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ORIGINAL PAPER

Worldwide molecular phylogeny of common estuarine polychaetes of the genus *Hediste* (Annelida: Nereididae), with special reference to interspecific common haplotypes found in southern Japan

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Abstract

The nucleotide sequences of two mitochondrial genes (16S rDNA and COI) were compared among all species of *Hediste*, including five nominal and two cryptic species (*H. atoka* and *H. diversicolor* both consisting of two cryptic species, *H. diadroma*, *H. japonica*, and *H. limnicola*), as well as a newly found undescribed species (*H.* sp.), to estimate their phylogenetic relationships. The analysis using 16S rDNA sequence supported the monophyly of all five nominal species and *H.* sp., with no detection of the genetic differentiation between the two cryptic species in both *H. atoka* and *H. diversicolor*. However, analysis using COI sequence detected a marked differentiation between the cryptic species, and indicated that the two forms of *H. atoka* were separated into distinct clades; form A was included in a clade together with *H. diversicolor*, *H. limnicola*, and *H.* sp., whereas form B was included in another clade together with *H. diadroma*. Based on the topology of our phylogenetic analysis using the combined data set of 16S rDNA and COI, a hypothesis on the evolutionary history of the worldwide speciation in *Hediste* is proposed. This hypothesis seems to correspond well with the geographical distributions of current species and their morphological differentiation, supporting the previous hypothesis that the unique epitokous swarming and planktic larval development evolved independently in *H. diadroma* and *H. japonica* in eastern Asia. We also show that no or few interspecific substitutions have occurred in sequences of nuclear DNA (18S rDNA, 28S rDNA, and histone H3) in *Hediste*.

Keywords 16S rDNA · COI · Cryptic speciation · Molecular systematics · Nuclear DNA

Introduction

Estuarine polychaetes of the genus *Hediste* differ from the closely related genus *Neanthes* in Nereididae by the presence of simple chaetae derived from falconers in the posterior neuropodia (Fong and Garthwaite 1994; Khlebovich 1996; Sato and Nakashima 2003). The *Hediste* species show circumboreal distributions in the North Temperate Zone of both the Pacific and Atlantic coasts, and consist of the following five nominal species (Sato 1999, 2004, 2017): *H. diversicolor* (O. F. Müller, 1776) distributed along both the Northeast and Northwest Atlantic (Smith 1977), *H. limnicola*

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(Johnson, 1903) along the Northeast Pacific (Smith 1958), and three species in Asia along the Northwest Pacific, i.e., *H. japonica* (Izuka, 1908), *H. diadroma* Sato and Nakashima, 2003, and *H. atoka* Sato and Nakashima, 2003. Recently, the invasion of the Asian species, *H. diadroma*, along the North American Pacific coasts was discovered (Nishizawa et al. 2014; Tosuji and Furota 2016). These five species are morphologically very similar to one another. In particular, the three Pacific species, *H. diadroma*, *H. atoka*, and *H. limnicola* are morphologically indistinguishable in sexually immature specimens, whereas *H. diversicolor* and *H. japonica* are distinguishable from all other species, even in immature specimens, by their unique chaetal morphology in the neuropodial infra-acicular fascicle: the absence of homogomph spinigers and the presence of homogomph falcigers, respectively (Smith 1958; Sato 2004).

On the other hand, the reproductive and developmental characteristics of the five species of *Hediste* are markedly diverse (Table 1), showing two contrasting life histories (catadromous and estuary-resident forms) (Sato 2017). The life cycle of the catadromous form is characterized by migration between adult habitats with low salinity and larval habitats with high salinity, with species-specific epitokous metamorphosis in combination with reproductive swarming in adults, and a true planktic larval phase in early development. The typical and non-typical catadromous forms of life cycle are adopted by only two Asian species, *H. diadroma* and *H. japonica*, respectively; their epitokous metamorphoses are markedly different from those into the typical heteronereis form prevailing in many marine nereidids (Sato 2017). Another life cycle of the estuary-resident form is characterized by the completion of life cycle within low-salinity regions, without epitokous metamorphosis and reproductive swarming in adults, lacking a true planktic larval phase in early development. This form is widespread in both Pacific (*H. atoka* and *H. limnicola*) and Atlantic species (*H. diversicolor*); *H. limnicola*, which inhabits not only estuaries but also freshwaters, has very specialized reproductive characteristics such as hermaphroditism, self-fertilization, and viviparity (Smith 1950).

Higher interpopulational genetic differentiation is expected in dioecious species with an estuary-resident life cycle, i.e., *H. atoka* and *H. diversicolor*. In fact, this hypothesis has been supported by an electrophoretic study on allozymes of Japanese populations of *H. atoka* in comparison with those of *H. diadroma* (Sato and Masuda 1997). Moreover, a recent analysis of the mitochondrial cytochrome oxidase subunit I (COI) DNA sequence suggested cryptic speciation into two parapatric forms had occurred in populations of *H. atoka* without morphological differentiation: form A constituted all Korean and most Japanese populations except for those in southern Japan, occupied by form B (Tosuji and Sato 2010). A similar degree of interpopulational genetic differentiation has been also revealed in the Atlantic species *H. diversicolor*, in which the existence of two cryptic species (species A and B) was suggested, by the analyses of allozymes and mitochondrial genes (Hateley et al. 1992; Fong and Garthwaite 1994; Abbiati and Maltagliati 1996; Scaps 2002; Breton et al. 2003; Virgilio and Abbiati 2006; Audzijonyte et al. 2008; Virgilio et al. 2009).

The first study on the phylogenetic relationships among the species of *Hediste* was carried out by an allozyme electrophoretic analysis, comparing *H. diversicolor*, *H. limnicola*, and *H. atoka* (as *H. japonica*), showing that *H. atoka* appears more closely related to *H. limnicola* than it is to *H. diversicolor* (Fong and Garthwaite 1994). Thereafter, Sato (1999) and Sato and Nakashima (2003) compared the atokous and epitokous morphology of three Japanese species (*H. japonica*, *H. diadroma*, and *H. atoka*) and proposed the hypothesis that an *H. atoka*-like species with an estuary-resident life cycle is the ancestral form, from which *H. japonica* and *H. diadroma*, with the unique epitoky and catadromous life cycle, were derived independently of each other (see also Sato 2017).

In the present study, we evaluate the phylogenetic relationships between all species of *Hediste* in the world, based on the nucleotide sequence of parts of two mitochondrial DNA sequences (16S rDNA and COI). We also sequenced three nuclear genes (18S rDNA, 28S rDNA, and histone H3) and show that these molecular markers are generally too conserved to be informative for resolving evolutionary relationships among species of *Hediste*.

Materials and methods

Collection of specimens

Specimens of *Hediste* were collected from intertidal flats in estuaries in East Asia (Japan and Korea), North America (USA), and Europe (Denmark, Finland, France, Italy, Germany, Norway, Portugal, and Great Britain) by excavating worms at low tide, fixing the specimens in 75–99% ethanol. These were then used for DNA isolation. As an outgroup species, specimens of *Neanthes* cf. *glandicincta* were collected from estuarine mudflats in Kagoshima Bay in southern Japan. Detailed collection data for all materials (256 specimens and one outgroup specimen) are shown in Online Resource 1. Specimens preserved in ethanol were soaked for 30 min in a phosphate-buffered saline (PBS) prior to DNA extraction. Total DNA was extracted using a DNeasy Tissue kit (Qiagen) or Wizard Genomic DNA Purification Kit (Promega). We used a 1–5-mm middle section from each worm for these extractions.

Table 1 Comparison of reproductive, developmental, and chromosomal characteristics of five *Hediste* species modified from Nishizawa et al. (2014) and Sato (2017)

Geographic range	Geographic range and species (diploid chromosome number)	Reproductive mode	Epitoke-specific chaetae	Egg diameter (μm)	Salinity favorable for early development	Remarks on early development	References
Eastern Asia	*H. japonica* (28)	Swarming	Absent	180–210	22–30	10-day planktic trochophore/nectochaeta1	Izuka (1908); Sato and Nakashima, (2003) Tosuji et al. (2004); Tosuji and Sato (2006)
	H. diadroma (28)	Swarming	Present	130–170	22–30	1-month planktic trochophore/nectochaeta	Kagawa (1955); Sato and Tsuchiya (1987), (1991) Sato and Ikeda (1992); Sato and Nakashima (2003)
	H. atoka (28)	Female spawns in burrow	Absent	200–250	9–21	No planktic larval life, hatching out at 3-chaetiger juvenile	Sato and Tsuchiya (1987), (1991) Sato and Ikeda (1992); Sato (2004)
North American Pacific coast	*H. limnicola* (26)	Hermaphrodite, self-fertilization	Absent	120–170* 210*	15–20	Viviparity, emerging from parent coelomat 15 to 30-chaetiger juvenile	Johnson (1903); Smith (1950); Fong and Pearse (1992); Khlebovich 1996; Tosuji et al. 2010
North Atlantic coasts	*H. diversicolor* (28)	Female spawns, broods embryos in burrow	Absent	200–250	5–27	10-week demersal trochophore/nectochaeta	Müller (1776); Dales (1950); Smith (1964); Christensen (1980); Bartels-Hardege and Zeeck (1990); Scaps (2002)

*Fong and Pearse (1992) reported the egg diameter as 210 μm, although Smith (1950) reported the egg diameter as 120–170 μm

For identification of sexually immature specimens of three Pacific species (*H. atoka*, *H. diadroma*, and *H. limnicola*), which are morphologically indistinguishable, we used the PCR-RFLP method (Tosuji and Sato 2012) and the species-specific multiplex PCR method (Tosuji and Sato 2008).

