Genetic population structure of *Hemigrammocypris rasborella* (Cyprinidae) inferred from mtDNA sequences

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Abstract The genetic population structure of the small cyprinid *Hemigrammocypris rasborella*, distributed widely in lowlands of western Japan, was examined using partial sequence data of mitochondrial DNA (mtDNA). Molecular phylogenetic analysis revealed that the populations of the western Kyushu region were markedly differentiated from all eastern populations, such that the groups would be comparable to different species; their divergence was inferred to have occurred in the Late Miocene–Pliocene. Also, a largely divergent mtDNA group (with divergence in the early Pleistocene) was found in the Sanyo and northeastern Shikoku regions, forming a secondary contact zone in the western Kinki with the eastern mtDNA group. To date, these aspects of the population structure of *H. rasborella* appear to be unique among lowland fishes in western Japan. Deeper understanding of the formation processes of freshwater faunas in western Japan will require further comparisons of the phylogeographic patterns and ecological traits of constituent species.

Keywords Cytochrome *b* (cyt*b*) · Molecular dating · Phylogeography · Secondary contact · Western Japan

Introduction

The Japanese Archipelago is an island system extending over 2,000 km in a northeast–southwest direction at the eastern margin of East Asia. It harbors unique, rich biota with regional heterogeneity. Strictly freshwater fishes, which live only in fresh waters throughout their life history, are some of the best organisms with which to investigate historical aspects of such biota, including relationships between geology and biota formation, because they are easily isolated by geological barriers such as mountains and straits (Avise 2000). Previous studies have shown that the freshwater fish fauna of northeastern Hokkaido is primarily divided from the fauna of southwestern areas, and that there is a secondary large change across the central highlands, or the region around the Fossa Magna, a great rift valley running north to south through central Honshu (Nishimura 1974; Watanabe 1998, 2012).

Western Japan, comprised of the regions west of the Fossa Magna, including the western part of Honshu, Shikoku, and Kyushu islands, is known to have especially rich freshwater fish fauna (Nishimura 1974; Watanabe 2012). There are two major cores of endemicity, as defined by unique species; i.e., the eastern part of western Japan (Lake Biwa and adjacent areas) and northeastern Kyushu (Watanabe 2012). Elucidation of the formation processes of the freshwater fish fauna of western Japan is hence an important step for understanding the origins and development of the freshwater biota of Japan.

Phylogeographic assessments of the population structure and historical dynamics of species using molecular genetic markers play an essential role in the study of historical biogeography, especially over relatively short geological time scales (the Neogene–Quaternary) (Avise 2000). Such approaches have been previously applied to several freshwater fishes distributed in western Japan. For example, in some cyprinids widely occurring in the lowlands of western Japan, the easternmost populations around the Ise Bay area (Fig. 1) exhibit large divergence from other populations, as evidenced by mitochondrial DNA (mtDNA) data (Watanabe et al. 2010a; Komiya et al. 2013). This suggests that a mountain system (the Suzuka Mountains), which forms a geological barrier dividing watersheds, has had a notable impact on population structure and the regional heterogeneity of freshwater fauna. This also suggests that gene flow existed among populations of those species in western areas after the uplifting of the mountains since the early Pleistocene (Kawabe 1994). On the other hand, Minamimedaka, *Oryzias latipes* (Oryziidae), shows a different population structure, with

its westernmost populations in western Kyushu possessing the most divergent mtDNA in western Japan (Takehana et al. 2003). Phylogeographic studies on freshwater fishes in western Japan have been fragmentary, and general patterns of population structures and their relations to geological patterns and processes have not been elucidated sufficiently. Accumulations and comparisons of phylogeographic information on more fish species (and other aquatic organisms) are necessary to clarify the faunal history of western Japan.

Hemigrammocypris rasborella is a small cyprinid fish that is endemic to Japan and is a representative lowland freshwater fish distributed across western Japan (Nakamura 1969; Kanagawa and Itai 1998). This species was once commonly found in ponds, marshes, and streams in its distribution range. However, due mainly to habitat degradation (e.g., urbanization and river improvement) and the introduction of predatory fishes, the species is declining throughout its range (Kanagawa and Itai 1998; Nakajima et al. 2006), and has been designated as a critically endangered species by Japanese national and local governments (e.g., Japan Ministry of the Environment 2003). Previous studies have revealed that some populations of this species have maintained their genetic diversity and that the species shows clear genetic population structure associated with watersheds in the eastern part of western Japan (Watanabe and Mori 2008; Watanabe et al. 2009). Variation in body shape is also reported among populations collected over small scales (¥100 km), suggesting possible genetic divergence and local adaptation among populations of this species (Akada and Yodo 2006). The phylogeographic patterns of this species throughout its entire range will provide important information on and insights into geographical divergence and interchange among regional populations and faunas in western Japan, as well as the species' conservation.

This study was conducted primarily to clarify the population structure of *H. rasborella* using mtDNA sequence data from specimens collected across the entire range of the species. Then, we examined whether or not cyprinids found in lowland habitats in western Japan share the same or similar population structures. Furthermore, estimating the divergence times among the detected major geographical groups, the distribution and isolation processes of this species are discussed with reference to potentially associated geological events.

Recently, Zarske (2013) claimed that *Barilius neglectus*, which was described by Stieler (1907) based on juveniles of German aquarium fish imported from Japan, is the senior synonym of *H.*

rasborella, which was originally described by Fowler (1910). However, because of the potential for taxonomic confusion, as raised in this study, we tentatively use *H. rasborella* in this paper.

