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Evolution of rotifers in saline and subsaline lakes: A molecular phylogenetic approach

Alison M. Derry[1]
Department of Zoology, University of Guelph, Guelph, Ontario, N1G 2W1 Canada; Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9 Canada

Paul D. N. Hebert
Department of Zoology, University of Guelph, Guelph, Ontario, N1G 2W1 Canada

Ellie E. Prepas[2]
Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9 Canada

Abstract

Evolutionary relationships within the phylum Rotifera are poorly understood despite the important role that they play in freshwater ecosystems. Phylogenetic analyses of DNA sequences from two mitochondrial genes, 16S rDNA and cytochrome oxidase I (COI), were employed to examine the extent of genetic divergence within populations of several common taxa. This work sought to verify the role of phenotypic plasticity versus genetic variation in explaining the morphological variation in some taxa. Deep genetic divergence (4.4% COI nucleotide sequence divergence) was detected between spined and unspined forms of *Keratella cochlearis,* which suggests that they represent different species. However, morphological variation in *K. hiemalis* appeared to be environmentally induced. The study also sought to ascertain the role of one environmental variable, salinity, in the patterning of sequence variation. Greater haplotype diversity and genetic divergence were observed among populations of halophilic *Brachionus plicatilis* than among freshwater *Keratella quadrata* populations from northern Canada. When COI DNA sequences for *B. plicatilis* haplotypes were compared with those from Spain, there was evidence for considerable genetic diversity within this species among closely located saline lakes in northern Canada.

Genetic differentiation among zooplankton populations often occurs on a local scale (De Meester 1996; Colbourne et al. 1997; Gómez et al. 2000, 2002*a;* De Meester et al. 2002) in spite of their potential for widespread genetic exchange through the passive dispersal of resting stages (e.g., Weider et al. 1999). Most zooplankton populations are derived from locally produced resting eggs (De Meester 1996), and the spatial boundaries of lakes and ponds are thought to restrict gene flow between populations (Slatkin 1985). Colonization history (founder effects), combined with local adaptations, has been shown to reduce gene flow among populations (De Meester et al. 2002; Gómez et al. 2002*a*). For example, the occupancy of temporally variable environments may influence the timing of sexual reproduction in cyclic parthenogenic organisms, enhancing genetic differentiation across geographic areas (Serra et al. 1997). These mechanisms coupled with extremely short generation times are thought to promote divergence of monogonont rotifer populations, which may occur in the absence of morphological change, resulting in cryptic species complexes (Serra et al. 1997; Gómez et al. 2002*a*).

Evolutionary relationships among rotifers are poorly understood despite their significance in aquatic ecosystems. Rotifers often dominate freshwater zooplankton communities, are important in nutrient recycling, and can alter trophic dynamics of planktonic communities (Pennak 1989). Rotifers belonging to the class Monogononta represent 90% of the species within phylum Rotifera (Pennak 1989). The morphological discrimination of these species is often difficult because of the extensive phenotypic variation caused by environmental and genetic factors (Serra et al. 1998). For example, *Keratella cochlearis* is a complex of morphs with varying degrees of posterior spine development, and it is unknown whether these forms are morphological variations of a single species or whether they represent different species. Similar confusion arises with many other rotifer species, such as *K. hiemalis* and *K. quadrata.* It is now accepted that molecular techniques will be critical in both clarifying species boundaries in such cases and in identifying the role that environmental factors play in determining the extent of local population divergence.

Salinity variation is one environmental factor that can promote local divergence of cyclic parthenogens (Weider and Hebert 1987; Wilson and Hebert 1992). Additionally, accelerated rates of molecular evolution have been reported for saline lake organisms, such as brine shrimp (Iwabe et al. 1996; Maley and Marshall 1998) and cladocerans (Hebert 1998; Hebert et al. 2002). Work on the halophilic rotifer

[1] Corresponding author (derrya@biology.queensu.ca).
[2] Present address: Faculty of Forestry and Forest Environments, Lakehead University, Thunder Bay, Ontario P7B 5E1, Canada.

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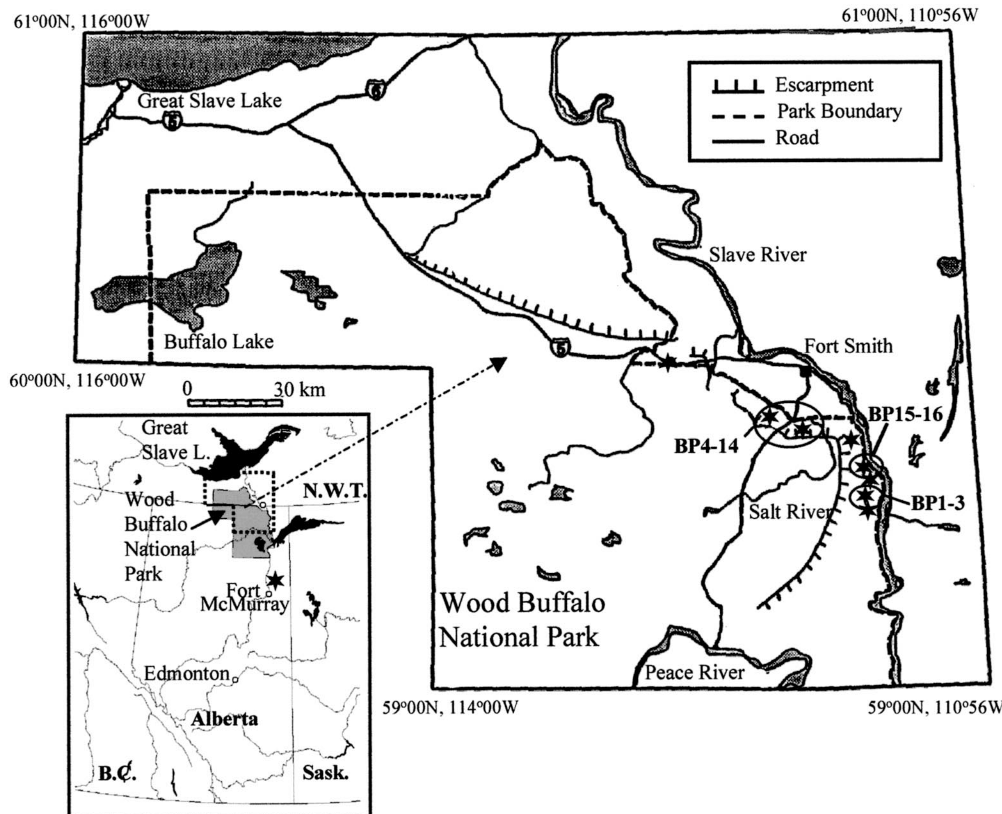

Fig. 1. Locations of lakes in Wood Buffalo National Park and near Fort McMurray, Alberta, Canada from which rotifers were collected. *Brachionus plicatilis* (BP) COI haploptypes clustered according to the lakes from which they were collected and are indicated on the map (BP1–3 = HC Lake; BP4–14 = Grosbeak Lake/Salt Pan Lake; BP15–16 = FP Lake).

