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PRIMARY RESEARCH PAPER

Validating the systematic position of *Plationus* Segers, Murugan & Dumont, 1993 (Rotifera: Brachionidae) using sequences of the large subunit of the nuclear ribosomal DNA and of cytochrome C oxidase

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Abstract Members of the family Brachionidae are free-living organisms that range in size from 170 to 250 microns. They comprise part of the zooplankton in freshwater and marine systems worldwide. Morphologically, members of the family are characterized by a single piece loricated body without furrows, grooves, sulci or dorsal head shields, and a malleate trophi. Differences in these structures have been traditionally used to recognize 217 species that are classified into seven genera. However, the validity of the species, *Plationus patulus*, *P. patulus macracanthus P. polyacanthus*, and *P. felicitas* have been confused because they were alternatively assigned in *Brachionus* or *Platyias*, when considering only morphological and ecological characters. Based on scanning electron microscope (SEM) images of the trophi, these taxa were assigned in a new genus, *Plationus*. In this study, we examined the systematic position of *P. patulus* and *P. patulus macracanthus* using DNA sequences of two genes: the cytochrome oxidase subunit 1 (*cox1*) and domains D2 and D3 of the large subunit of the nuclear ribosomal RNA (LSU). In addition, the *cox1* and LSU sequences representing five genera of Brachionidae (*Anuraeopsis*, *Brachionus, Keratella*, *Plationus,* and *Platyias*) plus four species of three families from the order Ploima were used as the outgroup. The maximum likelihood (ML) analyses were conducted for each individual gene as well as for the combined (*cox1* + LSU) data set. The ML tree from the combined data set yielded the family Brachionidae as a monophyletic group with weak bootstrap support (<50%). Five main clades in this tree had high (>85%) bootstrap support. The first clade was composed of three populations of *P. patulus* +*P. patulus macracanthus*. The second clade was composed of a single species of *Platyias*. The third clade was composed of six species of *Brachionus.* The fourth clade included a single species of the genus *Anuraeopsis*, and the fifth clade was composed of three species of the genus *Keratella*. The genetic divergence between *Plationus* and *Platyias* ranged from 18.4 to 19.2% for *cox1,* and from 4.5 to 4.9% for LSU, and between *Brachionus* and *Plationus,* it ranged from 16.9 to 23.1% (*cox1*), and from 7.3 to 9.1% (LSU). Morphological evidence, the amount of genetic divergence, the

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systematic position of *Plationus* within the family Brachionidae, and the position of *Plationus* as a sister group of *Brachionus* and *Platyias* support the validity of *Plationus patulus* and *P. patulus macracanthus* into the genus *Plationus*.

Keywords *Plationus* · *Cox1* · LSU · Maximum likelihood · Phylogeny

Introduction

The family Brachionidae Ehrenberg, 1838 (Monogononta) is one of the most diverse groups of rotifers with approximately 217 species classified into seven genera: *Anuraeopsis* Lauterborn, 1900, *Brachionus* Pallas, 1766, *Keratella* Bory de St. Vincent, 1822, *Notholca* Gosse, 1886, *Kellicotia* Ahlstrom, 1938, *Plationus* Segers, Murugan & Dumont, 1993 and *Platyias* Harring, 1913 (Segers, 2007). This family is diagnosed by a loricated body without furrows, grooves, sulci or dorsal head shields, lorica formed by one piece without covering all the body, a malleate trophi that consists of a pair of mallei and each malleus formed by a manubrium and a paired uncus with 4–7 teeth. The taxonomic position of some Brachionidae species has been controversial due to high phenotypic plasticity of their diagnostic morphological characters, which has impaired a robust phylogenetic hypothesis (Stelzer, 2002; Gilbert & Walsh, 2005; Gómez, 2005; Van der Stap et al., 2007). In particular, the validity of *Plationus patulus* (Müller, 1786), *P. patulus macracanthus* (Daday, 1905), and *P. polyacanthus* (Ehrenberg, 1834) has been questioned when only morphological characters are considered. These three taxa were originally described in the genus *Brachionus* and confirmed by subsequent morphological studies (Turner, 1940; Wulfert, 1965; Koste, 1978; Koste & Shiel, 1987). However, Ahlstrom (1940), Bartos (1959), Rudescu (1960), and Kutikova (1970) analyzed the taxonomic validity of *P. patulus*, *P. patulus macracanthus*, and *P. polyacanthus* and placed these taxa within *Platyias* because they present a foot with three pseudosegments as in the genus *Platyias*. A study based on ultrastructural characters placed these three taxa neither in *Platyias* nor *Brachionus*, but in the genus *Plationus* (Segers et al., 1993). In the most recent revision of Rotifera, the genus *Plationus* contains three species, *Plationus felicitas* (Wulfert, 1965), *P. patulus,* and *P. polyacanthus*, and two subspecies, *P. patulus patulus* and *P. patulus macracanthus* (Segers, 2007). However, some studies do not recognize the validity of these species and subspecies as members of *Plationus*, and still classify them with *Brachionus* or *Platyias* (Kotikova et al., 2005; Xian-Ling et al., 2006; Nandini et al., 2007; Kennari et al., 2008; Sarma et al., 2008). The aim of this study was to develop a phylogeny for five genera of Brachionidae with a particular interest in the systematic position of *Plationus patulus* and *P. patulus macracathus* based on gene sequences of cytochrome oxidase subunit 1 (*cox1*) and domains D2 and D3 of the large subunit of the nuclear ribosomal RNA (LSU).