Materials and methods

Samples. A total of 509 specimens of *Hemigrammocypris rasborella* were collected from 35 localities throughout the entire range of the species in Japan (Fig. 1; Table 1). These included 141 specimens from 12 localities that were used in Watanabe and Mori (2008) and Watanabe et al. (2009). The localities were grouped geographically into six regions: Shizuoka, Ise Bay, Kinki, Sanyo, Shikoku, and Kyushu (Fig. 1; Table 1). Collection of the specimens was conducted with permission from local governments when necessary by law (Mie and Kagawa Prefectures), and was accomplished by short-time trapping or netting in areas estimated to be inhabited by hundreds or more of the fish. In other cases, fish were temporarily collected and released after the non-invasive clipping of a small piece of a pelvic fin (several mm^2) for genetic analyses. Fish or fin clips were preserved in 100 % ethanol. Specimens from neighboring sites (e.g., irrigation ponds within an area) were pooled as a sample. A local sample from Tokushima was collected from a captive population that had been maintained at the Fisheries Research Division of Tokushima Agriculture, Forestry, and Fisheries Technology Support Center, Tokushima since 2007.

MtDNA sequencing and analyses. Total genomic DNA was isolated using a Genomic DNA Purification Kit (Promega, Madison, WI, USA) from fin clips preserved in 100 % ethanol. Polymerase chain reaction (PCR) amplification was performed using the primer pair L14724 (5′-TGA CTT GAA RAA CCA YCG YYG-3′) (Palumbi et al. 1991) and H15915 (5′-ACC TCC GAT CTY CGG ATT ACA AGA C-3′) (Aoyama et al. 2000) to obtain the nucleotide sequences of the mitochondrial cytochrome *b* gene (cyt*b*). The PCR conditions and sequencing method were as described in Watanabe and Mori (2008). The newly obtained sequences (690 bp of 3′-half of cytb) were deposited in the DNA Data Bank of Japan (DDBJ), GenBank, and the European Nucleotide Archive (EMBL) [accession numbers AB907301–907336; see Electronic Supplementary Material (ESM) Table S1]. The haplotype frequencies of each population were deposited in the Genetic

Diversity and Distribution Map (GEDIMAP) freshwater fish database (Watanabe et al. 2010b) (see Table 1 for ID numbers).

Genetic diversity indices, i.e., the number of haplotypes (k), haplotype diversity (h), and nucleotide diversity (π), were calculated for each local sample using ARLEQUIN v3.5 (Excoffier and Lischer 2010).

For phylogenetic analysis, the following three outgroup sequences were added: *Metzia formosae* (HM224304) and *Metzia lineata* (HM224305) both from Hanoi, Vietnam (Tang et al. 2010), and *Aphyocypris chinensis* (AB218688; Fukuoka, Japan; Saitoh et al. 2006), referring to the cyprinid phylogeny proposed by Tang et al. (2010).

The maximum-likelihood (ML) tree for mtDNA haplotypes was estimated using PAUP*4.0b10 (Swofford 2002), with the HKY+G model selected by Akaike's information criterion (AIC), implemented in jModeltest v2.1.1 (Darriba et al. 2012). The model parameters were as follows: base frequencies of A = 0.3102, C = 0.2687, G = 0.1367, and T = 0.2844; kappa = 10.2588; gamma shape = 0.2310. The robustness of the ML tree was assessed using the bootstrap method (BP) with 300 replicates.

A Bayesian approach was used to estimate the phylogenetic tree and the divergence times of lineages using the HKY+G model, selected by the Bayesian information criterion (BIC) in jModeltest, and the Yule (speciation) tree prior using BEAST v1.7.5 (Drummond and Rambaut 2007). We adopted the uncorrelated lognormal relaxed clock (Drummond et al. 2006). To estimate the time of the most recent common ancestors (tMRCA), two constraints were given: one is geological and the other is for evolutionary rate. As the geological constraint, the uplift of the Suzuka Mountains in central Honshu Island 1.0–1.5 million years ago (Mya) (Yokoyama 1988; Kawabe 1994) was applied for the relevant node age. This constraint was specified as a lognormal prior distribution, ranging from approximately 1.1 to 1.5 Mya in the 95 % range with mean = 1.3 Mya, log(SD) = 0.1, and offset = 0. Previous estimates of cyt*b* molecular substitution rates for teleosts range from ~0.3 to 1.5 %/million years (Myr)/lineage (e.g., Burridge et al. 2008; Watanabe and Takahashi 2010), and a rate of 0.76 %/Myr/lineage has been obtained for cyprinids (Zardoya and Doadrio 1999). We set a normal prior distribution (mean ± SD) for both the mean molecular rate and its standard error = 0.76 % and SD = 0.50 %, which is a lax constraint covering 0–1.58 % in the 95 % interval. All other model parameters used default priors. For each Markov-chain Monte

Carlo (MCMC) analysis, we performed two independent runs of 10 million generations. We sampled every 1,000th generation and removed 10 % of the initial samples as a burn-in period. The convergence of the chains to stationary distribution and large effective sample size (ESS; >200 were confirmed using TRACER v1.5 (Rambaut and Drummond 2009). The consensus tree was calculated using LogCombiner v1.6.2 and TreeAnnotator v1.6.2 in the BEAST package, and the tree was visualized using FigTree v1.3.1 (Rambaut 2009). The robustness of the Bayesian tree was evaluated by posterior probability (PP).

Results

A total of 70 haplotypes (690 bp) were obtained from the 509 specimens of *Hemigrammocypris rasborella* (ESM Table S1). The number of haplotypes (*k*) within local samples ranged from 1 to 8 (average ± SD, 2.7 ± 1.9) with h = 0–0.8667 (0.3679 ± 0.3018) and π = 0–0.0124 (0.001600 ± 0.002821). Twelve of the total 35 samples (34 %) were monomorphic.

The ML and Bayesian trees of mtDNA haplotypes showed similar tree topologies, and consistently revealed two deeply diverged clades (A and B) of H. rasborella (clade A, PP=100%, BP=94%; clade B, PP=100%, BP=100%), with 0.101±0.003 SD in average uncorrected *p* (pairwise sequence differences), and 0.163 ± 0.007 SD in HKY+G distance (Fig. 2; Table 2; ESM Fig. S1). Clade A consisted of haplotypes from the specimens collected from Honshu and Shikoku, whereas the clade B haplotypes were exclusively from Kyushu.

