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ORIGINAL PAPER

Genetic analysis reveals multiple cryptic invasive species of the hydrozoan genus *Cordylophora*

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Abstract Understanding the patterns and dynamics of biological invasions is a crucial prerequisite to predicting and mitigating their potential ecological and economic impacts. Unfortunately, in many cases such understanding is limited not only by ignorance of invasion history, but also by uncertainty surrounding the ecology, physiology, and even systematics of the invasive taxa themselves. The invasive, colonial euryhaline hydroid *Cordylophora* has invaded multiple regions outside of its native Ponto-Caspian range. However, extensive morphological and ecological plasticity has prevented consensus on both species-level classification within the genus and the environmental conditions conducive to establishment. The goal of this research was to explore the invasive history and species composition of the genus *Cordylophora* through molecular analyses. We addressed both issues using DNA sequence data from two mitochondrial loci [the small subunit 16S rRNA and cytochrome *c* oxidase subunit I (COI)] and one nuclear locus (28S large nuclear rRNA), generated from 27 invasive *Cordylophora* populations collected throughout the global range of the taxon. Phylogenetic analysis and comparisons of genetic distances between populations suggest the presence of multiple cryptic species within the genus. This conclusion is further supported by the observation of significantly different habitat preferences between invasive lineages. Geographic distribution of lineages is consistent with the introduction of multiple lineages to some non-native regions, indicating that repeated introductions may contribute to the current global distribution of *Cordylophora*. Applying molecular and morphological analyses to additional populations of *Cordylophora* is likely to assist in clarifying the taxonomy of this genus and in providing a better understanding of the invasive history of this hydroid.

Keywords *Cordylophora* · Cryptic species · Hydroid · Invasive species · Ponto-Caspian · Taxonomy

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Introduction

The assessment of economic and ecological impacts of non-indigenous species has grown in recent years

(Carlton 1996; Ruiz et al. 1999; Ojaveer et al. 2002; Wasson et al. 2005; Wonham and Carlton 2005). More specifically, the global impact of invasive Ponto-Caspian species (e.g., mussels, crustaceans and fish) on benthic invertebrate communities has received much attention because of the increased prevalence of these alien species and their ability to successfully invade and alter the composition and structure of benthic communities (Bially and MacIsaac 2000; Kolar and Lodge 2001; Vanderploeg et al. 2002; Ricciardi and Atkinson 2004; Leppäkoski 2005).

A sometimes overlooked Ponto-Caspian benthic invasive species is the euryhaline, colonial hydroid *Cordylophora caspia* (Pallas 1771). *Cordylophora* is an athecate hydroid (Family Oceaniidae, previously Clavidae; Schuchert 2004) occurring in freshwater and brackish habitats globally (Roos 1979; Folino 2000; Thorp and Covich 2001; Jankowski et al. 2008). *Cordylophora* most likely was transported from the Ponto-Caspian region via ship ballast and/or ship fouling (Pienimäki and Leppäkoski 2004; Streftaris et al. 2005). The first records of *Cordylophora* in the Baltic Sea are from the early 1800s (Leppäkoski 2005), and it was observed in Danish waters in 1895 (Jensen and Knudsen 2005). It continued to spread throughout Western Europe to the Loire estuary, France (NE Biscay, <1901; Goulletquer et al. 2002) and to inland German waters around 1858 (Nehring 2002). The hydroid was first found on the east coast of North America in Mystic Pond, Massachusetts in 1860 (Verrill et al. 1873) and was later discovered on the West coast of North America in the Puget Sound and San Francisco Bay areas circa 1920 (Cohen et al. 1998; Ruiz et al. 2000; Wonham and Carlton 2005). *Cordylophora* spp. supposedly invaded the Great Lakes via the St. Lawrence River System in 1956 (Mills et al. 1993). In freshwater systems, *Cordylophora* is becoming a prevalent biofouler, possibly due to changes in water quality (increased salts) and its ability to colonize various hard substrata including zebra mussels (Leppäkoski and Olenin 2000; Folino 2000; Smith et al. 2002; Folino-Rorem et al. 2006). It seems highly likely that the presence of zebra and quagga mussels in the Great Lakes and comparable freshwater habitats may enhance the range expansion and establishment of *Cordylophora* by at least providing a substrate for attachment (personal observations).

The ability to tolerate a wide range of salinities is typical for Ponto-Caspian organisms, which often contend with changes in salinity as they invade new habitats (Lee et al. 2003; Berezina and Panov 2004; Paavola et al. 2005). Indeed, the global distribution of *Cordylophora* continues to expand at least in part because of the ability of this species to proliferate in varying salinities (Folino 2000; Smith 2001; Bij de Vaate et al. 2002; Janssen et al. 2005). The published salinity range for *Cordylophora* is 0–40 PSU, though 15–17 PSU has been cited as the optimal range for brackish populations (Arndt 1984; Kinne 1958). Unfortunately, the factors explaining this hydroid's ability to become established in freshwater and brackish habitats remain unclear. Some researchers suggest that *Cordylophora* is one of a number of taxa that have recently undergone rapid evolutionary transitions to novel salinity regimes as a result of anthropogenic translocation outside of their native range (Lee and Bell 1999). Others have challenged this idea, however, noting the existence of both freshwater and brackish *Cordylophora* populations prior to global expansion of the taxon (Strayer 1999; Wolff 2000). While rapid adaptation is one possible explanation for the invasion of both freshwater and brackish habitats, *Cordylophora* may in fact be a truly euryhaline taxon capable of flourishing in a wide range of salinities. Alternatively, there may exist multiple species of *Cordylophora* with different habitat preferences.

