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FULL PAPER

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Molecular phylogeny of terrestrial holocarpic endoparasitic peronosporomycetes, *Haptoglossa* spp., inferred from 18S rDNA

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Abstract Phylogenetic relationships of seven isolates of the genus *Haptoglossa* parasitic on terrestrial nematodes within the Peronosporomycetes were analyzed using 18S rDNA sequence data with 21 peronosporomycetes, 2 marine stramenopilous flagellates, and 2 hyphochytridiomycetes. The marine stramenopilous flagellates and hyphochytridiomycetes were used as the outgroup. All *Haptoglossa* isolates formed a monophyletic clade and clustered with the marine genus *Eurychasma*. The clade of *Haptoglossa* and *Eurychasma* formed a sister-group to the clade that consisted of all other peronosporomycetes. These results suggest that the genus *Haptoglossa* and other terrestrial peronosporomycetes included in the two subclasses, the Saprolegniomycetidae and the Peronosporomycetidae, might have originally adapted to the terrestrial environment individually. In the maximum-likelihood (ML) analysis, the *Haptoglossa* clade was divided into three subclades, one aplanosporic species clade and two zoosporic species clades. Phylogenetic analyses of combined 18S rDNA and *cox*2 genes among five species of *Haptoglossa* supported the results of the ML analysis using 18S rDNA and suggested that zoosporic species may be separated into two lineages. This topology of the analysis may suggest that aplanosporic species diverged from zoosporic species.

Key words *Haptoglossa* · Molecular phylogeny · Nematode parasites · Oomycetes · Peronosporomycetes

Introduction

The class Peronosporomycetes (Oomycetes), which consists of absorptive heterotrophs that have two heterokont flagella on the zoospore, is placed in the Stramenopile (Patterson 1989). For the classification of this class, Sparrow (1976) emphasized that the Oomycetes was composed of two morphologically distinguishable major taxonomic groups, the saprolegnialean and peronosporalean "galaxies." These galaxies almost correspond to the subclasses defined by Dick et al. (1984), the Saprolegniomycetidae and the Peronosporomycetidae. In 2001, Dick erected a new class, Peronosporomycetes, consisting of three subclasses instead of the traditional class name of Oomycetes and subdivided into three subclasses (Saprolegniomycetidae, Peronosporomycetidae, and Rhipidiomycetidae). The existence of the former two subclasses in the Peronosporomycetes has been supported by phylogenetic analysis on DNA sequence data including 18S rDNA (Dick et al. 1999), 28S rDNA (Riethmuller et al. 1999), and the mitochondrial cytochrome *c* oxidase subunit 2 (*cox*2) locus (Hudspeth et al. 2000; Cook et al. 2001). Recent molecular phylogenetic studies revealed that some marine holocarpic peronosporomycetes that are endoparasitic in the Crustacea or algae were not included in any clade of two subclasses and formed a separate clade at the basal position of the Peronosporomycetes (Cook et al. 2001; Kupper et al. 2006; Sekimoto et al. 2007). Holocarpic endoparasitic peronosporomycetes have been discovered in both the marine and the terrestrial environment (Karling 1981). Molecular phylogenetic analyses have been mostly conducted for marine holocarpic peronosporomycetes (Cook et al. 2001; Kupper et al. 2006; Sekimoto et al. 2007), whereas it has been rarely tried for the terrestrial members (Cook et al. 2001).

The genus *Haptoglossa* is a terrestrial holocarpic peronosporomycetes that parasitizes nematodes and rotifers (Drechsler 1940; Barron 1980). The genus includes 11 species that can be divided into two groups by spore type, whether aplanosporic or zoosporic. The species included in the aplanosporic group form infection cells by several different ways whereas zoosporic species produce these cells by one way only (Glockling and Beakes 2002; Hakariya et al. 2007). Recently, we analyzed the phylogenetic position of seven isolates of the genus *Haptoglossa* using mitochondrial cytochrome *c* oxidase subunit 2 amino acid (COII) sequences with a data set of 34 peronosporomycetes (Hakariya et al. 2007). Our results revealed that all

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Haptoglossa isolates formed a single clade and appeared to be basal to the clade consisting of all other peronosporomycetes. In addition, the *Haptoglossa* clade was divided into two subclades, which correspond to different development patterns of the infection cell. However, spore types for propagation did not correspond to subclades.