Clade A was further discriminated into four regional clades (A1–A4) (Fig. 2; ESM Fig. S1). While clades A1, A2 and A3 formed a monophyletic group (PP = 96 %, BP = 50 %), their interrelationship was not fully resolved.

Clades A1 and A2 consisted exclusively of the haplotypes from Shizuoka and the Ise Bay area, respectively (Figs. 1, 2). Clade A3 included the haplotypes from the Kinki region and the eastern part of Shikoku (Tokushima Prefecture; locality #27). The clade A4 haplotypes originated from the western Kinki, northeastern Shikoku (Kagawa Pref.), and Sanyo regions. The clade A3 and A4 haplotypes occurred parapatrically or sympatrically in the western Kinki region, suggesting secondary contact of these divergent haplotype groups in this region.

The geological constraint associated with the uplifting of the Suzuka Mountains was given for the tMRCA of clades A1, A2 and A3. Using this and evolutionary rate constraints, the divergence times between the major clades were estimated (Fig. 2). The time at the calibration point was estimated at 1.27 Myr [95 % highest probability density (HPD), 1.04–1.53 Myr]. The divergence time between clades A1–A3 and clade A4 was estimated at 1.71 Myr (1.15–2.35 Myr). Divergence between clades A and B was estimated to have occurred at 5.89 Myr (3.78–8.17 Myr). The estimation of the divergence time between *H. rasborella* and *Metzia lineata* was 6.82 Myr (4.44–9.45 Myr). The estimated evolutionary rate was 0.88 %/Myr/lineage on average (95 % HPD, 0.58–1.19 %), and did not show large heterogeneity among the major clades (0.87–0.88 %).

Discussion

The present study successfully revealed, using mtDNA divergence, the major regional groups within *Hemigrammocypris rasborella* based on specimens collected from the entire distribution range of the species. Specifically, the populations in western Kyushu, the westernmost populations of the species, showed remarkable differentiation from the eastern populations, which are geographically separated from the Kyushu populations by a gap in distribution from westernmost Honshu to eastern Kyushu (Fig. 1). The two mtDNA groups were estimated to have diverged from each other ca. 3.8–8.2 Mya, during the Late Miocene–Pliocene, and this divergence is comparable to that from another species (*Metzia lineatus*) from the southern part of East Asia, which has not been necessarily proven to be *H. rasborella*'s closest relative.

Among the primary freshwater fishes that are widely distributed in western Japan, the largest intraspecific divergence of Kyushu populations, as shown in *H. rasborella*, is not common among the species that have been investigated to date. The divergence is sufficiently wide to be equivalent to the genetic differentiation (0.088 in GTR+I for ND1 gene) between two subspecies of the bitterling *Rhodeus atremius atremius* (distributed in Kyushu) and *Rhodeus atremius suigensis* (distributed in the Sanyo region) (Miyake et al. 2011). *Oryzias latipes* is another exception in which the most divergent haplotype group is found from western Kyushu to the Ryukyu Islands (Takehana et al. 2003), although the species shows some salinity tolerance. Rather, the largest divergence is

found in the easternmost populations (Ise Bay area and the east) of some cyprinids (e.g., *Biwia zezera*, Watanabe et al. 2010a; *Sarcocheilichthys variegatus*; Komiya et al. 2013). The Ise Bay area harbors several endemic species and populations, including freshwater fishes (Watanabe 2012) and marsh plants (Ueda 1989). These support the long, somewhat isolated history of the wetland environments in this region, which is probably associated with the formation of the Suzuka Mountains during the early Pleistocene. Also in the case of *H. rasborella*, the divergence of the around-Ise Bay population from its neighbors was significant and probably reflects the mountain formation (Watanabe and Mori 2008; Watanabe et al. 2009; present study). However, the differentiation of the Kyushu population from the others is much larger than this, indicating that the isolation of the species' populations between Kyushu and the eastern regions has been sustained over a long period during which other lowland fish species maintained gene flow between those regions. Variations in population structure and presence/absence among species can be attributable to differences in their dispersal abilities or historic local extinctions. Comparative analyses of faunal and phylogeographic patterns that explicitly incorporate the ecological traits of species are needed.

It would be possible to treat the two widely divergent *H. rasborella* groups as different species, or at least subspecies. No detailed morphological comparisons have been conducted for this species throughout its distribution range, except for those focusing on a restricted area (the Ise Bay area; Akada and Yodo 2006) or on specific characteristics (cephalic lateral line canal system; Takeuchi et al. 2011). Inclusive morphological and taxonomic studies, with particular focus on the status of the Kyushu populations, are necessary for *H. rasborella*. To resolve the taxonomy of this species, it is also important to determine the population from which the syntype specimens of *H. neglectus* originated (Stieler 1907; Zarske 2013).

The second largest differentiation was found between clade A4 and clades A1–3 (uncorrected *p*-distance, 0.024 on average; estimated divergence time, 1.2–2.4 Myr), which were collected from the middle part of the around Seto Inland Sea area (Sanyo, western Kinki, and northeastern Shikoku) and the eastern areas, respectively, with an overlap zone in the western Kinki region (Fig. 1). This pattern of population structure is also unique among those previously known for freshwater fishes. That is, although the Sanyo populations of some other freshwater fishes are also genetically unique, their differentiation from Kinki populations is not so large (the average uncorrected *p*-distance in cyt*b*: *Biwia zezera*, 1.0 %, calculated from Watanabe et al. 2010a; *Rhodeus ocellatus kurumeus*,