Materials and methods

Specimen collection

Rotifers were isolated from different water bodies in México (Table 1) and cultured from a single parthenogenetic female, which was maintained in EPA medium prepared by dissolving 96 mg $NaHCO_3$, 60 mg $CaSO_4$, 60 mg $MgSO_4$, and 4 mg KCl in a final volume of 1 l of distilled water. The cultures were transferred to new EPA medium every 3 days using plankton meshes with a pore size of 50 µm. All the species were maintained on a diet of algae *Chlorella vulgaris*, which was cultured axenically in the laboratory in transparent bottles using Bold's basal medium. Algae in the log-phase of their growth were harvested, centrifuged, and resuspended in distilled water. The algae density was estimated using a hemocytometer. The food level used for maintenance of the rotifers was 1×10^6 cells ml^{-1}.

DNA isolation

Rotifers were washed thoroughly in sterile distilled water, and pelleted by centrifugation prior to DNA extraction. Fifteen rotifers were digested overnight at 56°C in a solution containing 10 mM Tris–Hcl (pH 7.6), 20 mM $NaCl_2$, 100 mM EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant

Table 1 Specimen information and Genbank accesses

Family	Species	Locality	Coordinates		Genbank access LSU	Genbank access *cox1*
			North	West		
Brachionidae	*Anuraeopsis fissa*	Patzcuaro Lake, Michoacan	19°32′ 50.41″	101°38′ 31.2″	*GQ890451	*GQ890449
	Brachionus calyciflorus	Chapultepec Lake, Mexico City	19°25′ 18.5″	99°11′ 06.7″	*GQ890452	*DQ664504
	Brachionus havanaensis	Xochimilco Lake, Mexico City	19°16′ 20.55”	99°06′ 18.9″	*GQ890453	*DQ664505
	Brachionus plicatilis	Gulf of Mexico, Veracruz	18°28′18.9”	92°39′14.9”	*GQ890454	*DQ664507
	Brachionus rubens	Aragon Lake, Mexico City.	18°59′ 3.56”	91°58′0.3”	*GQ890455	*DQ664506
	Brachionus falcatus	Rodeo Lake, Cuernavaca.	20°54′ 15.5”	90°20′ 34.4″	*GQ890456	*DQ664508
	Brachionus urceolaris[1]	Nd	Nd	Nd	DQ089726	DQ089740
	Brachionus urceolaris[2]	Nd	Nd	Nd	DQ089740	DQ089726
	Keratella quadrata[1]	Xochimilco Lake, Mexico City.	19°16′ 20.55”	99°06′ 18.9″	*GQ890462	*GQ890450
	Keratella quadrata[2]	Nd			DQ297735	DQ297774
	Keratella americana	Xochimilco Lake, Mexico City.	19°16′ 20.55”	99°06′ 18.9″	*GQ890457	*GQ890446
	Keratella tropica	Xochimilco Lake, Mexico City	19°16′ 20.55”	99°06′ 18.9″	*GQ890458	*GQ890447
	Platyias quadricornis	Chimalipan, State of Mexico.	24° 29′ 00″	97°45′ 00″	*GQ890459	*GQ890448
	Plationus patulus[3]	Nd	Nd	Nd	DQ297750	DQ297786
	Plationus patulus[1]	Santa Elena, State of Mexico	19° 53′ 55″	99°32′ 9.9’	AY829084	AF416995
	Plationus patulus[2]	Chicoasen Lake, Chiapas	16°56′ 9.51″	93°06′ 9.90″	*GQ890460	*DQ664503
	Plationus patulus macracanthus	Morelia, Mexico	18°42′ 13.4″	95°45′ 27.9″	*GQ890461	*DQ664502
Mytilinidae	*Mytilina ventralis*	Nd	Nd	Nd	DQ297747	DQ297783
Notommatidae	*Notommata allantois*	Nd	Nd	Nd	DQ297748	DQ297784
Lepadellidae	*Lepadella rhomboides*	Nd	Nd	Nd	DQ297740	DQ297779
	Lepadella patella	Nd	Nd	Nd	DQ297739	DQ297778