The aim of this study is to investigate whether the results of our former work based on COII amino acid sequence data are supported by phylogenetic analyses using 18S rDNA sequence data. Especially, we focused on clarifying the evolutionary relationships between the genus *Haptoglossa* and marine holocarpic endoparasitic genera, and also on reevaluating interspecific relationships within the genus.

Materials and methods

Haptoglossa isolates

Isolation and maintenance of all *Haptoglossa* isolates followed the methods described by Hakariya et al. (2007). Seven isolates of *Haptoglossa* were used in this study (Table 1). *Haptoglossa heterospora* NA01, *Haptoglossa* sp. 1 KG01, and *Haptoglossa* sp. 2 SZ01 are the isolates used in the previous study (Hakariya et al. 2007). The other four isolates are newly established strains. *Haptoglossa* sp. 3 KG02 and SZ03, which are distinguishable from any known species by producing a chlamydospore-like structure, are different species from the *Haptoglossa* sp. 3 used in the our previous study (Hakariya et al. 2007). All aplanosporic species used in this study form small and large aplanospores that produce small and large infection cells, respectively (heterospora type). All zoosporic species used in this study form single-size zoospores that encyst to produce a single type of infection cells (zoospora type).

Amplification of DNA

Two methods were used for amplification of DNA in this study. For the *Haptoglossa zoospora* Y11 and the *Haptoglossa* sp. 2 SZ01, total genomic DNA was extracted from the thalli following the modified cetyltrimethylammonium bromide (CTAB) method described by Matsuda and Hijii (1999) for ectomycorrhizae. Primers NS1 (White et al. 1990) and PCR-B (Spencer et al. 2002) were used to amplify the almost complete 18S rDNA. Polymerase chain reaction (PCR) was achieved using HotStarTaq Master Mix (Qiagen, Hilden, Germany). Each PCR tube contained a total 50 µl mixture (21 µl distilled water, 25 µl master mix, 3 µl template DNA, and 0.5 µl each primer; final, 0.25 µM). PCR amplification was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) as follows: an initial denaturation of 94°C for 15 min is followed by 45 cycles consisting of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by one extension period of 72°C for 10 min. For the other isolates, the DNA fragment was amplified by the modified direct PCR method developed by Hakariya et al. (2007). Three primer pairs, NS1 and NS2, SR-4 and NS4, and NS5 (White et al. 1990) and PCR-B, were used to amplify almost complete 18S rDNA. PCR amplification were performed in a GenAmp PCR system 2400 (Perkin Elmer, Waltham, MA, USA), which was an initial denaturation of 95°C for 15 min followed by 45 cycles consisting of 94°C for 20 s and 50°C for 1 min, followed by one extension period of 71°C for 10 min. PCR products were purified with a QiAquick PCR purification Kit (Qiagen).

DNA sequencing

Sequencing reactions were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) using a ABI PRISMR Big Dye Terminator Cycle Sequencing Kit with AmpliTaqR DNA polymerase (FS enzyme) (Applied Biosystems, Foster City, CA, USA), following the protocols supplied by the manufacturer. These reactions were performed on each template using primers NS1, NS4, SR-4, and PCR-B (Nakayama et al. 1998). The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Table 1. *Haptoglossa* isolates analyzed in this study