This observed capacity of *Cordylophora* to thrive in both freshwater and brackish environments has contributed in part to inconsistencies in taxonomic nomenclature and uncertainty over the systematic status of the genus (see review Folino 2000; Schuchert 2004). The current taxonomic classification of the genus includes at least eight species (*albicola*, *annulata*, *caspia*, *dubia*, *inkermanica*, *lacustris*, *pusilla*, *whiteleggi*), though it is not clear whether these are distinct species or whether synonyms exist. For example, some authors refer to *C. caspia* and *Cordylophora lucustris* as being synonymous, while others believe that although they appear morphologically identical, they are in fact different species with different habitat preferences, *C. caspia* being a brackish water species and *C. lacustris* being freshwater (Folino 2000; Schuchert 2004).

Here we explore both the molecular taxonomy and invasive history of *Cordylophora* using DNA

sequence data from two mitochondrial loci [the small subunit 16S rRNA and cytochrome *c* oxidase subunit I (COI)] and one nuclear locus (28S large nuclear rRNA), generated from 27 invasive *Cordylophora* populations collected throughout the global range of the taxon. We determined phylogenetic relationships and assessed degrees of genetic differentiation, based on DNA sequence variation, between *Cordylophora* lineages in order to determine the likelihood of multiple morphologically cryptic species within the taxon. We also examined phylogenetic patterns in relation to the geographic distribution and apparent salinity tolerances of these populations. We discuss the relevance of our results with respect to both the invasion history of *Cordylophora* and the need for taxonomic revision of this genus.

Methods

Collection of *Cordylophora* tissue

Both fresh and brackish water colonies of *Cordylophora* were collected for genetic analysis (Table 1). Colonies were either preserved in 95% ethanol or collected live and returned to the laboratory, where fresh tissue was later removed for DNA extraction. When multiple samples were removed from single sites, care was taken to collect spatially discrete specimens in order to limit the likelihood of sampling twice from the same colony.

Molecular methods

Three to ten hydranths (feeding polyps) from either live or ethanol-preserved colonies were pooled and whole genomic DNA was extracted using DNeasy columns (Qiagen). Hydranths were removed from single uprights in order to ensure that all processed tissue came from a single colony. In some cases, quiescent colonies were collected and lacked hydranths; for these samples, several connected stolons were removed and crushed manually with sterile plastic pestles in order to free tissue within the outer perisarc prior to DNA extraction.

PCR amplification was conducted using primers COIF-PR115 (TCWACNAAYCAYAARGAYATTGG) and COIR-PR114 (ACYTCNGGRTGNCCRAARARYCA) for COI (Folmer et al. 1994), SHA (ACGGAATGAACTCAAATCATGT) and SHB (TCGACTGTTTACCAAAAACATA) for 16S (Cunningham and Buss 1993), and LSUD1F (ACCCGCTGAATTTAAGCATA) and LSUD4Ra (AACCAGCTACTAGRYGGTTCGAT) for 28S (Sogin and Edman 1990). Cycling parameters for both COI and 28S amplification consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 60 s at 50°C, and 90 s at 72°C, with a final 15 min extension at 72°C. For 16S amplification we modified the cycling parameters of Cunningham and Buss (1993): 5 min denaturation at 94°C followed by 35 cycles of 20 s at 94°C, 45 s at 50°C, and 120 s at 68°C, with a final extension of 10 min at 68°C.

Sequence analysis

All PCR products were sequenced directly in both forward and reverse directions using amplification primers. Sequences were aligned using ClustalX (Thompson et al. 1997) and trimmed to the length of the shortest available sequence, resulting in 532 basepairs of aligned sequence for COI, 525 for 16S, and 785 for 28S. Phylogenetic relationships were determined by Bayesian inference using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Analysis was performed assuming a Generalized Time Reversible model with gamma distribution of substitution rates and a proportion of invariant sites (GTR + I + G). The search was run with four chains for 10^6 generations, with sampling every 100 generations and 2,500 trees discarded as burnin. In addition, phylogenetic inference under the parsimony criterion was performed in PAUP* v. 4.0 (Swofford 2000), employing a heuristic search with tree bisection-reconnection (TBR) branch swapping. Statistical support for nodes was assessed by bootstrapping (1,000 replicates). Since the mitochondrial genome represents a single genetic locus, and since preliminary analyses revealed concordant tree topologies between COI and 16S (not shown), these two loci were combined into a single mitochondrial haplotype for final phylogenetic analysis. In order to reduce computation time, both mitochondrial (COI/16S) and nuclear (28S) datasets were reduced by removing duplicate sequences within populations. Trees were rooted using sequences from three outgroup species, all members of order Anthomedusae, suborder