Sequences marked with an *asterisk* were obtained in this study

Nd not determined

using the DNAzol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions.

Amplification and sequencing of DNA

The two genes, *cox1* and LSU were amplified using the polymerase chain reaction (PCR). A fragment of the mitochondrial *cox1* (618 bp) was amplified using the forward 5′-AGTTCTAATCATAA(R)GATAT(Y) GG-3′ and the reverse primer 5′-TAAACTTCAG GGTGACCAAAAAATCA-3′ (Folmer et al., 1994). The domains D2 + D3 (766 bp) of the LSU rDNA were amplified using the forward 5′-CAAGTAC CGTGAGGGAAAGTTGC-3′ and the reverse primer 5′-GTCGATAGGACTCCCTTTG-3′ (García-Varela & Nadler, 2005). The PCR reaction mixture (25 µl) consisted of 1 µl of 10 µM of each primer, 2.5 µl of 10× buffer, 1.5 µl of $MgCl_2$, 15 Mm, 0.5 µl of dNTP′s 10 mM, 14.25 µl of water, and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters included denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing at 40°C (*cox1*)

and 50°C (LSU) for 1 min, and extension at 72°C for 1 min, followed by a post-amplification incubation at 72°C for 7 min. Each PCR product was purified using Millipore columns (Amicon, Billerica, Massachusetts). Purified products were cloned by ligation into pGEM-T vector (Promega, Madison, Wisconsin) and used to transform competent *Escherichia coli* (JM109). Positive clones were identified by blue/white selection, and clone (insert) size was confirmed by PCR of DNA extracts prepared from bacterial (clone) colonies. Liquid cultures for minipreps were grown in Luria medium containing 50 μg/ml of ampicillin. Plasmids for DNA sequencing were prepared using commercial miniprep kits (Qiaprep, Qiagen, Valencia, California). At least two plasmids of each ligation were sequenced for both DNA strands using universal (vector) and internal primers. Sequencing reactions were performed using ABI Big Dye (PE Applied Biosystems, Boston, Massachusetts) terminator-sequencing chemistry, and reaction products were separated and detected using an ABI 310 capillary DNA sequencer. Contigs were assembled, and base-calling differences were resolved using Codoncode Aligner version 3.0.1 (Codoncode Corporation, Dedham, Massachusetts). All the sequences have been deposited in the Genbank (access numbers in Table 1).