0.9 %; Abe et al. 2013) as in *H. rasborella* (2.6 %). The distribution of clade A3 haplotypes (Kinki and eastern Shikoku) suggests gene flow via the eastward paleo-river system that flowed from the eastern Seto Inland Sea region into the Pacific Ocean through the Kii Channel during the Pleistocene regression periods (Fig. 1; Kuwashiro 1959; Ota et al. 2004). The clade A4 haplotypes, on the other hand, would have originated from regional populations differentiated from those with the clade A3 haplotypes. Since the former's distribution area belongs to the eastward paleo-river basin (Ota et al. 2004), the differentiation between A4 and A1–3 might be caused by division of tributaries of the eastward paleo-river. However, this seems difficult to explain the long isolation (>1 Myr) and interrelationships among the clades A1–4. Instead, the clade A4 haplotypes might be associated with the westward paleo-river that flowed into the Pacific Ocean through the Bungo Channel between Shikoku and Kyushu Islands (Fig. 1), although the species does not presently occur in the westward paleo-river basin. The partially overlapped distributions of clades A3 and A4 in the western Kinki region suggest the range expansion of either or both clades and secondary contact between the differentiated populations (Avise 2000). Analysis using sensitive nuclear DNA markers will reveal the complex distribution processes of isolation, gene flow and local extinction histories around the Seto Inland Sea.

In conclusion, we clarified the unique aspects of the phylogeographic pattern of *H. rasborella* compared with those of previously studied lowland freshwater fishes in Japan. The distribution processes of lowland freshwater fishes are generally thought to be affected by geological factors, such as isolation by mountain uplift, range expansion following sea regression, and isolation and reduction following sea-level rise. Simultaneously, the effects of such geological factors are, at least partly, dependent on ecological traits of the species. Deeper understandings of the historical processes of regional freshwater faunas require comparisons of the distributions and phylogeographic patterns of constituent species with incorporation of their ecological traits.

The clear genetic differentiation among regional populations of *H. rasborella* emphasizes the necessity to protect them separately as important management units. Most of the populations of *H. rasborella* now survive in small, isolated ponds, which were not necessarily the main habitat of this species in the past (i.e., lowland marshes and creeks). Special attention should be paid to reintroduction plans for this species, as well as potential losses of intra-populational genetic diversity.

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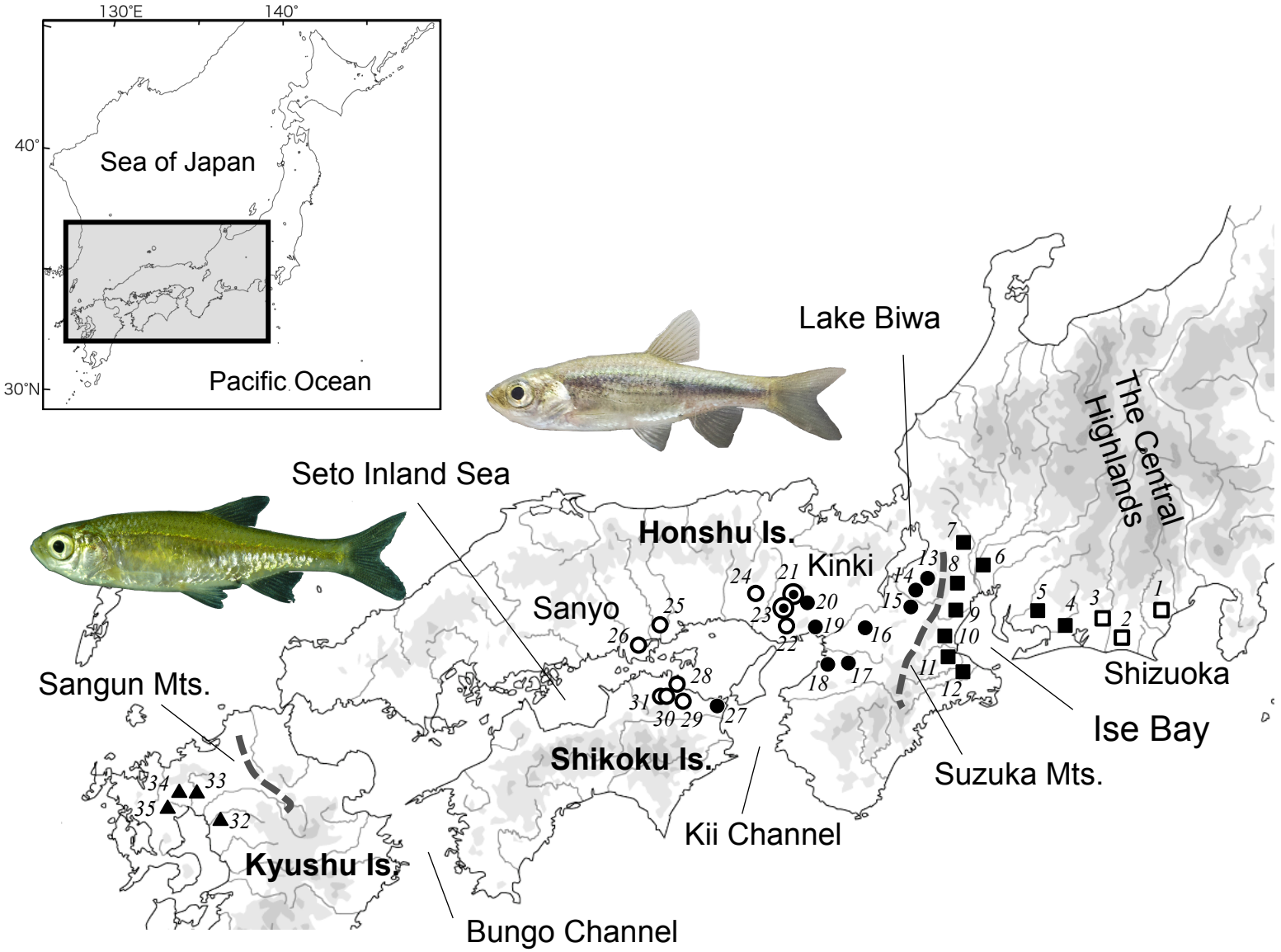
Figure legends