Table 1 *Cordylophora* specimens used in the current study

Sample ID	Collection site	Latitude	Longitude	*N*	Salinity	Clade
A	Antioch, CA, USA	38°1′24.89″N	121°49′39.87″W	2	1.3	2B
BH	Burnham Harbor, Chicago, IL, USA	41°51′12.37″N	87°36′37.35″ W	9	0.1	1A
C	Huinay, Chile	41°28′38.74′S	72°55′54.62″W	1	Brackish	2A
CB	Coos Bay, OR, USA	43°25′47.06″N	124°13′47.49″W	1	11	2B
CL	Cayuga Lake, NY, USA	42°27′30.48″N	76°30′52.2″W	9	0.2	1A (5)/1B (4)
CR	Columbia River, OR, USA	46°15′49.00″N	124°4′56.99″W	1	0	1B
DP	DesPlaines River, Joliet, IL, USA	41°31′30.70″N	88°5′11.13″W	1	0.5	1A
E	Squamscott River, Exeter, NH, USA	42°58′59.02″N	70°56′54.63″W	10	10	2A (1)/1B (9)
F	Canet St. Nazaire Lake, France	42°40′27.25″N	3°0′0.82″E	1	Brackish	2A
FN	59th Street Marina, IL, USA	41°47′18.69″N	87°34′29.86″W	8	0.4	1A
G	Ryck River, Greifswald, Germany	54°5′58.98″N	13°23′15.38″E	1	5	2A
GW	Grafham Waters, UK	52°17′52.83″N	0°18′50.42″W	4	0	1A
H	Illinois River, Henry, IL, USA	41°6′36.81″N	89°21′4.02″W	1	1	1A
I	Shannon River, Limerick, Ireland	52°39′56.33″N	8°37′50.44″W	1	2	2A
J	Jackson Landing, Durham, NH, USA	43°7′53.05″N	70°53′1.35″W	7	25	1B
LB	Lake Balaton, Tihany, Hungary	46°54′51.98″N	17°53′17.46″E	1	0.45	1B
LO	Lake Ontario, Rochester, NY, USA	43°15′10.98″N	77°36′27.66″W	6	0.3	1A
LS	LaSalle Lake, Marseilles, IL, USA	41°17′39.44″N	88°37′41.09″W	1	0	1A
N	Waal River, Nijmegen, Netherlands	51°50′59.34″N	5°52′22.02″E	5	0.3	1A (2)/1B (3)
NM	Lamprey River, Newmarket, NH, USA	43°4′55.59″N	70°56′5.18″W	3	1.2	1B
NR	Napa River, CA, USA	38°11′49.87″N	122°18′57.59″W	1	16	2B
PB	Pittsburg, CA, USA	38°2′23.95″N	121°53′13.98″W	6	2.9	2B (5)/1B (1)
PR	Petaluma River, CA, USA	38°10′1.48″N	122°32′19.89″W	14	22	2B
P	Panama Canal, Gamboa, Panama	9°6′54.86″N	79°42′20.00″W	1	0	1A
SL	Seneca Lake, New York, USA	42°37′42.44″N	76°55′6.55″W	11	0.3	1B
V	James River, Jamestown, VA, USA	37°12′22.93″N	76°44′15.66″W	1	0.5	1A
WH	Woods Hole, MA, USA	41°31′37.46″N	70°40′10.72″W	1	3	1B
Outgroups						
T. rubra, New Zealand						
L. octona, France						
C. multicornis, France						

Salinity values are listed in PSU; precise salinity values for samples from Chile and France are unknown

Fillifera: *Turritopsis rubra* (Oceaniidae), *Leuckartia octona* (Pandeidae), and *Clava multicornis* (Hydractiniidae).

In addition to phylogenetic analyses, we determined pairwise genetic distances between all *Cordylophora* samples by calculating the uncorrected number of changes between sequences (*p*-distance). These calculations were conducted independently for all three loci. To determine if the observed genetic differentiation between *Cordylophora* clades was consistent with independent species status, we compared these measures to similar measures calculated for other hydrozoan taxa. For 16S, genetic distance measures were drawn directly from a published study on the anthomedusan genus *Coryne* (Schuchert 2005), and were independently calculated from sequences generated for the closely related oceaniid genus *Turritopsis* (Miglietta et al. 2007; sequences kindly provided by the authors). Differentiation at the COI locus was compared to interspecific distances determined by analysis of published COI sequences from the leptomedusazoan family Campanulariidae (Govindarajan et al. 2006; GenBank accession numbers AY789881 through AY789916). The 28S

distances were compared to those between species of the genus *Hydra* (Anthomedusae; Hydridae), and were calculated from unpublished sequences drawn from GenBank (Hemmrich et al., accession numbers EF059950 through EF059957). All sequence analyses were conducted using MEGA v. 3.1 (Tamura et al. 2007).

Comparison of salinity tolerances

Precise salinity values (in PSU) were obtained from all collection sites with the exception of Huinay, Chile and Canet St. Nazaire, France. We tested for correlation between salinity tolerance and phylogenetic affinity by comparing mean observed collection site salinity values both between major lineages 1 and 2 and between all four lineages (1A, 1B, 2A, and 2B). Since these values were not normally distributed among samples in lineage 1, and since variances in those values differed between clades, we utilized a non-parametric Mann–Whitney *U* test to assess overall significance of differences between means. Significance of differences between individual means was determined using Tukey's HSD test. The Chilean and French samples were excluded from this analysis as no precise salinity measurements were available from these sites, although both are known to be brackish. In order to determine a correlation between salinity and phylogeny for all *Cordylophora* samples (including France and Chile), salinity values were placed into two categories (<0.5 PSU, fresh; >0.5 PSU, brackish) and significance was determined using Fisher's exact test (in the case of the two-lineage comparison) or Pearson's chi square (in the case of the four-lineage comparison). All statistical tests were conducted in JMP1.0 (SAS).

Results

Phylogenetic analysis

Phylogenetic analysis reveals considerable genetic diversity within the genus *Cordylophora*. Bayesian inference of phylogeny based on mitochondrial sequence data reveals four monophyletic clades (1A, 1B, 2A, and 2B), all with 100% posterior probabilities (Fig. 1a). These clades nested within two more inclusive clades (1 and 2), also reciprocally monophyletic with 100% posterior probability. Tree topology was similar in the analysis of nuclear 28S sequence (Fig. 1b). Nodal support was substantially reduced due to the lower genetic divergence at the nuclear locus; only four 28S haplotypes were observed among the 109 individuals tested, whereas 13 combined mtDNA haplotypes were observed. Despite the deep divergences apparent within the genus *Cordylophora*, all sequences formed a monophyletic group relative to three species from other filliferan genera; as expected, the oceaniid species *T. rubra* falls out as the nearest neighbor to *Cordylophora*, with 100% posterior probability support in analysis of 28S data. Results of Bayesian phylogenetic inference were supported by maximum parsimony analysis of mtDNA data, which revealed a virtually identical tree topology (Fig. 2). As in the case of Bayesian analysis, statistical support for all major lineages was 100%, based on 1,000 bootstrap replicates.