Multiple sequence alignment and phylogenetic analysis

Sequences of the *cox1* and LSU generated in this study were aligned with four additional sequences downloaded from Genbank. These four sequences represented outgroup taxa, and the resulting data sets included 21 taxa each. Both the *cox1* and LSU data sets were aligned using PSalign (Sze et al., 2006), and the LSU data set was re-aligned with MAFFT ver. 6.611b (Katoh et al., 2005) to correct for the poorly aligned regions. The L-INS-i pairwise alignment settings (–localpair–maxiterate 1000) were implemented for MAFFT. The initial *cox1* alignment included 658 sites for 12 taxa. However, 40 sites were removed from the initial alignment because the sequences obtained from the Genbank for another nine taxa were partial (618 sites). The second alignment contained 618 sites for all the 21 taxa. This second *cox1* data set was translated to protein sequences to detect any possible reading frame errors. Geneious Pro 4.0.4 (Drummond et al., 2009) was used to combine LSU and *cox1* data sets.

The best fit-model for each data set and the combined data set of both genes (*cox1* + LSU) were inferred using the Akaike information criterion (AIC) with Modeltest version 3.7 (Posada & Crandall, 1998). Maximum likelihood (ML) analyses for the three data sets were conducted using GARLI 0.96 (Zwickl, 2006). Bootstrap analyses were conducted using GARLI and Grid computing (Cummings & Huskamp, 2005) through "The Lattice Project" (Bazinet & Cummings, 2009) which includes clusters and desktop computers in one encompassing system (Myers et al., 2008). A Grid service for GARLI was developed using a special programming library and associated tools (Bazinet et al., 2007). Based on the model of Cummings et al. (2003) who used an earlier Grid computing system (Myers & Cummings, 2003), we distributed required files among hundreds of computers, where the analyses were then conducted asynchronously in parallel. Clade support was assessed by bootstrap resampling with 2,000 (ML) bootstrap replicates for each data set and the combination of both data sets. The trees were illustrated using FigTree (FigTree program version v1.1.2). Uncorrected pairwise distances were calculated in PAUP*(Swofford, 2000). In order to compare trees representing specific alternative phylogenetic hypotheses, topological constraints were defined on the tree obtained from ML analysis of the combined (*cox1* + LSU) data set. Differences between unconstrained (best) and constrained trees representing alternative hypotheses were evaluated using the Shimodaira and Hasegawa (HS) test (Shimodaira & Hasegawa, 1999) and calculated in PAUP*. A χ^2-test was estimated for the combined data set to determine the heterogeneity of nucleotide frequencies across taxa using the "basefreq" option implemented in PAUP*. All the alignments and data sets are available from the corresponding author on request.

Results

Base composition and genetic divergence

The DNA fragments of *cox1* and LSU were amplified, cloned, and sequenced for 21 taxa representing five genera: *Anuraeopsis* (1 sp), *Brachionus* (6 spp; 2 populations), *Keratella* (3 spp and 2 populations), *Plationus* (1 sp; 3 populations and 1 subspecie) and

Platyias (1 sp) of Brachionidae plus four species from Order Ploima Hudson and Gosse, 1886, which were used as outgroups (See Table 1.). Length of the PCR products among congeneric species of Brachionidae ranged from 505 to 766 bp for LSU and was 658 bp for *cox1*. However, nine *cox1* sequences obtained from Genbank were 618 bp. In order to compare sites across all the sequences, we removed some sites at the beginning and the end of the alignment, resulting in a final alignment of 618 bp. Nucleotide frequencies for the combined (*cox1* + LSU) data set were 0.244 (A), 0.144 (C), 0.222 (G), and 0.387 (T). Heterogeneity of nucleotide frequencies across taxa was: $\chi^2 = 48.997$, $P = 0.28$. This result indicated that rDNA nucleotide frequencies were not significantly heterogeneous across taxa, which was advantageous because ML inference methods perform optimally when nucleotide frequencies are homogeneous (Omilian & Taylor, 2001). The genetic divergence estimated from the combined (*cox1* + LSU) data set within populations ranged from 0.07 to 9.8%, among congeneric species it ranged from 8.6 to 13.9%, and among genera from 9.6 to 19.3%.

