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International Journal for Parasitology 35 (2005) 793–801
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Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal gastropod, *Batillaria cumingi* (Crosse)☆

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Received 29 November 2004; received in revised form 14 February 2005; accepted 22 February 2005

Abstract

Cryptic species of the digeneans, *Cercaria batillariae* (Heterophyidae) and an undescribed philophthalmid, were detected using polymerase chain reaction-based restriction fragment-length polymorphism methodology and sequence analyses. These digeneans were all collected from the same species of gastropod first intermediate host, *Batillaria cumingi* (=*Batillaria attramentaria*). The mitochondrial *cytochrome oxidase subunit 1* gene (approximately 800 bp) and *nuclear internal transcribed spacer 1* gene (approximately 400 bp) were used for species level discrimination. Restriction fragment-length polymorphism analyses of *cytochrome oxidase subunit 1* gene showed that *C. batillariae* included 10 distinguishable fragment patterns, and the philophthalmid included five patterns. On the basis of subsequent sequence analyses, the restriction fragment length polymorphism patterns of *C. batillariae* were grouped into eight phylogenetically distinct lineages and those of the philophthalmid into three phylogenetically distinct lineages. There was no evidence of gene flow among the different lineages due to the lack of heterozygosity within the observed *internal transcribed spacer 1* gene fragment patterns. This suggests that all of these lineages are different species. Most of these species were widespread, but some exhibited restricted geographic distributions. We discuss the implications of these findings for host specificity of these trematodes. These results demonstrate the utility of genetic analysis to distinguish species of morphologically similar trematodes. Hence, trematode species diversity may often be underestimated when species identifications are limited to morphological features.

Keywords: Cryptic species; *Batillaria cumingi*; *Cercaria batillariae*; Restriction fragment-length polymorphism; Cytochrome oxidase subunit 1; Internal transcribed spacer 1

1. Introduction

Cryptic species (or 'sibling species') are common in all major taxa (Knowlton, 1993). Although such species are genetically distinct from each other, they are morphologically very similar. Parasitic taxa, due to their restricted morphology, appear to often manifest cryptic species (e.g. McManus and Bowles, 1996; Hung et al., 1999; Huspeni, 2000. A molecular genetic analysis of host specificity, continental geography, and recruitment dynamics of a larval trematode in a salt marsh snail, PhD Dissertation. University of California, Santa Barbara; Macnish et al., 2002; Donald et al., 2004). This is unfortunate since the accurate identification of species is crucial for virtually any ecological or evolutionary investigation. Cryptic parasite species may differ in traits important to host-parasite interactions, such as host susceptibility (e.g. Reversat et al., 1989), pathogenesis (e.g. Homan and Mank, 2001; Haque et al., 2003), and epidemiology (e.g. Murrell and Pozio, 2000).

☆Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AY626457-AY262550.

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doi:10.1016/j.ijpara.2005.02.014

Molecular genetic methods can distinguish otherwise cryptic species (Avise, 2004). Molecular methods are often used to identify species of bacteria and protozoans lacking major distinguishing morphologies (e.g. Perkins, 2000; Jenga et al., 2001). Molecular techniques can also be applied to helminths to enable identification of cryptic species and to reveal their phylogenetic relationships (e.g. Bowles and McManus, 1993; Bowles et al., 1995; Morgan and Blair, 1998; Hung et al., 1999; Huspeni, 2000. A molecular genetic analysis of host specificity, continental geography, and recruitment dynamics of a larval trematode in a salt marsh snail. Ph.D. Dissertation. University of California, Santa Barbara; Bell et al., 2001; Macnish et al., 2002; Olson et al., 2003; Donald et al., 2004).

A comprehensive molecular genetic analysis of an assemblage of parasites infecting a single host species over a wide geographic range will help define the role of cryptic species in host-parasite interactions. The rich assemblage of larval trematodes in the Northeast Asian mud snail, *Batillaria cumingi* (=*Batillaria attramentaria*), provides an excellent system to examine parasites within a single host species. This host snail has a broad geographic distribution throughout eastern Asia (Hasegawa, 2000) and is very common on mud flats of Japan (Adachi and Wada, 1999). Further, *B. cumingi* has been introduced to the west coast of North America (Byers, 1999). This snail is infected by at least nine morphologically recognized species of digenean trematodes (Ito, 1957; Shimura and Ito, 1980; Rybakov and Lukomskaya, 1988; Harada, 1989; Harada and Suguri, 1989; Hechinger, unpublished data). All the described species are in taxa that use shorebirds as final hosts and a variety of invertebrates and fishes as second intermediate hosts.

Here, by examining genetic variation in two of trematode morphospecies, we identify a previously unrecognized substantial diversity of cryptic species of trematodes infecting a single host snail species. We also examine the phylogenetic relationships between the cryptic species groups and report on their geographic distributions in Japan. One of the species we studied is the larval heterophyid, *Cercaria batillariae* Shimura and Ito, 1980. Members of the Heterophyidae use fishes as second intermediate hosts (Yamaguti, 1975; Schell, 1985). The other species is an unnamed philophthalmid. Species of the Philophthalmidae (such as *Cloacitrema* spp. and *Parorchis* spp.) typically encyst on hard substrates. For both species that we studied, the life cycle is completed via trophic transmission of second intermediate hosts by bird final hosts.