Fig. 1 Sampling localities of *Hemigrammocypris rasborella*. *Numbers of localities* correspond to those in Table 1. *Open squares* the localities where clade A1 haplotypes were collected, *closed squares* clade A2 haplotypes, *close circles* clade A3 haplotypes, *open circles* clade A4 haplotypes, *circled bullets* clade A3 and A4 haplotypes, *triangles* clade B haplotypes. Photographs: *H. rasborella* from Hyogo Prefecture, western Kinki, by K. Tominaga (*right*), and Saga Prefecture, northwestern Kyushu, by J. Nakajima (*left*), both uncatalogued

Fig. 2 Bayesian phylogenetic tree of *Hemigrammocypris rasborella* with outgroups based on the mtDNA cytochrome *b* sequences (690 bp) with the HKY+G model. The tree is dated by the uncorrelated lognormal relaxed clock model with a node-age constraint (*) and molecular evolutionary rate (see text). The *numbers at nodes* correspond to Bayesian posterior probabilities on the left and ML bootstrap probabilities on the right. *Bars* show credibility intervals as 95 % HPD


130°E
140°
40°
30°N
Sea of Japan
Pacific Ocean
Lake Biwa
The Central Highlands
Seto Inland Sea
Honshu Is.
Kinki
Sanyo
Sangun Mts.
Shikoku Is.
Kyushu Is.
Kii Channel
Bungo Channel
Suzuka Mts.
Ise Bay
Shizuoka
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2
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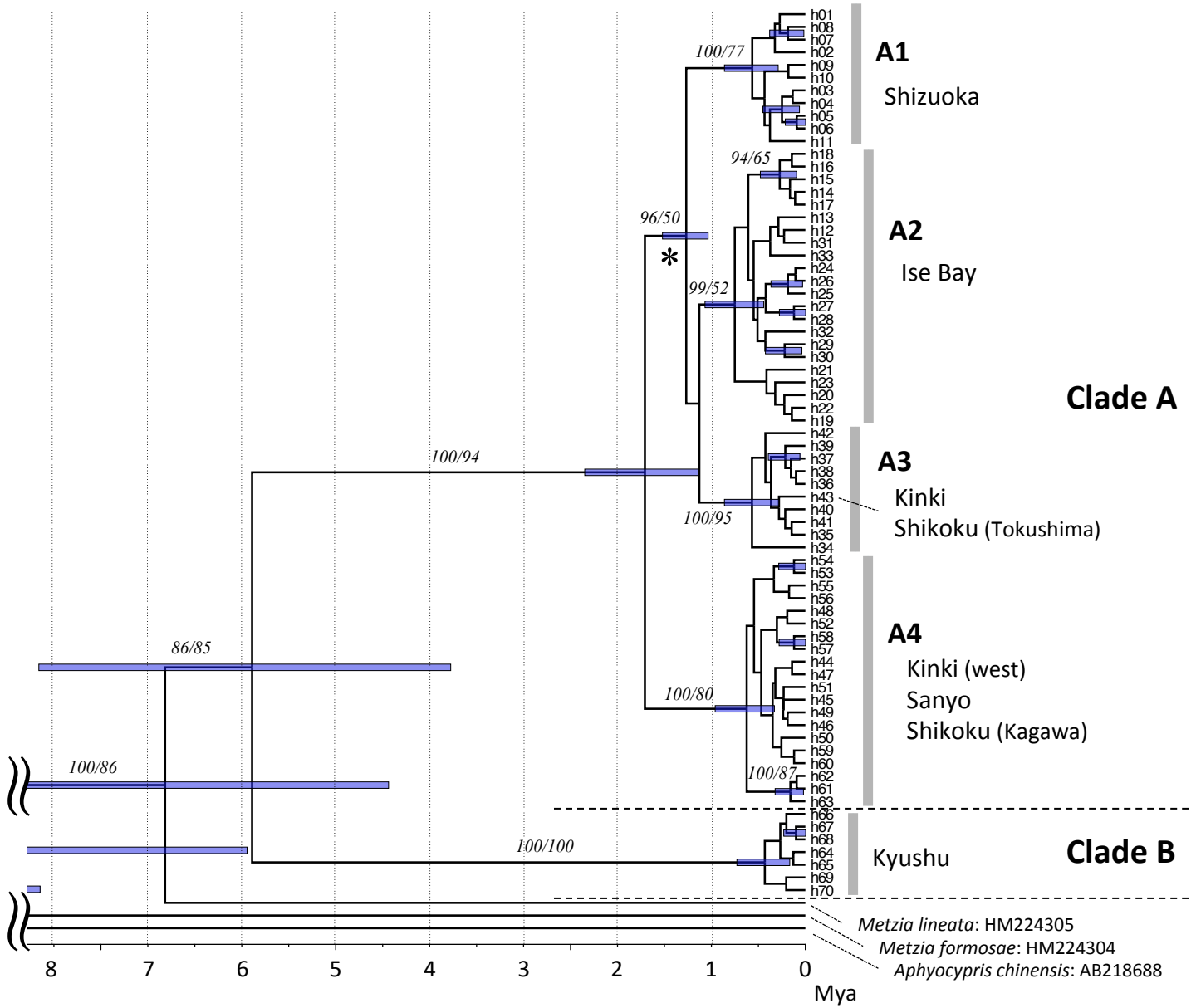
Fig. 1


h01
h08
h07
h02
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h14
h17
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h46
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h59
h60
h62
h61
h63
h66
h67
h68
h64
h65
h69
h70
100/77
94/65
96/50
*
99/52
100/94
100/95
86/85
100/80
100/86
100/87
100/100
A1
Shizuoka
A2
Ise Bay
Clade A
A3
Kinki
Shikoku (Tokushima)
A4
Kinki (west)
Sanyo
Shikoku (Kagawa)
Kyushu
Clade B
Metzia lineata: HM224305
Metzia formosae: HM224304
Aphyocypris chinensis: AB218688
8
7
6
5
4
3
2
1
0
Mya

