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FULL PAPER

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A molecular phylogeny of *Haptoglossa* species, terrestrial peronosporomycetes (oomycetes) endoparasitic on nematodes

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Abstract Phylogenetic relationships of seven *Haptoglossa* isolates were analyzed by using mitochondrial COII amino acid sequences with a data set of 34 peronosporomycetes. *Haptoglossa* isolates formed a single clade and appeared to be basal to the clade consisting of all other peronosporomycetes. The *Haptoglossa* clade was divided into two subclades: one clade consisted of five aplanosporic isolates and the other included one aplanosporic and one zoosporic isolate. These results indicate that the genus *Haptoglossa* is monophyletic, and patterns of infection cell formation reflect more the phylogenetic relationship between the species than patterns of sporogenesis.

Key words *Haptoglossa* · Mitochondrial cytochrome *c* oxidase subunit · Molecular phylogeny · Nematode parasites · Peronosporomycetes (Oomycetes)

Introduction

For many years, a large group of fungus-like chromists that consists of water molds, downy mildews, and probably their relatives was classified with true fungi as the class Oomycetes in the phylum Oomycota (Dick 1990). Dick et al. (1984) proposed two subclasses in the class, i.e., Peronosporomycetidae and Saprolegniomycetidae, based on the biochemical and ultrastructural differences. Subsequently, Dick et al. (1989) proposed a new subclass Rhipidiomycetidae in the class. Dick (1999) considered that the term Oomycetes is taxonomically imprecise and later adopted a new term, Peronosporomycetes; the three subclasses he had erected were then transferred to the new class (Dick 2001). However, recent molecular studies using small subunit (SSU) rDNA (Dick et al. 1999), large subunit (LSU) rDNA (Petersen and Rosendahl 2000; Riethmuller et al. 1999), and the mitochondrial *cox2* locus (Cook et al. 2001; Hudspeth et al. 2000, 2003) have rather supported the old two-subclass system of Oomycetes proposed by Dick et al. earlier (1984).

Recently, the phylogeny of peronosporomycetous organisms has been studied vigorously utilizing the evolutionary history of the mitochondrial *cox2* locus by Hudspeth and coworkers (Cook et al. 2001; Hudspeth et al. 2000, 2003). Cook et al. (2001) analyzed the *cox2* genes of three terrestrial and three marine species of the genus *Lagenidium*. The revealed phylogenetic relationships suggested that all the species examined were included in the Peronosporomycetidae clade. These authors also analyzed the same genes of four haliphthoracean taxa and found that the family was polyphyletic and that *Haliphthoros* and *Halocrusticida* formed a sister clade with the clade of the subclasses proposed by Dick et al. in 1984. Their studies strongly suggested that molecular analyses for endoparasitic peronosporomycetous organisms are important in understanding the whole phylogeny of the Peronosporomycetes.

The genus *Haptoglossa* was erected by Drechsler based on a single species, *H. heterospora* (Drechsler 1940) (Fig. 1). The genus consists of obligate endoparasites of nematodes or rotifers inhabiting in soil or litter. Eleven holocarpic species have been described to date. The genus is characterized by production of unique infection cells (gun cells) that physically rupture the host cuticle at infection. The species are divided into two groups by patterns of sporogenesis, whether aplanosporic or zoosporic. Aplanosporic species form infection cells by two or three different ways whereas zoosporic species produce the cells by one way only (Fig. 2).