Phylogenetic analysis

cox1

The *cox1* data set included 21 taxa with 618 bp. The best substitution model for this data set was the General Time Reversible (GTR) (Rodríguez et al., 1990), with a proportion of invariable sites of 0.3176 and a gamma distribution of 0.2696 (+G) (Yang, 1994). The maximum likelihood analysis (ML) yielded a single best tree with a −ln likelihood of 5720.0786 (Fig. 1). This tree yielded Brachionidae as a monophyletic assemblage with poor bootstrap support (<50%). The genus *Plationus* was composed of two clades. The first contained *Plationus patulus macracanthus* + *Plationus patulus*[3] and the second contained two populations of *Plationus patulus* from Mexico. However, both clades were poorly supported (<50% bootstrap). The six species of *Brachionus* were monophyletic and had a bootstrap support of 59%. The estimated genetic divergence ranged from 0 to 17. 4% within populations, from 14.5 to 22.3% among congeneric species, and from 16.9 to 25.8% among genera of Brachionidae.

LSU

The LSU data set included 21 taxa with 766 bp. The best substitution model for this data set was the GTR, with a proportion of invariable sites of 0.4706 and a gamma distribution of 0.7473 (+G). The maximum likelihood analysis (ML) yielded a single best tree with a −ln likelihood of 3791.0747 (Fig. 2). This tree yielded Brachionidae as monophyletic, but with weak bootstrap support (<50%). The genera *Anuraeopsis*, *Brachionus, Keratella*, *Plationus*, and *Platyias* were

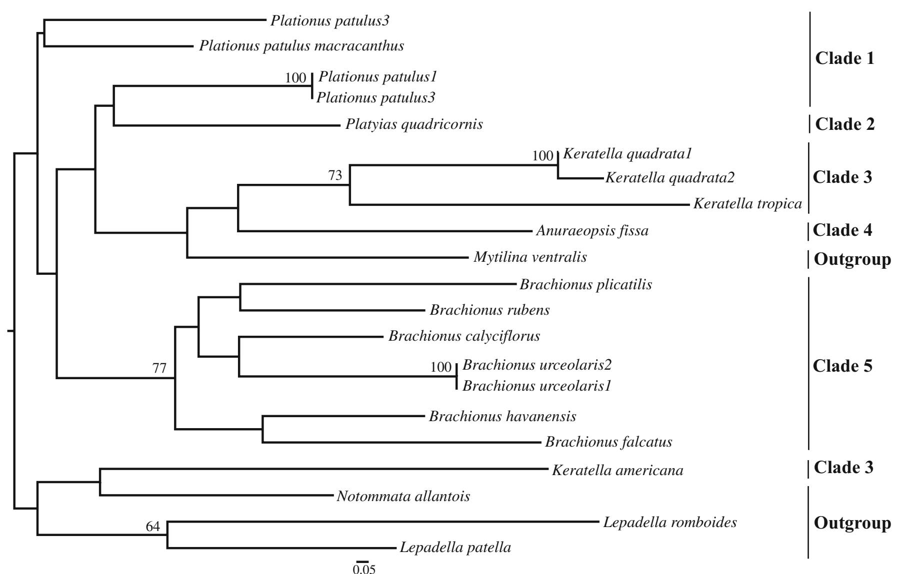

Fig. 1 Maximum likelihood tree (−ln likelihood of 5720.1) inferred from the *cox1* data set. Numbers near internal nodes show ML bootstrap clade frequencies