Primer design, experimental condition, and SNP genotyping for Hediste atoka

The tetra-primer ARMS–PCR procedure (Newton et al. 1989; Ye et al. 2001) was used to detect the genotype of the varieties of *H. atoka* (forms A and B) at an SNP locus using COI gene sequences (Table 2 and Fig. 1). Primers were designed using the primer design computer program Primer1 (Collins and Ke 2012) and slightly modified. The reaction mixture (5 μL) consisted of 0.125 U *Taq* DNA polymerase (BioAcademia, Japan), 0.5 μL 10× reaction buffer, 0.4 μL 2.5 mM dNTP mixture, 0.35 μL primers (Table 2), and 0.1–0.5 ng template DNA with/without 0.5 M betaine solution (Sigma-Aldrich). The mixing ratio of the primers was as follows: Fi:Ri1:Ri2:299–325FO1:299–325FO2:590-567RO1:590–567RO2:590–567RO3:590–567RO4 = 40:64:16:4:2:6:6:3:3. The cycling program was as follows: initial denaturation at 95 °C for 2 min, followed by 37 cycles of 95 °C for 1 min, 56.5 °C for 1 min, 72 °C for 1 min, and 72 °C for 2 min. An aliquot of the PCR product was separated by a 5% polyacrylamide gel using TAE buffer and detected by staining with SYBR Gold nucleic acid gel stain (Molecular Probes). The alleles were detected on the basis of the estimated amplicon sizes, 112 bp (form A), 221 bp (form B), and 291 bp (outer amplicon) (Fig. 2).

DNA sequencing

The 16S rDNA fragments were amplified using the primers 16SarL and 16SbrH (Palumbi 1996) or with 12412F primer (present study) combined with 16SbrH primer. The COI gene was amplified either using the LCO1490 and HCO2198 primers (Folmer et al. 1994) or with the LCO1490 primer combined with HCO709 primer (Blank et al. 2008). The amplifications were performed using an Ex *Taq* (Takara Bio, Japan). The cycling profile was as follows: initial denaturation at 95 °C for 2 min; 38 cycles at 95 °C for 30 s, then at 45 °C for 45 s and at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. All products were verified on a 1% agarose gel and purified with the Plus Gel Elution Kit (GMbiolab). The cycle sequencing reaction was carried out using BigDye 3.1 (Applied Biosystems) as a direct sequencing method with the amplification primers or sequencing primers (12415SF and 12897SR for 16S rDNA, 17SL and 733SH for COI) (present study) and all sequences were checked and corrected by visual inspection. The nuclear 18S rDNA was amplified using the 18SF35 and 18SR1779 primers (Struck et al. 2002). The amplifications were performed using an Ex *Taq*. The cycling profile was as follows: initial denaturation at 95 °C for 2 min; 38 cycles at 95 °C for 30 s, then at 45 °C for 30 s and at 72 °C for 2 min, with a final extension step at 72 °C for 7 min. The amplicons were verified on a 1% agarose gel and purified with the Plus Gel Elution Kit. Then, they were sequenced using the direct sequencing method with 18SF35, 18F509, 18F997, 18R925, 18R1256, and 18SR1779 primers (Struck et al. 2002) and all sequences were checked and corrected by visual inspection. The nuclear 28S rDNA (D1, D4-7b, and D9-10 regions) and histone H3 were amplified and sequenced as same as the method for 18s rDNA using the following primers; 28SD1F/28SD1R (Brown et al. 1999), 28Srd4.8a/28Srd7b1 (Whiting 2002), 28SD9–10F/28SD9–10R (Hills and Dixon 1991) and H3F/H3R(1) (Colgan et al. 2000). The primers used in this study are displayed in Table 2.

The base-called data obtained from an ABI DNA sequencer were assembled to get contig sequences using the sequence analysis software Genetyx-Mac ver. 19 for manual editing.

The nucleotide sequences were deposited in DDBJ (accession numbers LC323003–LC323104, LC323646–LC323647, LC378710–LC378715, LC380654–LC380663, LC381232–LC381234, and LC381864–LC381865).

Sequence alignment and data analysis

All sequences were aligned using the multiple sequence alignment software MAFFT ver. 7.310 (Katoh and Standley 2013) with L-INS-i method. When samples had the same sequence, the duplicate sequences were removed.

The best-fitting model of nucleotide substitution for a maximum likelihood (ML) tree was selected by ModelFinder Plus (Kalyaanamoorthy et al. 2017) using Akaike information criterion (AIC) (Akaike 1973). This is general time reversible model (GTR) (Tavaré 1986) using a free rate model with two categories (+R2) (Yang 1995; Soubrier et al. 2012) that generalizes the +G model by relaxing the assumption of gamma-distributed rates for the 16S rDNA. The model selected was TPM2 using a discrete gamma distribution (+G) and by assuming that a certain fraction of sites were evolutionarily invariable (+I) for COI.

MrModeltest v. 2.3 (Posada and Crandall 1998) was used to select Bayesian inference (BI) tree. The best substitution models were GTR +G +I, for 16S rDNA and COI genes as determined by AIC and hierarchical likelihood ratio tests (hLRTs). Phylogenetic inference was conducted for sequences of 16S rDNA and COI individually, and this data was combined to create a concatenated data set. Each of the protein-coding and non-coding nucleotide sequence regions was used as separate partitions.

IQ-tree 1.5.4 (Nguyen et al. 2015) was used to obtain phylogenetic trees constructed with ML supported with 1000 bootstrap. MrBayes v. 3.1.6 (Ronquist et al. 2012) was used

Table 2 List of primers used in this study

	Target	Name	Sequence	Reference
Amplification primer	16S			
		16SarL	CGCCTGTTTATCAAAAACAT	Palumbi (1996)
		16SbrH	CCGGTCTGAACTCAGATCACGT	Palumbi (1996)
		12412F	CAAAAACATCGCCTGTTG	Present study**
	COI			
		LCOI1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
		HCOI2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
		HCO709	AATWAGAATRTAKACTTCWGGGTG	Blank et al. (2008)
	18S			
		18SF35	TCTCAAAGATTAAGCCATGCA	Struck et al. (2002)
		18SR1779	TGTTACGACTTTTACTTCCTCTA	Struck et al. (2002)
	28S D1			
		28SD1F	ACCCSCTGAAYTTAAGCAT	Brown et al. (1999)
		28SD1R	AACTCTCTCMTTCARAGTTC	Brown et al. (1999)
	28S D4-7b			
		28Srd4.8a	ACCTATTCTCAAACTTTAAATGG	Whiting (2002)
		28Srd7b1	GACTTCCCTTACCTACAT	Whiting (2002)
	28S D9-10			
		28SD9-10F	AAGGTAGCCAAATGCCTCATC	Hillis and Dixon (1991)
		28SD9-10R	GTGAATTCTGCTTCATCAATGTAGGA AGAGCC	Hillis and Dixon (1991)
	Histone H3			
		H3F	ATGGCTCGTACCAAGCAGAC	Colgan et al. (2000)
		H3R (1)	ATATCCTTRGGCATRATRGTGAC	Colgan et al. (2000)
Sequence primer*	16S			
		12415SF	AAACAYCGCCTRTTGAAC	Present study**
		12897SR	CATGTAGTGATTTAATGGTTG	Present study**
	COI			
		17SL	CAACMAATCATAAAGATATTKGTA	Present study**
		733SH	GRTGWCCAAAAAATCAGAA	Present study**
	18S			
		18SF35	TCTCAAAGATTAAGCCATGCA	Struck et al. (2002)
		18SF509	CCCCGTAATTGGAATGAGTACA	Struck et al. (2002)
		18SF997	TTCGAAGACGATCAGATACCG	Struck et al. (2002)
		18SR925	GATCCAAGAATTTCACCTCT	Struck et al. (2002)
		18SR1256	AGCTCTCAATCTGTCAATCCT	Struck et al. (2002)
		18SR1779	TGTTACGACTTTTACTTCCTCTA	Struck et al. (2002)
ARMS primer	*H. atoka*			
		Fi	CCCYTAGCAAGAAATATTGCTAAC	Present study
		Ri1	RRTCTACTGATGGTCCRTCA	Present study
		Ri2	TCCACTGATGGTCCRTCA	Present study
		299-325FO1	ATCCCTWACTCTTCTTTTATCAAGTGC	Present study
		299-325FO2	ATCGTTAACTCTTCTTTTATCAAGTGC	Present study
		590-567RO1	TCCRGCTAGAACTGGTAAGCTAAG	Present study
		590-567RO2	YCCAGCTAATACTGGTAAACTAAG	Present study
		590-567RO3	CCCAGCTAGTACTGGTAAGCTAAG	Present study
		590-567RO4	TCCAGCTAATACTGGTAAGCTAAG	Present study

*Sequence primers for 28S D1, 28S D9-10, and histone H3 were same as the amplification primers