2. Materials and methods

2.1. Study sites and sample collection

Samples of *B. cumingi* were collected from 15 muddy shore and estuary sites in Japan (Fig. 1 and Table 1) on daytime low tides between September and November 2003. Snails were identified following Adachi and Wada (1998). Snails were dissected under a stereo-microscope and trematode species were identified based on previous work on cercariae from *B. cumingi* (Ito, 1957; Shimura and Ito, 1980; Harada, 1989; Harada and Suguri, 1989; Hechinger, unpublished data). The parasites were fixed with 70% ethanol and stored at −20 °C for molecular analysis. DNA of the two most prevalent species, *C. batillariae*

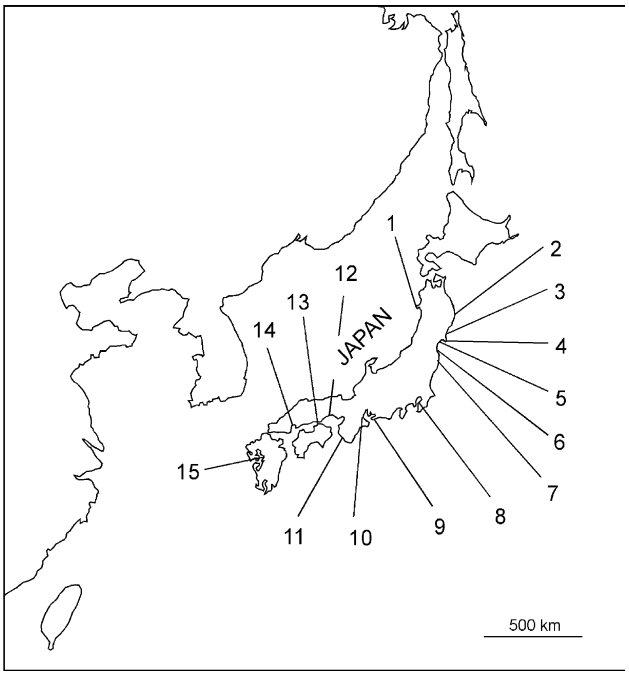

Fig. 1. Map of Japan Islands with sample localities. Details of each locality are shown in Table 1.

Table 1
Collection localities and numbers of individual parasites used in restriction fragment-length polymorphism and sequence analyses of the cytochrome oxidase subunit 1 and internal transcribed spacer 1 genes. Numbers to the left of the slash are individuals of *Cercaria batillariae* and the numbers to the right are individuals of the philophthalmid

Site	Name	RFLP (*CO1*)	RFLP (*ITS1*)	Sequence analysis (*CO1* and *ITS1*)
1	Toga Bay	17/0	–	4/0
2	Yamada Bay	12/1	12/1	10/1
3	Nagazura Bay	10/0	–	5/0
4	Mangoku Bay	39/0	39/0	5/0
5	Matsushima Bay	28/0	–	4/0
6	Torinoumi	37/0	37/0	6/0
7	Matsukawa Bay	36/0	36/0	8/0
8	Obitsu River	18/6	–	5/2
9	Shiogawa	0/5	–	0/2
10	Kumode River	44/1	44/1	11/1
11	Tanabe Bay	36/0	–	3/0
12	Ibo River	8/34	8/34	2/5
13	Kasuga River	5/22	5/22	5/6
14	Hiroshima Bay	61/23	61/23	14/4
15	Ariakekai	3/2	–	2/2

(Heterophyidae) and the undescribed philophthalmid, was isolated using a modification of the procedure described in Doyle and Doyle (1987). We recognized the philophthalmid as a morphospecies using characters of the rediae and cercariae (such as the presence of profuse dark cystogenous glands). The larval trematodes from a single snail are assumed to be the genetically identical products resulting from the asexual reproduction of an individual trematode. A few rediae were separated from host snail tissue and homogenized in a solution of 300 ml of 2×hexadecyltrimethylammonium bromide buffer and 10 mg/ml proteinase K, incubated at 60 °C for approximately 1 h, extracted once with phenol/chloroform (v:v, 1:1) and precipitated with 2 volumes of ethanol. The DNA pellets were briefly washed in 75% ethanol, air-dried for approximately 30 min, and dissolved in 50 ml of H_2O.

2.2. DNA PCR-RFLP analysis

To discriminate genotypes, we performed RFLP analyses of the mitochondrial DNA *cytochrome oxidase subunit 1* (*CO1*) gene (~800 bp) of 447 larval trematode individuals taken from separate snails from 15 sites. To estimate levels of gene flow among individuals with different genotypes, we performed restriction fragment length polymorphism (RFLP) analyses of the nuclear DNA *internal transcribed spacer 1* (*ITS1*) gene (~400 bp) of 323 larval trematode individuals from eight sites (Table 1).

Published sequences from a range of digenean organisms *Paragonimus westermani* (GenBank accession no. AF219379), *Fasciola hepatica* (GenBank accession no. AF216697) and *Schistosoma japonicum* (GenBank accession no. AF215860) were used to develop trematode-specific primers for PCR amplification of *CO1*. Comparative analysis of these published sequences revealed identification of highly conserved t-RNA regions adjacent to the *CO1* region for the reverse primer, CO1-R trema (5′-CAACAAATCATGATGCAAAAGG-3′). As a forward primer for *CO1*, we used JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) of Bowles and McManus (1993), described previously for the studies of other parasite species. To amplify the *ITS1* gene, we used two other primers described in Bowles and McManus (1993), BD1 (5′-GTCGTAACAAGGTTCCGTA-3′) and 4S (5′-TCTAGATGCGTTCGAAGTGTCGATG-3′).