Species	Strain no.	Spore type	Development pattern of infection cell[a]	Gene	GenBank accession no.	Reference
Haptoglossa heterospora	NA01	Aplanosporic	Heterospora type	18S rDNA	AB425199	This study
Haptoglossa heterospora	NA01	Aplanosporic	Heterospora type	*cox*2	AB253781	Hakariya et al. 2007
Haptoglossa sp. 1	KG01	Aplanosporic	Heterospora type	18S rDNA	AB425201	This study
Haptoglossa sp. 1	KG01	Aplanosporic	Heterospora type	*cox*2	AB253784	Hakariya et al. 2007
Haptoglossa zoospora	Y11	Zoosporic	Zoospora type	18S rDNA	AB425202	This study
Haptoglossa zoospora	Y11	Zoosporic	Zoospora type	*cox*2	AB437405	This study
Haptoglossa zoospora	SZ02	Zoosporic	Zoospora type	18S rDNA	AB425200	This study
Haptoglossa sp. 2	SZ01	Zoosporic	Zoospora type	18S rDNA	AB425203	This study
Haptoglossa sp. 2	SZ01	Zoosporic	Zoospora type	*cox*2	AB253786	Hakariya et al. 2007
Haptoglossa sp. 3	KG02	Zoosporic	Zoospora type	18S rDNA	AB425204	This study
Haptoglossa sp. 3	SZ03	Zoosporic	Zoospora type	18S rDNA	AB425205	This study
Haptoglossa sp. 3	SZ03	Zoosporic	Zoospora type	*cox*2	AB437406	This study

[a] Development pattern of infection cell is represented in Hakariya et al. (2007)

Alignment and phylogenetic analysis

The BioEdit program for the sequence data management and phylogenetic analysis (Hall 1999) was used for preliminary multiple alignments of nucleotide sequences. Final alignments were manually adjusted. Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. Phylogenetic analyses were carried out using PAUP* 4.0b10 (Swofford 2003). The neighbor-joining (NJ) topologies were calculated with the HKY 85 (Hasegawa et al. 1985) model. For maximum-likelihood (ML) analysis, the tree topologies were inferred using the model "TrN + I + G" (Tamura and Nei 1993), which was selected as the best-fit model of nucleotide substitution by the Akaike information criterion (AIC) in Modeltest 3.7 (Posada and Crandall 1998). A heuristic search procedure using the tree bisection-reconnection branch-swapping algorithm was performed to find the optimal ML tree topology. To estimate clade support, the bootstrap procedure of Felsenstein (1985) was employed with 1000 replicates in NJ analyses and 100 replicates in ML analysis. User-defined alternative tree topologies were compared to the ML tree by the approximately unbiased (AU) test using the CONSEL program (Shimodaira and Hasegawa 2001) to evaluate how much more strongly the sequence data supported the best tree compared to alternative trees.

To obtain a more robust inference on the phylogenetic relationships among the species of the genus *Haptoglossa*, a data set consisting of 18S rDNA and mitochondrial *cox*2 sequences was analyzed using two different models (concatenate and separate models). The analyses using each model were performed by the BASEML program in the PAML package (Yang 1997) and carried out by exhaustively examining the 105 alternative trees possible for the five species of *Haptoglossa* with an outgroup. In the analysis using the concatenate model, the tree with the highest log-likelihood in total was selected as the best tree by comparing of the sum of the site-by-site log-likelihoods for each tree. In the analysis using a separate model, the results based on individual sites were combined by summing up the estimated log-likelihoods using the TOTALML program in the MOLPHY package. The adequacy of models was compared by using the AIC (Akaike 1974). Bootstrap proportion (BP) for each node of the best tree with the best log-likelihood score among alternatives was calculated by applying the resampling estimated log-likelihood (RELL) method (Kishino et al. 1990) with 5000 resamplings to the alternative trees mentioned earlier.

Results and discussion

Phylogenetic relationships within the class Peronosporomycetes

In both NJ and ML analyses, the Peronosporomycetes clade was strongly supported by high bootstrap values (100%/100% of NJ/ML bootstrap value; Figs. 1, 2). Trees obtained by NJ and ML analyses showed similar topology within the Peronosporomycetes clade. The clade was divided into four major groups: *Haptoglossa* and *Eurychasma* clade (44%/75%), a group of marine holocarpic endoparasites of the Crustacea (consisting of *Haliphthoros*, *Halodaphnea*, and *Halocrusticida*), the Peronosporomycetidae clade (100%/99%), and the Saprolegniomycetidae clade (82%/51%). The latter two clades formed a clade, as shown in previous studies (Dick et al. 1999; Riethmuller et al. 1999; Hudspeth et al. 2000; Sekimoto et al. 2007). The group of marine holocarpic endoparasites of the Crustacea is a paraphyletic group. The separation of the group of marine holocarpic endoparasites of the Crustacea from the clade composed of these two subclasses is supported by high bootstrap values (100%/100%). The clade of *Haptoglossa* and *Eurychasma* is the sister-group to a clade consisting of other peronosporomycetes. In both NJ and ML analyses, the branching order among four major groups in the Peronosporomycetes is not in conflict with previous studies (Cook et al. 2001; Cavalier-Smith and Chao 2006; Kupper et al. 2006; Sekimoto et al. 2007).