**Based on the sequence of *Perinereis nuntia* (JX644015 and NC_020609)

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form A        1 TACYTTATATTTTATCTTCGGAATRTGATCYGGYCTTCTAGGAACATCAATAAGTCTTTT    60
form B        1 TACTTTATATTTTATCTWCGGDATATGATCMGGYCTTYTAGGAACATCAATAAGYCTTYT    60

form A       61 AATTCGAGCTGAATTAGGACARCCTGGNTCCYTAYTAGGAAGAGATCAACTTTATAAYAC   120
form B       61 AATTCGAGCTGAATTAGGACARCCTGGRTCCYTAYTAGGMAGWGATCAACTTTATAACAC   120

form A      121 AATTGTTACCGCACAYGCATTTTTAATAATTTTYTTYCTAGTAATACCAGTAATAATTGG   180
form B      121 RATTGTTACYGCACATGCATTCTTAATAATTTTCTTTCTAGTAATACCAGTAATAATTGG   180

form A      181 AGGATTYGGAAAYTGACTTGTHCCTYTAATAYTRGGRGCCCCTGATATAGCATTYCCTCG   240
form B      181 RGGATTCGGTAACTGACTTGTWCCTTTAATAYTAGGRGCCCCTGATATAGCWTTTCCWCG   240
                                                 ------------------------->
form A      241 TCTTAAYAAYATAAGATTYTGRYTHCTTCCTCCATCCCTTACTCTTCTTTTATCAAGTGC   300
form B      241 TCTTAACAATATAAGATTTTGRCTWCTTCCDCCATCSYTAACTCTTCTTTTATCAAGTGC   300

form A      301 AGCTGTTGAAAAAGGTGTYGGCACWGGATGRACHGTRTAYCCRCCYTTAGCHAGAAATAT   360
form B      301 AGCTGTTGAAAAAGGAGTCGGYACWGGATGRACAGTRTAYCCRCCCYTAGCAAGAAATAT   360
                      <-------------------              ------------------
form A      361 TGCTCATGCYGGACCATCAGTRGAYYTHGCDATYTTYTCYCTTCATTTAGCAGGAGTDTC   420
form B      361 TGCTCAGGCYGGACCATCAGTRGATCTYGCAATTTTYTCTCTTCACYTAGCAGGWGTATC   420
                ------>
form A      421 RTCWATTWTAGGAGCATTAAAYTTTATYACYACAGTTATYAATATRCGRTCHAAAGGTCT   480
form B      421 ATCDATTATAGGAGCCTTAAACTTTATTACTACAGTTATYAACATGCGGTCCAAAGGKCT   480

form A      481 TCGTCTTGAACGAGTYCCATTATTTGTRTGATCHGTAATAATTACHGCTATTYTWYTHYT   540
form B      481 ACGTCTTGAACGAGTCCCATTATTTGTWTGATCCGTAATAATTACAGCTATTTTAYTACT   540

form A      541 HCTTAGCTTACCAGTTCTAGCYGGAGCRATTACTATACTTCTYACWGATCGWAATTTAAA   600
form B      541 CCTTAGYTTACCAGTAYTAGCTGGRGCRATTACYATACTTCTCACTGACCGAAATTTAAA   600
                 <-----------------------
form A      601 YACTGCATTYTTYGAYCCTGCYGGDGGNGGWGACCCTATTCTTTATCARCAYYTATTC    658
form B      601 CACKGCATTCTTTGACCCTGCKGGTGGAGGAGACCCAATTCTTTATCAACAYTTATTC    658
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Fig. 1 ARMS primers to detect genotype of the varieties of *Hediste atoka* (forms A and B). ARMS outer primer sequences are indicated with gray shade, and inner primer sequences are indicated highlighted with shade (arrow for orientation). The primers were designed from accession numbers AB603842–AB603870 (form A) and AB603871–AB603887 (form B)

to obtain BI of phylogenetic trees. Eight Metropolis-coupled Markov chain Monte Carlo algorithms were run, starting with random initial trees and sampling every 100 generations. The analyses were allowed to continue until the average standard deviation of split frequencies (ASDSF) reached below 0.01. As a result of this task, for 16S rDNA, 1,000,000 (ASDSF = 0.009010), for COI, 11,500,000 (ASDSF = 0.008392), for the combined data, 1,000,000 (ASDSF = 0.006127) generations were obtained. The first 25% of the sampled trees were excluded as burn-in samples, and burn-in value for each analysis was assessed using the software Tracer1.6 (Rambaut et al. 2014).

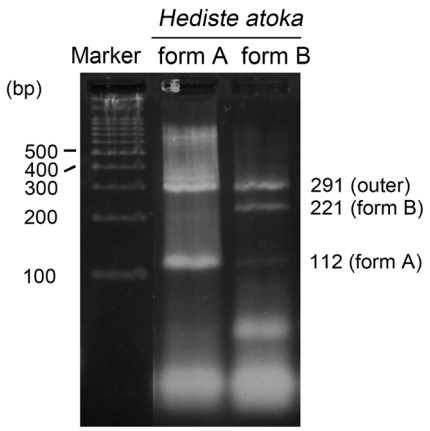

Fig. 2 An example of ARMS–PCR diagnosis of the two forms of *Hediste atoka*. An example of the gel image showing the existence of amplified DNA products in form A (112 bp) and form B (221 bp)

The maximum parsimony (MP) trees were analyzed by PAUP* 4.0 (Swofford 2003) using the heuristic search with 1000 bootstrap replicates. The phylogenetic network was constructed using FigTree ver.1.4.3.

We used Arlequin version 3.512 (Excoffier and Lischer 2010) to calculated pairwise fixation index (F_{ST}) values (Weir and Cockerman 1984) to estimate the level of genetic divergence between populations.

Results

Detection of an unknown cryptic species

In both analyses using 16S rDNA and COI sequences, the specimens collected from the Han River System in western Korea (a total of 13 specimens) constituted a clearly separated clade from any other of the previously known species or forms. Therefore, we designated this clade as *Hediste* sp., which seemed to be an unknown cryptic species.

Phylogenetic analysis using 16S rDNA sequence

The 16S rDNA dataset consisted of 57 nucleotide sequences (haplotypes) from 160 specimens (one of them belonged to the outgroup species) containing 429 characters, of which 322 were conserved across all taxa (75.1%), 99 were variable (23.1%) and 50 were parsimony informative (11.7%). When the outgroup was excluded, the dataset had 344 conserved

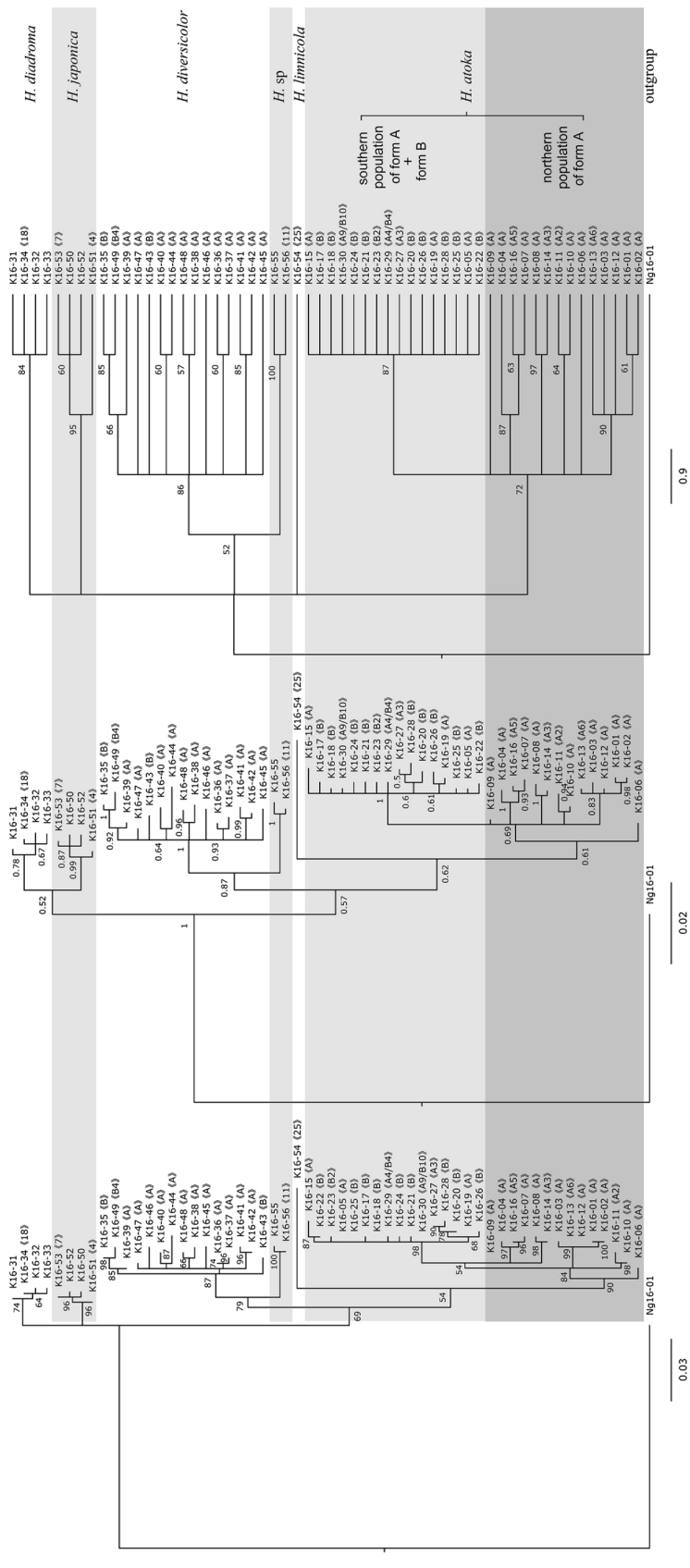

Fig. 3 Maximum likelihood (left), Bayesian inference (middle), and maximum parsimony (right) phylograms for *Hediste* species derived from the analyses of 429-bp fragment of mitochondrial 16S rDNA sequence. *Neanthes* cf. *glandicincta* is used as an outgroup for rooting the tree. In the maximum likelihood and maximum parsimony tree, bootstrap values from 1000 replications are indicated on the branches and only greater than or equal to 50% are shown. In the Bayesian tree, posterior probability values are indicated on the branches and only greater than or equal to 0.5 are shown. The scale bar corresponds to the substitutions per nucleotide site. The letters in brackets (A or B) indicate species A or B for *H. diversicolor*; and form A or B for *H. atoka*, respectively. The numbers in brackets indicate the number of individuals (no number means single individual)