The type species is aplanosporic, but Drechsler (1940) assumed the species to be a member of the Peronosporomycetes because its thalli were similar to those of members of genera *Ectrogella* and *Aphanomycopsis*. The first zoosporic species, *H. zoospora*, was found in 1973 by Davidson and Barron, who supported the opinion that the genus was placed in the Peronosporomycetes from the results of

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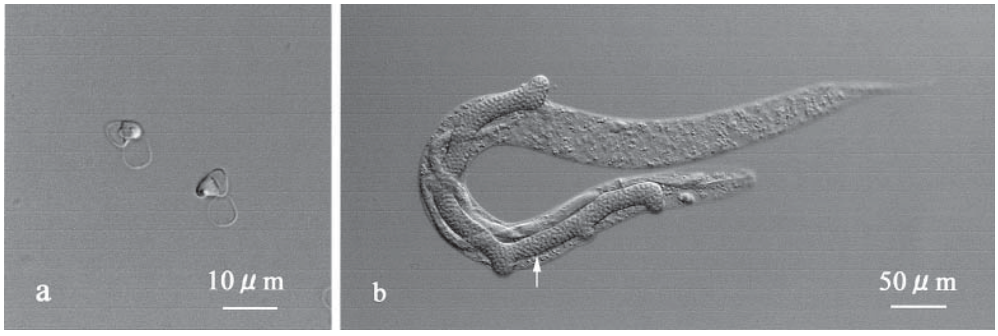

Fig. 1. Characteristic structures of *Haptoglossa heterospora*. **a** Two infection cells still attached to empty aplanospores. **b** A single holocarpic thallus (*arrow*) with exit papillae within a dead nematode body

type	sporogenesis	infection cell formation	species
zoospora type	zoospores only	zoospores encyst and produce uniform infection cells	*H. zoospora, H. mirabilis, H. humicola, H. intermedia, H. dickii, H. northumbrica,* ***Haptoglossa* sp. 2**
heterospora type	two different sized aplanospores	each aplanospore form a morphologically distinct infection cell	*H. heterospora, H. heteromorpha,* ***Haptoglossa* sp. 1**
elegans type	two different sized aplanospores	1. large aplanospores produce two morphologically distinct infection cells 2. small aplanospores produce single infection cells	*H. elegans*
polymorpha type	two different sized aplanospores	1. large aplanospores produce single type infection cells 2. small aplanospores produce two morphologically distinct infection cells	*H. polymorpha*
erumpens type	single sized aplanospores	aplanospores produce two morphologically distinct infection cells	*H. erumpens*
?	single sized aplanospores	aplanospores produce uniform infection cells	***Haptoglossa* sp. 3**

Fig. 2. Development pattern of infection cell in *Haptoglossa* spp.

observation of zoospores with two flagella (Davidson and Barron 1973).

Other opinions, however, have existed about the systematic position of the genus *Haptoglossa*. Robb and Lee (1986a,b) suggested that the genus was related to plant parasitic plasmodiophorids based on the similarity of the infection mode, using infection cells with a similar specialized structure. Beakes and Glockling (1998) observed the anterior flagella of zoospores of *H. dickii* by transmission electron microscopy and found that the flagella have no tripartite tubular hairs. Then, they suggested that there are some relationships between *Haptoglossa* and plasmodiophorids. From these reports, Dick (2001) regarded the members of *Haptoglossa* as plasmodiophorids and classified the genus to his new class, Plasmodiophoromycetes, in the subphylum Peronosporomycotina.

Recent comprehensive studies on the molecular phylogeny of Eukaryota, however, do not support the opinion just described. Cavalier-Smith (1996/7, 1998) and Cavalier-Smith and Chao (1996/7) revealed that plasmodiphorids and peronosporomycetous organisms are phylogenetically different groups and that the former is classified into the class Phtomyxea within the phylum Cercozoa. Glockling and Beakes (2001) suggested that *Haptoglossa* is either within or close to the Peronosporomycetes on the basis of their later ultrastructural observations of zoospores.

