



# Geometric morphometric on a new species of Trichodinidae. A tool to discriminate trichodinid species combined with traditional morphology and molecular analysis

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## ABSTRACT

This study reports a new Trichodinidae, *Trichodina bellottii* n. sp., parasitizing the pearly fish *Austrolebias bellottii* from Buenos Aires, Argentina. Its small subunit ribosomal ribonucleic acid (SSU rDNA) was sequenced for the first time. Based on the results from morphological identification, SSU rDNA sequencing, and Elliptical Fourier analysis, the new species was identified and compared with similar species. Phylogenetic analysis revealed that the genetic distances among the new species and similar species reached interspecific levels, furthermore, the phylogenetic study also validated the identification of *T. bellottii* n. sp. and its placement in the genus *Trichodina*. To be able to quantitatively describe the differences in shapes with similar species, this study used the elliptical Fourier analysis by first time in this genus.

## 1. Introduction

Ciliated protozoan Trichodinidae are among the main etiological agents of mortality in farmed fish (Martins et al., 2015). They can be considered opportunistic ectoparasites and are present in both marine and inland fish species. Trichodinids ciliates are able to invade their hosts within a short period of time, especially fish that are kept under less than optimal conditions (Lom, 1995).

Until now, in Argentina have been reported *Trichodina* spp. (Viozzi, 1996; Cremona and Figueras, 2004; Cremona et al., 2005), *Trichodina marplatensis* Martorelli et al., 2008, *Trichodina scalensis* Marcotegui and Martorelli, 2009, *Trichodina puytoraci* Lom, 1962, *Trichodina lepsi* Lom, 1962, *Trichodina jadratica* Lom and Laird, 1969, *Trichodina murmanica* Poljansky, 1955, and *Dipartiella simplex* Raabe, 1959 (Marcotegui and Martorelli, 2009). While freshwater fish have been found to be infected with *Trichodina jenynsii* Marcotegui et al., 2016 from *Jenynsia multi-dentata* (Cyprinodontiformes: Anablepidae), *Trichodina corydori* Marcotegui et al., 2016, and *Trichodina cribbi* Dove and O'Donoghue, 2005 from *Corydoras paleatus* (Siluriformes: Callichthyidae) (Marcotegui et al., 2016).

Traditionally, species of trichodinid has been identified by denticles morphology. According to Tang et al. (2017), during the last few years, not many trichodinids have been sequenced, preventing their effective

molecular identification.

On the other hand, geometric morphometric data have been utilized to identify genera, species, and local population mainly on fish, snails, and plants (pollen) (Ibañez et al., 2007; Ramirez-Perez et al., 2010; Bonhomme et al., 2013; Sobrepeña and Demayo, 2014; Karahan et al., 2014; Pavlov, 2016). Shape is defined by Kendall (1989) and Small (1996) as “the total of all information invariant under translations, rotations, and isotropic rescaling”. While traditional morphometry use lengths, wide, and angles, geometric morphometrics considers shape as a whole, taking into account all the geometrical relationships of the input data (Bonhomme et al., 2014). Geometric morphometric data have the advantage of providing a consistent set of shape variables for hypothesis testing and provide the graphic analyses that quantify and visualize morphometric variation within and between organisms (Tracey et al., 2006). Elliptical Fourier functions represent a precise method for describing and characterizing outlines, efficiently capturing shape information in a quantifiable manner (Kuhl and Giardina, 1982; Lestrel, 1997).

During a survey of parasites on the annual fish *Austrolebias bellottii* (Cyprinodontiformes: Rivulidae) from temporary ponds, we found only one kind of specimens of trichodinids parasitizing their external surface. The aim of this paper is to identify these specimens using traditional morphology, molecular data, and geometric morphometric for

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the first time in this genus.