Brachionus plicatilis has shown that it is a complex of at least three sibling species (Ciros-Pérez et al. 2001; Gómez et al. 2002*b*), with varying salinity, thermal, and seasonal preferences (Ciros-Pérez et al. 2001). It is probable that there are additional sibling species in this complex (Ortells et al. 2000; Gómez et al. 2002*b*). A mitochondrial DNA (mtDNA) study of these organisms showed considerable genetic divergence (2.8% uncorrected COI nucleotide sequence divergence) between *B. plicatilis* populations from Spanish salt lakes, which was thought to reflect their origin from different glacial refugia (Gómez et al. 2000).

The objectives of this study were to address whether phenotypic plasticity has concealed reproductively isolated species of rotifers and to determine whether rotifers inhabiting saline environments show greater population divergence than freshwater species. This study employed DNA sequences to evaluate the extent of population divergence in four of the most common rotifers from inland waters. Specifically, this investigation examines the role of genetic factors in explaining variance in spine development among members of the genus *Keratella* (*K. cochlearis, K. hiemalis,* and *K. quadrata*). The second genus selected for analysis, *Brachionus,* has been the target of prior molecular studies. The present analysis sought to ascertain the extent of genetic divergence among populations of *B. plicatilis* from saline lakes in one region of North America. We hypothesized that greater intraspecific genetic diversity would be detected in this species because of the prior evidence for accelerated rates of molecular evolution in halophilic taxa.

Materials and methods

Sample collection—Rotifers were collected using 64-μm Nitex tow net at the point of maximum lake depth in nine lakes that spanned a salinity gradient (556 to 26,318 mg L^{-1} total dissolved solids (TDS), summer means; Derry et al. in press). These lakes were located within an 85-km radius in Wood Buffalo National Park, Alberta, Canada (59°30′N, 111°80′W) (Fig. 1). In addition, *Brachionus urceolaris, Keratella cochlearis robusta,* and one *Keratella quadrata* population were obtained from three mine-tailing ponds 330 km south of these lakes, near Fort McMurray, Alberta (56°55′N, 111°29′W) (Fig. 1). Individuals of *B. calyciflorus* from Gainesville, Florida (29°N, 82°W) (Snell et al. 1991) were used as an outgroup for other *Brachionus* species. Members of the genus *Asplanchna* sp. were collected from southern Ontario (44°N, 80°W) and served as an outgroup in other phylogenetic analyses. Vertical net hauls were performed in lakes that were sufficiently deep (2 to 10 m) for vertical migration of zooplankton, while horizontal tows were conducted in shallow (0.3 to 0.5 m) lakes. Rotifers were iden-

Table 1. GenBank accession numbers for 16S DNA sequences isolated from rotifers.

Species	Haplotype	Accession number
Asplanchna sp.	1	AF499036
Brachionus calyciflorus	1	AF499037
Brachionus plicatilis	2	AF499040
	5	AF499039
	11	AF499038
Brachionus urceolaris	1	AF499041
Keratella cochlearis var. *faluta*	1	AF499042
Keratella cochlearis var. *robusta*	1	AF499043
Keratella cochlearis var. *tecta*	1	AF499044
	2	AF499047
Keratella hiemalis	1	AF499045
Keratella quadrata	1	AF499046
Synchaeta sp.	2	AF499050
	3	AF499049
Synchaeta c.f. *pectinata*	1	AF499048
	2	AF499051

tified to genus for *Synchaeta* and to species for other taxa using the keys in Stemberger (1979).

Molecular techniques—Total DNA was extracted from single rotifers using proteinase K methods (Schwenk 1996), and 15-μl extracts were stored frozen at -20°C. Two mitochondrial (mt) genes with different rates of molecular evolution were employed to examine levels of genetic variability within and between selected rotifer families. Cytochrome oxidase subunit I (COI) was used to investigate variation among conspecific populations, while 16S rDNA (16S) was used to examine deeper phylogenetic relationships (Palumbi 1996). The polymerase chain reaction (PCR) was used to amplify a 447 base pair (bp) fragment of the 16S gene with the primer pair 16S-AR and 16S-BR (Palumbi 1996). Each 50-μl PCR reaction contained 7 μl of DNA template, 4.5 μl of 10× PCR buffer (Roche), 2.2 μl of 50 μmol L^{-1} $MgCl_2$, 0.25 μl of each 10 μmol L^{-1} dNTP (C, G, A, T), 1 μl of each 10 μmol L^{-1} primer, 0.4 μl of 1 : 10 Taq DNA polymerase (Qiagen), and 32.9 μl of sterile, double-distilled water. PCR was also employed to amplify a 633 bp fragment of cytochrome c oxidase subunit I with the LCOI490 and HCO2918 primers (Folmer et al. 1994). Each 50-μl PCR reaction used to amplify the COI fragment consisted of the same ingredients as 16S, with the exception of 3 μl of DNA template and 36.9 μl of water. Procedures for PCR amplification involved one cycle of 1 min at 94°C; 40 cycles of 1 min at 94°C, 1.5 min at 45°C, and 1.5 min at 72°C; followed by one cycle of 5 min at 72°C in an MJ Research PCR machine (PTC-100). PCR products were gel purified (1.6% agarose) using the Qiaex II kit (Qiagen). DNA fragments were sequenced with an ABI Prism 377 automated sequencer (Applied Biosystems), with primers 16S-AR and LCO1490 respectively, and the Taq FS dye rhodamine sequencing kit (Perkin-Elmer). All sequences were checked for accidental amplification of contamination DNA by searching the Genbank/EMBL database (Altschul et al. 1997).

Table 2. GenBank accession numbers for COI DNA sequences isolated from rotifers.