In this study, we attempted to reveal the phylogenetic position of the genus *Haptoglossa* using the mitochondrial cytochrome *c* oxidase subunit 2 (COII) amino acid sequences. We focused on the monophyly of *Haptoglossa*, the molecular phylogenetic relationships between *Haptoglossa* spp. and other groups of Peronosporomycetes, and on find-

Table 1. *Haptoglossa* isolates analyzed in this study

Species	Sampling sites	Substratum	Spore type	Spore release apparatus	Accession number	Strain number
Haptoglossa heterospora Drechsler A	Tokyo	Forest soil	Aplanosporic	Papilla	AB253780	TK01
Haptoglossa heterospora Drechsler B	Nagano	Farmyard soil	Aplanosporic	Papilla	AB253781	NA01
Haptoglossa heterospora Drechsler C	Tokyo	Soil	Aplanosporic	Papilla	AB253782	TK02
Haptoglossa heterospora Drechsler D	Tokyo	Decayed grass	Aplanosporic	Papilla	AB253783	TK03
Haptoglossa sp. 1	Kagoshima	Litter	Aplanosporic	Short exit tubes with a vesicle	AB253784	KG01
Haptoglossa sp. 2	Shizuoka	Plant debris	Zoosporic	Short exit tubes	AB253786	SZ01
Haptoglossa sp. 3	Niigata	Pine litter	Aplanosporic	Short exit tubes	AB253785	NI01

ing some phylogenetic characters reflecting the interspecific relationships within the genus.

Materials and methods

Haptoglossa species were isolated by a modified baited plate method (Barron 1977). About 10 ml of heavy water suspension of live nematodes, *Caenorhabditis elegans*, and a pinch of substratum such as soil or litter were added to the surface of 2% water agar plates. The plates were incubated on a laboratory bench and observed regularly to find the nematodes infected with *Haptoglossa* species. The infected nematodes were transferred to new water agar plates containing live nematodes with pipettes or fine needles. *Haptoglossa* isolates were maintained by three-member culture (*Escherichia coli*, *C. elegans*, *Haptoglossa* spp.).

Seven isolates were established and used in this study (Table 1). Four of these were identified as *H. heterospora* (see Fig. 1), but the other three isolates did not fully agreed with any known species (Table 1, Fig. 2) in important characteristics. It is likely that they are undescribed *Haptoglossa* species. Therefore, they are indicated as sp. 1, sp. 2, and sp. 3, respectively, in this article.

Amplification of DNA

DNA fragment was amplified by a method modified from the direct polymerase chain reaction (PCR) method developed by Suyama et al. (1996) for pollen DNA. The thalli of *Haptoglossa* that were included in the dead host body were picked up with 24 µl distilled water and added to a PCR tube with a micropipette. The PCR tube that contained thalli was subjected to five cycles of freezing (liquid nitrogen) and thawing (65°C in hot water bath) to break the cell wall of the thalli. PCR was performed using a HotStarTaq Master Mix (Qiagen, Hilden, Germany). Each PCR tube contained a total 50 µl mixture (24 µl distilled water with thalli, 25 µl master mix, and 0.5 µl each primer; final, 0.25 µM). The Peronosporomycetes-specific primers from total DNA were as previously described (Hudspeth et al. 2000). PCR conditions for amplification were performed in a GenAmp PCR system 2400 (Perkin Elmer): an initial denaturation of 95°C for 15 min is followed by 45 PCR cycles consisting of 94°C for 20 s and 58°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

Direct sequencing of PCR products was done in an Applied Biosystems 377 DNA sequencer (Perkin Elmer) using a PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's protocol. The Peronosporomycetes-specific primers were used for sequencing in both directions. The sequenced DNA was purified with a DyeEx Spin Kit (Qiagen) according to the manufacturer's instructions.