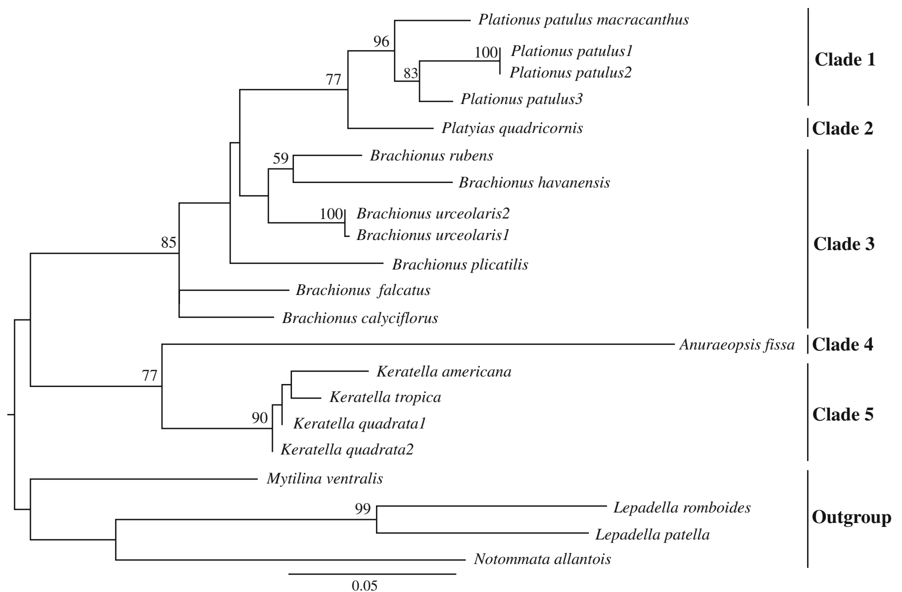

Fig. 2 Maximum likelihood tree (−ln likelihood of 3791.0) inferred from the LSU data set. Numbers near internal nodes show ML bootstrap clade frequencies

monophyletic with high bootstrap support ranging from 77 to 96%. The estimated genetic divergence ranged from 0.14 to 3.14% within populations, from 1.1 to 8.5% among congeneric species and from 4.5 to 15.2% among genera of Brachionidae.

cox1 + LSU

The combined (*cox1* + LSU) data set included 21 taxa with 1384 bp. The best substitution model for this combined data set was the GTR, with a proportion of invariable sites of 0.495 and a gamma distribution of 0.908 (+G). The ML analysis yielded a single tree with a −ln likelihood of 9809.5 (Fig. 3). This tree yielded the same general topology as the LSU tree (Fig. 2), but with more resolved nodes and higher bootstrap values. The first clade is composed of three populations of *P. patulus* + *P. patulus macracanthus* with a bootstrap support of 90%. The second clade was composed of a single species of *Platyias* with a bootstrap support of 85%. The third clade is composed of six species of *Brachionus* with a bootstrap support of 98%. The fourth clade included a single species of the genus *Anuraeopsis* with a bootstrap support of 91%. Finally, the fifth clade was composed of three species of the genus *Keratella*, which had a bootstrap support of 100%.

Discussion

The maximum likelihood tree (Fig. 3) inferred from a combined data set (*cox1* + LSU) that included five recognized genera of Brachionidae, suggested that this family is monophyletic albeit with poor bootstrap support (<50%). This phylogenetic hypothesis is in contrast with a previous study based on morphological and molecular characters, which suggested that Brachionidae is paraphyletic also with poor bootstrap values (Sorensen & Giribet, 2006). The inclusion of more species and sequences of another nuclear or mitochondrial genes would be necessary to clarify the monophyly or paraphyly of Brachionidae.

The genus *Plationus* was described by Segers et al. (1993) and included three species *Plationus patulus*, *P. polyacanthus*, and *P. macracanthus* which have been alternatively assigned to *Brachionus* or *Platyias* (Müller, 1786; Ahlstrom, 1940; Bartos, 1959; Rudescu, 1960; Kutikova, 1970; Kotikova et al., 2005; Xian-Ling et al. 2006; Nandini et al., 2007; Kennari et al., 2008; Sarma et al., 2008). Later another two taxa—*P. patulus patulus* and *P. felicitas*—were added to the genus *Plationus* (Segers, 2007). In this study, we analyzed two taxa representing the genus *Plationus*, including the type species *P. patulus* with three populations and the subspecies *P. patulus macracanthus*. The genetic divergence estimated within the three