Species	Haplotype	Accession number
Asplanchna sp.	1	AF499052
Brachionus calyciflorus	1	AF499053
Brachionus plicatilis	1	AF499054
	2	AF499065
	3	AF499064
	4	AF499057
	5	AF499063
	6	AF499061
	7	AF499058
	8	AF499060
	9	AF499059
	10	AF499062
	11	AF499068
	12	AF499069
	13	AF499066
	14	AF499067
	15	AF499055
	16	AF499056
Brachionus urceolaris	1	AF499070
	2	AF499072
	3	AF499071
Keratella cochlearis var. *faluta*	1	AF499073
Keratella cochlearis var. *robusta*	1	AF499074
	2	AF499075
Keratella cochlearis var. *tecta*	1	AF499076
	2	AF499087
Keratella hiemalis	1	AF499077
	2	AF499085
	3	AF499086
Keratella quadrata	1	AF499078
	2	AF499081
	3	AF499080
	4	AF499084
	5	AF499079
	6	AF499083
	7	AF499082
Synchaeta sp.	1	AF499090
	2	AF499092
	3	AF499091
Synchaeta c.f. *pectinata*	1	AF499088
	2	AF499093
	3	AF499089

Phylogenetic analyses—16S sequences were aligned with Sequence Navigator (ABI Prism, Applied Biosystems, Perkin Elmer) using the clustal alignment default parameters. Segments of the 16S gene were omitted where alignments were ambiguous as a result of gap hypervariability. As a result, 387 bp were used in the phylogenetic analyses of the 16S sequence data with gaps treated as missing data because of the difficulty of modeling insertions and deletions (GenBank accession numbers AF499036 to AF499051; Table 1). The COI sequences were aligned with Seqapp 1.9a sequence editor (ftp://iubio.bio.indiana.edu/molbio/seqapp/) using default parameters, and an unambiguous alignment was produced because of the absence of gaps (GenBank accession numbers AF499052 to AF499093; Table 2). COI sequences were obtained from GenBank database for *Bra-*

chionus plicatilis from Spain (accession numbers AF266853 to AF266950; Gómez et al. 2000) and Australia (AF387279 to AF387280), as well as for *B. ibericus* (AF387270 to AF387280) and *B. rotundiformis* (AF387287 to AF387293) (Gómez et al. 2002*b*). These sequences were used to provide a more comprehensive understanding of geographic divergence within the *B. plicatilis* species complex. Phylogenetic trees were generally rooted with an outgroup taxon (*Asplanchna* sp.) of a species belonging to the same order, but a different family than the family under study. For comparisons of intraspecific divergence within the *B. plicatilis* species complex, phylogenetic trees were rooted with the freshwater species, *B. calyciflorus.* All characters were treated as unordered and equally weighted because neither transitions nor transversions appeared to be saturated for 16S or COI.

Analyses were conducted with PAUP 4.02b (Swofford 1998) except where otherwise noted. A chi-square goodness-of-fit test was performed on the sequence data for each gene region to determine whether shifts in nucleotide composition occurred among taxa. Whereas base frequencies were homogeneous among 16S sequences (homogeneity, $\chi^2 = 15.2$, df = 45, $p > 0.99$), nucleotide composition was variable for COI (homogeneity, $\chi^2 = 397.3$, df = 129, $p < 0.01$) when all taxa were included. COI base composition was also heterogeneous for comparisons within the *B. plicatilis* species complex when data from GenBank were included (homogeneity, $\chi^2 = 232.2$, df = 171, $p < 0.01$). The Kimura two-parameter distance model (Kimura 1980) was employed to correct for the possibility of multiple superposed substitution events in the 16S sequences because the assumption of homogeneous base composition was met and there was no evidence of transitional saturation (Kumar et al. 1993). This simple measure was chosen because more complex distance models yielded similar topologies for phylogenetic trees, and variances are lower when fewer parameters are estimated. The Log Det distance transformation (Lake 1994) was employed to correct pairwise differences between COI sequences involving all of the taxa from this study and for comparisons within the *B. plicatilis* species complex because this measure compensates for base heterogeneity. Matrices of distance measures with standard errors were determined with MEGA 1.02 (Kumar et al. 1993) for the Kimura two-parameter model and with PAUP 4.02b for Log Det calculations. Mean nucleotide diversity (π), the average proportion of pairwise nucleotide base differences at a particular sequence position, was calculated for selected interspecific comparisons (Hartl 2000).

Both phenetic (neighbor joining) and cladistic (maximum parsimony) analyses were employed in the phylogenetic studies. Whereas phenetic techniques are based on pairwise distances among taxa, cladistic methods infer common ancestry and operate on phylogenetically informative sites. A phylogenetically informative site is a character that is both different from the outgroup (variable) and shared within the taxonomic group in question at a particular position within a set of aligned sequences. Matrices of distance measures were used to estimate neighbor-joining (NJ) phenograms with confidence limits determined with 1,000 bootstrap pseudoreplicates for 16S rDNA and COI nucleotide sequences. Maximum parsimony (MP) analyses of phylogenetically informative sites employed heuristic searches with a starting tree obtained by 1,000 replicates of random stepwise sequence addition. Optimal trees were found with the tree bisection–reconnection (TBR) branch swapping algorithm and the MulTrees and steepest descent options invoked in PAUP 4.02b. Groups appearing in ≥70% of the bootstrap replicates were considered well supported (Hillis and Bull 1993). Confidence was assessed in the cladistic analyses both a priori, by estimation of the g_1 skewness statistic from 100,000 random tree length distributions, and a posteriori, by bootstrap analysis with 1,000 pseudoreplicates. A monophyletic clade is a taxonomic set of common descendents (e.g., species) that cluster on a common branch of a phylogenetic tree.

Results

16S Comparisons among rotifer taxa—Sequence variability: The sequence alignment was 387 base pairs (bp), of which 150 bp were variable and 113 bp were phylogenetically informative using cladistic criteria. Mean base frequencies were 0.32 (A), 0.14 (C), 0.18 (G), and 0.36 (T), and there was no evidence of heterogeneity in nucleotide composition among taxa (homogeneity $\chi^2 = 15.22$, df = 45, $p > 0.99$). Table 3 shows the number of 16S haplotypes per species and the number of individuals detected with each haplotype.

Phylogenetic analyses: NJ analysis indicated that members of the families Brachionidae and Synchaetidae showed an average of 23.0% sequence divergence at 16S, while members of the two brachionid genera, *Brachionus* sp. and *Keratella* sp., showed a mean divergence of 22.1% (Table 4). The two freshwater species (*B. calyciflorus* and *B. urceolaris*) and the single saltwater species (*B. plicatilis*) showed 8.0% divergence, while the two freshwater species were 4.1% divergent. *Keratella cochlearis* showed 17.3% sequence divergence from *K. hiemalis* and *K. quadrata,* while *K. hiemalis* was 10.7% divergent from *K. quadrata.*

Maximum parsimony heuristic searches yielded two equally parsimonious trees of length 297 (consistency index [CI] = 0.72, homoplasy index [HI] = 0.28, retention index [RI] = 0.83). These trees were equivalent with respect to the position of clades, differing only in the branch arrangement for haplotypes 2 and 5 of *B. plicatilis,* and this variation was collapsed in the 70% majority rule tree (Fig. 2). The g_1 skewness statistic was highly significant ($g_1 = -0.80$, $g_{1\ crit} = -0.20$, $p = 0.01$), which indicates strong phylogenetic signal in the 16S data set. Both phenetic and cladistic analyses of the 16S sequence data were congruent with respect to branch arrangement, and the bootstrap support for these nodes was strong (>70%). Resolution of the two families and three genera were in agreement with prior morphological assignments, and all three genera were monophyletic (Fig. 2).