Alignment and phylogenetic analysis

The sequence data management and phylogenetic analysis BioEdit program (Hall 1999) was used for preliminary multiple alignments of both nucleotide and amino acid sequences. Final alignments were manually adjusted to ensure that codon alignments were maintained. Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. In all analyses, we estimated phylogenetic relationships based on the COII amino acid sequence. Both neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-parsimony (MP) analyses were carried out using PAUP* 4.0b10 (Swofford 2003). Maximum-parsimony trees were generated using the heuristic search option with tree-bisection-reconnection (TBR) branch swapping. In the maximum-likelihood (ML) analysis, an initial tree search was done by applying proml in PHYLIP 3.65 (Felsenstein 2005) with the JTT model (Jones et al. 1992) for the amino acid substitution process, assuming homogeneous rates across sites. Based on the best tree obtained, an Γ-shaped parameter (α) of the discrete Γ-distribution with four categories that approximates sites was estimated by codeml in PAML 3.15 (Yang 1997). By using the α value, a further tree search with the JTT + Γ model with four site rate categories was done by proml in PHYLIP. To estimate clade support, the bootstrap procedure of Felsenstein (1985) was employed with 1000 replicates in MP and NJ analyses and 100 replicates in ML analysis.

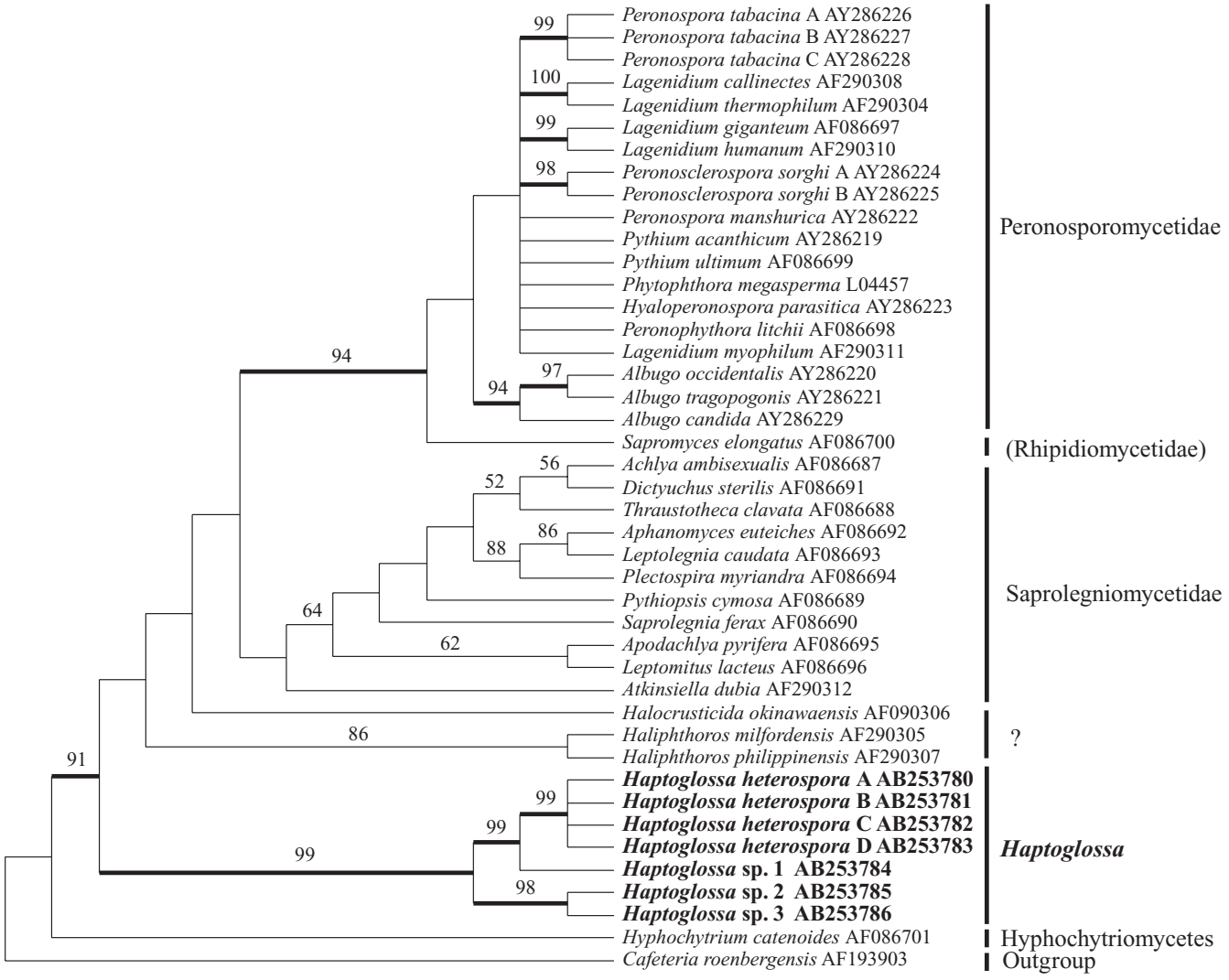

Fig. 3. Phylogenetic positions of *Haptoglossa* species among the Peronosporomycetes inferred from the maximum-parsimony (MP) analysis of COII sequences. Bootstrap values above 50% from 1000 replicates are indicated for corresponding branches. Branches significantly supported by bootstrap value above 90% are shown with *thick lines*