All PCR reactions were run in a 20 μl reaction mixture containing <200 ng of template DNA, 1 μl of each 2 μM primer, 1.6 μl of each 2.5 mM dNTP, 1 unit of Taq DNA polymerase (Takara rTaq™, Takara Biomedicals, Japan), 2 μl of pH 8.3 buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM $MgCl_2$), and 14–15 μl of water. PCR reactions ran for 35 cycles under the following conditions: denaturing at 94 °C for 30 s, annealing at 45 °C for 30 s, and extension at 72 °C for 60 s. The 35 cycles were preceded by an initial denaturing at 94 °C for 1 min, and followed by a final extension of 72 °C for 7 min.

Five micro litre of the unpurified PCR products were digested for 10 h by the four-base cutting restriction enzyme, endonuclease *Mse* 1 (New England Biolabs). The restricted fragments were separated using electrophoresis in 4% Tris-acetate EDTA (TAE) agarose gels at 50 V for 4 h. Bands were visualized by staining with ethidium bromide. Genotypes were identified based on the fragment patterns and scored individually for each DNA region.

2.3. DNA sequencing and estimation of phylogenetic relationships

An individual possessing a particular *CO1* RFLP pattern was selected from each sample and its *CO1* gene was sequenced. The *ITS1* gene was also sequenced for the individual possessing a particular CO1 RFLP pattern. A total of 107 individuals were used for the sequence analysis (Table 1). The PCR products of the samples were purified and sequenced using an automated sequencer (HITACHI SQ5500). Sequences were aligned using CLUSTALX (Thompson et al., 1997) using default parameters and we manually corrected, by eye, poorly aligned regions. All insertions and deletions (indels) were removed from the alignment before phylogenetic analysis. Phylogenetic trees were constructed for both the *CO1* and *ITS1* genes, using the software package of PAUP* (Swofford, 2001. PAUP*: Phylogenetic Analysis Using Parsimony (and other methods), Version 4.0b10. Sinauer, Sunderland, MA). We generated trees using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) algorithms. We based the NJ analyses on Kimura's two-parameter distances because more complicated models may sometimes yield inconsistent results in NJ analyses when a large number of sequences (operational taxonomic units) are compared (Li, 1997; Nei and Kumar, 2000). Maximum parsimony analysis was conducted using heuristic searches. For both NJ and MP trees, support values for internal nodes were generated by bootstrap resampling with 1000 replicates. Groups of haplotypes were identified on the basis of phylogenetic relationships between the haplotypes. An ML analysis was also performed to more thoroughly investigate the relationships among the groups by taking a subset of sequences from each terminal clade. The best models for ML analysis (HKY+I+G for CO1 and HKY+ G for ITS1 sequence) were selected by the MODELTEST program (Posada and Crandall, 1998). The parameters for the model were also selected by Modeltest. One hundred replications for the likelihood bootstrap were calculated with a heuristic search. *Paragonimus westermani* was chosen as the outgroup (sequence data from GenBank accession no. AF219379 for *CO1*, and GenBank accession no. AF040934 for *ITS1*) based on a higher level phylogenetic study of the Digenea (Olson et al., 2003).

2.4. Estimation of species richness

We sought to determine whether many additional cryptic species might exist undiscovered by our study. We used two methods to do this for *C. batillariae* and the philophthalmid. First, we inspected the shapes of both sampling-site-based and individual-based randomized species accumulation curves (Gotelli and Colwell, 2001). These curves indicate the average rate of discovery of new cryptic species for varying sample effort (numbers of sites or individuals sampled). If such a curve levels-out, it suggests that few more species will be discovered by additional sampling. For these curves, there is no dependency on sample order, because sample order is randomly chosen each iteration. To generate sampling-site-based species accumulation curves, we plotted the average number of cryptic species observed for varying numbers of sites randomly sampled from each species dataset (1000 iterations, sampling without replacement). We generated individual-based species accumulation curves similarly, but randomly sampled individual parasites from each species dataset. Randomized sampling-site-based curves would tend to have greater variance than individual-based curves, since sampling effort (the number of individuals sampled) varied among sites. The second way we assessed the existence of potentially undiscovered cryptic species was to compare our observed total cryptic species richness to extrapolated estimates of total species richness. We used a non-iterative bootstrap species richness estimator (Smith and Van Belle, 1984) to extrapolate total species richness on both site-based and individual-based data. Species richness estimates and curves were computed using the computer program, ESTIMATES (Colwell, 2000. Estimates: Statistical Estimation of Species Richness and Shared Species from Samples. Version 6.0b1. User's Guide and application published at: http://purl.oclc.org/estimates).

3. Results

Of the eight trematode species reported from *B. cumingi* in Japan (Shimura and Ito, 1980; Harada, 1989; Harada and Suguri, 1989; Hechinger, unpublished data), we found *C. batillariae* (72.4% of the observed trematodes) and the philophthalmid (18.2%) to be the most prevalent. Mixed infections were rarely observed (0.4% of all infections). Other species contributed a small proportion of total trematodes recovered (Fig. 2).