COI comparisons among rotifer taxa—Sequence variability: Sequence alignments and amino acid translations were unambiguous, since there were no gaps or nonsense codons among the 84 COI sequences (Table 5). The aligned sequences corresponded to nucleotide position 1543 to 2173

Table 3. Species, number of individuals sequenced, number of haplotypes, locations of sample collection, and number of different lakes from which samples were collected at each location for 16S rotifer sequences.

Species	Number of 16S haplotypes	Number of individuals sequenced	Locations (number of different lakes)
Brachionus calyciflorus	1	4	Gainesville, Florida
Brachionus plicatilis	3	5	Wood Buffalo National Park (3)
Brachionus urceolaris	1	1	Fort McMurray, Alberta (1)
Keratella cochlearis	4	5	Wood Buffalo National Park (2), Fort McMurray, Alberta (1)
Keratella hiemalis	1	1	Wood Buffalo National Park (1)
Keratella quadrata	1	3	Wood Buffalo National Park (1), Fort McMurray, Alberta (1)
Synchaeta sp.	2	2	Wood Buffalo National Park (1)
Synchaeta c.f. *pectinata*	2	5	Wood Buffalo National Park (3)

of *Drosophila yakuba* (Folmer et al. 1994). The length of the nucleotide sequence alignment was 630 bp, of which 294 bp were variable for distance-based analysis and 284 were phylogenetically informative using cladistic criteria. Base frequencies among rotifer species were heterogeneous and averaged at 0.21 (A), 0.20 (C), 0.18 (G), and 0.41 (T) (homogeneity, $\chi^2 = 397.32$, df = 129, $p < 0.001$). When translated to amino acids using the *Drosophila* mtDNA code, 54 of 210 positions were variable and 51 were phylogenetically informative according to MP.

Phylogenetic analyses: The average nucleotide sequence divergence between members of the families Brachionidae (A, B) and Synchaetidae (C) was 29.9% (Fig. 3), while the two brachionid genera differed by 29.1% (Table 6). Freshwater and saltwater *Brachionus* species showed a sequence divergence of 24.6%. Pairwise nucleotide sequence divergence between selected species of *Brachionus* ranged from 20 to 25%, those of *Keratella* from 23 to 27%, while *Synchaeta* isolates ranged from 1 to 20%.

The various isolates of *Keratella cochlearis* clustered according to the presence (*K. cochlearis* var. *faluta* [f-1] and *K. cochlearis* var. *robusta* [r-1 and r-2]) or absence (*K. cochlearis* var. *tecta* [t-1 and t-2]) of posterior spines (Fig. 3), with 4.4% mean COI nucleotide sequence divergence (Table 6). The divergence of spined and unspined *K. cochlearis* types was also apparent at the amino acid level, as the two groups showed 0.9% divergence (Table 6). By comparison, different morphs of *K. hiemalis* (one- and two-spined) showed only 0.21% sequence divergence and no amino acid divergence. *K. quadrata* showed low intraspecific nucleotide divergence, ranging from 0.1 to 0.7%, and little amino acid divergence (0 to 0.5%). *K. quadrata* 7 was from the most geographically distant location (330 km away) but showed only slightly greater mean nucleotide sequence divergence from the other haplotypes (0.5%, Fig. 3). The average nucleotide diversity (π) of seven haplotypes identified among 27 *K. quadrata* sequences was 0.09, and the mean number of nonsynonymous changes between these haplotypes was just 0.3.

Table 4. Mean 16S divergence ± SE between phylogenetic clusters of rotifers identified by NJ.*

Clade	*B. calyciflorus*	*B. plicatilis*	*B. urceolaris*	*K. cochlearis*	*K. hiemalis*	*K. quadrata*	*Synchaeta* sp.	*S.* c.f. *pectinata*
B. calyciflorus	—	—	—	—	—	—	—	
B. plicatilis	$0.0861 \pm 9.8\times10^{-4}$	$\mathbf{0.0053 \pm 1.5\times10^{-3}}$	—	—	—	—	—	
B. urceolaris	0.0406	$0.0739 \pm 9.7\times10^{-4}$	—	—	—	—	—	
K. cochlearis	$0.2154 \pm 3.0\times10^{-3}$	$0.2085 \pm 1.3\times10^{-4}$	$0.2082 \pm 1.8\times10^{-3}$	$\mathbf{0.0120 \pm 1.5\times10^{-3}}$	—	—	—	
K. hiemalis	0.2186	$0.2279 \pm 1.2\times10^{-3}$	0.2257	$0.1.624 \pm 1.7\times10^{-3}$	—	—	—	
K. quadrata	0.2191	$0.2406 \pm 2.1\times10^{-3}$	0.2296	$0.1842 \pm 8.1\times10^{-4}$	0.01067	—	—	
Synchaeta sp.	$0.1641 \pm 3.5\times10^{-3}$	$0.1673 \pm 2.0\times10^{-3}$	$0.1661 \pm 8.4\times10^{-3}$	$0.2906 \pm 3.9\times10^{-3}$	$0.2975 \pm 2.3\times10^{-2}$	$0.3105 \pm 1.6\times10^{-2}$	**0.0803**	
S. c.f. *pectinata*	0.1638	$0.1675 \pm 2.0\times10^{-3}$	0.1610	$0.2823 \pm 1.8\times10^{-3}$	0.2974	$0.2928 \pm 2.0\times10^{-3}$	$0.0688 \pm 4.0\times10^{-3}$	**0.0026**

* The mean nucleotide sequence divergence within clusters is shown on the diagonal (bold). The mean nucleotide divergence between clusters is shown below the diagonal. The distance estimates were corrected with Kimura's (1980) two-parameter model.