Fig. 2

Table 1 Localities and sample size of *Hemigrammocypris rasborella* analyzed

Population code		River system	Locality (Year)	Habitat type	*n*	Clade					Remarks	GEDIMAP ID
						A-1	A-2	A-3	A-4	B		
Shizuoka												
1	Fujieda	Seto R.	Fujieda, Shizuoka (2006)	P	15	15	–	–	–	–	6. Seto (wild)[a]	P836
2	Iwata	Ota R.	Iwata, Shizuoka (2006)	P	15	15	–	–	–	–	4. Ota Pond2 (wild)[a]	P844
3	Hamakita	Tenryu R.	Hamakita, Shizuoka (2006)	P	13	13	–	–	–	–	1. Tenryu (wild)[a]	P841
Ise Bay												
4	Shinshiro	Toyo R.	Hitokuwada, Shinshiro, Aichi (2004, 2005)	P	8	–	8	–	–	–	10. Shinshiro[b]	P543
5	Okazaki	Yahagi R.	Okazaki, Aichi (2004)	P	10	–	10	–	–	–	9. Okazaki[b]	P542
6	Shippo	Nikko R.	Shippo, Aichi (2004)	C	17	–	17	–	–	–	8. Shippo[b]	P541
7	Ogaki	Ibi R.	Kogetsu, Ogaki, Gifu (2004)	C	15	–	15	–	–	–	6. Ogaki[b]	P539
8	Inabe	Inabe R.	Inabe, Mie (2005)	P	7	–	7	–	–	–	5. Inabe[b]	P538
9	Kameyama	Suzuka R.	Kameyama, Mie (2009)	P	24	–	24	–	–	–		P1761
10	Tsu	Kumozu R.	Katadahaseba, Tsu, Mie (2005)	P	11	–	11	–	–	–	4. Tsu[b]	P537
11	Matsusaka	Kushida R.	Awaso, Matsusaka, Mie (2005)	P	12	–	12	–	–	–	2. Matsusaka1[b]	P535
12	Watarai	Miya R.	Watarai, Mie (2005)	P	16	–	16	–	–	–	1. Watarai[b]	P534
Kinki												
13	Eigenji	Echi R., L. Biwa	Eigenji, Shiga (2008)	P	24	–	–	24	–	–		P1762
14	Hino	Hino R., L. Biwa	Hino, Shiga (2008)	P	37	–	–	37	–	–		P1763
15	Minakuchi	Yasu R., L. Biwa	Minakuchi, Shiga (2008)	P	23	–	–	23	–	–		P1764
16	Ikoma	Yamato R.	Ikoma, Nara (2003)	P	2	–	–	2	–	–	11. Ikoma[b]	P544
17	Taishi	Yamato R.	Taishi, Osaka (2009)	P	8	–	–	8	–	–		P1765
18	Sakai	Yamato R.	Sakai, Osaka (2010)	P	8	–	–	8	–	–		P1766

19	Nishinomiya	Muko R.	Nishinomiya, Hyogo (2007)	P	16	–	–	16	–	–		P1767
20	Sanda_1	Muko R.	Kashita, Sanda, Hyogo (2005, 2007)	P	16	–	–	16	–	–		P1768
21	Sanda_2	Muko R.	Aino, Sanda, Hyogo (2008, 2009)	P	16	–	–	8	8	–		P1769
22	Hasetani	Akashi R.	Hasetani, Kobe, Hyogo (2007, 2009)	P	15	–	–	–	15	–		P1770
23	Miki_1	Kako R.	Kuchiyokawa, Miki, Hyogo (2009)	P	13	–	–	9	4	–		P1771
24	Kasai	Kako R.	Shimoakuta, Kasai, Hyogo (2009)	P	5	–	–	–	5	–		P1772
Sanyo												
25	Okayama	Asahi R.	Higashi-ku, Okayama, Okayama (2009, 2010)	C	44	–	–	–	44	–		P1773
26	Kurashiki	Kurashiki R.	Minami-ku, Okayama, and Kurashiki, Okayama (2009)	C	39	–	–	–	39	–		P1774
Shikoku												
27	Naruto	Yoshino R.	Otsu, Naruto, Tokushima (2011)	C	14	–	–	14	–	–	c	P1775
28	Sanuki	Kabe R.	Sanuki, Kagawa (2010)	P	8	–	–	–	8	–		P1776
29	Higashikagawa	Yoshino R.	Higashikagawa, Kagawa (2010)	P	6	–	–	–	6	–		P1777
30	Miki_2	Shin R.	Miki, Kagawa (2010)	P	8	–	–	–	8	–		P1778
31	Takamatsu	Kasuga R.	Takamatsu, Kagawa (2010)	P	15	–	–	–	15	–		P1779
Kyushu						–	–	–	–	–		
32	Takada	Yabe R.	Takada, Miyama, Fukuoka (2011)	C	8	–	–	–	–	8		P1780
33	Kase	Kase R.	Kase, Saga, Saga (2008)	C	7	–	–	–	–	7		P1781
34	Taku	Rokkaku R.	Nouso, Taku, Saga (2011)	C	8	–	–	–	–	8		P1782
35	Kashima	Shiota R.	Kashima, Saga (2011)	C	6	–	–	–	–	6		P1783

Habitat type: *P* pond, *C* creek

[a] Watanabe et al. (2009)

[b] Watanabe and Mori (2008)