## 2. Materials and methods

### 2.1. Morphology

Fish were collected from the Natural Reserva Punta Lara (34° 48' S, 58° 00' W) between 2015 and 2017. A total of 32 specimens of *Austrolebias bellottii* were examined. Fish were killed by spinal severance and examined for parasites. Fresh skin smears were made from the hosts. Smears with trichodinids were air dried and Foissner's modifications of Klein's dry silver nitrate technique (Foissner, 1992) was used to impregnate the specimens. The sequence and method for the description of adhesive disc and denticle elements follow the recommendations of Lom (1958) and Van As and Basson (1992). Live specimens, stained with methylene blue or orcein, were used for nuclear observations and details of the infraciliature. Examinations of prepared slides were made with an Olympus BX51 microscope and the measurements were made with Image J software. The photomicrographs were taken using a microscope at 100 × magnification. The description of each species is based on 20 stained and mounted specimens. All measurements are presented in micrometres, the arithmetic mean is followed by (±) standard deviation. In case of the number of denticles and radial pins per denticle, minimum and maximum values are given, followed in parentheses by the mode. The span of the denticle is measured from the tip of blade to the tip of the ray. Body diameter is measured as the adhesive disc plus border membrane. The type and voucher materials have been deposited in the Museo de La Plata, Argentina, Invertebrate Collection (MLP, coll. Nos. MLP000 to MLP000).

### 2.2. DNA extraction, amplification and sequencing

For the genetic analysis, the total genomic DNA of 10 specimens fixed in alcohol 96% was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol.

The fragment of the partial SSU rDNA gene was amplified with Polymerase Chain Reaction (PCR) in an Eppendorf Mastercycler thermal cycler using forward primers ERIB1 (5'-ACC TGG TTG ATC CTG CCA G-3') and ERIB10 (5'-CTT CCG CAG GTT CAC CTA CGG-3'). The reaction was made with GoTAQ Master Mix (Promega) according to the manufacturer's protocol. Thermocycling conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 58 °C for 1 m, 72 °C for 2 min; and a final extension at 72 °C for 10 min.

The PCR products were sequenced using an ABI 3730XLs sequencer, Macrogen Inc. (Korea).

### 2.3. Phylogenetic analysis

The sequences were optimized by eye using the platform Geneious Pro v5.1.7 (Drummond et al., 2016) and compared with sequences from GenBank. Alignments were assembled using the online version of MAFFT v.7 (Kato and Standley, 2013). Then, to edit out poorly aligned regions of the rDNA we used the online program Gblocks v0.91 (Castresana, 2000; Talavera and Castresana, 2007) with relaxed parameters. A phylogenetic analysis was conducted using the compared sequences rooted by *Epystylis urceolata* (AF335516) for outgroup. The best partitioning scheme and substitution model for the DNA partition were chosen under the Bayesian Information Criterion (Schwarz, 1978) using the 'greedy' search strategy in Partition Finder v.1.1.1 (Lanfear et al., 2012, 2014). The appropriate nucleotide substitution model implemented for the matrix resulting after the Gblock program was Trn + I + G (Tamura and Nei, 1993).

Phylogenetic reconstruction was carried out using Bayesian Inference (BI) through MrBayes v.3.2.1, and using the Maximum Likelihood method (Ronquist et al., 2012). The resulting phylogenetic

trees were reconstructed using two parallel analyses of Metropolis-Coupled Markov Chain Monte Carlo (MCMC) for  $2.0 \times 10^7$  generations each, to estimate the posterior probability (PP) distribution. Topologies were sampled every 1000 generations. Once the average standard deviation of split frequencies was determined, it was less than 0.01, as suggested by MrBayes 3.2. Two separate runs were carried out and the last 10,000 trees from each run were combined after establishing in Tracer v.1.5. The robustness of the clades was assessed using Bayesian PP, where  $PP > 0.95$  was considered strongly supported. A majority consensus tree with clade lengths was reconstructed for each run after discarding the first 15,000 sampled trees in both analyses.

Additionally, the proportion (p) of absolute nucleotide sites (p-distance) (Nei and Kumar, 2000) was obtained to compare the genetic distance between lineages and with the outgroup. The p-value matrix was obtained using MEGA v.6.0 (Tamura et al., 2013), with variance estimation, with the bootstrap method (500 replicates) and with a nucleotide substitution (transition C transversions) uniform rate.

The bootstrap consensus tree bases on ML inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 354 positions in the final dataset.

Two generated sequences from the new species were submitted to the National Center for Biotechnology Information (NCBI) GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under accession numbers XXXXXX-XXXXX, and XXXXXXXXXX.