Phylogenetic position of the genus *Haptoglossa* in the Peronosporomycetes

The genera *Haptoglossa* and *Eurychasma* formed a clade that was the sister-group to a clade consisting of other peronosporomycetes (Figs. 1, 2). Monophyly of *Haptoglossa* and *Eurychasma* should be concluded carefully, because the clade was not supported firmly by bootstrap value in NJ and ML analyses (44%/75%). Branching order among the *Haptoglossa* clade, *Eurychasma*, and the other peronosporomycetes clade is ambiguous. The probability of other branching orders was tested using the AU test to evaluate how much more strongly the 18S rDNA sequence data supported the ML tree than the two alternative trees (Table 2). The ML tree (tree 1) was compared to two alternative trees, tree 2 (the *Haptoglossa* clade was sister with a clade consisting of *Eurychasma* and the other peronosporomycetes clade) and tree 3 (*Eurychasma* was sister to a clade consisting of the *Haptoglossa* clade and the other peronosporomycetes clade). The results of the AU test show that tree 3 is significantly worse than the ML tree (Table 2). Although the branching order among three clades within the Peronosporomycetes clade was not clearly resolved, the result of the AU test suggests that the genus *Haptoglossa* is not the group that diverged first in the Peronosporomycetes. Our results also indicate all peronosporomycetes that diverged earlier than the Peronosporomycetidae and Saprolegniomycetidae are holocarpic endoparasites, and they are marine, except the genus *Haptoglossa*. This result supports the hypothesis by Cavalier-Smith and Chao (2006) and Kupper et al. (2006) that the Peronosporomycetes arose from the sea. If the origin of the Peronosporomycetes was in the sea, the genus *Haptoglossa* and other terrestrial peronosporomycetes included in the two subclasses might have adapted to terrestrial environment individually.