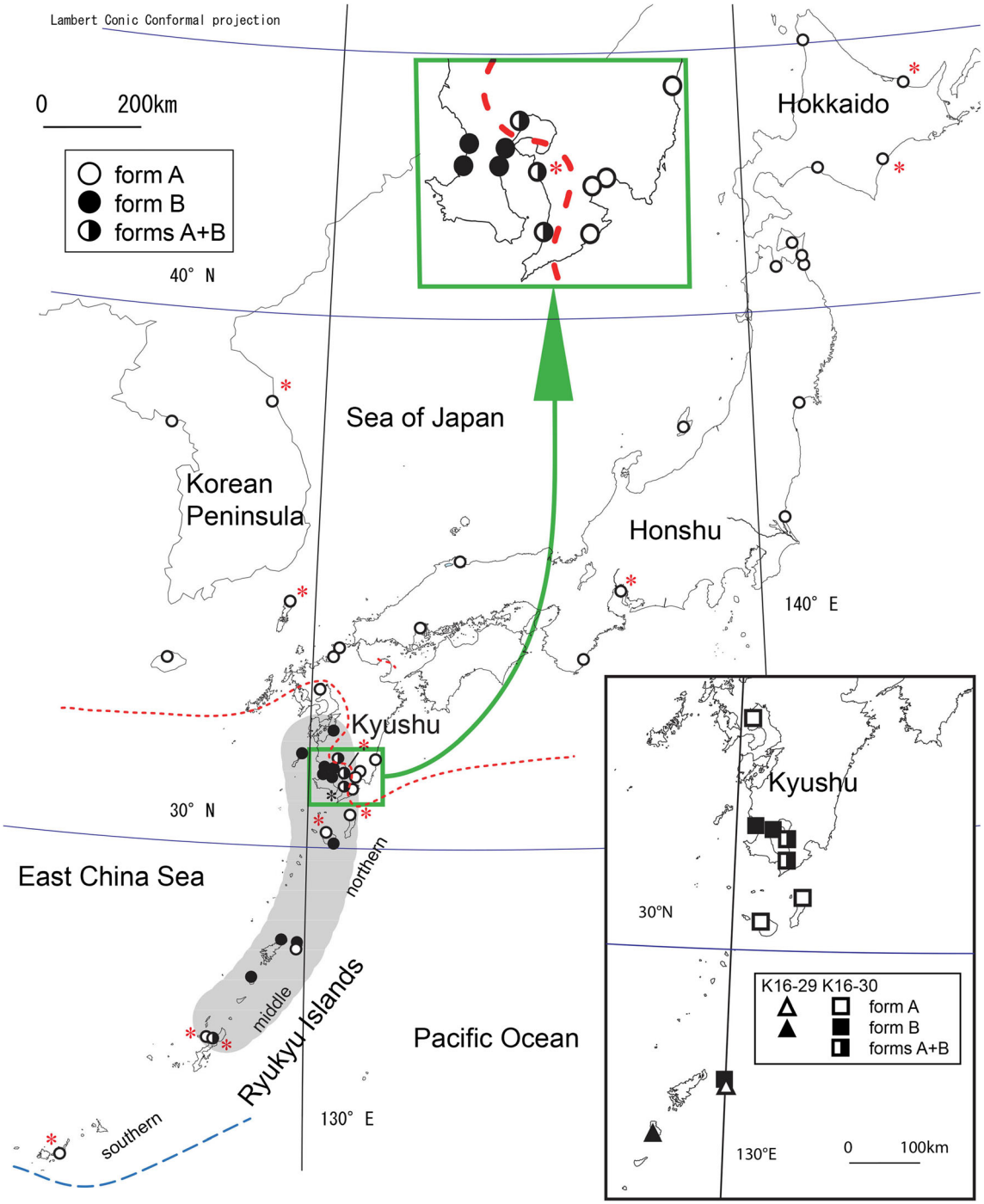

Fig. 4 Distribution of all haplotypes of the forms A and B of *Hediste atoka*. Two haplotypes of the mitochondrial 16S rDNA sequence (K16-29 and K16-30) which were shared by the two forms (inset figure). Form B of *H. atoka* is distributed only in southern Japan (southwestern Kyushu and the northern and middle Ryukyu Islands), whereas form A of this species has a wide distribution covering the whole of Japan (from Hokkaido to the Ryukyu Islands) and Korea. The gray area shows the distributional range of form B of *H. atoka* based on Tosuji and Sato (2010) and the present study. Dotted line indicates the border between the range of the northern and southern populations of form A of *H. atoka*. Dashed line indicates the southern limit of the southern population of form A of *H. atoka*. An asterisk indicates new records of form A of *H. atoka* in the present study

characters (80.2%), 77 variable characters (17.9%), and 47 parsimony informative characters (11.0%).

In all of the ML, BI, and MP trees, haplotypes from each of all six species (*H. atoka*, *H. diadroma*, *H. diversicolor*, *H. japonica*, *H. limnicola*, and *H.* sp.) constituted independent species-specific clades, supporting the monophyly of these species (Fig. 3). The clade of *H.* sp. was consistently recovered sister to *H. diversicolor* including both species A and B.

Table 3 Pairwise F_{ST} values among the *Hediste* species based on the dataset of 16S rDNA sequence (1), COI sequence (2), and both of 16S rDNA and COI sequences (3)

	H. atoka (A)	*H. atoka* (B)	*H. diadroma*	*H. diversicolor* (A)	*H. diversicolor* (B)	*H. japonica*	*H. limnicola*	*H.* sp.
1								
Form A of *H. atoka*	0.00000							
Form B of *H. atoka*	0.23231	0.00000						
H. diadroma	0.73139	0.94413	0.00000					
Species A of *H. diversicolor*	0.64027	0.82387	0.82470	0.00000				
Species B of *H. diversicolor*	0.67484	0.90116	0.94950	0.37637	0.00000			
H. japonica	0.67407	0.92345	0.95231	0.74998	0.90518	0.00000		
H. limnicola	0.83059	0.96928	0.99534	0.91894	0.98091	0.99115	0.00000	
H. sp.	0.73724	0.94692	0.98651	0.77227	0.93003	0.96829	0.99813	0.00000
2								
Form A of *H. atoka*	0.00000							
Form B of *H. atoka*	0.73910	0.00000						
H. diadroma	0.80036	0.11177	0.00000					
Species A of *H. diversicolor*	0.72364	0.82594	0.88913	0.00000				
Species B of *H. diversicolor*	0.73674	0.82946	0.89822	0.63493	0.00000			
H. japonica	0.80601	0.87514	0.91689	0.89067	0.91872	0.00000		
H. limnicola	0.62966	0.84647	0.90444	0.82354	0.85651	0.92664	0.00000	
H. sp.	0.75694	0.89212	0.93212	0.87617	0.92052	0.96919	0.93769	0.00000
3								
Form A of *H. atoka*	0.00000							
Form B of *H. atoka*	0.69496	0.00000						
H. diadroma	0.80091	0.68331	0.00000					
Species A of *H. diversicolor*	0.70503	0.81416	0.86861	0.00000				
Species B of *H. diversicolor*	0.71952	0.84369	0.90144	0.58821	0.00000			
H. japonica	0.70350	0.89438	0.92829	0.83711	0.88405	0.00000		
H. limnicola	0.79575	0.90388	0.92498	0.85675	0.90626	0.93679	0.00000	
H. sp.	0.77316	0.93683	0.95171	0.84814	0.91628	0.95241	0.96770	0.00000

All *P* values are significant sufficiently

The clade of *H. limnicola* was recovered sister to *H. atoka* including both forms A and B, in the ML and BI trees. The clade of *H. diadroma* was recovered sister to *H. japonica* in the BI tree.

In all trees, the large clade of *H. atoka* was subdivided into several groups, which, however, did not support the monophyly of the forms A and B. The following two common haplotypes were shared by the two forms of *H. atoka*: haplotype K16-29 (LC323060) obtained from each of four specimens of the forms A and B; haplotype K16-30 (LC323061) from nine specimens of form A and 10 specimens of form B.