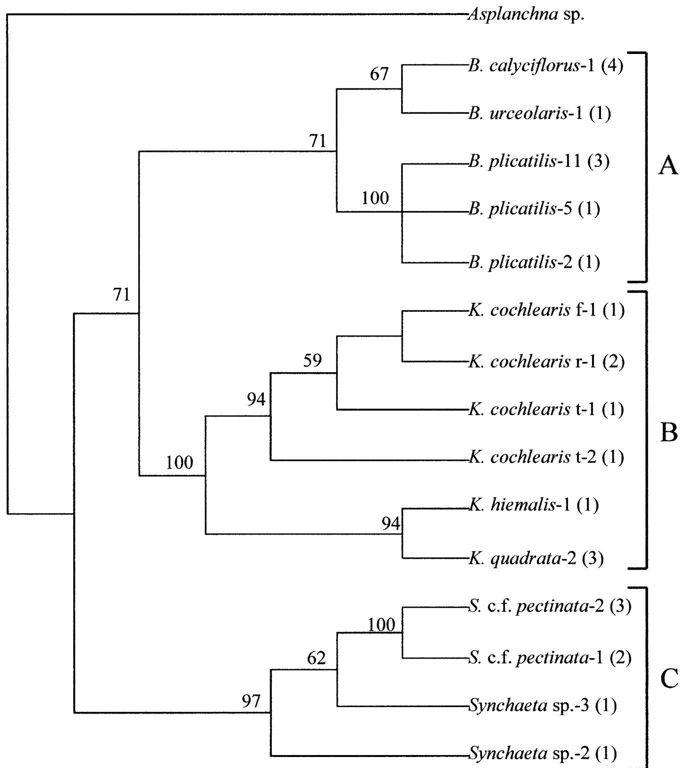

Fig. 2. Seventy percent majority rule consensus of two equally parsimonious trees for 15 16S rotifer sequences rooted with *Asplanchna* sp. Bootstrap support (1,000 pseudoreplicates) for major clades is indicated above the nodes. Numbers at the terminal branches give the haplotype and correspond with the numbering assigned to equivalent COI taxa. Numbers in brackets after the terminal branches indicate the number of sequences obtained for each haplotype. Capitalized letter clades indicate monophyletic clusters representing different genera: A = *Brachionus* sp., B = *Keratella* sp., and C = *Synchaeta* sp.

Sequence divergence at COI between haplotypes of *Brachionus plicatilis* ranged from 0.1 to 1.4%, with haplotypes 15 and 16 demonstrating the greatest mean divergence (0.9%) from the other isolates (Table 6). The average nucleotide diversity (π) of the 16 haplotypes of *B. plicatilis* from Wood Buffalo National Park, Canada, was 0.74, and the mean number of amino acid substitutions between haplotypes was 0.8. Four amino acid variants of *B. plicatilis* were detected (Fig. 3: clades a, b, and c), showing 0.5 to 0.9% amino acid sequence divergence (Table 6). These variants clustered according to the lakes from which they were collected. Clade a was obtained from saline Hay Camp lake (Fig. 1), while clade c was collected from a subsaline lake (Forgotten Pass), which was only 10 km away (Fig. 1). Clade b was collected from two interconnected saline lakes (Grosbeak and Salt Pan) that were 45 km away from the other two lakes (Fig. 1). Haplotypes 15 and 16 (clade c) showed a mean nucleotide sequence divergence of 0.5% from clade a and 0.9% from clade b.

COI amino acid NJ and MP trees were congruent with 16S and COI nucleotide trees for recent evolutionary events (genus and species) but did not resolve some of the deeper taxonomic relationships. One thousand random addition search replicates yielded a single COI amino acid MP tree of length 92 (CI = 0.77, HI = 0.23, RI = 0.96). *K. hiemalis* was more closely allied with *K. quadrata* (2.3%) than with *K. cochlearis* (6.5%) according to the amino acid sequence data (Table 4), a result congruent with the 16S MP tree. However, the COI amino acid phenetic and cladistic analyses failed to resolve the families Brachionidae and Synchaetidae. Phenetic and cladistic COI amino acid analyses were congruent with 16S data for all other deep internal nodes, and bootstrap values were high for recent evolutionary events.

COI comparisons within Brachionus plicatilis—Sequence variability: *Brachionus plicatilis* COI haplotypes detected in this study (Table 5) were aligned with *B. plicatilis* haplotypes from Spain (Gómez et al. 2000) and Australia, as well as with *B. ibericus* and *B. rotundiformis* (Gómez et al. 2002*b*). The length of the sequence alignment (nucleotide position 1543 to 2141 in *Drosophila yakuba*) was 603 bp, of which 227 bp were variable and 206 bp were phyloge-

Table 5. Species, number of individuals sequenced, number of haplotypes, locations of sample collection, and number of different lakes from which samples were collected at each location for COI rotifer sequences.

Species	Number of COI haplotypes	Number of individuals sequenced	Locations (number of different lakes)
Brachionus calyciflorus	1	2	Gainesville, Florida
Brachionus plicatilis	16	22	Wood Buffalo National Park (4)
Brachionus urceolaris	4	7	Fort McMurray, Alberta (1)
Keratella cochlearis	5	9	Wood Buffalo National Park (3), Fort McMurray, Alberta (1)
Keratella hiemalis	3	7	Wood Buffalo National Park (2)
Keratella quadrata	7	26	Wood Buffalo National Park (4), Fort McMurray, Alberta (1)
Synchaeta sp.	3	5	Wood Buffalo National Park (1)
Synchaeta c.f. *pectinata*	3	7	Wood Buffalo National Park (3)