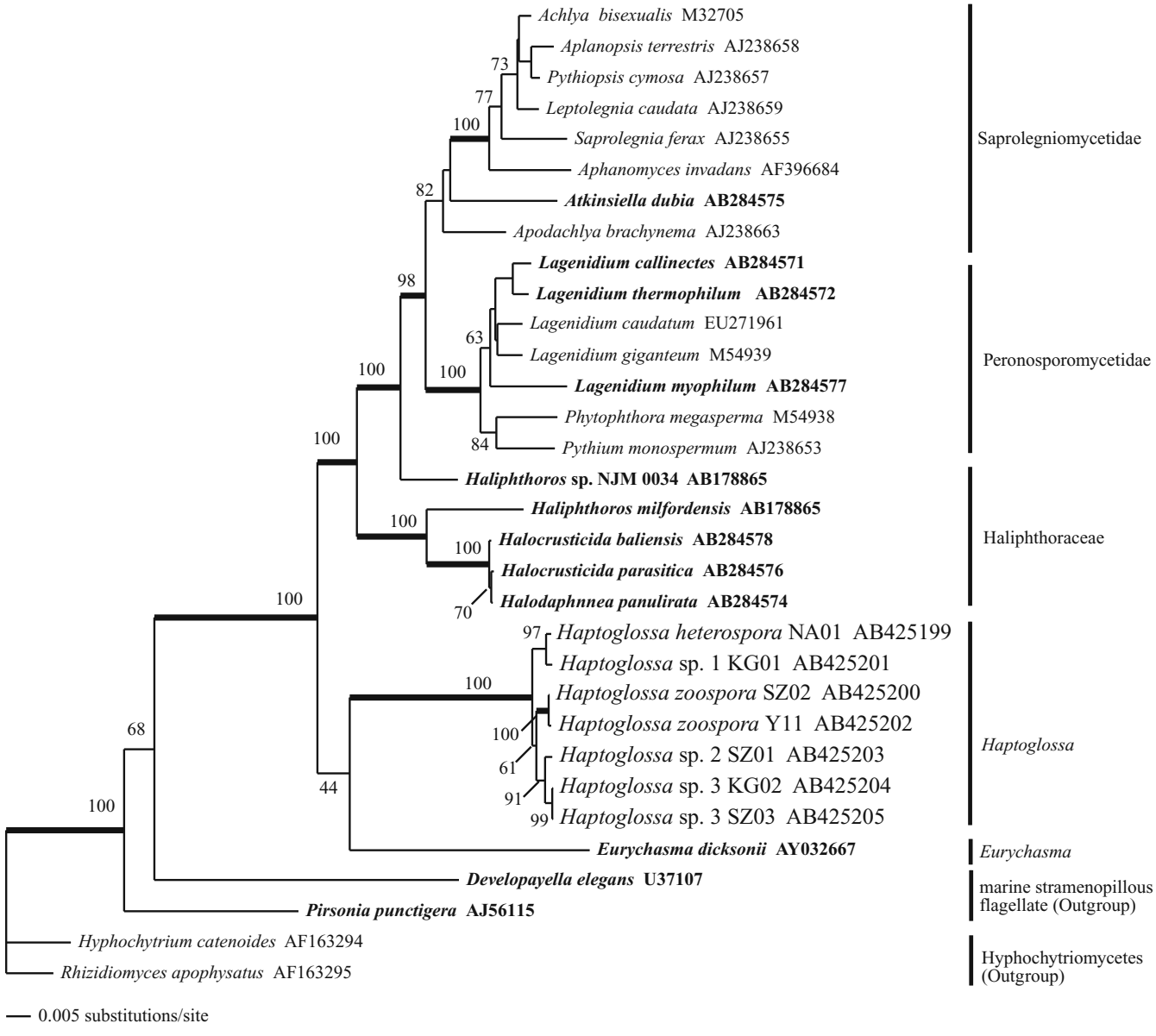

Fig. 1. Phylogenetic positions of *Haptoglossa* species among the Peronosporomycetes inferred from the neighbor-joining (NJ) analysis of 18S rDNA. Bootstrap values greater than 50% from 1000 replicates are indicated for corresponding branches. Branches significantly supported by bootstrap value above 95% are shown with *thick lines*. Organisms from the marine environment shown in *bold*

Table 2. Results of approximately unbiased (AU) tests of alternative topologies against the best one found in maximum-likelihood (ML) analysis

	Tree 1	Tree 2	Tree 3
Topology	Pero Hapto Eury Outgroup	Pero Hapto Eury Outgroup	Pero Eury Hapto Outgroup
P value	0.612	0.388	0.087
Significantly worse	–	No	Yes

Hapto, *Haptoglossa* clade; Eury, *Eurychasma dicksonii*; Pero, other peronosporomycetous organisms clade