Within *H. atoka*, all 12 haplotypes of form B, including the two common haplotypes, constituted a monophyletic clade, together with four haplotypes specific to form A (haplotype K16-05, K16-15, K16-19, and K16-27), which were all obtained in southern Japan, from western Kyushu to Okinawa-jima Island, including new records from four islands in the Ryukyu Islands (Tanegashima, Yakushima, Okinawa-jima, and Ishigaki-jima); here, the population of form A belonging to this clade is designated as the southern population of form A of *H. atoka*. The common haplotypes shared by the two forms were distributed in western and southern Kyushu and the northern and middle Ryukyu Islands in southern Japan, which is within the range of the southern population of form A, mostly overlapping that of form B. On the other hand, the other 14 haplotypes of form A, which were obtained from a wide area in Japan and Korea, from northern Hokkaido to northern Kyushu, formed a paraphyletic group including six subdivided clades in all of ML, BI, and MP trees; here, this group is designated as the northern population of *H. atoka* (Fig. 4).

The clade of *H. diversicolor* was also subdivided into several groups, which did not support the monophyly of species A and B designated by Audzijonyte et al. (2008).

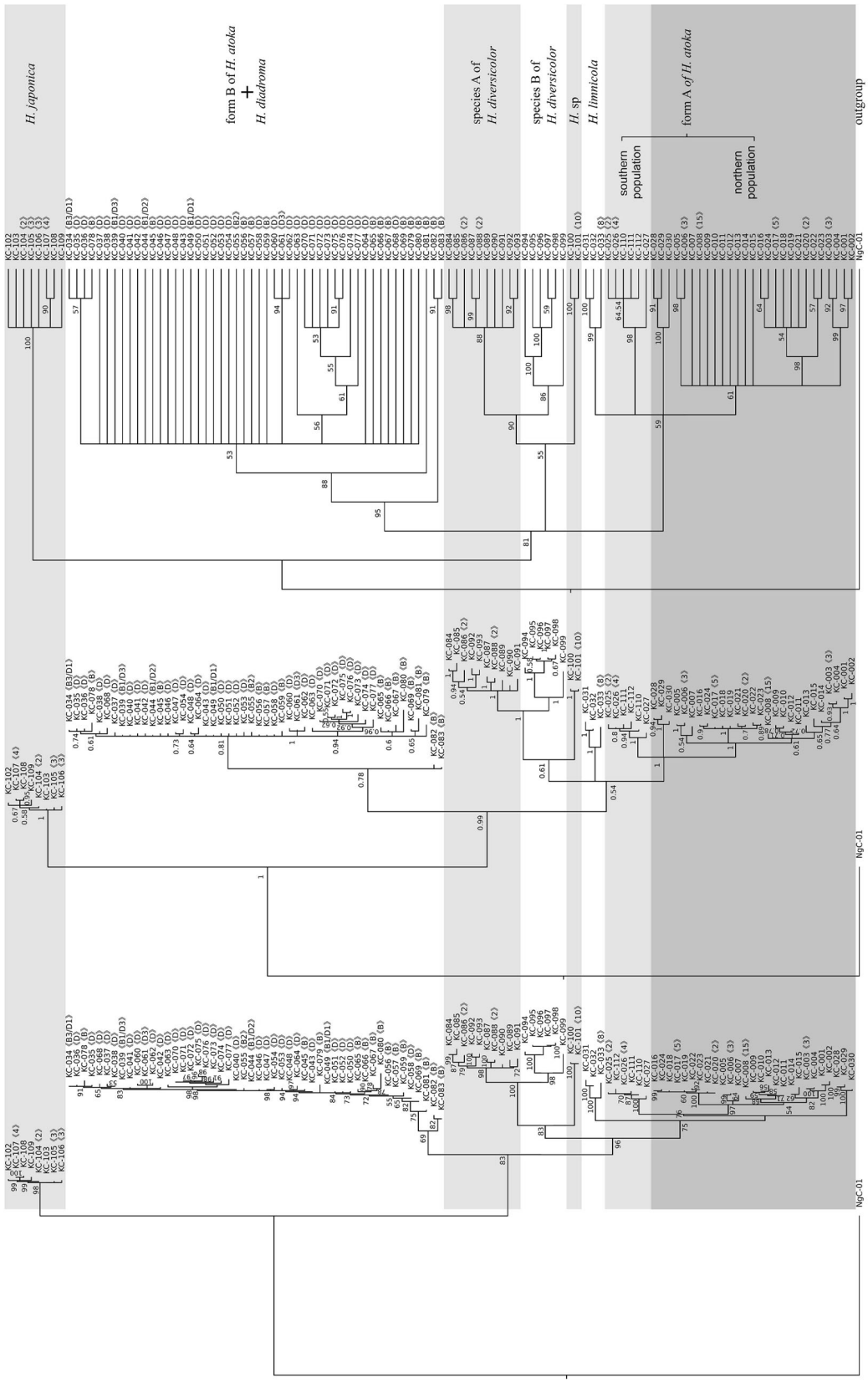

Fig. 5 Maximum likelihood (left), Bayesian inference (middle), and maximum parsimony (right) phylograms for *Hediste* species derived from the analyses of the 570-bp fragment of mitochondrial COI sequence. *Neanthes* cf. *glandicincta* is used as an outgroup for rooting the tree. In the maximum likelihood and maximum parsimony tree, bootstrap values from 1000 replications are indicated on the branches and only greater than or equal to 50% are shown. In the Bayesian tree, posterior probability values are indicated on the branches and only greater than or equal to 0.5 are shown. The scale bar corresponds to the substitutions per nucleotide site. The letters in brackets (B or D) indicate forms B of *H. atoka* or *H. diadroma*, respectively. The numbers in brackets indicate the number of individuals (no number means single individual)

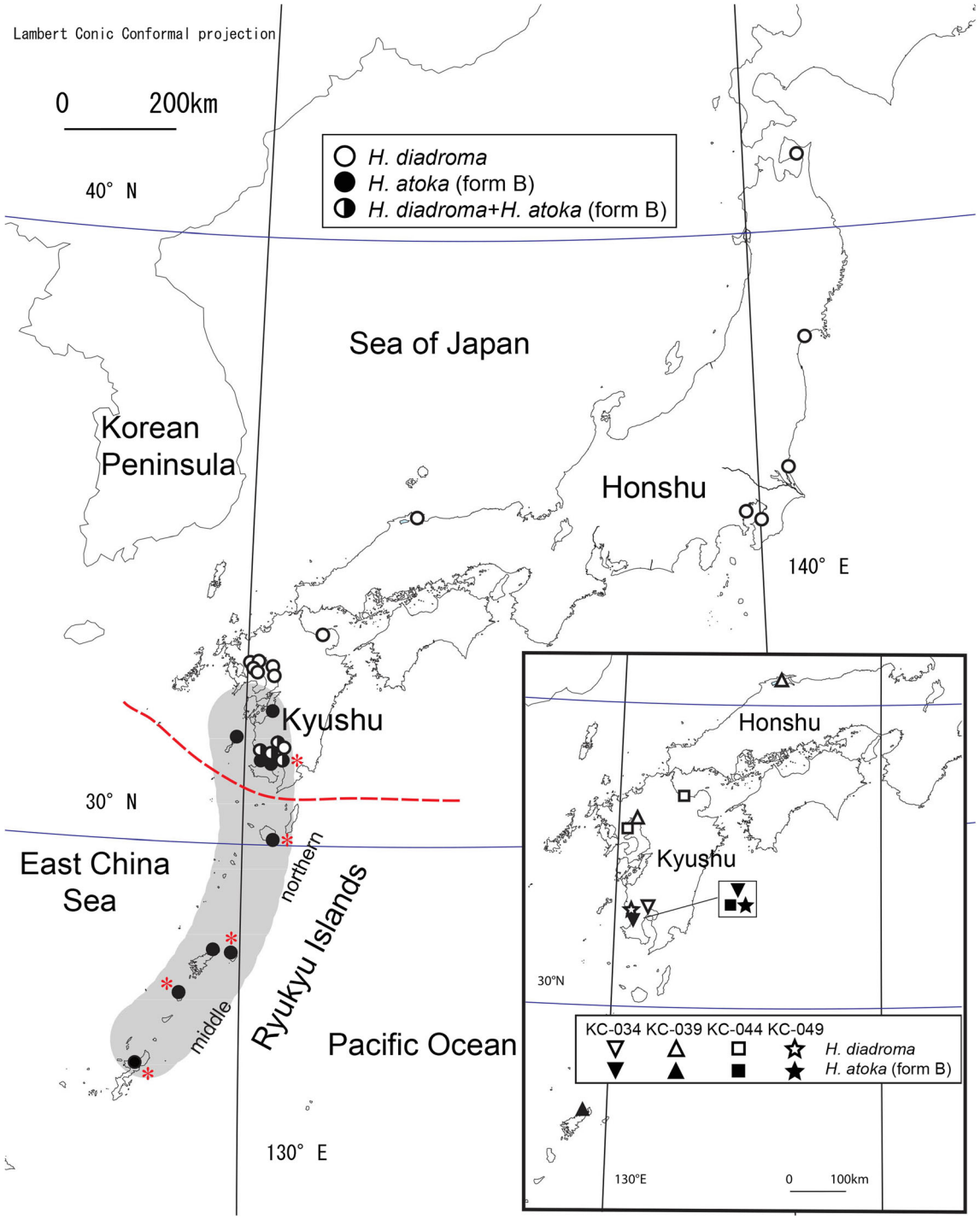

Fig. 6 Distribution of all haplotypes of form B of *Hediste atoka* and *H. diadroma*. Four haplotypes of the mitochondrial COI gene (KC-034, KC-039, KC-044, and KC-049) which were shared by form B of *H. atoka* and *H. diadroma* in southern Japan (inset figure). Form B of *H. atoka* is distributed only in southern Japan (southwestern Kyushu and the northern and middle Ryukyu Islands), whereas *H. diadroma* has a wide distribution covering Honshu and Kyushu in Japan. The gray area shows the distributional range of form B of *H. atoka*, based on Tosuji and Sato (2010) and the present study. Dashed line indicates the southern border of the range of *H. diadroma*. An asterisk indicates new records of form B of *Hediste atoka* in the present study