Genetic differentiation

Genetic divergence between major lineages 1 and 2 was pronounced at mitochondrial loci; the minimum *p*-distance observed was 6.04% for 16S and 12.35% for COI (Table 2). Divergence between minor lineages was also high, with minimum *p*-distances of 3.51% (16S) and 7.83% (COI) between clades 1A and 1B, and 3.31% (16S) and 9.57% (COI) between clades 2A and 2B. Variation at the nuclear 28S locus was considerably lower than for mtDNA loci. The greatest distance observed between minor lineages was 0.66% (between 1A and 1B), and distances between lineages 1 and 2 ranged from 1.98 to 2.51%.

The degree of interclade genetic differentiation in *Cordylophora* is generally within the range of interspecific differentiation in other hydrozoan taxa (Table 3). Analysis of 16S sequences drawn from a recent study of the closely related oceaniid genus *Turritopsis* (Miglietta et al. 2007) reveals a minimum uncorrected *p*-distance between any two *Turritopsis* species of 3.61%, with a mean distance of 8.51%. Similarly, the minimum uncorrected *p*-distance between any two species of the anthomedusan genus *Coryne* (family Corynidae) was reported as 3.7%, with a mean of 11.19% (Schuchert 2005). In *Cordylophora*, the minimum distance observed between major clades 1 and 2 was 6.04% (mean = 6.61%), and between any two clades was 3.31%.

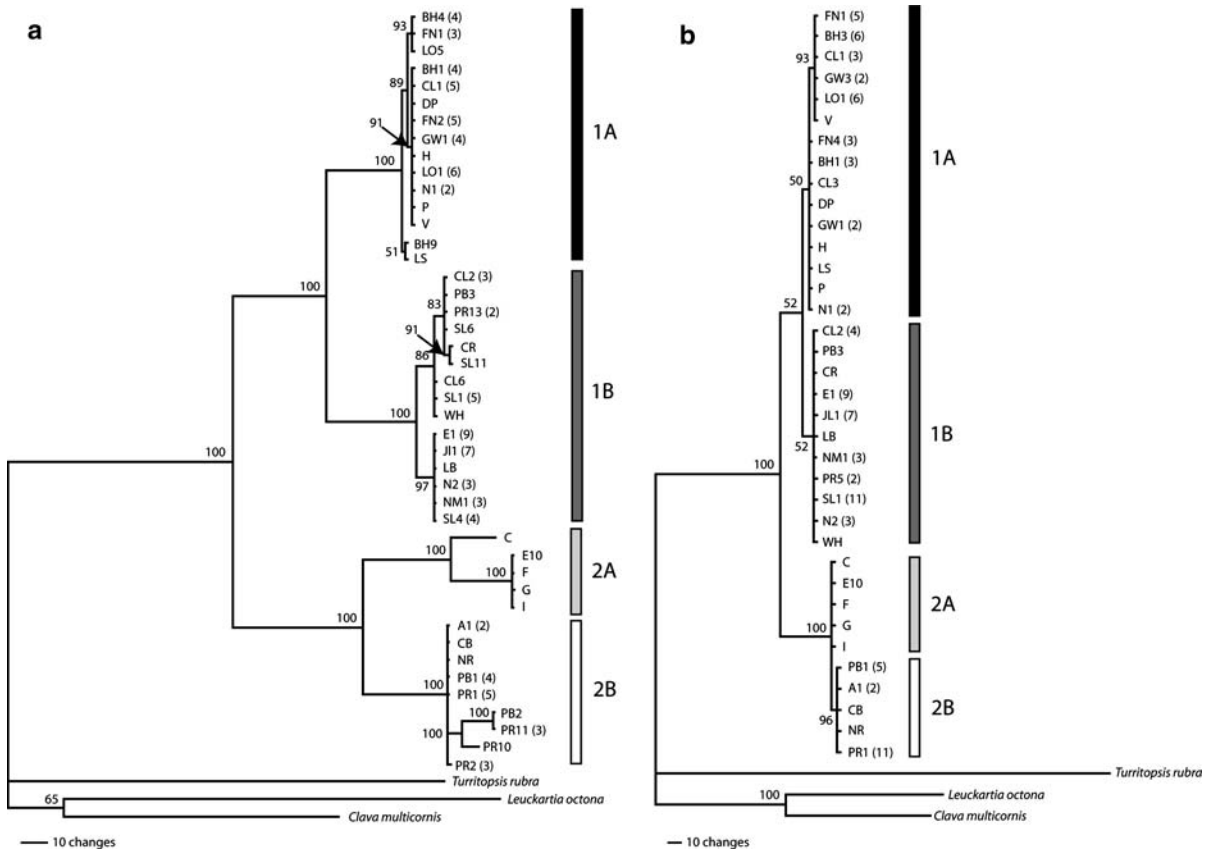

Fig. 1 Phylogenetic trees determined by Bayesian inference, based on combined mtDNA data (**a**) and nuclear 28S data (**b**). Note difference in scale in branch lengths. Posterior probabilities are indicated for all nodes. *Shaded bars* indicating *Cordylophora* lineages correspond to those in Fig. 3. *Numbers* in parentheses indicate multiple individuals with the same sequence occurring within populations

The differentiation between clades 1 and 2 was well within the range observed for interspecific distances in *Turritopsis* (3.61–12.11%). Analysis of COI sequences published for the leptomedusazoan family Campanulariidae (Govindarajan et al. 2006) revealed intrageneric, interspecific uncorrected *p*-distances ranging from 8.5 (in the genus *Bonneviella*) to 20.24% (genus *Orthopyxis*). By comparison, the minimum distance observed between *Cordylophora* clades 1 and 2 was 12.35%, and the minimum distance observed between any two *Cordylophora* clades was 7.83%. Similar patterns of genetic differentiation were observed at the nuclear 28S rRNA locus, although divergence here was considerably lower than that observed at mtDNA loci. Uncorrected *p*-distances between clades 1 and 2 ranged from 1.98 to 2.51%. In comparison, interspecific distances calculated for the genus *Hydra* (Anthomedusae; Hydridae) range from 0.16 to 3.91%, with a mean of 1.78%.