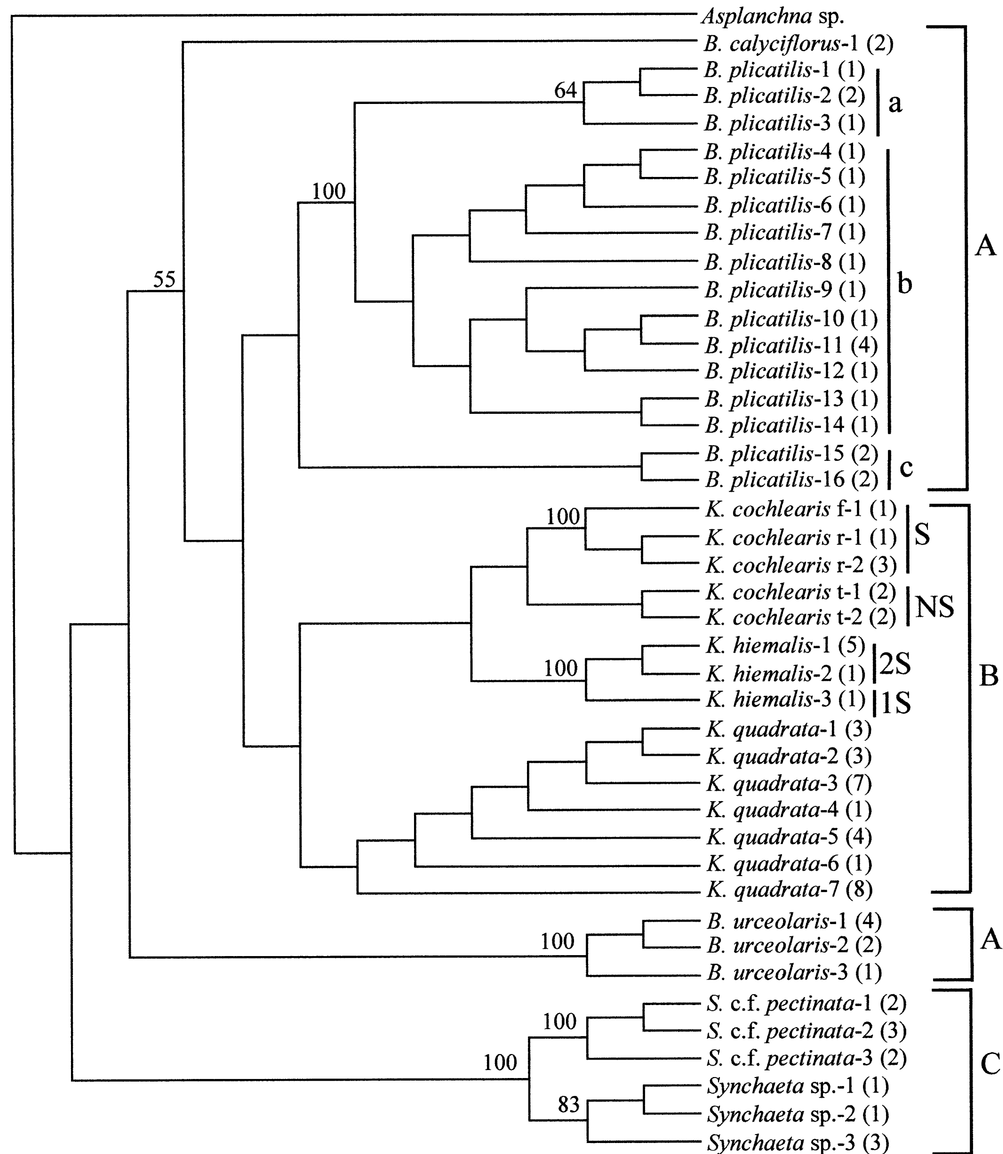

Fig. 3. Neighbor-joining phenogram of 42 COI rotifer nucleotide sequences based on Log Det distances (Lake 1994) and rooting with *Asplanchna* sp. Bootstrap support (1,000 pseudoreplicates) for major clades is indicated above the nodes. Numbers at the terminal branches give the nucleotide haplotype and correspond with the numbering assigned to equivalent 16S taxa. Numbers in brackets after the terminal branches indicate the number of sequences obtained for each haplotype. Capitalized letter clades indicate clusters representing different genera: A = *Brachionus,* B = *Keratella,* and C = *Synchaeta.* Lowercase letter clades indicate *Brachionus plicatilis* haplotypes that clustered according to lakes from which the animals were collected: clade a = Hay Camp Lake, clade b = Grosbeak/Salt Pan lakes, and clade c = Forgotten Pass Lake. Spined (S) and unspined (NS) subspecies of *Keratella cochlearis* as well as one- (1S) and two- (2S) spined morphs of *K. hiemalis* are indicated.

netically informative using cladistic criteria. The base composition of these sequences was heterogeneous ($\chi^2 = 232.2$, df = 171, $p < 0.01$) with 0.20 (A), 0.21 (C), 0.20 (G), and 0.39 (T). Nucleotide sequences translated to 201 amino acids according to *Drosophila* mtDNA code, of which 17 amino acid positions were variable, and only seven characters were phylogenetically informative for cladistic analysis.

Phylogenetic analyses: The NJ and MP phylogenetic trees for the COI nucleotide sequence data revealed that haplotypes collected from Canadian salt lakes clustered with *B. plicatilis* sequences from Spain and Australia rather than with *B. ibericus* or *B. rotundiformis* within the *B. plicatilis* species complex (Fig. 4). While Canadian and Spanish populations of *B. plicatilis* had an average nucleotide sequence divergence of 4.1%, Canadian and Australian varieties were divergent by 5.7%. Average nucleotide sequence divergence between the two major Spanish lineages was 2.6%, which represents divergence among two different glacial refugia (Gómez et al. 2000). Nucleotide sequence divergences with-

Table 6. Mean COI divergence ± standard errors among rotifer species identified by NJ.*

Clade	*B. calyciflorus*	*B. plicatilis*	*B. urceolaris*	*K. cochlearis*	*K. hiemalis*	*K. quadrata*	*Synchaeta* sp.	*S.* c.f. *pectinata*
B. calyciflorus	—	0.0379± 4.7×10^{-4}	0.0509	0.1111	0.1111	0.0979± 6.7×10^{-4}	0.1358± 3.1×10^{-3}	0.1173± 4.1×10^{-3}
P. plicatilis	0.2339± 4.4×10^{-4}	**0.0060± 2.2×10^{-4}**	0.0550± 2.3×10^{-4}	0.1198± 1.8×10^{-4}	0.1213± 2.6×10^{-4}	0.1067± 2.3×10^{-4}	0.1409± 6.3×10^{-4}	0.1315± 3.6×10^{-4}
B. urceolaris	0.2017± 1.0×10^{-3}	0.2511± 3.3×10^{-4}	—	0.1065	0.1157	0.0965± 3.6×10^{-4}	0.1450± 1.3×10^{-3}	0.1296± 2.0×10^{-3}
K. cochlearis	0.2710± 5.3×10^{-3}	0.3146± 7.5×10^{-4}	0.2921± 6.8×10^{-4}	**0.0277± 6.8×10^{-3}**	0.0833	0.0654± 2.8×10^{-4}	0.1250± 1.8×10^{-3}	0.1173± 1.6×10^{-3}
K. hiemalis	0.2582± 1.5×10^{-3}	0.3059± 4.9×10^{-4}	0.2785± 7.3×10^{-4}	0.2610± 8.4×10^{-4}	**0.0021± 5.1×10^{-4}**	0.0227± 3.7×10^{-4}	0.1482± 2.3×10^{-3}	0.1358± 2.8×10^{-3}
K. quadrata	0.2724± 1.1×10^{-3}	0.2738± 2.9×10^{-4}	0.2893± 6.7×10^{-4}	0.2676± 5.3×10^{-4}	0.2315± 6.1×10^{-4}	**0.0039± 4.3×10^{-4}**	0.1303± 1.5×10^{-3}	0.1180± 1.8×10^{-3}
Synchaeta sp.	0.2710± 1.6×10^{-2}	0.3208± 8.6×10^{-4}	0.2633± 1.0×10^{-2}	0.3071± 5.5×10^{-3}	0.2984± 4.6×10^{-3}	0.3197± 6.4×10^{-3}	**0.1409± 1.7×10^{-2}**	0.0463± 2.9×10^{-3}
S. c.f. *pectinata*	0.2269± 2.8×10^{-3}	0.3272± 8.5×10^{-7}	0.2440± 1.2×10^{-3}	0.2648± 2.3×10^{-2}	0.2836± 2.7×10^{-3}	0.3063± 2.0×10^{-3}	0.1705± 5.5×10^{-3}	**0.0343± 2.0×10^{-2}**

* The mean uncorrected (*p* distance) amino acid sequence divergence between clusters is shown above the diagonal. The mean nucleotide sequence divergence within clusters is shown on the diagonal (bold), while the mean nucleotide sequence divergence between clusters is shown below the diagonal. The nucleotide distance estimates were corrected with the Log-Det transformation (Lake 1994).

in these two Spanish lineages, however, were similar to that observed among *B. plicatilis* haplotypes (0.1 to 1.4%) from Canada. Canadian *B. plicatilis* haplotypes had average nucleotide divergences of 25.6% and 22.8%, respectively, from *B. ibericus* and *B. rotundiformis.* The phylogenetic signal was strong for the nucleotide data ($g_1 = -0.56$, $g_{1\ crit} < -0.12$, $p = 0.01$). Maximum parsimony heuristic searches yielded 75 parsimonious trees of length 387 (CI = 0.74, HI = 0.26, RI = 0.77). These MP trees varied in haplotype branch arrangement within Spanish and Canadian clades of *B. plicatilis,* but all trees resolved these two groups of populations. Additionally, resolution among *B. plicatilis, B. ibericus,* and *B. rotundiformis* was conserved among MP trees.