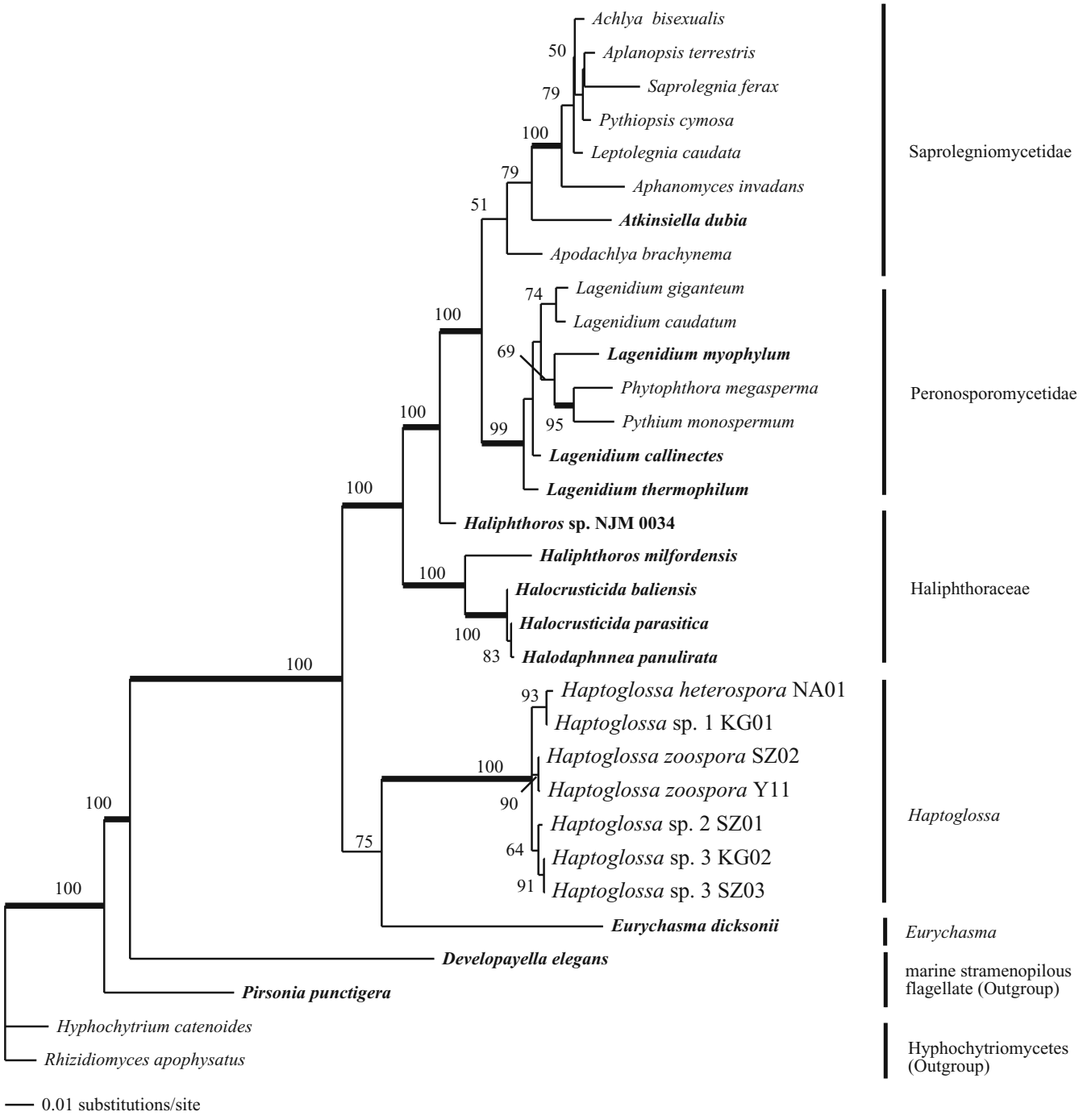

Fig. 2. Phylogenetic positions of *Haptoglossa* species among the Peronosporomycetes inferred from the maximum-likelihood (ML) analysis of 18S rDNA. Bootstrap values greater than 50% from 100 replicates are indicated for corresponding branches. Branches significantly supported by bootstrap value above 95% are shown with *thick lines*. Organisms from the marine environment shown in *bold*

Phylogenetic relationships within the genus *Haptoglossa*

In NJ and ML analyses all isolates of *Haptoglossa* formed a single clade, which was supported by high bootstrap values (100%/100%). In the *Haptoglossa* clade, there were three subclades, the aplanosporic species clade (*H. heterospora* and *Haptoglossa* sp. 1), the zoosporic species clade (*Haptoglossa* sp. 2 and *Haptoglossa* sp. 3), and another zoosporic clade of *H. zoospora*. These three subclades were supported by high bootstrap values, above 91%, except the former zoosporic species subclade in ML analysis (64%). The topologies of the two analyses resulted in different branching orders. In the NJ analysis, all zoosporic subclades formed a single clade. The clade and the aplanosporic clade formed a sister-group. In ML analysis, three subclades of *Haptoglossa* isolates seemed to branch almost at the same time. To infer more precisely phylogenetic relationships among the five species of the *Haptoglossa* with an outgroup,