Geographic distibution of *Cordylophora* lineages

Cordylophora lineages generally exhibit broad geographic distributions (Fig. 3). Only one lineage (2B) had limited distribution in our sampling, occurring only on the Pacific coast of North America. Lineages 1A and 1B were the most widespread, with the former found in eastern North America, Central America, and Europe and the latter found throughout North America and Europe. In many cases, identical combined mitochondrial haplotypes were observed in disparate geographic regions; for instance, a single haplotype from lineage 1A was observed in the United Kingdom, Netherlands, Panama, Virginia, and the US Great Lakes region.

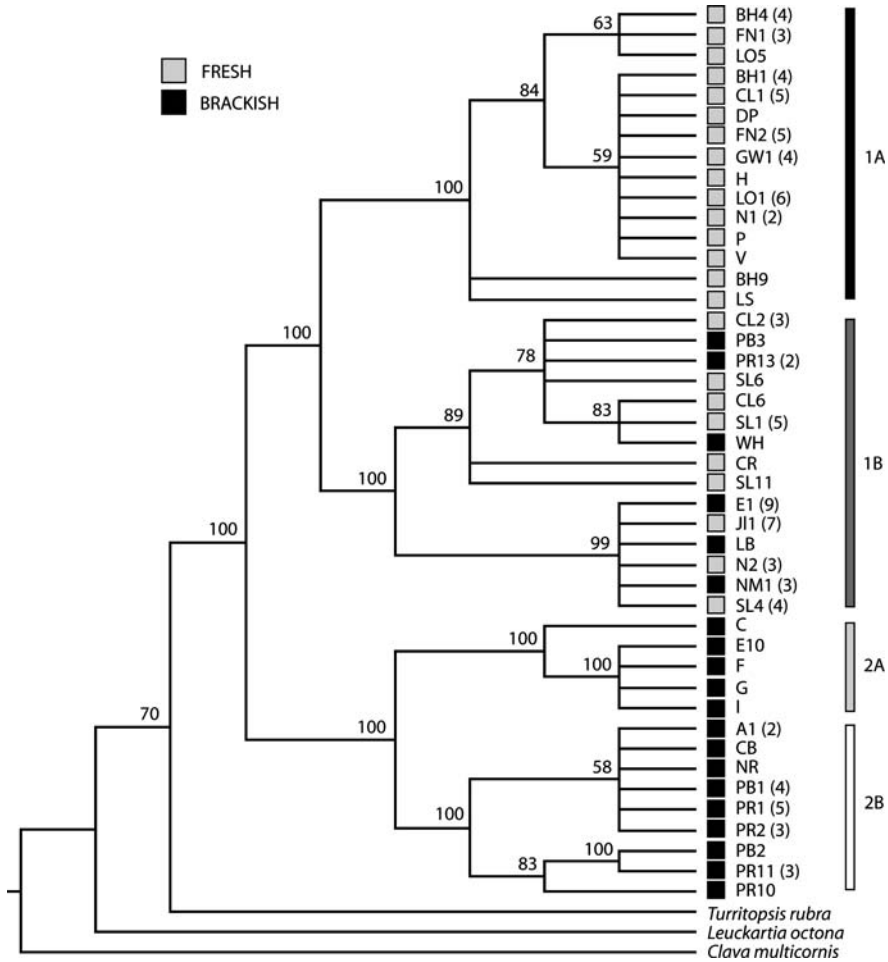

Fig. 2 Single most parsimonious tree based on mtDNA haplotype data. Nodal support is indicated as percent of 1,000 bootstrap replicates. Collection site salinities are indicated with *shaded boxes*

Several populations were found to include individuals belonging to multiple *Cordylophora* lineages. The populations from both the Netherlands and Cayuga Lake (New York, USA) were composed of individuals from lineages 1A and 1B; in both cases there was roughly equal representation from both clades. At Exeter (New Hampshire, USA) and Pittsburg (California, USA), individuals from both major lineages 1 and 2 were observed.

Variation in salinity tolerance between *Cordylophora* lineages

Phylogenetic affinity proved to be a good predictor of salinity tolerance in *Cordylophora* (Fig. 2). All specimens falling within major lineage 2 were collected from brackish water environments (observed salinity >0.5 PSU), and all those from lineage 1A were collected from fresh water (<0.5 PSU). Lineage 1B, in contrast, was composed of individuals collected from both fresh and brackish water. The correlation between lineage membership and collection site salinity was highly significant, both when comparisons were made between major lineages 1 and 2 ($P = 0.0002$, Fisher's exact test) and when comparisons were made between all lineages ($P < 0.0001$, Pearson chi square). Mean values of observed collection site salinities were also significantly different between lineages (Fig. 4; Mann–Whitney U test, $P = 0.0012$ for comparison of lineages 1 and 2, $P = 0.0031$ for comparisons of all lineages). When all four lineages were compared, only lineages 1A and 2B had significantly different salinity tolerances.

Table 2 Genetic distances within and between *Cordylophora* clades

	COI			16S			28S		
	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
Intra-clade									
1A	nd	0.7	0.21	nd	0	0	nd	0.13	0.07
1B	nd	2.09	0.93	nd	0.78	0.22	nd	0	0
2A	nd	6.43	0.65	nd	1.36	0.55	nd	0	0
2B	nd	3.13	2.57	nd	0.58	0.07	nd	2.75	0
1	nd	9.22	4.63	nd	4.09	1.93	nd	0.66	0.32
2	nd	10.78	3.69	nd	4.09	1.29	nd	0.26	0.05
Inter-clade									
1A, 1B	7.83	9.22	8.57	3.51	4.09	3.71	0.53	0.66	0.6
2A, 2B	9.57	10.78	9.88	3.31	4.09	3.8	0.13	0.26	0.14
1, 2	12.35	15.3	14.36	6.04	7.8	6.61	1.98	2.51	2.27