The values of pairwise F_{ST} calculated between all taxa indicated that the differentiation between the form A and B of *H. atoka* (0.23231) and species A and B of *H. diversicolor* (0.37637) are extremely low in comparison with all other combinations (0.64027–0.99534). The Korean undescribed species *H.* sp. was relatively close to form A of *H. atoka* (0.73724) and species A of *H. diversicolor* (0.77227) when compared with the other species (0.93003–0.99813) (Table 3).

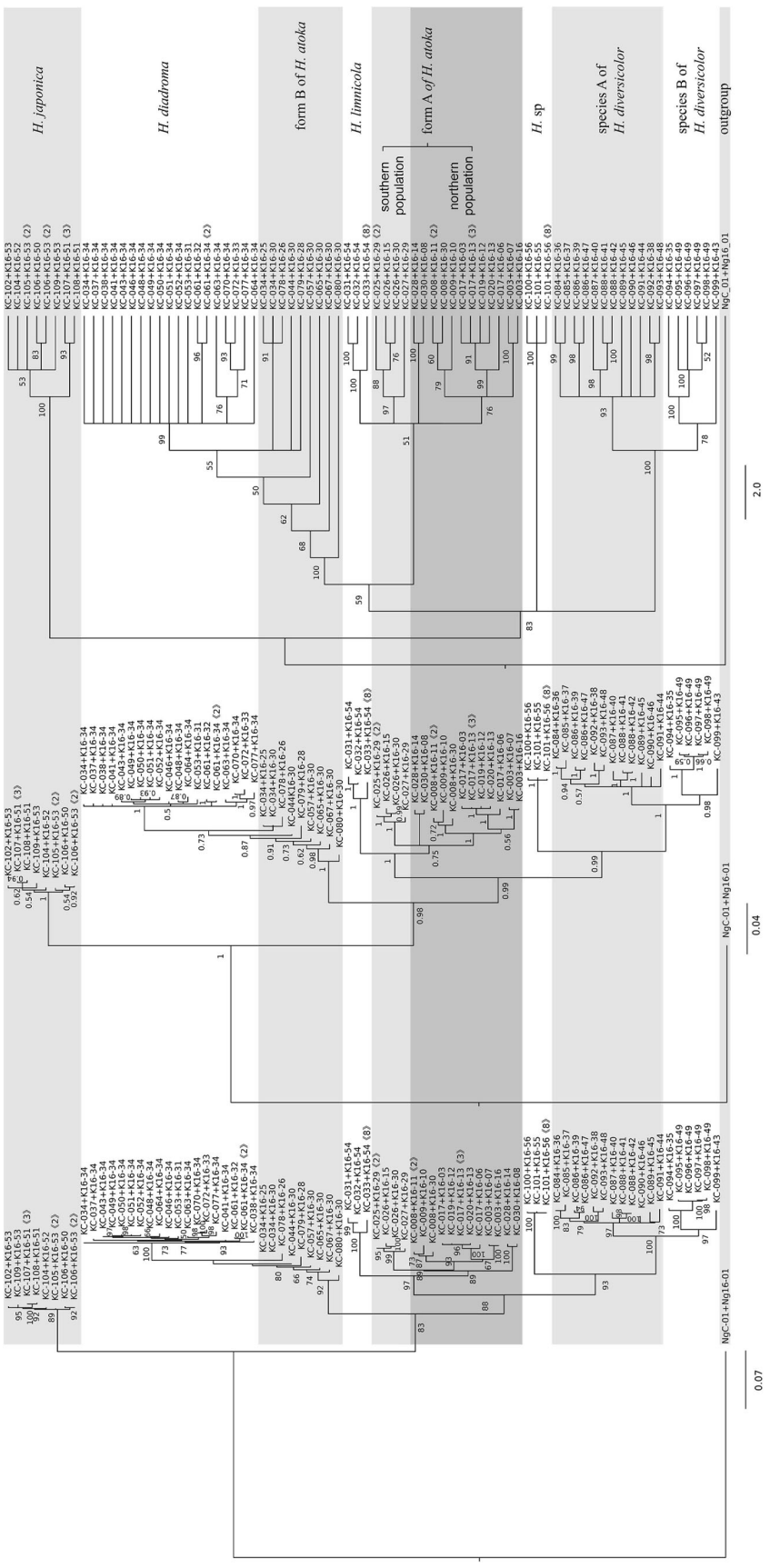

Fig. 7 Maximum likelihood (left), Bayesian inference (middle), and maximum parsimony (right) phylograms for *Hediste* species derived from the analyses of the combined data of sequences of the 429-bp fragment of 16S rDNA and 429-bp fragment of COI sequences. *Neanthes* cf. *glandicincta* is used as an outgroup for rooting the tree. In the maximum likelihood and maximum parsimony tree, bootstrap values from 1000 replications are indicated on the branches and only greater than or equal to 50% are shown. In the Bayesian tree, posterior probability values are indicated on the branches and only greater than or equal to 0.5 are shown. The scale bar corresponds to the substitutions per nucleotide site. The numbers in brackets indicate the number of individuals (no number means single individual)

Phylogenetic analysis using COI sequence

The COI dataset consisted of 113 nucleotide sequences (haplotypes) from 178 specimens (one of them belonged to the outgroup species) with 570 characters, of which 357 were conserved (62.6%), 213 were variable (37.4%), and 171 were parsimony informative (30.0%). When the outgroup was excluded, the dataset had 370 conserved characters (64.9%), 200 variable characters (35.1%), and 171 parsimony informative characters (30.0%).

All of the ML, BI, and MP trees showed almost the same topology (Fig. 5). Haplotypes of each of the four species (*H. diversicolor*, *H. limnicola*, *H. japonica*, *H.* sp.) constituted an independent species-specific clade, supporting the monophyly of these species, with the basal clade comprising *H. japonica*. The clade of *H. diversicolor* was subdivided into two monophyletic groups corresponding to two cryptic species (species A and B designated by Audzijonyte et al. 2008). The clade of *H.* sp. was a sister clade of *H. diversicolor*.

Conversely, haplotypes of each of the two Asian species (*H. diadroma* and *H. atoka*) did not constitute a species-specific monophyletic clade, in contrast to the analysis of the 16s rDNA sequence. Haplotypes of the form A of *H. atoka* constituted a monophyletic clade including several subdivided clades, in which haplotypes of the southern population constituted a monophyletic clade exclusively, whereas those of the northern population constituted a paraphyletic group. The clade form A of *H. atoka* formed a sister group together with the clade of *H. limnicola* in the ML tree, though they constituted a paraphyletic group within a large monophyletic clade including both the form A of *H. atoka* and *H. limnicola* in the BI and MP trees. Haplotypes of both the form B of *H. atoka* and *H. diadroma* constituted another large monophyletic clade. The following four haplotypes were shared by form B of *H. atoka* and *H. diadroma*: haplotype KC-034 (AB603872 and AB996698) obtained from three specimens of form B of *H. atoka* and a specimen of *H. diadroma*; haplotype KC-039 (AB603887 and AB996709) from a specimen of form B of *H. atoka* and three specimens of *H. diadroma*; haplotype KC-044 (AB603878 and AB996712) from a specimen of form B of *H. atoka* and two specimens of *H. diadroma*; haplotype KC-049 (AB603876 and AB996700) from a specimen of form B of *H. atoka* and a specimen of *H. diadroma*. Most of these common haplotypes were distributed in the western coast of Kyushu, southern Japan, where the distributions of the two species overlap (Fig. 6); all specimens of form B of *H. atoka* have been obtained from a limited area in southern Japan, from southwestern Kyushu to Okinawa-jima Island in the middle of the Ryukyu Islands, with new records from 4 islands in the Ryukyu Islands (Yakushima, Kikaijima, Tokunoshima, and Okinawa-jima). On the other hand, all specimens of *H. diadroma* have been obtained from a wide range in Japan, from southern Hokkaido to southern Kyushu, without any records from the Ryukyu Islands as shown in the previous study (Sato and Nakashima 2003).

Three Pacific taxa (form A of *H. atoka*, *H. limnicola*, and *H.* sp.) and an Atlantic species (*H. diversicolor*) formed a large monophyletic group in the ML and MP trees (Fig. 5).