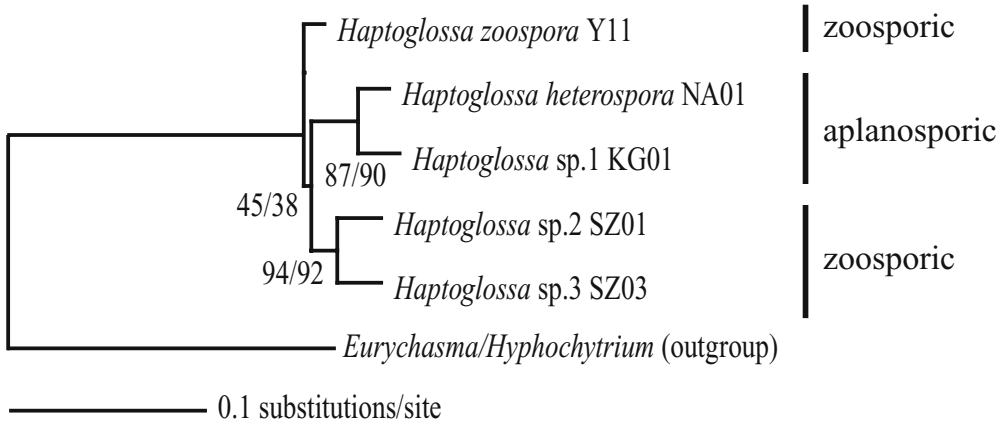

Fig. 3. Phylogenetic tree based on concatenate and separate models using 18S rDNA and *cox*2 nucleotide sequences. The topology shows the ML tree obtained from the separate model. The branch length was obtained from the concatenate model. In the ML analysis of the separate model, *Eurychasma dicksonii* and *Hyphochytrium catenoides* were used as an outgroup on the analyses of 18S rDNA and *cox*2, respectively. Bootstrap proportions based on separate and concatenate models are attached to the internal branches

we performed ML analyses of combined phylogeny using a concatenate model for 18S rDNA + *cox*2 data set and a separate model for 18S rDNA and *cox*2 data set. Comparison of the AIC values for the ML tree selected the separate model as better. The concatenated analysis gave an AIC value of 10738.12, and the AIC value of separated analysis was reduced to 9768.6. This result indicates that the separate model approximates the underlying evolutionary process better than the concatenated model, which assumes homogeneity of substitution process across genes.

The ML tree of the separate model showed three lineages, which are congruent with the NJ and ML trees using 18S rDNA, an aplanosporic clade consisting of *H. heterospora* and *Haptoglossa* sp. 1, a zoosporic clade that consisted of *Haptoglossa* sp. 2 and sp. 3, and a zoosporic isolate *H. zoospora* (see Fig. 3). The ML tree of the separate model also showed that *H. zoospora* was positioned basal to the clade consisting of aplanosporic and zoosporic clades. The topology of the analysis may suggest that the genus arose from zoosporic and aplanosporic species derived from zoosporic species. However, separation of *H. zoospora* from other *Haptoglossa* species was not supported by bootstrap proportion value (44%). This result may suggest that zoosporic species are not a monophyletic group, although the branching order of *Haptoglossa* species was not clearly revealed. Further studies on the molecular analysis using more isolates are needed to clarify this problem.

In our previous study we suggested that the development pattern of infection cell may reflect the phylogeny of the genus (Hakariya et al. 2007). Our results of this present study, however, do not clearly support this idea. Our former study also indicated that the spore type for propagation does not reflect phylogeny in the genus (Hakariya et al. 2007). Our results here support this suggestion. We have not yet detected what characters reflect the phylogenetic relationships within the genus *Haptoglossa*. To find the characters, further studies are needed by observing and comparing more carefully the morphology, life cycle, and ultrastructure of more species. Especially, we expect that detailed observations of the entire life cycle of the genus will lead to this goal.

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