Results

The mitochondrial COII amino acid data set consisted of 184 aligned sites, which was deduced from the *cox2* nucleotide data set that consisted of 552 aligned sites exclusive of PCR primer and indel regions. Ninety-four sites were phylogenetically informative by parsimony criteria. The 50% majority rule consensus of six MP trees found is shown in Fig. 3.

In the MP tree (Fig. 3), all known peronosporomycetes taxa and *Haptoglossa* spp. analyzed formed a clade that was strongly supported by a high bootstrap value (91%). The clade was divided into four major groups: the *Haptoglossa* clade, *Haliphthoros* and *Halocrusticida* group, Peronosporomycetidae clade, and Saprolegniomycetidae clade. The *Haliphthoros* and *Halocrusticida* group is paraphyletic in Fig. 3, but they formed a single clade in other analysis, as shown in Figs. 4 and 5. Among the four groups, the *Haptoglossa* clade was basal and appeared to be the sister-group of the clade of the remaining Peronosporomycetes taxa.

The *Haptoglossa* clade was divided into two subclades. The clade of *H. heterospora* and sp. 1 consists of aplanosporic species, and the other included both zoosporic (sp. 2) and aplanosporic species (sp. 3). Both clades were supported by very high bootstrap values (99% and 98%, respectively).

Trees inferred by NJ (Fig. 4) and ML (Fig. 5) analyses showed similar topologies to the MP tree (Fig. 3). All known peronosporomycetes taxa and *Haptoglossa* spp. analyzed formed a clade. The clade was supported by NJ analysis (98%), but it was not supported by ML analysis (<50%). The *Haptoglossa* clade in NJ and ML analyses was divided into two subclades, and the members of individual clades were the same in the MP tree.