The values of pairwise F_{ST} calculated between all taxa indicated that the differentiation between form B of *H. atoka* and *H. diadroma* (0.11177) is extremely low. On the other hand, the differentiation of pairwise F_{ST} values between the two forms (A and B) of *H. atoka* (0.73910) and between the two species (A and B) of *H. diversicolor* (0.63493) are comparable to those between all other combinations of distinct species (0.62966–0.92664). The Korean undescribed species *H.* sp. was relatively close to form A of *H. atoka*

Table 4 Number of haplotypes of *Hediste* species and samples examined

	16S rRNA		COI	
	Number of haplotypes*	Number of samples	Number of haplotypes*	Number of samples
Form A of *H. atoka*	20 (44.4)***	45	33 (55.0)	60
Form B of *H. atoka*	12 (48.0)***	25	19 (86.4)**	22
H. diadroma	4 (19.0)	21	35 (87.5)**	40
Species A of *H. diversicolor*	12 (100.0)	12	10 (83.3)	12
Species B of *H. diversicolor*	3 (50.0)	6	6 (100.0)	6
H. japonica	4 (30.8)	13	8 (50.0)	16
H. limnicola	1 (4.0)	25	3 (30.3)	10
H. sp.	2 (16.7)	12	2 (18.2)	11
Total	56 (35.2)***	159	112 (63.3)**	177

*The numbers in parentheses indicate the number of haplotypes per 100 samples

**Four haplotypes were shared

***Two haplotypes were shared

(0.75694) in comparison with all other taxa (0.87617–0.96919) (Table 3).

Phylogenetic analysis using the combined data set of COI and 16S rDNA sequences

The combined dataset involved 77 nucleotide sequences (haplotypes) from 101 specimens (both genes from all taxa; one of them belonged to the outgroup species) containing 999 characters, of which 691 were conserved (69.2%), 302 were variable (30.2%), and 223 were parsimony informative (22.3%). When the outgroup was excluded, the dataset had 728 conserved characters (72.9%), 265 variable characters (26.5%), and 221 parsimony informative characters (22.1%).

All of the ML, BI, and MP trees showed essentially the same topology (Fig. 7), which is similar to that of the analysis using the COI gene alone. Haplotypes of each of five species (*H. diadroma*, *H. diversicolor*, *H. limnicola*, *H. japonica*, *H.* sp.) constituted species-specific clades, supporting the monophyly of these species, with the basal clade being *H. japonica*. The clade of *H. diversicolor* was subdivided into two monophyletic groups corresponding to species A and B designated by Audzijonyte et al. (2008). The clades of *H. diversicolor* and *H.* sp. formed a sister group in the ML and BI trees.

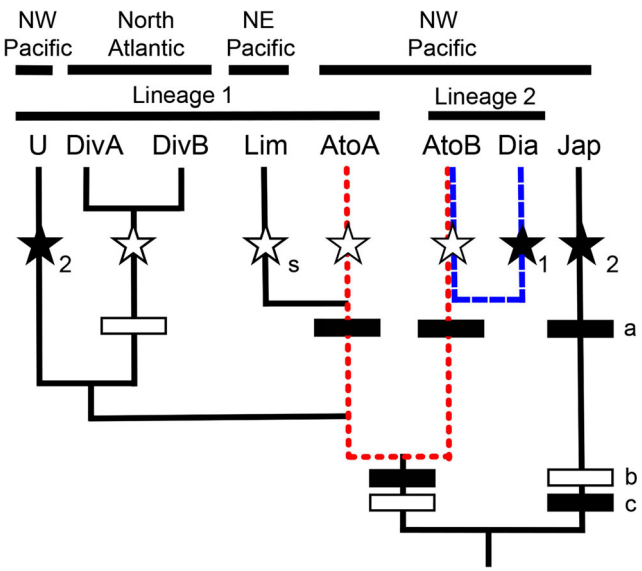

Fig. 8 Hypothesis of the phylogeny of *Hediste*, based on the topology of the phylogenetic analysis using the combined data set of two mitochondrial DNA sequences (16S rRNA and COI). Geographical distributions of the current seven species are shown at the top. AtoA, form A of *H. atoka*; AtoB, form B of *H. atoka*; Dia, *H. diadroma*; DivA, species A of *H. diversicolor*; DivB, species B of *H. diversicolor*; Jap, *H. japonica*; Lim, *H. limnicola*; U, undescribed species (*H.* sp.). Dotted and dashed lines indicate the presence of common haplotypes of 16S rDNA and COI sequences, respectively. Stars indicate reproductive characteristics (Sato 1999; Sato and Nakashima 2003; our unpublished data): black star, reproduction with some epitokous metamorphosis with (1) or without (2) addition of epitoke-specific sesquigomph spinigers; white star, reproduction without any epitokous metamorphosis; s, specialized mode with hermaphrodite, self-fertilizing, and viviparity. Rectangles indicate the different morphology in lower neurochaetae of atokes (Smith 1958; Sato and Nakashima 2003): present (black) or absent (white) of homogomph spinigers (a), heterogomph spinigers (b), homogomph falcigers (c); no morphological characteristic of *H.* sp. is included

Haplotypes of *H. atoka* did not form a clade. Haplotypes of form A of *H. atoka* constituted a monophyletic clade (the ML and BI trees) or a paraphyletic group (the MP tree), consisting of a monophyletic clade together with the clade of *H. limnicola*. In any case, the form A of *H. atoka* was subdivided into several clades, in which haplotypes of the southern population constituted a monophyletic clade exclusively, whereas those of the northern population constituted a paraphyletic group. On the other hand, haplotypes of form B of *H. atoka* form a paraphyletic group with respect to *H. diadroma*.

Three Pacific taxa (form A of *H. atoka*, *H. limnicola*, and *H.* sp.) and two Atlantic taxa (species A and B of *H. diversicolor*) formed a large monophyletic group in the ML and BI trees.

The values of pairwise F_{ST} calculated between all taxa indicated that differentiation between the two species (A and B) of *H. diversicolor* was lowest (0.58821), and that the differentiation between the two forms (A and B) of *H. atoka* (0.69496) and between the form B of *H. atoka* and *H. diadroma* (0.68331) were relatively low in comparison with all other combinations (0.70350–0.93679) (Table 3). The Korean undescribed species *H.* sp. was relatively close to the form A of *H. atoka* (0.77316) in comparison with all other taxa (0.84814–0.96770).

Nucleotide sequence of nuclear DNA

The nucleotide sequences of 18S rDNA were examined for a total of 48 individuals from seven taxa (Online Resource 2). They contained 1682–1683 nucleotides. Most sequences were exactly identical as haplotype K18-01, except sequences in four individuals of form B of *H. atoka* collected from the same location (Kaminokawa River, Kagoshima, Japan), which belonged to another haplotype K18-02 with only one nucleotide (0.06%) replaced and one nucleotide (0.06%) inserted.

The nucleotide sequences of the D1 region of 28S rDNA were examined for a total of 21 individuals from six taxa (Online Resource 2). They contained 338–339 nucleotides. The sequences from most taxa were identical to haplotype K28D1-01, except sequences from *H. limnicola*, which belonged to another haplotype K28D1-02 with only one nucleotide (0.3%) inserted.

The nucleotide sequences of the D4–7b region of 28S rDNA was examined for a total of 10 individuals from six taxa (Online Resource 2). They contained 802–804 nucleotides, constituting three haplotypes: the nucleotide sequences of both species A and B of *H. diversicolor* belonged to haplotype

K28D4-7-01; those of form A of *H. atoka*, *H. diadroma*, and *H. limnicola* belonged to haplotype K28D4-7-02; and those of *H. japonica* belonged to haplotype K28D4-7-03. Only one nucleotide (0.12%) was variable between K28D4-7-02 and K28D4-7-03. K28D4-7-01 had four nucleotide (0.50%) changes and two nucleotide (0.25%) deletions compared to K28D4-7-02.

The nucleotide sequences of the D9–10 region of 28S rDNA was examined for a total of 14 individuals from both forms A and B of *H. atoka* and *H. diadroma* (Online Resource 2). They contained 624 nucleotides, constituting two haplotypes: the nucleotide sequences of both forms A and B of *H. atoka* belonged to haplotype K28D9-10-01; those of *H. diadroma* belonged to another haplotype K28D9-10-02, with only one nucleotide (0.16%) replaced.

The nucleotide sequences of histone H3 were examined for a total of 41 individuals from seven taxa (Online Resource 2). They contained 291 nucleotides, constituting five haplotypes: the nucleotide sequences of both forms A and B of *H. atoka* belonged to haplotype KH3-01; those of a part of specimens of *H. diadroma* belonged to haplotype KH3-02; those of the other specimens of *H. diadroma*, both species A and B of *H. diversicolor* and *H. limnicola* belonged to haplotype KH3-03; those of *H. japonica* belonged to haplotypes KH3-04 and KH3-05. Only four nucleotides (1.37%) were replaced between the five haplotypes. The amino acid sequences derived from the all haplotypes were identical.