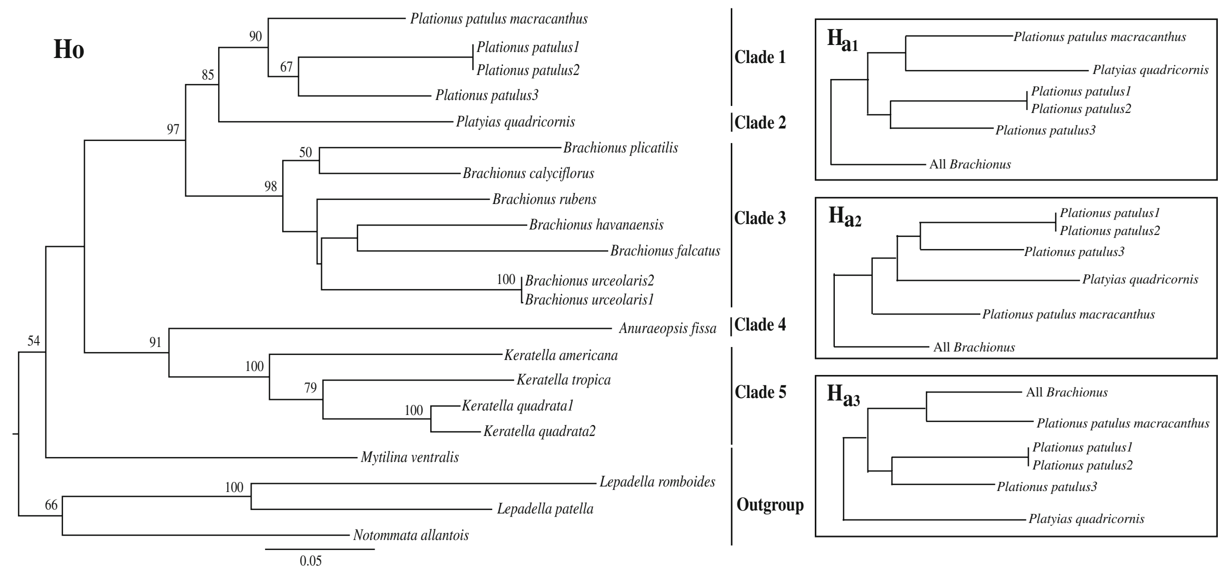

Fig. 3 Maximum likelihood tree, $\mathbf{H}_o$ (−ln likelihood of 9809.5) inferred from combined *cox1* + LSU rDNA data set. Numbers near internal nodes show ML bootstrap clade values. Differences in −ln likelihood among the three alternative hyphoteses: $\mathbf{H}a_1$ (−ln likelihood of 9818.98; $P = 0.055$*) $\mathbf{H}a_2$ (−ln likelihood of 9819.05; $P = 0.052$*), and $\mathbf{H}a_3$ (−ln likelihood of 9812.65; $P = 0.178$*). * $P > 0.05$ not significant

populations of *P. patulus* ranged from 0 to 17.4% for *cox1* and from 0 to 3.1% for LSU, and among the three populations of *P. patulus* with the subspecies *P. patulus macracanthus* ranged from 14.5 to 17.4% for *cox1* and from 3.1 to 4.2% for LSU. This genetic divergence for *cox1* is similar to other congeneric comparisons within this family, for example, among species of *Keratella* divergence was as high as 12% (Gómez et al., 2002; Gómez, 2005), and from 20 to 25% among species of *Brachionus* (Derry, 2003). The two taxa of *Plationus* analyzed in this study were recovered as a monophyletic group, consistent with its recognition as a separate genus. However, in order to test the taxonomic validity of *Plationus*, three alternative hypotheses were proposed (Fig. 3). These hypotheses were evaluated through ML analyses using the combined data set (*cox1* + LSU). Based on the Shimodaira & Hasegawa (1999) test as executed in PAUP*, the three alternative hypotheses ($\mathbf{H}_{a1}$, $\mathbf{H}_{a2}$, and $\mathbf{H}_{a3}$) were significantly worse than the best tree ($\mathbf{H}_o$) represented in Fig. 3. The close phylogenetic position of *Plationus* with *Brachionus* and *Platyias* in our combined tree is consistent with a previous phylogenetic study inferred from molecular and morphological characters (Sorensen & Giribet, 2006). The close phylogenetic position of *Brachionus*, *Plationus*, and *Platyias* has been supported by three morphological synapomorphies features (pseudosegmented foot, presence of an eye, and specialized trophi) (Wulfert 1965; Segers et al., 1993). The systematic position of *Plationus* as independent genera within Brachionidae in our phylogenetic analysis, was previously supported by three autapomorphies (lorica inserting terminally, anterior processes of the rami are present, and proximal cavities of the manubria closed) (Segers et al., 1993).

