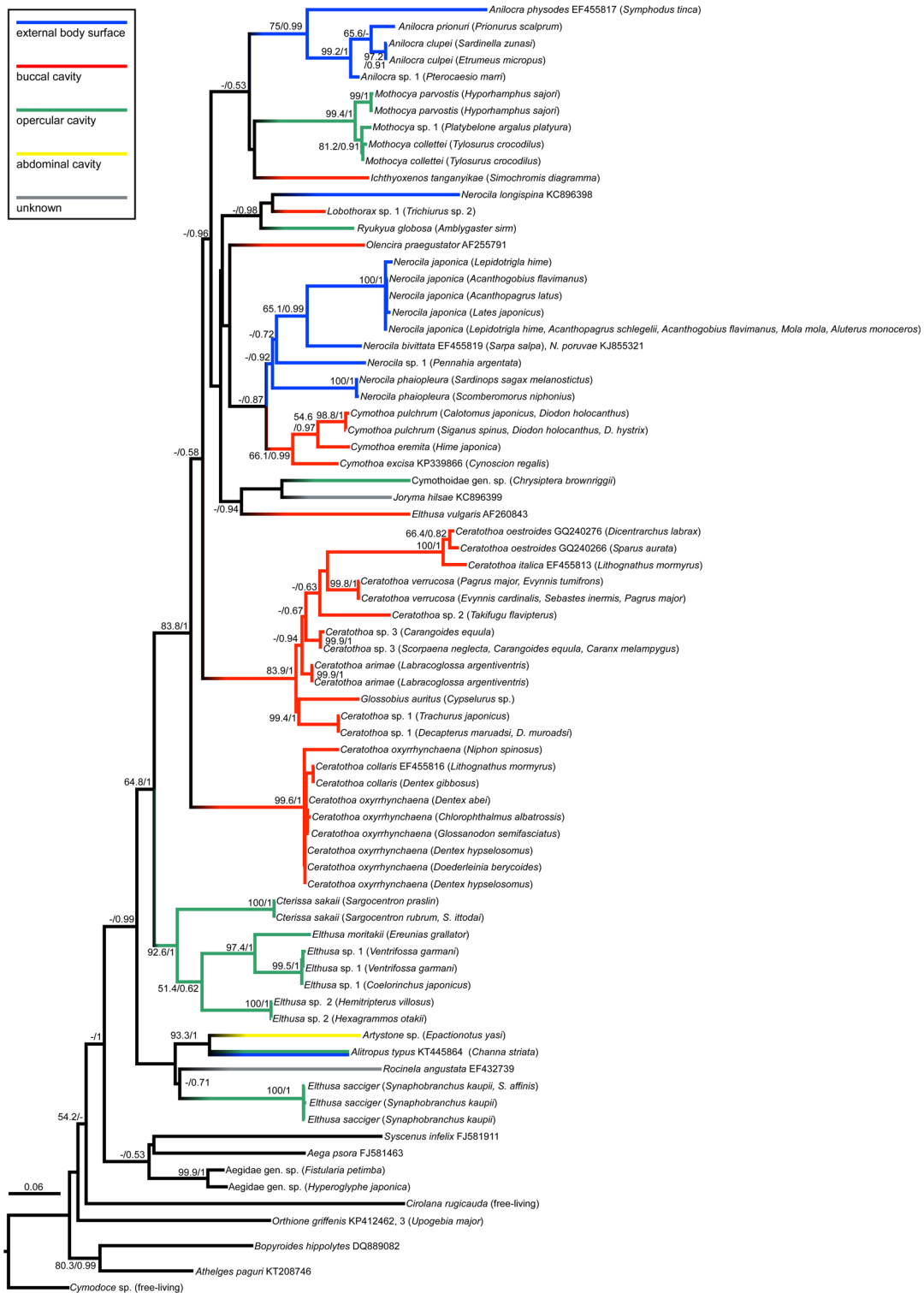


Fig. 3 Maximum likelihood tree of the family Cymothoidae based on mitochondrial COI sequence data. The numbers adjacent to the branches are the maximum likelihood bootstrap values followed by

the Bayesian posterior probabilities. Values >50 and 0.5 (bootstrap values and probabilities, respectively) are shown. The host fish species are shown in parentheses