The RFLP patterns of the CO1 gene of *C. batillariae* were discriminated into 10 types (HA to HJ) and those of the philophthalmid were discriminated into five types (PA to PE) (Table 2). The categorization of individuals based on RFLP patterns was mostly consistent using either *CO1* genes or the *ITS1* genes (Table 2). The only discrepancies are readily explained by lower rates of evolution of *ITS1* gene compared to *CO1* (Morgan and Blair, 1998; Bell et al., 2001) and by using the nucleotide sequence derived phylogenies for

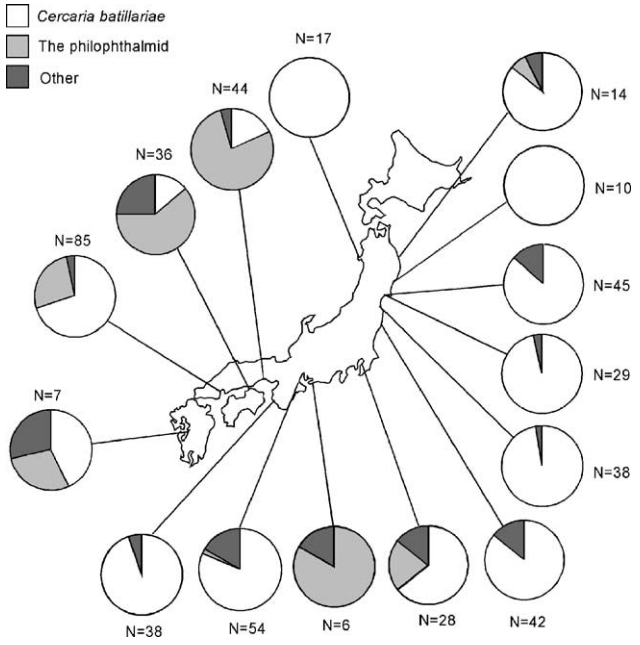

Fig. 2. Distributions of the morphologically recognized trematode species found in *Batillaria cumingi*.

interpretation (see below). There was no evidence of gene flow among the different RFLP types due to the observed absence of heterozygosity at the *ITS1* region.

Sequence analysis of the *CO1* gene revealed three to 12 haplotypes for each RFLP type of *C. batillariae* and one to five haplotypes for each RFLP type of the philophthalmid. In total, 70 CO1 haplotypes were found in *C. batillariae*, and 13 in the philophthalmid (GenBank accession numbers AY626457-AY626539). The inferred

Table 2
Relationships between the restriction fragment length polymorphism types found in cytochrome oxidase subunit 1 (HA–HJ for *Cercaria batillariae* and PA–PE for the philophthalmid) and internal transcribed spacer 1 genes (H1–H5 for *C. batillariae* and P1, P2 for the philophthalmid)

Species	RFLP types (*CO1*)	RFLP types (*ITS1*)	Lineages
Cercaria batillariae	HA	H1	HL1
	HB	H2	HL6
	HC	H2	HL6
	HD	H3	HL5
	HE	H3	HL5
	HF	H4	HL3
	HG	H5	HL2
	HH	H3	HL7
	HI	H3	HL4
	HJ	H3	HL8
The philophthalmid	PA	P1	PL1
	PB	P1	PL1
	PC	P1	PL1
	PD	P2	PL2
	PE	P1	PL3

The lineages that involve each restriction fragment length polymorphism type were also shown (HL1–HL8 for *C. batillariae* and PL1–PL3 for the philophthalmid).