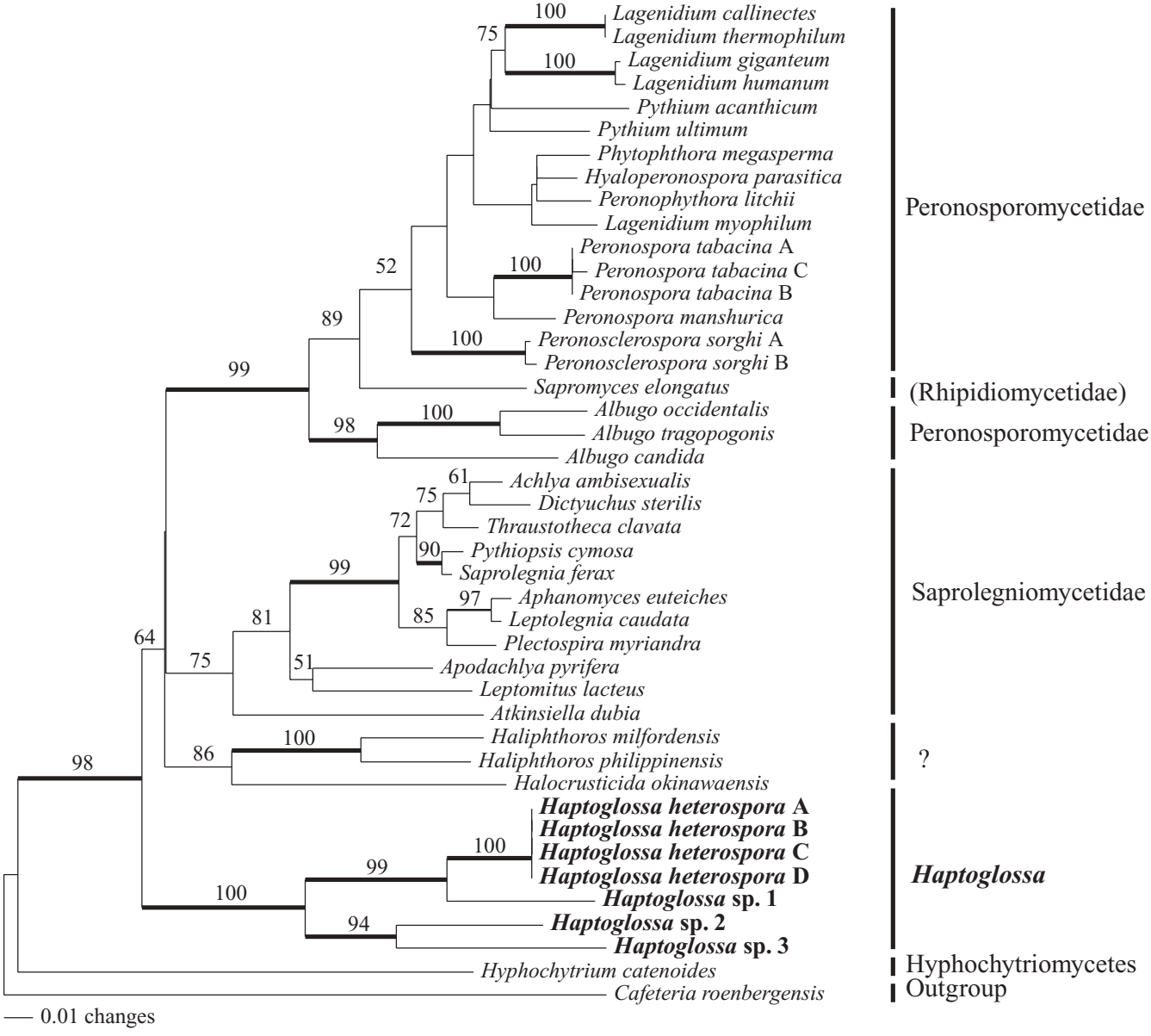

Fig. 4. Phylogenetic positions of *Haptoglossa* species among the Peronosporomycetes inferred from the neighbor-joining (NJ) analysis of COII sequences. Bootstrap values above 50% from 1000 replicates are indicated for corresponding branches. Branches significantly supported by bootstrap value above 90% are shown with *thick lines*

Discussion

This is the first molecular phylogenetic study on the nematode parasitic genus *Haptoglossa*. The results strongly indicate the monophyly of the genus. Seven isolates consisting of four *H. heterospora* and three distinct unidentified isolates formed a single clade supported by high bootstrap values (>86%) in all analyses. Glockling and Beakes (2000) suggested that the genus belongs to a lineage of its own based on ultrastructural study of zoospores and infection cells. Our results in this study supported their opinion.