Distances are expressed as uncorrected percent nucleotide difference (*p*-distance). Maximum and mean distances are shown for all comparisons; minimum distances were only calculated for inter-clade comparisons

nd, not done

Table 3 Minimum interspecific genetic distances for hydrozoan taxa

Taxon	COI	16S	28S
Cordylophora	12.35	6.04	1.98
	7.83	3.31	0.13
Turritopsis		3.61	
Coryne		3.70	
Companularidae[a]	8.5–20.25		
Hydra			0.16

All distances are expressed as uncorrected percent nucleotide difference (*p*-distance). For *Cordylophora*, two values are shown for each locus; the top value indicates the minimum distance observed between clades 1 and 2, the bottom value indicates the minimum distance observed between any two clades

[a] For Companularidae, intrageneric interspecific distances were calculated for multiple genera

Discussion

Multiple cryptic invasive species of the genus *Cordylophora*

Our results indicate that multiple evolutionarily divergent lineages of *Cordylophora* have invaded outside of the Ponto-Caspian region and that in some cases these lineages have established in the same non-native region, suggesting multiple cryptic invasions. Phylogenetic tree topology reveals the presence of well-supported, reciprocally monophyletic clades within the genus *Cordylophora* (Fig. 1), and levels of differentiation observed at mitochondrial DNA loci are consistent with interspecific differentiation observed in other hydrozoan taxa.

At both mitochondrial loci, relevant comparisons suggest that the genetic divergence within *Cordylophora* may be commensurate with independent species status for all reciprocally monophyletic clades (Table 3). There is no agreed level of genetic differentiation that would decisively indicate independent species status for two individuals. However, the reciprocal monophyly between lineages 1 and 2, along with the very high levels of genetic differentiation observed between those two lineages, argues strongly for their status as independent species. Data from a recent taxonomic revision of the genus *Turritopsis* based on the mitochondrial 16S locus (Miglietta et al. 2007) provide perhaps the most appropriate framework for comparison, as this genus is traditionally placed along with *Cordylophora* within the family Oceaniidae. Genetic differentiation between *Cordylophora* clades 1 and 2 (minimal uncorrected *p*-distance 6.04%) is clearly within the range of interspecific distances within *Turritopsis* (3.61–12.11%). In addition, the minimal pairwise uncorrected *p*-distance between any of the reciprocally monophyletic *Cordylophora* clades was 3.31%, while the minimal distance between any two

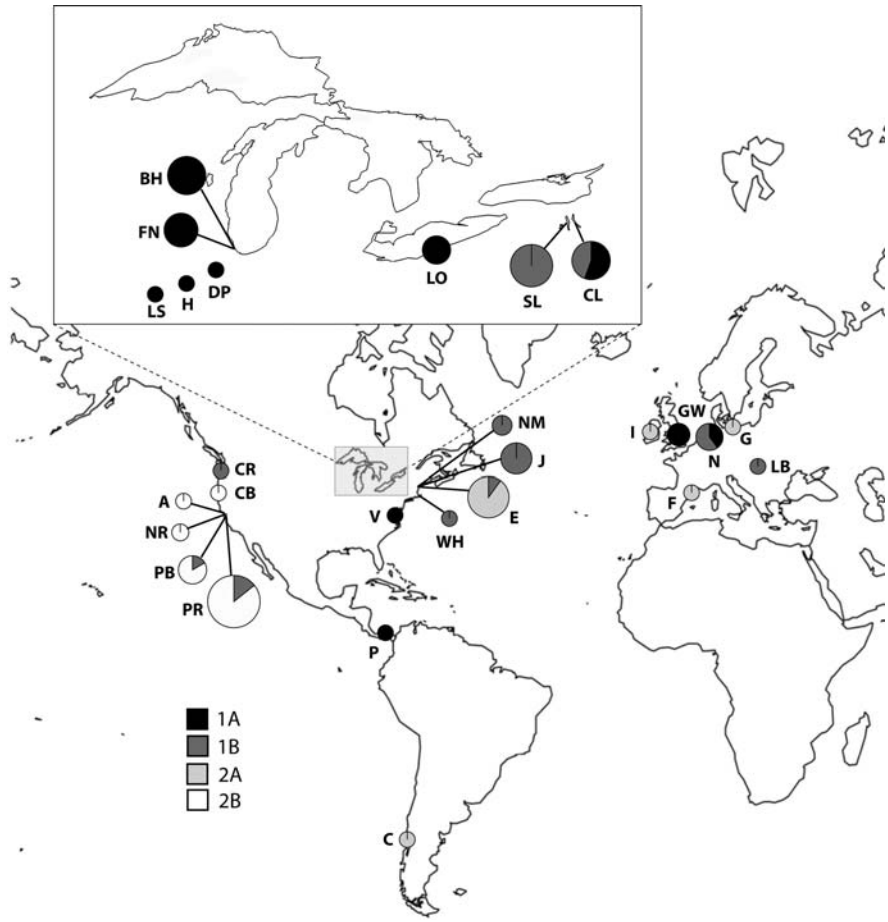

Fig. 3 Global distribution of *Cordylophora* lineages. Sample IDs correspond to those presented in Table 1, *shades* indicating lineage correspond to Fig. 1. Pie charts have been scaled to reflect sample size. The Great Lakes region is shown as an *inset* for clarity of presentation

Turritopsis species was 3.61%, suggesting that even minor lineages deserve further investigation as putative independent species. Similar comparisons at the 28S locus indicate that the degree of nuclear genetic differentiation within *Cordylophora* is also consistent with the existence of multiple cryptic species within the taxon (Table 3). This differentiation at nuclear loci is consistent with observations made by Schable et al. (2008), who found that 11 nuclear microsatellites designed for lineage 1 failed to cross-amplify with individuals from lineage 2. Such limited microsatellite transferability between related species is a commonly reported phenomenon (Selkoe and Toonen 2006).