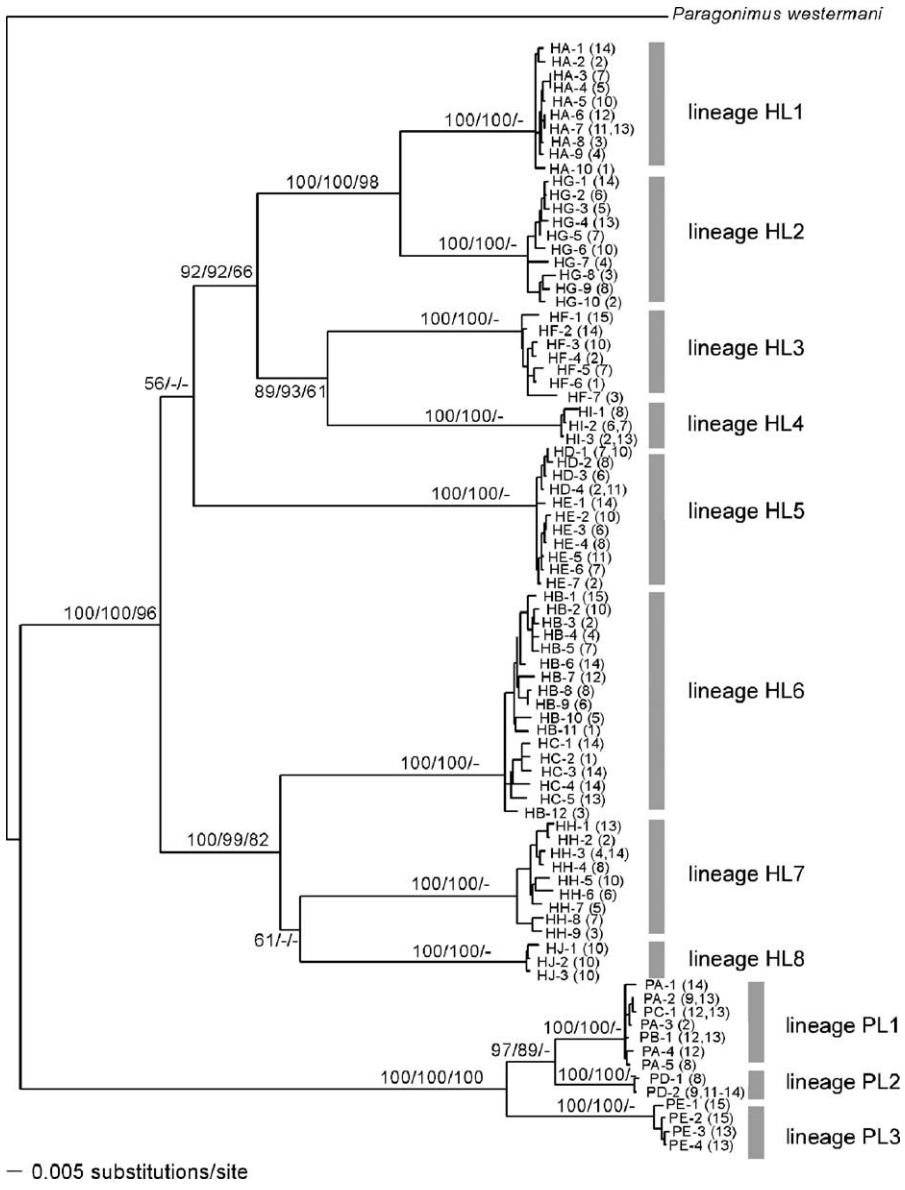

Fig. 3. Neighbor-joining tree based on mitochondrial *cytochrome oxidase subunit 1* sequences (792 bp). Trees generated using maximum parsimony and maximum likelihood had the same topology. Numbers above nodes are the bootstrap support for that clade from the different analyses in order (values <50% not shown): NJ/MP/ML. HL1–HL8 indicate lineages of *Cercaria batillariae*, and PL1–PL3 indicate lineages of the philophthalmid. The numbers in parentheses indicate the population locality (Fig. 1 and Table 1) where each haplotype was found.

phylogenetic relationships among the *CO1* haplotypes are shown in Fig. 3. The CO1 haplotype phylogenies estimated by the ML algorithm and MP algorithm were consistent with phylogeny generated by the NJ algorithm. In total, eight monophyletic CO1 lineages (HL1–HL8, see Fig. 3) were identified for *C. batillariae* and three monophyletic lineages (PL1–PL3, see Fig. 3) were identified for the philophthalmid. Relationships of these 11 monophyletic clades were supported by high bootstrap values (100%).

Individuals with the same *CO1* RFLP type possessed very similar *CO1* sequences (sequence divergences were from 0% to 2.0%) and always belonged to the same *CO1* haplotype clade. Sometimes, a monophyletic *CO1* lineage was characterized by a single *CO1* RFLP type. Other times, a monophyletic *CO1* lineage was characterized by several *CO1* RFLP types. The different *CO1* RFLP types, HB/HC and HD/HE of *C. batillariae* were closely related phylogenetically, and PA, PC and PB of the philophthalmid were also closely related (Fig. 3). As mentioned above, the few discrepancies between categorizing individuals based on *CO1* or *ITS1* RFLP types are explainable when mapped onto this sequence-based phylogeny. For instance, for *C. batillariae*, *ITS1* RFLP type H3 is apparently a plesiomorphic (ancestral) trait observed in lineages HL4, HL5, HL7 and HL8 (Table 2 and Fig. 3).

High levels of sequence divergence were found among the different lineages. Sequence divergences among the CO1 lineages ranged from 9.2 to 28.1% for *C. batillariae*

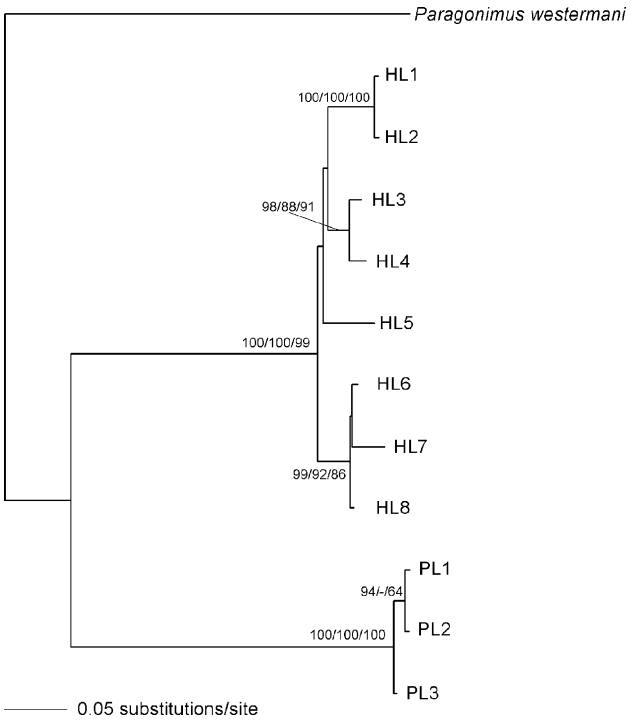

Fig. 4. Neighbor-joining tree of the 11 lineages based on nuclear *internal transcribed spacer 1* sequences (436 bp). Trees generated using maximum parsimony and maximum likelihood had the same topology. Each lineage was identified in the *CO1* tree (see Fig. 3). Numbers above nodes are the bootstrap support for that clade from the different analyses (NJ/MP/ML, values <50% not shown).