Discussion

Between-gene comparisons—Past morphological studies have placed the genera *Keratella* and *Brachionus* in the family Brachionidae, while *Synchaeta* sp. are in the family Synchaetidae (Pennak 1989). The tree topologies derived through NJ and MP analyses of 16S and COI sequence data were largely congruent with these conclusions. However, COI nucleotide data varied from 16S in the placement of *Keratella hiemalis* and in the positioning of freshwater *Brachionus* taxa in the NJ tree. As expected, the COI amino acid trees resolved these uncertain branch positions according to 16S, but were poor at resolving deep evolutionary relationships (e.g., families).

The similarity in levels of nucleotide sequence divergence between the Brachionidae and Synchaetidae (Tables 4 and 6) likely reflects homoplasy (Page and Holmes 1998). This was particularly likely for COI, which evolves much faster than 16S (1.4 to 2.3% versus 0.4 to 0.9% per million years) (e.g., Knowlton and Weight 1998; Schubart et al. 1998). COI bootstrap values could not be resolved for deep nodes between families and genera (Fig. 3). It is likely that 18S rRNA, a more slowly evolving nuclear gene (0.1% per million years) (Spears et al. 1992), would provide better resolution of familial boundaries than 16S rRNA. The rapidly evolving COI gene was, however, very appropriate for resolution of recent evolutionary relationships at the population level.

Phenotypic plasticity among keratella species—Because environmental and genetic factors cause morphological variability in rotifers, the delineation of species boundaries is often difficult (Pennak 1989). *Keratella cochlearis* has long been recognized as a complex of morphs that vary in their degree of posterior spine development (Dumont 1983). Long-spined individuals dominate oligotrophic and cold habitats, while morphs with shorter or no spines are abundant in eutrophic, warm environments (e.g., Hillbricht-Ilkowska 1983). Although spine development in *K. cochlearis* is induced by predators (Stemberger and Gilbert 1984; Conde-Porcuna et al. 1993), the biometric analysis of Hofmann (1983) did not detect any transitional forms between spined and unspined morphs, which suggests genetic divergence rather than phenotypic plasticity. Our COI sequence data support this conclusion, indicating that the *K. cochlearis* complex is comprised of two clades: a spined clade consisting of two ''subspecies'' (*K. cochlearis* var. *robusta* and *K. cochlearis* var. *faluta*) and an unspined clade comprised of *K. cochlearis* var. *tecta.* The marked genetic divergence (4.4% nucleotide sequence divergence) between the spined and unspined morphs suggests that *K. cochlearis* is, in fact, a species complex.

The taxonomy of *K. hiemalis* has been much debated because some European populations have low morphological variability (Ruttner-Kolisko 1993), while the taxon shows much phenotypic variation in North America (Stemberger in the addendum for Ruttner-Kolisko 1993). Our results suggest that this variation reflects phenotypic plasticity, since we did