Comparisons between taxa of intra- and inter-specific variation must be interpreted with some caution, as differences in life history may significantly impact dispersal, gene flow, and the levels of genetic variability observed. For instance, taxa such as *Cordylophora* with sessile sporosacs may tend towards higher intraspecific variability than those like *Turritopsis* with planktonic medusae. However, unless levels of intra- and inter-specific genetic variation differ dramatically between *Cordylophora* and other hydrozoan taxa, our DNA sequence data offer strong support for the hypothesis that multiple species exist among invasive populations of *Cordylophora*.

Geographic distribution of *Cordylophora* lineages

The geographic distribution of *Cordylophora* mtDNA haplotypes indicates that evolutionarily independent lineages have frequently been introduced to the same non-native sites (Fig. 3). On the Pacific coast of North America representatives from both lineages 1 and 2 have been observed, and in one case both were collected from the same site (Pittsburg, CA). Individuals deriving from both major lineages also co-occurred at Exeter, NH in the eastern US. Similarly,

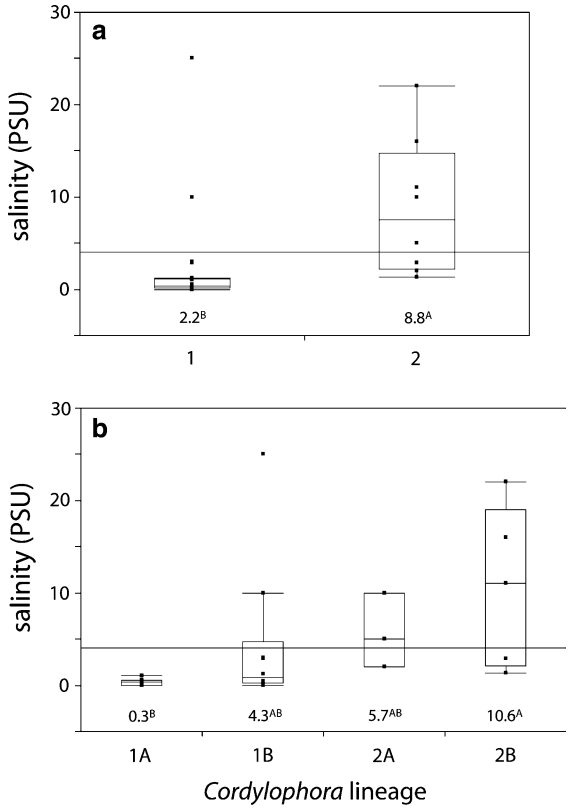

Fig. 4 Comparison of collection site salinities between *Cordylophora* lineages. **a** Comparison of major clades 1 and 2; **b** comparisons of all clades. Mean values are shown beneath box plots. *Superscripts* indicate statistical significance of comparisons; means that are not significantly different (Tukey's HSD, $\alpha = 0.05$) are connected by the same letter

the US Great Lakes region harbors both lineage 1A and 1B, and these occurred together within the same population in Cayuga Lake, one of the Finger Lakes in New York.

Other recent genetic research has demonstrated that multiple introductions may be the rule rather than the exception for many aquatic invasive species. For instance, a number of species likely to have been transported in ballast water from the Ponto-Caspian to the North American Great Lakes appear to have been introduced multiple times; these include the spiny waterflea *Bythotrephes longimanus* (Colautti et al. 2005), the zebra and quagga mussels *Dreissena polymorpha* and *D. bugensis* (Stepien et al. 2005), and the round and tubenose gobies *Apollonia melanastomus* and *Proterorhinus semilunaris* (Stepien and Tumeo 2006). The possibility of multiple introductions of *Cordylophora* species via similar routes is thus not surprising. Mixed-lineage populations such as those at Pittsburg, CA and Exeter, NH may either be the result of multiple independent introductions from different native sites or co-introduction of multiple lineages. Without knowledge of the distribution of *Cordylophora* lineages in the native range, it is impossible to determine the likelihood of these lineages being transported together in a single introduction event.

Cordylophora lineages 1A, 1B, and 2A all have very broad geographic ranges (Fig. 3). These distributions may be a result of the frequency of introductions associated with ballast water transport from the Ponto-Caspian region, the ease with which *Cordylophora* can be transported by contemporary vectors of introduction, and the broad range of recipient habitats conducive to successful establishment. The available genetic data are insufficient to reconstruct fully the invasion history of *Cordylophora*, and it is unclear how much of the observed distribution is the result of post-introduction population expansion either by natural dispersal or by secondary anthropogenic introductions. Unlike lineages 1A, 1B, and 2A, lineage 2B was found only on the Pacific coast of North America. This narrow observed non-native distribution may be the result of limited sampling effort, or it may reflect a lower frequency of introduction events involving this lineage, either because of relative rarity in the native range or reduced invasiveness.

Ecological differences between *Cordylophora* lineages

Although there appears to be little correspondence between phylogenetic relationships and geographic distribution of *Cordylophora* lineages, we have found a very strong correlation between phylogenetic affinity and apparent salinity tolerance. Introduced populations from *Cordylophora* lineage 2 were collected in all cases from brackish sites, while all populations from lineage 1A were collected from freshwater sites; lineage 1B was roughly evenly distributed between brackish and freshwater collection sites (Fig. 2). Lineages 1A and 2B, in particular, were found in habitats with significantly different mean salinities (Fig. 4b), and this was reflected in an overall difference between the salinities of collection sites for lineages 1 and 2 (Fig. 4a). The results

indicate two possibilities: either the native Ponto-Caspian range harbors multiple cryptic species of *Cordylophora* with significantly different habitat preferences (i.e., a freshwater species and a brackish water species), or different lineages of a taxon that is generally euryhaline in its native range possess different capacities to establish in freshwater habitats.