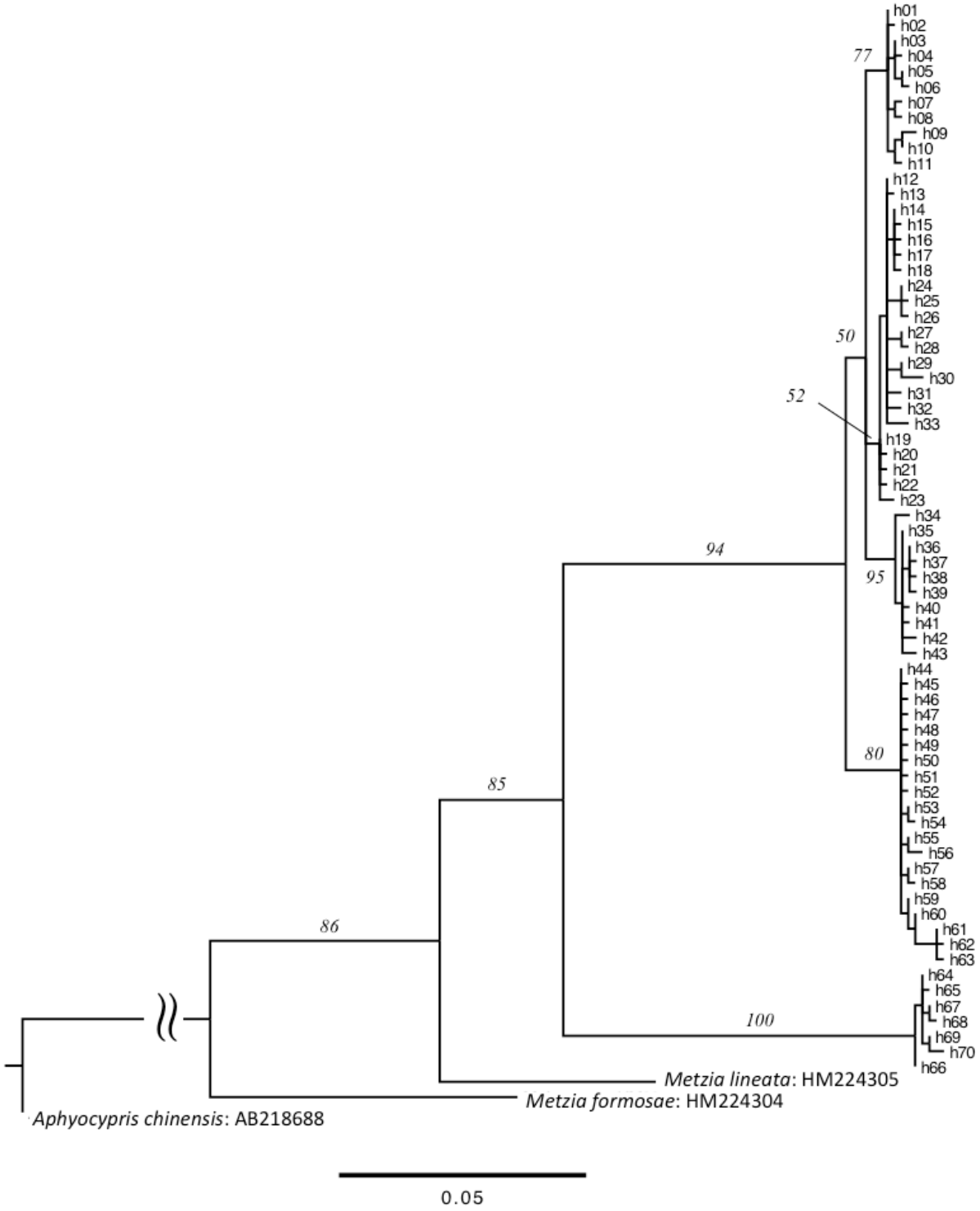
[c] Collected from a captive population founded in 2007

Table 2 Genetic distances among the major clades of *Hemigrammocypris rasborella* with the outgroup species

	A	A1	A2	A3	A4	B	*M. lineata*	*M. formosae*	*A. chinensis*
A	–	–	–	–	–	0.163 ± 0.007	0.151 ± 0.004	0.234 ± 0.007	0.370 ± 0.011
A1	–	–	0.013 ± 0.002	0.017 ± 0.002	0.025 ± 0.002	0.157 ± 0.005	0.150 ± 0.004	0.234 ± 0.006	0.368 ± 0.005
A2	–	0.012 ± 0.002	–	0.016 ± 0.003	0.026 ± 0.003	0.159 ± 0.004	0.151 ± 0.004	0.238 ± 0.004	0.363 ± 0.009
A3	–	0.016 ± 0.001	0.016 ± 0.002	–	0.029 ± 0.002	0.169 ± 0.003	0.147 ± 0.003	0.240 ± 0.005	0.363 ± 0.007
A4	–	0.022 ± 0.002	0.023 ± 0.002	0.026 ± 0.002	–	0.169 ± 0.004	0.154 ± 0.002	0.227 ± 0.004	0.382 ± 0.006
B	0.101 ± 0.003	0.097 ± 0.002	0.099 ± 0.002	0.104 ± 0.001	0.104 ± 0.002	–	0.165 ± 0.003	0.229 ± 0.004	0.380 ± 0.011
Metzia lineata	0.095 ± 0.002	0.093 ± 0.002	0.095 ± 0.002	0.093 ± 0.001	0.096 ± 0.001	0.100 ± 0.001	–	0.164 ± –	0.325 ± –
Metzia formosae	0.126 ± 0.002	0.123 ± 0.002	0.127 ± 0.001	0.128 ± 0.001	0.124 ± 0.001	0.123 ± 0.001	0.099 ± –	–	0.325 ± –
Aphyocypris chinensis	0.157 ± 0.003	0.155 ± 0.001	0.156 ± 0.002	0.156 ± 0.001	0.161 ± 0.001	0.156 ± 0.002	0.145 ± –	0.149 ± –	–

Lower diagonal uncorrected *p* distance; *upper diagonal* HKY+G distance

Data shown as average ± SD



ESM Fig. S1 The maximum likelihood tree of *Hemigrammocypris rasborella* with outgroups based on the mtDNA cytochrome *b* sequences (690 bp) with the HKY+G model. The *numbers at nodes* indicate ML bootstrap probabilities