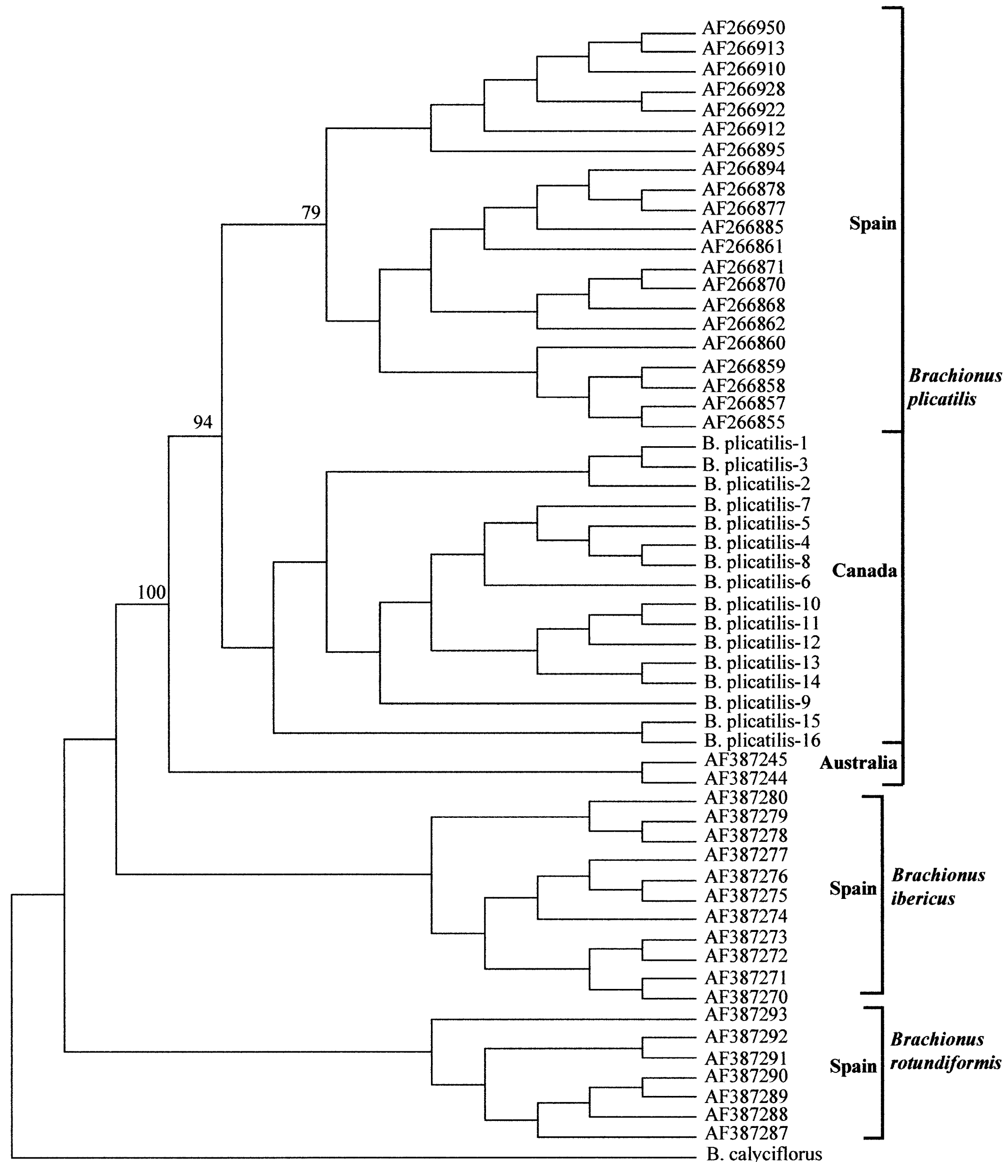

Fig. 4. Neighbor-joining phenogram of 57 COI nucleotide sequences from the *Brachionus plicatilis* species complex based on Log Det distances (Lake 1994) and rooting with *B. calyciflorus.* Accession numbers AF266855 to AF266950 belong to 21 *B. plicatilis* haplotypes collected by Gomez et al. (2000) from Spanish salt lakes and AF387244 to AF387245 were collected from Australia (Gómez et al. 2002*b*). *B. plicatilis* 1 to 16 were collected from salt lakes in Wood Buffalo National Park, Canada. Numbers at the terminal branches give nucleotide haplotypes for populations from Canada and correspond with the numbering assigned on other 16S and COI trees. *B. ibericus* (AF387270 to AF387280) and *B. rotundiformis* (AF387287 to AF387293) were collected by Gómez et al. (2002*b*) from Spain. Bootstrap support (1,000 pseudoreplicates) for major clades is indicated above the nodes.

not detect any genetic divergence between one-spined and two-spined morphs of *K. hiemalis.* This morphological variation may be determined by chemical cues exuded by predatory copepods and *Asplanchna* sp., as for *K. testudo* (Stemberger and Gilbert 1987).

Both 16S and COI genes revealed a deep genetic divergence between the summer species, *K. quadrata,* and its winter counterpart, *K. hiemalis* (Stemberger 1979; Ruttner-Kolisko 1993). *K. quadrata* is usually recognized by its two long, curved posteriolateral spines, but this characteristic is highly variable, causing confusion with the shorter spined *K. hiemalis* (Ruttner-Kolisko 1993). Our results indicate that these two taxa are clearly distinct.

Genetic divergence among rotifer populations—Substantial sequence divergence was present at COI among *Brachionus plicatilis* populations from different saline lakes within a small geographic area of northern Canada. These

haplotypes clustered according to their lake of origin with overlap only apparent between two interconnected lakes. Studies of other inland zooplankton have also revealed little genetic structure among populations from interconnected lakes (De Meester 1996; Straughan and Lehman 2000) and localization of haplotypes in unconnected ponds (Gómez et al. 2000). Zooplankton possess high dispersal potential, and experimental work suggests that these organisms are rapid colonizers of new ponds (Jenkins 1995). Genetic research supports evidence for rapid colonization of new environments by passively dispersing aquatic organisms (Weider et al. 1999) but restricted gene flow among established populations (e.g., DeMeester 1996; Colbourne et al. 1997; Gómez et al. 2000, 2002*a;* De Meester et al. 2002). Processes proposed to explain this paradox in planktonic pond populations include selection, founding events, population age, life history, inbreeding, and drift (e.g., Gómez et al. 2002*a*). The magnitude of genetic divergence among *B. plicatilis* haplotypes from northern Canada suggests that these lineages last shared a common ancestor 300,000 to 700,000 years ago (e.g., Knowlton and Weight 1998; Schubart et al. 1998). However, their habitats have existed for less than 8,000 years (Smith 1994). Founder effects associated with colonization events, in combination with local adaptations, likely contributed to the genetic divergence that we observed among nearby *B. plicatilis* populations (De Meester et al. 2002; Gómez et al. 2002*a*).

Freshwater populations of *Keratella quadrata* showed much less COI nucleotide divergence between populations (0.2% average) than in *B. plicatilis* (0.8% average). In addition, the average nucleotide diversity (π) of *B. plicatilis* haplotypes was eight times higher and the mean number of nonsynonymous changes was more than twice as great as between *K. quadrata* haplotypes. The low intraspecific divergence of freshwater *K. quadrata* compared to halophilic *B. plicatilis* may be a result of inherent differences, such as dispersal ability (Jenkins 1995), colonization history, and differences in local adaptations (DeMeester et al. 2002; Gómez et al. 2002*a*). Further, salinity has been shown to increase rates of molecular evolution in halophilic plankton (Hebert et al. 2002).

B. plicatilis is a complex of at least three sibling species: *B. plicatilis, B. ibericus,* and *B. rotundiformis* (Ciros-Pérez et al. 2001; Gómez et al. 2002*b*). These species possess different salinity, temperature, and seasonal preferences (Ciros-Pérez et al. 2001). *B. plicatilis* is found at low to high salinities (3 to 45 g L^{-1}) and low temperatures (<25°C) and is present in the water column during the spring, fall, and autumn. *B. ibericus* occurs in waters with medium to high salinities (8 to 50 g L^{-1}) and high temperatures (>15°C) during the spring and summer. *B. rotundiformis* typically occurs in habitats with medium to high salinities (10 to 57 g L^{-1}) and high temperatures (10 to 30°C) during the spring, summer, and autumn. Although additional sibling species within the *B. plicatilis* complex will likely be found with further research (Ortells et al. 2000; Gómez et al. 2002*b*), the present results suggest that strains from northern Canada belong to *B. plicatilis* (Fig. 4). Lineages of *B. plicatilis* from Canada showed an average nucleotide sequence divergence of 4.1% and 5.7%, respectively, from Spanish and Australian lineages. The average sequence divergence between the two major Spanish lineages, which derived from different glacial refugia, was about twice as great as that observed in the Canadian populations. However, the mean sequence divergence within each of these clades was similar to that detected in Canada.

The results of this study are congruent with some conclusions from prior taxonomic work. However, species boundaries among some morphologically variable rotifers need revision. For example, *Keratella cochlearis* shows a deep genetic divergence between spined and unspined forms. Environmental variation is one of many factors that may contribute to the patterning of genetic diversity among zooplankton populations. For example, greater genetic diversity was detected among populations of the halophilic *Brachionus plicatilis* among closely located saline lakes than in the freshwater species *Keratella quadrata.* However, mechanisms responsible for divergence in zooplankton populations are complex. Founder effects from colonization events, stochastic events, connectivity patterns of habitats, and local adaptations all likely influence population divergence of rotifers. Molecular phylogenetics can be broadly applied to monogonont rotifers to improve our understanding of factors that control dispersal, colonization, and gene flow among their populations.

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