There is some precedent in the existing literature for the former possibility. Traditionally, there has been confusion with regards to the taxonomic status of at least two *Cordylophora* species, *caspia* and *lacustris* (Folino 2000; Smith 2001; Schuchert 2004). Some authors equate the two species (Roch 1924; Cohen et al. 1998; Smith 2001) while others suggest that *C. caspia* occurs in brackish habitats while *C. lacustris* occurs in freshwater (Folino 2000; Smith 2001). Recently, *lacustris* is considered an invalid name with *caspia* being the more commonly used and valid species (Cairns et al. 2002). The diagnostic morphological features describing *C. caspia* (Schuchert 2004) and *C. lacustris* (Gosner 1971) are comparable, suggesting possible synonymy of the two taxa. More recently, the majority of references to *Cordylophora* in brackish or freshwater habitats use the species name *caspia* (Wasson et al. 2005; Ricciardi 2006). Still, it may be that the distinction between *lacustris* and *caspia* is based on differences in ecological tolerance that reflect underlying genetic differentiation and reproductive incompatibility. Although the results presented here do not fully resolve the relationship between *C. caspia* and *C. lacustris*, the likely existence of multiple species among the invasive populations analyzed, along with the highly significant correlation between phylogenetic affinity and salinity tolerance, suggests that the *caspia/lacustris* distinction deserves close re-examination.

Certain lines of evidence suggest that *Cordylophora* can acclimate to novel salinity regimes. *Cordylophora* colonies form a dormant menont stage in colder temperatures with regeneration occurring with increasing spring temperatures (Roos 1979; Folino 2000). This menont stage could conceivably serve as a 'resistant propagule' and enhance the success of the invasive transition (Panov et al. 2004; Ricciardi 2006). Preliminary laboratory experiments demonstrated that menonts from a freshwater population of *Cordylophora* (southern Lake Michigan) maintain their regenerative capacity when reared in 0, 2, 4, 8 and 12 PSU, suggesting that portions of *Cordylophora* in this dormant stage could be transported to new habitats of varying salinity, regenerate and become established (N. C. Folino-Rorem, unpublished results). In addition, laboratory evidence indicates that colonies of a freshwater population from the Des Plaines River in Joliet, Illinois are capable of growing in 24 PSU after a gradual acclimation in salinity (N. C. Folino-Rorem, unpublished results; see also Kinne 1958, 1964). It has already been suggested that native euryhaline populations of *Cordylophora* already possessed the ability to acclimate to freshwater prior to broad anthropogenic range expansion, and therefore had an advantage for becoming established when introduced to freshwater habitats (Strayer 1999; Wolff 2000). Additional studies are clearly needed to determine the degree to which ecophysiological plasticity contributes to *Cordylophora*'s capacity to invade both freshwater and brackish water habitats, and to determine whether inherited differences in such plasticity can explain the observed frequency with which certain *Cordylophora* lineages invade different habitats.

Although differences in plasticity and acclimation ability may explain the observed distributional patterns, it is also possible that the invasive success of *Cordylophora* may be due in part to rapid adaptation within introduced populations. Lee and Bell (1999) have suggested that the ability of *Cordylophora*—which they consider a euryhaline taxon in its native range—to invade freshwater ecosystems indicates rapid evolutionary change in response to novel environmental conditions experienced in the non-native range. Winkler et al. (2008), building on earlier work (Lee et al. 2003, 2007) have recently demonstrated the differential capacity to invade fresh water habitats of evolutionarily distinct lineages of the copepod *Eurytemora affinis*. An alternative interpretation of our own genetic data is that a similar differential capacity exists between *Cordylophora* lineages. This hypothesis would posit that preference for brackish water is the ancestral state for the *Cordylophora* lineage, and that only recently have freshwater populations arisen—and then only in those lineages that had evolved the capacity to adapt to freshwater conditions. This hypothesis is difficult to square with our failure to observe a single case in which individuals from lineage 1A exhibit the putative ancestral state. However, the hypothesis

bears further scrutiny, a task that would again clearly require additional genetic and ecological research into *Cordylophora* in its ancestral range.

Conclusions

Our findings strongly recommend further clarification of the taxonomy of this widely introduced taxon in order to better understand its invasion history and its capacity for additional future invasions. Although genetic data alone cannot determine species boundaries, particularly when only few loci are included in the analysis, the results presented here clearly support a hypothesis of multiple species within the genus *Cordylophora*. Clarification of species-level classification has been hindered by lack of consensus on the degree to which morphological plasticity may confound identification of diagnostic features (Arndt 1984, 1989; Kinne 1958, 1964; Folino 2000; Smith et al. 2002), and Schuchert (2004) has already proposed that populations of *Cordylophora* may exhibit similar morphologies while being different genetically. We concur, and suggest that currently recognized morphological features of *Cordylophora* spp. may be important but not sufficient for species-level classification of this hydroid. Assessing morphological similarities and differences for additional samples of *Cordylophora* from around the world in conjunction with molecular species identification will address the role of morphological plasticity in the taxonomy of this hydroid.

To better understand the patterns of invasion by this taxon, further observations of invasive *Cordylophora* populations should pay particular attention both to the extent of population expansion relative to salinity, and to the genetic identities of populations. While it is known that multiple *Cordylophora* lineages co-occur in the same localities, it is not known whether or not different lineages exhibit different distributions relative to habitat salinity gradients. Similar observations from the native range would reveal the likelihood of independent lineages co-occurring in potential source populations, and thus may clarify invasion history by addressing the degree to which multiple *Cordylophora* species are introduced via independent introduction events as opposed to being released simultaneously by the same vector.

In addition, experimental assessment of the morphological and ecophysiological responses of *Cordylophora* species to varying salinity regimes may help clarify the degree to which plasticity plays a role in the broad salinity tolerance of the genus, the possible genetic basis of salinity tolerance, and the evolutionary potential of *Cordylophora* species in adapting to novel salinity regimes. Understanding the genetic properties of the genus *Cordylophora* as they relate to adaptive ability and invasive success in fresh and brackish habitats is of importance both to the systematics of the genus and to defining the ecological roles of this species in its introduced range.

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