ESM Table S1 Haplotype frequency and genetic diversity of the local samples of *Hemigrammocypris rasborella*

Population code			Clade							
		n	A-1	A-2	A-3	A-4	B	*k*	*h*	π
Shizuoka										
1	Fujieda[a, c]	15	h05(7), h06(3), h10(3), h11(2)					4	0.7333	0.003423
2	Iwata[a]	15	h01(6), h03(1), h04(1), h07(2), h08(1), h09(4)					6	0.7905	0.002816
3	Hamakita[a]	13	h02(12), h07(1)					3	0.1538	0.000669
Ise Bay										
4	Shinshiro[b]	8		h12(8)				1	0.0000	0.000000
5	Okazaki[b]	10		h12(2), h13(1), h14(2), h19(2), h33(3)				5	0.8667	0.003349
6	Shippo[b]	17		h14(11), h15(1), h16(1), h17(1), h18(1), h20(1), h22(1)				7	0.5956	0.001662
7	Ogaki[b]	15		h14(1), h19(10), h21(1), h23(3)				4	0.5429	0.001573
8	Inabe[b]	7		h29(4), h30(3)				2	0.5714	0.002484
9	Kameyama[a]	24		h31(24)				1	0.0000	0.000000
10	Tsu[b]	11		h12(5), h32(6)				2	0.5455	0.001581
11	Matsusaka[b]	12		h27(7), h28(5)				2	0.5303	0.000769
12	Watarai[b]	16		h24(13), h25(1), h26(2)				3	0.3417	0.000519
Kinki										
13	Eigenji	24			h35(17), h41(7)			2	0.4312	0.000625
14	Hino	37			h35(23), h39(1), h42(13)			3	0.5030	0.001515

15	Minakuchi	23	h35(9), h36(10), h40(4)			3	0.6561	0.001180
16	Ikoma[b]	2	h34(2)			1	0.0000	0.000000
17	Taishi	8	h36(8)			1	0.0000	0.000000
18	Sakai	8	h36(8)			1	0.0000	0.000000
19	Nishinomiya	16	h36(10), h37(6)			2	0.5000	0.000725
20	Sanda_1	16	h36(16)			1	0.0000	0.000000
21	Sanda_2	16	h36(8)	h44(8)		2	0.5333	0.012367
22	Hasetani	15		h48(2), h54(13)		2	0.2476	0.001077
23	Miki_1	13	h38(9)	h53(4)		2	0.4615	0.012040
24	Kasai	5		h44(5)		1	0.0000	0.000000
Sanyo								
25	Okayama	44		h44(30), h45(2), h47(1), h50(3), h57(2), h58(2), h59(4)		7	0.5275	0.001002
26	Kurashiki	39		h44(26), h46(3), h49(1), h51(1), h52(2), h57(3), h59(2), h60(1)		8	0.5506	0.001001
Shikoku								
27	Naruto	14	h43(14)			1	0.0000	0.000000
28	Sanuki	8		h55(8)		1	0.0000	0.000000
29	Higashikagawa	6		h56(6)		1	0.0000	0.000000
30	Miki_2	8		h61(8)		1	0.0000	0.000000
31	Takamatsu	15		h61(6), h62(4), h63(5)		3	0.7048	0.001297
Kyushu								
32	Takada	8			h64(4), h66(1), h67(2), h68(1)	4	0.7500	0.001501
33	Kase	7			h67(7)	1	0.0000	0.000000
34	Taku	8			h64(5), h65(1), h67(2)	3	0.6071	0.000983

35	Kashima	6	h64(1), h69(3), h70(2)	3	0.7333	0.002029	

[a] Data from Watanabe K, Kanagawa N, Kakioka R, Itai T, Mori S (2009) Genetic diversity and conservation units in wild and captive populations of endangered freshwater fishes: a case of *Hemigrammocypris rasborella* in Shizuoka, Japan. Ichthyol Res 56:411–416

[a] Data from Watanabe K, Mori S (2008) Comparison of genetic population structure between two cyprinids, *Hemigrammocypris rasborella* and *Pseudorasbora pumila* subsp., in the Ise Bay basin, central Honshu, Japan. Ichthyol Res 55:309–320

[c] Haplotype frequency is partly corrected from that in Watanabe et al. (2009)

DDBJ/EMBL/GenBank accession nos. of haplotypes: *h01* AB469829, *h02* AB469830, *h03* AB469838, *h04* AB469839, *h05* AB469840, *h06* AB469841, *h07* AB469831, *h08* AB469832, *h09* AB469833, *h10* AB469836, *h11* AB469835, *h12* AB354663, *h13* AB354664, *h14* AB354670, *h15* AB354669, *h16* AB354667, *h17* AB354666, *h18* AB354668, *h19* AB354657, *h20* AB354659, *h21* AB354658, *h22* AB354660, *h23* AB354662, *h24* AB354673, *h25* AB354675, *h26* AB354674, *h27* AB354676, *h28* AB354677, *h29* AB354678, *h30* AB354679, *h31* AB469843, *h32* AB354671, *h33* AB354672, *h34* AB354680, *h35* AB907301, *h36* AB907302, *h37* AB907303, *h38* AB907304, *h39* AB907305, *h40* AB907306, *h41* AB907307, *h42* AB907308, *h43* AB907309, *h44* AB907310, *h45* AB907311, *h46* AB907312, *h47* AB907313, *h48* AB907314, *h49* AB907315, *h50* AB907316, *h51* AB907317, *h52* AB907318, *h53* AB907319, *h54* AB907320, *h55* AB907321, *h56* AB907322, *h57* AB907323, *h58* AB907324, *h59* AB907325, *h60* AB907326, *h61* AB907327, *h62* AB907328, *h63* AB907329, *h64* AB907330, *h65* AB907331, *h66* AB907332, *h67* AB907333, *h68* AB907334, *h69* AB907335, *h70* AB907336