The trees indicate that the genus *Haptoglossa* is close to the Peronosporomycetes because the *Haptoglossa* clade is included in the Peronosporomycetes clade (see Figs. 3–5). The *Haptoglossa* clade was positioned basal in the Peronosporomycetes clade in all trees and formed a sister-clade with the clade of other peronosporomycetous organisms. The inclusion of the *Haptoglossa* clade within the Peronosporomycetes clade was supported by a high bootstrap value in MP (91%) and NJ analyses (98%), but this value was less than 50% in ML analysis. The results of the ML analysis suggested that the *Haptoglossa* clade belonging to the Peronosporomycetes is not conclusive. We need ultrastructural data to ascertain that the genus *Haptoglossa* is included in the Peronosporomycetes.

It has been clarified through ultrastructural studies of zoospores endoparasitic genera *Haptoglossa* and *Haliphthoros* have cytological features in common with peronosporomycetidous and saprolegniomycetidous organisms

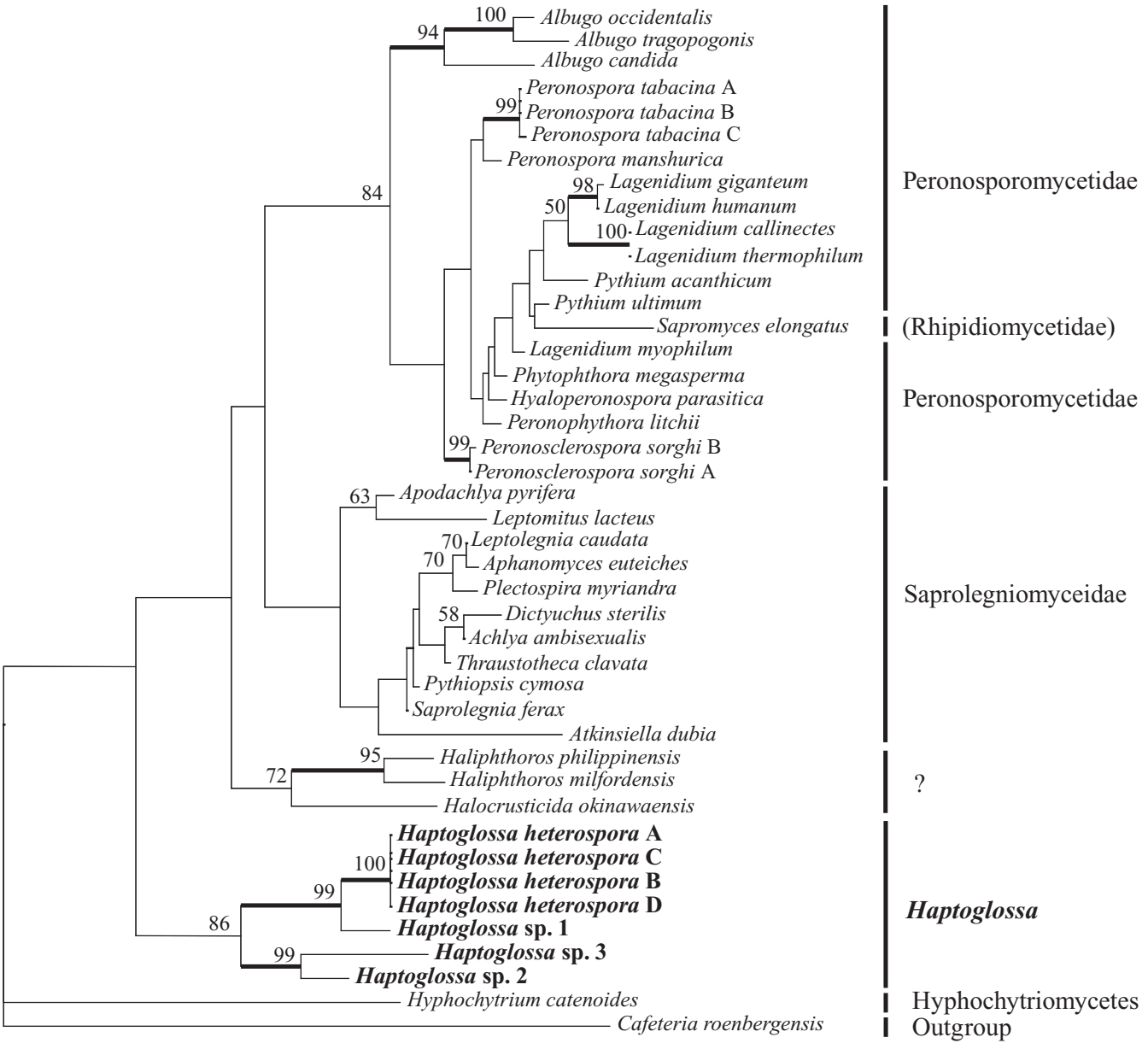

Fig. 5. Phylogenetic positions of *Haptoglossa* species among the Peronosporomycetes inferred from the maximum likelihood (ML) analysis of COII sequences. Bootstrap values above 50% from 100 replicates are indicated for corresponding branches. Branches significantly supported by bootstrap value above 90% are shown with *thick lines*

(Glockling and Beakes 2000, 2001; Overton et al. 1983). However, sexual reproduction has not been found in any such genus. In addition, Beakes and Glockling (1998) reported that *H. dickii* has no tripartite tubular hairs on the anterior flagella of zoospores. Considering our results with the information, these endoparasitic genera appear to be lying close to the Peronosporomycetes sensu stricto, which consist of two subclasses as proposed by Dick et al. (1984). On the other hand, there is still room for doubt in the phylogenetic relationships among the genus *Haptoglossa*, the *Haliphthoros*/*Halocrusticida* group, and the Peronosporomycetes sensu stricto.