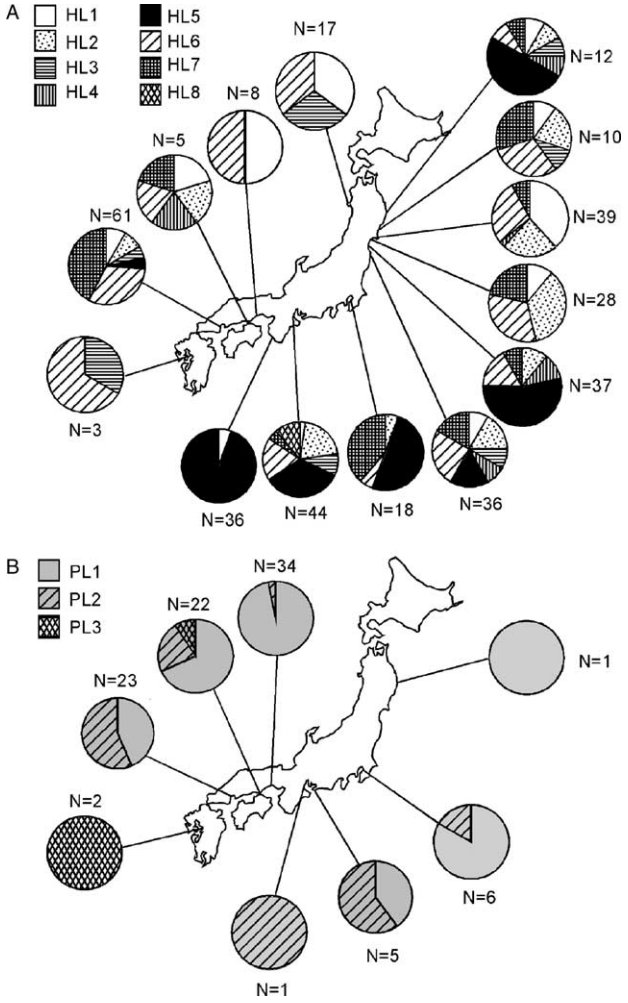

Fig. 5. Distribution of the genetically distinct lineages of *Cercaria batillariae* (A) and the philophthalmid (B).

and 5.0 to 10.3% for the philophthalmid. As is the case with other studies (Morgan and Blair, 1998; Bell et al., 2001), sequence divergences of *ITS1* among the lineages were much lower than that of *CO1* (1.2–11.5% for *C. batillariae* and 0.6–1.4% for the philophthalmid). The phylogenetic relationships among these lineages were mostly consistent between the *CO1* and *ITS1* genes (Figs. 3 and 4) (GenBank accession numbers AY626540-AY626550). The only observed difference between the mitochondrial and nuclear gene phylogenies is the minor change in the sister-group relationship of the HL7 lineage, which has low bootstrap support in each phylogeny (Figs. 3 and 4).

The lineages of *C. batillariae* and the philophthalmid varied in geographic distribution (Fig. 5). The lineages HL1 and HL6 were found at most of the sampling sites. Other lineages were rare or absent in many samples. The lineage HL5 was recovered from populations in the middle to northern Pacific coast of Japan (Torinoumi, Obitsu River, Kumode River, Tanabe Bay). The distributions of the lineages HL8 and PL3 were very restricted; HL8 was found only in the Kumode River, and PL3 only in the Kasuga River and at Ariakekai. No clear geographic pattern was detected for the phylogenies of the lineages (Fig. 5).

The randomized site-based and individual-based species accumulation curves (Fig. 6) suggest that our sampling effort was sufficient to detect all cryptic species present. For each morphospecies, the position of our sampling effort approaches the asymptote, and is well past the steeply rising portion of the curves for both site-based curves (14 sites for *C. batillariae* and eight sites for the philophthalmid) and individual-based curves (354 individuals *C. batillariae* and 94 individuals for the philophthalmid) (Fig. 6A and B). Further, the species accumulation curves have converged with the bootstrap species richness estimates. The bootstrap estimator computed total species richness as 8.35 (site-based data) and 8.01 (individual-based data) for *C. batillariae*, and 3.1 (site-based data) and 3.02 (individual-based data) for the philophthalmid.