Discussion

Molecular phylogeny of Hediste based on mitochondrial DNA sequences

The present study is the first attempt to estimate the phylogenetic relationship among all species of Hediste currently known in the world, based on the analyses of two mitochondrial DNA sequences. Our analysis using the 16S rDNA sequence supported the monophyly of each of all nominal species established in previous taxonomic studies (e.g., Sato and Nakashima 2003), though it did not support the monophyly of each of two cryptic species in *H. diversicolor* (Audzijonyte et al. 2008) and two forms of *H. atoka* (Tosuji and Sato 2010). In another analysis using the COI gene, however, each of the two cryptic species in *H. diversicolor* formed a monophyletic group as shown in Audzijonyte et al. (2008), and more surprisingly, the two forms of *H. atoka* were completely separated into distinct clades; form A of *H. atoka* was included in a large clade together with *H. limnicola H. diversicolor*, and *H.* sp., whereas form B of *H. atoka* was included in another large clade together with *H. diadroma*. The different results of the two sequences (16S rDNA and COI) analyzed here may be caused by the different rates of nucleotide substitution between the genes; the rate may be faster in COI than in 16S rRNA. This is supported by the fact that the number of haplotypes is larger in the COI than in 16S rDNA in most taxa, with an extreme case in *H. diadroma*, where the frequency of haplotypes of the COI gene was 4.59 times larger than that of the 16S rDNA sequence (Table 4).

Our data revealed that two common haplotypes of the 16S rDNA sequence were shared by the southern population of form A and the form B of *H. atoka*, and four common haplotypes of the COI were shared by form B of *H. atoka* and *H. diadroma*, showing that most of these interspecific common haplotypes are distributed in southern Japan, from western Kyushu to the Ryukyu Islands. This result suggests that the speciation between the two forms of *H. atoka*, and the subsequent one between form B of *H. atoka*, and *H. diadroma* occurred around western Kyushu or the Ryukyu Islands in southern Japan.

The high values of pairwise F_{ST} between the undescribed Korean species *H.* sp. and the other nominal species indicate that *H.* sp. is well differentiated genetically from the other species. *Hediste* sp. is currently known only from the Han River estuary in Korea, and is morphologically indistinguishable from *H. atoka* in a sexually immature stage, but distinguishable from it in a mature stage, where *H.* sp. shows a unique epitokous metamorphosis during reproductive swarming (our unpublished data). The taxonomic description of this species will be provided in another paper.

Hypothesis of the speciation in Hediste

We propose here a hypothesis on the probable evolutionary history of the worldwide speciation in *Hediste*, based on the topology of our phylogenetic analysis using the combined data set of two mitochondrial DNA sequences (COI and 16S rDNA) (Fig. 7), which seems to correspond well to the geographical distributions of current species and the extent of their morphological differentiation (Fig. 8). Our result suggests that the origin of *Hediste* seems to be located in the Northwest Pacific (i.e., eastern Asia). The oldest differentiation probably occurred in the ancient Yellow Sea dividing the strain of *H. japonica* from the stem strain. *Hediste japonica*, currently distributed mainly along the Korean coast of the Yellow Sea, with several isolated distributions in western Japan (Sato and Sattmann 2009; Sato 2017), is clearly distinguishable from all other congeners by the morphology of neurochaetae, neuropodial postchaetal lobes, and a unique epitokous metamorphosis (Sato and Nakashima 2003).

Thereafter, the dichotomous differentiation of the stem strain seems to have occurred to produce two stem lineages around southern Japan; the direct descendents of stem lineages 1 and 2 are the forms A and B of *H. atoka*, respectively. The genetic differentiation between forms A and B is comparable to that between the distinct species, with high pairwise F_{ST}

value (0.69054) (Table 3). The fact that the southern population of form A and the form B share some common haplotypes of 16s rDNA sequence suggests that the form B was derived from the southern population of form A. No difference has been detected between the two forms of *H. atoka* in adult morphology, reproduction lacking epitokous metamorphosis, and early development without a true planktic phase, though they may differ slightly in spawning behavior (Sato 2017). The highest similarity in these biological aspects implies that both forms of *H. atoka* have preserved the original characteristics of the common ancestor *Hediste* since the separation of the two stem lineages. In the present study, three sympatric habitats of the two forms were newly found in southern Kyushu (Kagoshima Bay) and one was found in the Ryukyu Islands (Okinawa-jima Island) (Fig. 4), and significantly, they were the first sympatric habitats to be found.

From stem lineage 1, the Korean undescribed species (*H.* sp.) and two circumboreal species (*H. diversicolor* and *H. limnicola*), which are distributed in the North Atlantic and the North West Pacific, respectively, seem to be descended as follows: (1) the common ancestor of *H. diversicolor* which is morphologically distinguishable from any other congener by the absence of homogomph spinigers in lower neurochaetae (Smith 1958), and *H.* sp. (morphology not yet described) are descended from the Asian stem lineage 1 in an earlier period; then, *H. diversicolor* was descended from the ancestor that invaded the North Atlantic across the Arctic basin in a period with climatic warming to allow the successful trans-Arctic dispersal, while *H.* sp. was descended from the resident ancestor in Asia; (2) *H. limnicola*, which is morphologically indistinguishable from *H. atoka*, was relatively recently descend from the Asian ancestor that invaded the North East Pacific along the Bering Strait in another period to allow for successful boreal dispersal. In *H. limnicola*, the most specialized reproductive mode with hermaphrodite, self-fertilizing, and viviparity evolved (Smith 1950). Because this species seems to have originated from a small branching population separated from the large Asian one, such a rapid and drastic differentiation in the reproductive mode may have occurred, accelerated by genetic drift.

The circumboreal speciation caused by the trans-Arctic dispersal has been suggested in various taxa including many familiar taxa such as the algae *Laminaria*, the bivalves *Mytilus* and *Macoma*, the gastropods *Littorina* and *Nucella*, and echinoderm *Asterias* (Vermeji 1991; Väinölä 2003): much of the present fauna in North Atlantic littoral and shallow waters is of Pacific ancestry; following the long-term isolation of the northern Atlantic and Pacific biota from the early Cenozoic period, the opening of the Bering Strait in the Pliocene ca. 3.5 million years ago allowed an exchange of the independently evolved boreal taxa across the current Arctic basin; due to climatic cooling since the Pliocene, the trans-Arctic dispersal rout has been largely closed again, followed by effective isolation between the Atlantic and Pacific populations, which were differentiated as vicarious distinct species.

Conversely, *H. diadroma*, which is widely distributed throughout Japan except for the northern and southern extremes (northern Hokkaido and the Ryukyu Islands, respectively) (Sato and Nakashima 2003; Tosuji and Sato 2012), seems to have been descended from stem lineage 2 in an area around the western coast of Kyushu most recently; this species is so closely related with form B of *H. atoka* that these taxa are indistinguishable by the comparison of COI gene alone in the present study; they are also almost indistinguishable in the morphology of sexually immature worms (atokes), but *H. diadroma* shows a unique epitokous metamorphosis (Sato and Nakashima 2003).

Our hypothesis based on the present molecular data corroborate the previous hypothesis on the phylogeny of three Asian species (*H. japonica*, *H. diadroma*, and *H. atoka*) based on their characteristic morphology and reproductive and developmental modes: an *H. atoka*-like species with an estuary-resident life cycle is the ancestral form, from which *H. japonica* and *H. diadroma* with the unique epitoky and catadromous life cycle were derived independently of each other (Sato 1999; Sato and Nakashima 2003; Sato 2017). At present, we conclude that the epitoky of *Hediste*, which is markedly different from the typical heteronereis form prevailing in many marine nereidids, seems to have evolved independently three times in *H. japonica*, *H. diadroma*, and *H.* sp. only in Asia.

Interspecific differentiation in nuclear DNA sequences of Hediste

There is no previous study on the interspecific comparison of sequences of nuclear DNA in *Hediste*. Our result demonstrates that five nuclear DNA sequences (18S rDNA, three regions of 28S rDNA and histone H3) are almost invariable, with very few inter- and intra-specific differences detected. This result is in contrast to that marked interspecific differences were detected in the sequences of 18S and 28S rDNA between congeneric species in Spionidae (Abe et al. 2016).

Our analyses of 28S rDNA and histone H3 could detect some interspecific differences. The sequences of D9–10 region of 28S rDNA showed a slight but consistent difference between *H. atoka* (both forms A and B) and *H. diadroma* in agreement with our result on the sequences of the mitochondrial gene of not COI but 16S rDNA.

On the other hand, according to our result on D4–7b region of 28S rDNA, the following three groups were distinguishable: *H. diversicolor*, *H. japonica*, and the others (*H. atoka*, *H. diadroma*, *H. limnicola*). This grouping is inconsistent with the result on D9–10 region.

The sequences of histone H3 were most variable, showing a total of five haplotypes. A consistent difference between *H.*

atoka (both forms A and B) and *H. diadroma* was detected as the result on D9–10 region.

Glasby et al. (2013) demonstrated that the sequences of histone H3 could detect the interspecific difference between morphologically very similar congeneric species in three genera of Nereididae (*Nereis*, *Pseudonereis*, *Perinereis*), as well as the sequences of mitochondrial COI, though the mitochondrial COI gene appears to have evolved more rapidly than nuclear histone H3 gene. However, our result indicates that the sequences of histone H3 and other regions of nuclear DNA are much less differentiated between species of *Hediste* in comparison with other genera of Nereididae and Spionidae. Therefore, it seems that we cannot estimate the phylogenetic relationship between species of *Hediste* by the data of nuclear DNA sequences.

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Compliance with ethical standards

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