### 2.4. Geometric morphometric

Based on morphological similarity and the high degree of variation in the morphology of denticle, the new species was compared with *T. pseudoheterodontata* Tang et al., 2017, and the related species *Trichodina heterodontata* Duncan, 1977, and *T. paraheterodontata* Tang and Zhao, 2013. A total of 21 denticle silhouette of trichodinids from *A. bellottii* were drawn from microphotographs of specimens stained with silver nitrate. Denticles of *T. heterodontata* (n = 19), *T. paraheterodontata* (n = 21) and *T. pseudoheterodontata* (n = 25) were redrawn from previous studies (Duncan, 1977; Albaladejo and Arthur, 1989; Bondad-Reantaso and Arthur, 1989; Van As and Basson, 1989; Basson and Van As, 1994; Al Rasheid et al., 2000; Asmat, 2004; Dove and O'Donoghue, 2005; Dias et al., 2009; Martins et al., 2010; Benites de Pádua et al., 2012; Miranda et al., 2012; Tang and Zhao, 2013; Valladão et al., 2014; Tang et al., 2017). Black and white denticle images were saved in bitmap (.bmp) format.

Statistical analyses and visualization were performed in R (R Core Team, 2014). Elliptic Fourier analysis was conducted using SHAPE v. 1.3 and R packages (Iwata and Ukai, 2002). The denticles bitmap images were converted to binary images and the contour of each denticle was digitized using the chain code. Files obtained from SHAPE (.chc), were imported using Momocs v0.9 (Bonhomme et al., 2014). Before performing Elliptical Fourier Descriptor analysis, an estimation of the number of harmonics to perform was undertaken using hquant function. The number of harmonics sufficient to reach approximately 99% of the maximum cumulated Fourier power was determined from the relationship between the harmonic numbers and their cumulated power (Fourier power spectrum). Qualitative analysis of the ability of different numbers of harmonics to recapitulate shape was performed using the hqual function. Fourier analysis was performed using the

eFourier function, which the outlines were normalized for rotation, translation, size, and orientation using the first ellipse. Harmonic coefficients from the resulting Coe object were then used for subsequent statistical analyses.

Linear Discriminant Analysis (LDA) on harmonic coefficients was performed using the *lda* function from MASS package. MANOVA (Multivariate analysis of variance) test was performed using harmonic coefficients matrix obtained from SHAPE.

### 3. Results

#### 3.1. Taxonomic summary

Type host: *Austrolebias bellottii*.

Type locality: Reserva Natural Punta Lara (34° 48' S, 58° 00' W).

Site of infection: external surface (skin and fins).

Type specimens: XXXXXX.

Etymology: The specific epithet “bellottii” is coined from the name of host species.

#### 3.2. Morphological description

*Trichodina bellottii* n. sp. (Fig. 1: A-F, Fig. 2: A-B).

All measurements were done on 20 specimens. Medium sized freshwater *Trichodina*, body diameter  $67.57 \pm 4.03$ ; diameter of adhesive disc  $57.342 \pm 4.93$ ; width of border membrane  $4.99 \pm 0.88$ ; diameter of denticle ring  $36.10 \pm 3.15$ ; number of denticles 21–26 (23); number of radial pins per denticle 11–12 (11); span of denticle  $18.20 \pm 1.5$ ; length of denticle  $8.02 \pm 0.77$ ; blade broad and sickle-shaped, length  $5.09 \pm 0.66$ , distal blade surface smooth and a little curved, not in parallel with border membrane; tangent point small, rounded, situated only slightly below distal surface; some specimens with a notch on the proximal anterior margin; apex present beyond Y +1 axes; anterior and posterior surfaces curved and did not parallel with each other; blade apophysis visible in some specimens; deepest point of blade posterior surface at the same level of apex. Blade connection thick and posterior projection present, encasing in an indentation of posterior denticle. Central part very developed extending half way to Y-1 axis, some specimens fit irregularly into preceding denticle, others specimens with a rounded point fitting into preceding denticle. Central part  $3.69 \pm 0.45$  in width, and the shape of central part above and below the X-axis is dissimilar. Indentation on lower central part. Ray connection well developed. Ray robust and relatively long, slightly curved to Y-1 axis, and tapers to a rounded point; ray apophysis present, length of ray  $8.511 \pm 1.21$ . Ratio between denticle above and below X-axis different to one. Macronucleus horse-shoe