4. Discussion

We detected eight genetically distinct lineages in *C. batillariae* and three for the philophthalmid morphospecies. There was no evidence of gene flow among these lineages, even when different lineages occurred in the same locality. Heterozygosity was absent among the most closely

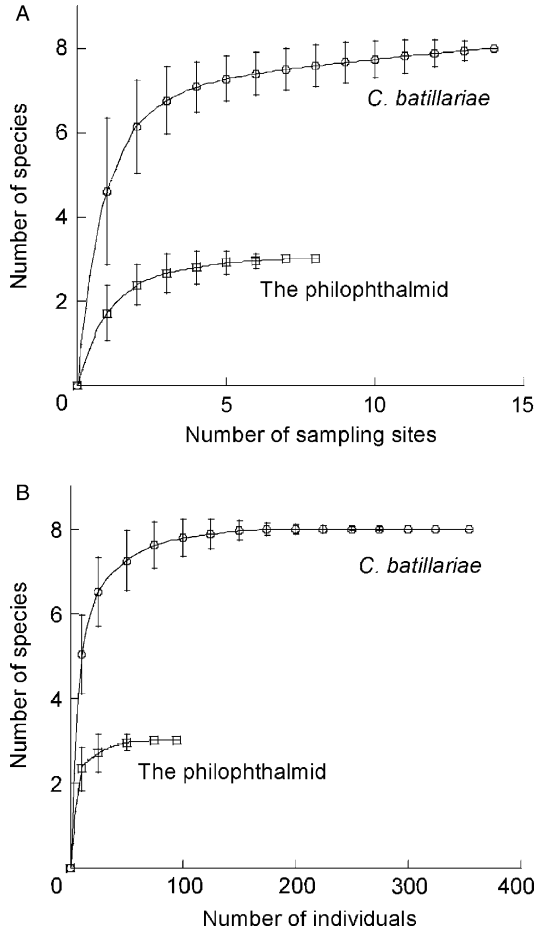

Fig. 6. Species accumulation curves of cryptic species of *Cercaria batillariae* and the philophthalmid. Curves were generated by randomly sampling 1000 times, from our datasets, varying amounts of sites (A), or individual parasites (B). The error bars represent ±1 S.D.

related lineages, even when found at the same sample sites (HL1 and HL2 in *C. batillariae,* and PL1 and PL2 in the philophthalmid). Further, the level of the sequence divergences of the *CO1* gene among these lineages within each morphospecies (9.2–28.1% for *C. batillariae*, and 5.0–10.3% for the philophthalmid) is similar to that observed in congeneric interspecific sequence divergences of the *CO1* gene (6–24%) (Bowles et al., 1995; Morgan and Blair, 1998; Bell et al., 2001). These independent lines of evidence strongly suggest that all of the 11 lineages represent different species. Hence, *C. batillariae* includes at least eight species and the philophthalmid includes at least three species.

These results expand previous work indicating that cryptic species are common among marine invertebrates (reviewed by Knowlton, 1993). For example, genetic studies of trematode cryptic species infecting the California mud snail, *Cerithidea californica*, demonstrated that cercariae with subtle or no apparent morphological differences can be readily genetically distinguished (Huspeni, 2000. A molecular genetic analysis of host specificity, continental geography, and recruitment dynamics of a larval trematode in a salt marsh snail. PhD Dissertation. University of California, Santa Barbara). Also, Donald et al. (2004) discovered three or four cryptic species within a single morphospecies in a recent molecular analysis of larval trematodes in New Zealand and Australia.

The discovery of eight cryptic species of *C. batillariae* within Japan is remarkable in that, to our knowledge, it is the highest number of cryptic species ever detected for a trematode species. Further, since the host snail, *B. cumingi,* has a geographic range extending beyond Japan, perhaps other cryptic species occur elsewhere. However, it seems improbable that additional cryptic species escaped our detection within the region we sampled, since our analysis of detected species richness as a function of sampling effort suggests that our effort was sufficient to detect most, if not all, of the cryptic species. For *C. batillariae* and the philophthalmid, not only did the species accumulation curves approach the asymptotes (Fig. 6), but they also converged with the bootstrap estimation of richness values. For the philophthalmid this conclusion must be slightly tempered due to the few individuals encountered at several locations.

Although our study was not designed to investigate how variation in life history affects genetic structure, we speculate on the biological basis for differences in the number of cryptic species within *C. batillariae* and the philophthalmid morphospecies. Fig. 6B shows that for any given number of individual parasites sampled, *C. batillariae* had greater cryptic species richness than did the philophthalmid. A priori, less genetic differentiation was predicted for the philophthalmid than for *C. batillariae*. Firstly, these two trematode species differ in second intermediate host use. Philophthalmids encyst on the surfaces of hard substrates (e.g. crab exoskeletons, bivalve shells, or plants) (Yamaguti, 1975; Schell, 1985). These ‘ectometacercariae’ will even readily encyst on artificial substrates, including the Syracuse watch glasses in which we dissected the snails. In contrast, as for most heterophyids, *C. batillariae* is more host-specific and encysts only in tissues of teleost fishes. To the extent that greater host specificity drives greater levels of speciation (Brooks and McLennan, 1993), we would expect the *C. batillariae* complex to have a greater speciation rate than would a philophthalmid. Secondly, these two trematode species likely differ in adult longevity. Adult stages of some types of trematodes, such as heterophyids, are generally short-lived (often less than 2 weeks), while adults of other types of trematodes, such as philophthalmids, may live more than 1 year (Ginetsinskaya, 1968). Since final host wetland birds will cover more distance over time, greater adult trematode longevity likely homogenizes genetic variation among local parasite populations. This is because these parasites have a greater dispersal distance, and a high dispersal rate will generally enhance gene flow (reviewed by Bohonak, 1999). Thus, we would expect less allopatric speciation to occur in the philophthalmid than in the heterophyid.