The pattern of the life cycle of individual species is a candidate of the character in which the interspecies relationship is reflected. The patterns of the life cycle in *Haptoglossa* species are various and complicated, so that they have been used as the major character in species delimitation. As shown in Fig. 2, *Haptoglossa* species are divided into two subgroups by their life cycles. One is the group of zoosporic species, in which the endozoic thalli become zoosporangia from which zoospores are released and soon encyst. Cysts germinate to form infection cells and infect new hosts. The other group is the aplanosporic species group: the endozoic thalli become sporangia in this case and form one or two kinds of spores by size. Patterns of infection cell formation are varied in this group (see Fig. 2). Therefore, the species delimitations of zoosporic species are mainly based on characters other than the pattern of infec-

tion cell formation, such as morphology of infection cells, whereas those of aplanosporic species are mainly distinguished by using the pattern of infection cell formation and morphology of infection cells. Although various life cycles can be observed within the genus, it would be appropriate to consider that zoosporic species are more primitive.

In this study, zoosporic *Haptoglossa* sp. 2 formed a clade with aplanosporic *Haptoglossa* sp. 3, and aplanosporic *H. heterospora* and *Haptoglossa* sp. 1 formed another clade. The former two species show a similar pattern of infection cell formation, a single type of infection cell causing a kind of sporangiospore. This observation suggests that the pattern of sporogenesis, whether zoosporic or aplanosporic, is not an important character when discussing the intrageneric phylogeny of the genus *Haptoglossa*. Rather, it seems to be important whether a large or a small spore is produced, looking at Figs. 3, 4, and 5. However, only one-half the number of types was analyzed in this study (see Fig. 2). More isolates and analyses are needed to discuss the intrageneric phylogeny of *Haptoglossa*.

In this article, we first revealed the monophyly of the genus *Haptoglossa* and also clarified that the genus is included in the Peronosporomycetes clade using COII amino acid sequences. The seven isolates formed two well-supported subclades, both of which include aplanosporic species. The pattern of sporogenesis would seem to be less important than that of infection cell formation when discussing the interspecies relationships, but to reveal the phylogenetic relationships among species, more comprehensive molecular and ultrastructural studies are required.

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