The genus *Brachionus* is a group of microscopic organisms that inhabit a variety of freshwater and marine systems. The variation of the shape and size of the lorica (body), and the number, shape, and size of the anterior spines have been traditionally used to recognize and separate species (Segers, 2002). Moreover, recently a complex of cryptic species has been detected using genetic information (Ciros-Pérez et al., 2001; Gómez et al., 2002; Suatoni et al., 2006). Sequences of the *cox1* were generated for five species of *Brachionus* and aligned with other congeneric species previously reported. The genetic divergence estimated among six species of *Brachionus* with *cox1* ranged from 14.8 to 22.3%, and within the two populations of *B. urceolaris* was 0%. This molecular marker has been previously used to

separate other congeneric species of *Brachionus*, which showed a genetic divergence up to 12% (Gómez et al., 2002; Gómez, 2005), as well as in the range from 20 to 25% (Derry, 2003). The genetic divergence estimated within the two populations of *B. urceolaris* was 0.14%, and among species of *Brachionus*, it ranged from 3.5 to 8.5% for LSU, which was useful to separate congeneric species. The phylogenetic tree inferred from a combined data set (*cox1* + LSU) showed the monophyly of the six congeneric species of *Brachionus*. This clade was supported with a bootstrap value of 98% (Fig. 3).

Keratella is a cosmopolitan genus composed of approximately 53 species (Segers, 2007). The three species sequenced here (*K. americana* Carlin, 1943; *K. tropica* Apstein, 1907; *K. quadrata* Müller, 1786) were aligned with another population of *Keratella quadrata* showing a genetic divergence among species from 20.3 to 22.6% for *cox1* and from 1.1 to 2.8% for LSU. The range of genetic divergence estimated among congeneric species of *Keratella* for *cox1* is similar to other reported for other *Keratella* species from Canada, which ranged from 23 to 27% (Derry, 2003). The genetic divergence estimated within the two populations of *K. quadrata* was 7% for *cox1* and 0.28% for LSU. Derry (2003) found a genetic divergence of 4.4% for *cox1* between the spined and unspined morphs of *K cochlearis* Gosse, 1851, suggesting these morphs as a species complex. The phylogeny inferred with the LSU and combined data sets, indicated that the three congeneric species of *Keratella* comprise a clade with a strong bootstrap support (Figs. 2, 3).

In this study, a nuclear ribosomal gene (LSU) was used for the first time as a molecular marker to determine differences/similarities among the species of Brachionidae. The LSU tree (Fig. 2) showed better resolution and bootstrap support for the five clades and within populations than the *cox1* tree (Fig. 1). This phenomenon can be due to the fact that *cox1* evolves 1.81 times much faster than LSU according to the results of this study. In the *cox1* tree (Fig. 1), nodes pertaining to the family and genera were not resolved. The rapid evolving *cox1* gene can be better used for population studies or to detect species complexes in rotifers (Gómez, 2005; Gómez et al., 2007). It is likely that a more slowly evolving gene as the LSU region can be better to resolve the families and genera relationships. Therefore, when combining both genes (*cox1* + LSU), the resolution at the generic level and support of the nodes were higher than the trees inferred with *cox1* and LSU genes alone.

Conclusion

The family Brachionidae is composed of an assemblage of genera with a long history of controversies. Relationships among representatives of this family have been examined using morphological and molecular characters (Sorensen & Giribet, 2006). However, this study includes a more complete representation of the family (5 out of 7 genera sampled). The analysis of this study reveals that Brachionidae is a monophyletic assemblage with weak support (<50%) and is composed of five main clades representing the five genera (*Anuraeopsis*, *Brachionus*, *Keratella*, *Plationus*, and *Platyias*). The morphological evidence, the amount of genetic divergence, the systematic position of *Plationus* within the family Brachionidae, and the position of *Plationus* as a sister group of *Brachionus* and *Platyias*, all these support the validity of *Plationus patulus* and *P. patulus macracanthus* into the genus *Plationus*. Nevertheless, what is still needed is the inclusion of more genera, such as *Notholca* and *Kellicottia* and more species of *Anuraeopsis*, to have a more comprehensive phylogeny of Brachionidae which will produce a robust classification scheme and a better understanding of these diverse group of rotifers.

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