Kojima et al. (2004) found that *B. cumingi* comprised two distinctive genetic groups (with a 1.3% *CO1* sequence

divergence). These were largely allopatric, one primarily being present on the Sea of Japan coast, and the other on the Pacific coast. The Sea of Japan is the semi-enclosed sea, which is connected with the neighboring Pacific Ocean by relatively shallow and narrow straits and may have been completely isolated during glacial periods. Thus, vicariance biogeography likely explains the genetic structure of *B. cumingi*, as we would expect, considering its low dispersal ability (it has direct-developing larvae (Adachi and Wada, 1999)). Regarding the trematode, *C. batillariae*, we have one sample from the Sea of Japan and it included three of the eight cryptic species (Fig. 2). Each of these cryptic species was also present in snails belonging to the Pacific coast *B. cumingi* clade (Fig. 2). Thus, our preliminary evidence suggests that neither cryptic snail host lineages, nor geologic history, were significant barriers to at least some of the trematode lineages. This is consistent with the findings of Huspeni (Huspeni, 2000. A molecular genetic analysis of host specificity, continental geography, and recruitment dynamics of a larval trematode in a salt marsh snail, PhD Dissertation. University of California, Santa Barbara.). He investigated two cryptic species of trematodes parasitizing three species of *Cerithidea* snails over a geographic range extending along 1000 s of km of coastline and in two ocean basins. Both trematode lineages used all three host species. He attributed the wide range of the lineages to be due to the migratory behavior of some of the bird final hosts.

We also would expect that geologic history would impart less genetic structure to the trematodes we studied than to their snail host because the trematodes disperse widely in bird final hosts. However, to robustly evaluate this, more samples are needed to map parasite lineages onto host lineages, and to more precisely record the geographic distribution of the various cryptic species. Also, we emphasize that our data is spread over 11 different species. We plan to sample these species more extensively to better assess gene flow among populations within these species.

In other systems where exploration of a morphospecies has revealed unexpected cryptic diversity, the genetic species have been shown to have different host use patterns, each being more host specific than previously considered for the morphospecies (e.g., Reversat et al., 1989; Jousson et al., 2000). Morphologically-identified *C. batillariae* has been found in at least 10 fish second intermediate host species of two families in Japan, and were recovered from a variety of tissues in these fishes (Hechinger and Kuris, unpublished data). This apparent broad host specificity may be due to not recognizing cryptic species and may be resolved when the cryptic species can be identified in the second intermediate host fishes. Differential parasitism of certain fishes by the eight genetically distinctive species of *C. batillariae* could also include more site specificity than previously detected. Finally, we have noticed some morphological variation among the metacercariae and cercariae of *C. batillariae* (Hechinger, unpublished data). We predict that some of this morphological variation will correspond to the genetically identified cryptic species.

Cercaria batillariae was introduced to the West Coast of North America along with the invasion by its first intermediate host snail, *B. cumingi* (Torchin et al., in press). *Batillaria cumingi* was accidentally introduced to North America in the early 1900's along with Japanese seed oysters (Bonnot, 1935). Most of these oysters were imported from Miyagi Prefecture in northern Japan (Anderson et al., 2004), where *C. batillariae* is very prevalent (sometimes $>90\%$, Fig. 2), and at some sites the only species infecting *B. cumingi* (this report; Kuris et al., unpublished data). It is not surprising that the most common trematode species (of the eight morphologically recognized species) would be the one to be introduced to North America (Torchin et al., 2003, in press). However, given the 'filters' parasites encounter during the introduction of their host (Torchin et al., 2002, 2003) it is unlikely that all of the eight cryptic species of *C. batillariae* are established in North America. We predict that the introduced *C. batillariae* in North America is a subset of the cryptic species found at our northern collection sites in Japan. Future genetic analysis will enable a precise determination of which cryptic species of *C. batillariae* invaded North America and perhaps resolve its origin within the native range in Japan.

We did not detect any mixed infections of *C. batillariae* lineages in individual snails (even at sites where prevalence was high ($>90\%$ (Fig. 2)). Trematodes, including heterophyids, generally engage in interference competition, via intraguild predation, that leads to competitive exclusion of subordinate competitors (Kuris and Lafferty, 1994). Therefore, we suspect that competition among these eight lineages is severe, and likely significantly impacts the abundance and distribution of some of these lineages.

In conclusion, molecular genetic methods enabled detection of a previously unappreciated diversity of trematodes in *B. cumingi*. Restriction fragment-length polymorphism and nucleotide sequence analyses of the *CO1* and *ITS1* genes distinguished eight cryptic species of *C. batillariae* and three cryptic species of an undescribed philophthalmid morphospecies. Recognition of this morphologically difficult to detect, but phylogenetically significant, diversity is crucial to understanding parasite systematics, the evolution of host-parasite interactions, host specificity, site specificity, competitive interactions and the invasion biology of these trematodes.

Acknowledgements

We thank Minoru Miura and Mamoru Fujikawa for their assistance on the sampling of *B. cumingi*. We also thank two anonymous reviewers and Alice Nguyen for providing useful comments on the manuscript. This study was supported by grants from Japan Society for the Promotion of Science and a grant from the US National Science

Foundation through the NIH/NSF Ecology of Infectious Disease Program (DEB-0224565) to AMK.

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