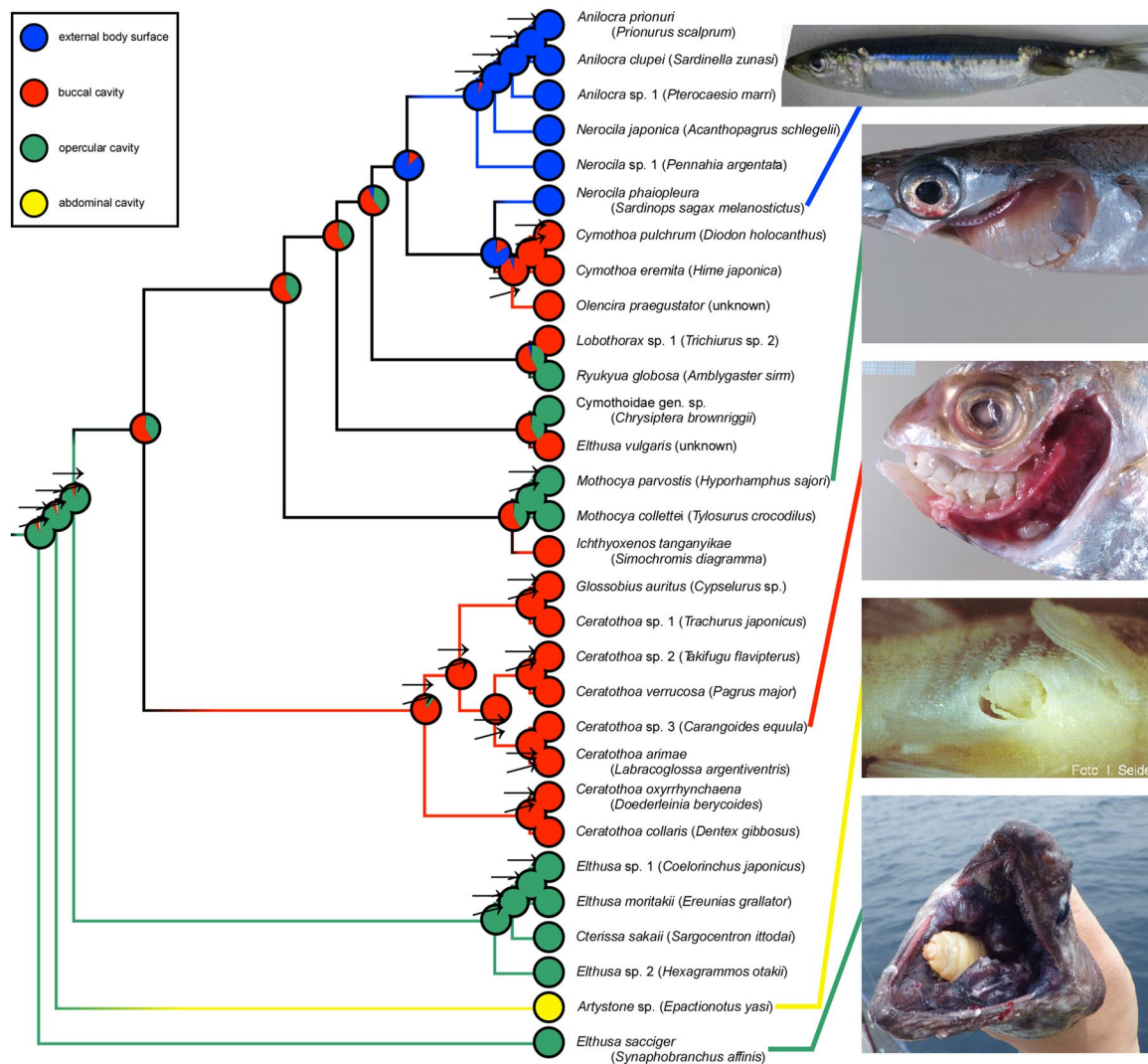


Fig. 4 Reconstruction of the Cymothoidae attachment mode using a maximum likelihood tree based on combined data on mitochondrial 16S rRNA and COI sequences. The pie charts indicate the relative likelihoods of the attachment mode for each clade. *Asterisks* indicate

nodes where only one state was significant upon comparison of log-likelihoods. The host fish species are shown in *parentheses*. The photographs of *Artystone* sp. and *Elthusa sacciger* are reproduced with the kind permission of I. Seidel and H. Hirasaka, respectively

to be the opercular cavity-dwelling. The ability to live in the buccal cavity and on external body surfaces evolved subsequently.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical standard All experiments were conducted in accordance with the Ethical Guidelines for Animal Experiments of Ehime University.

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