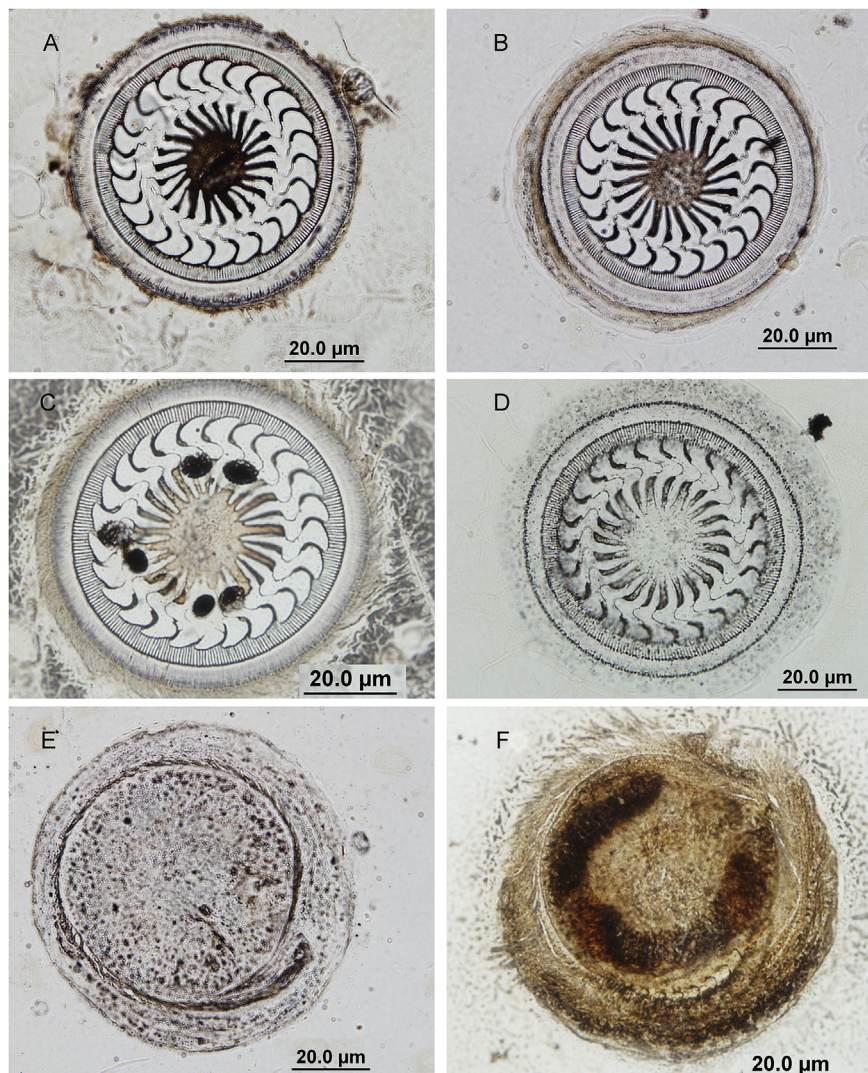
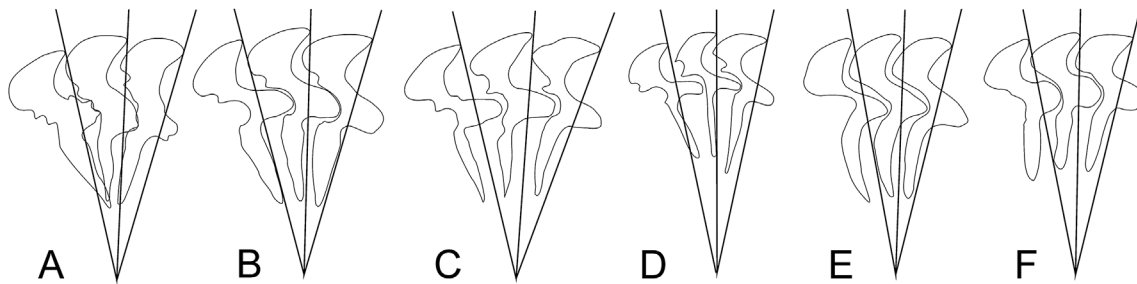
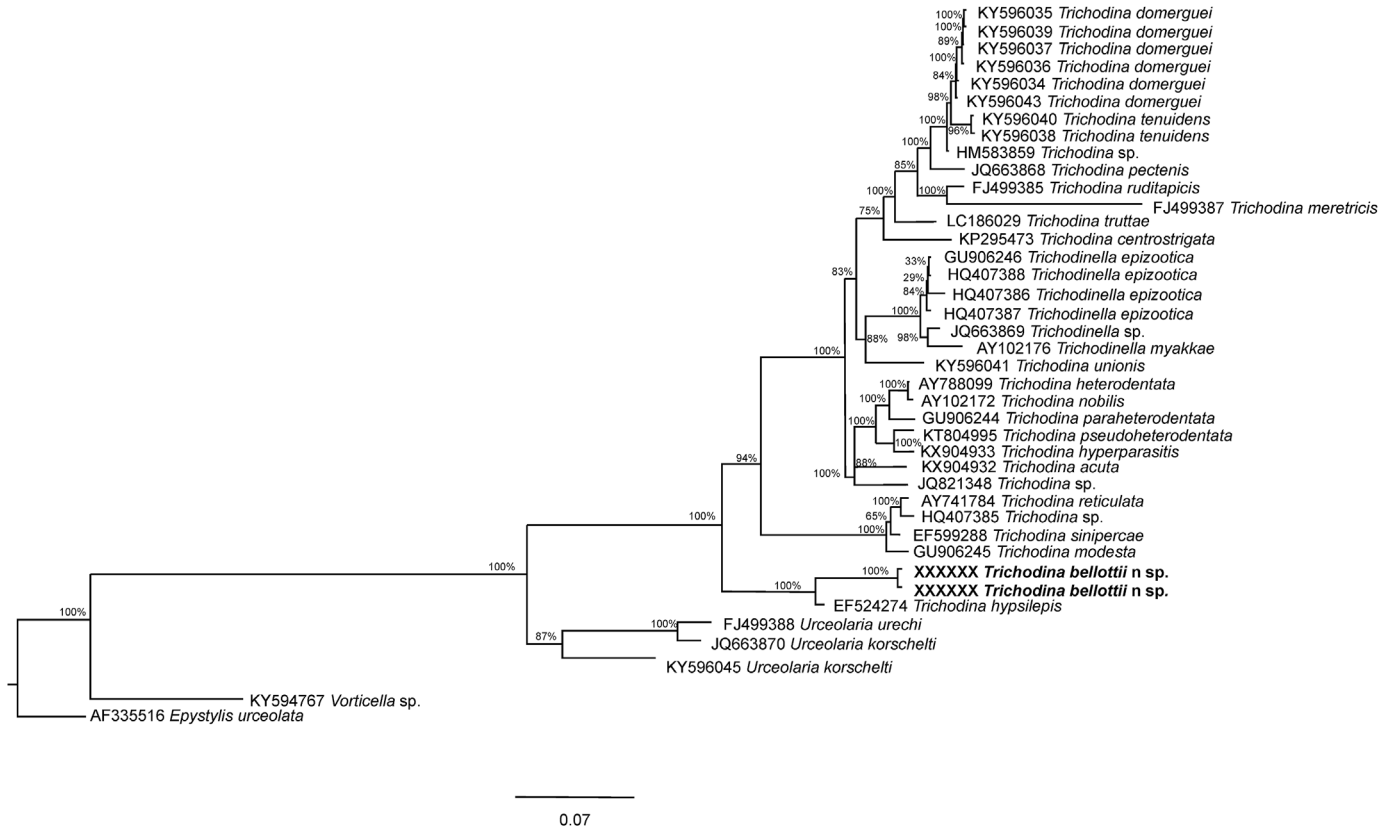


Fig. 1. Microphotographs of *Trichodina bellottii* n. sp. from *Austrolebias bellottii*. (A–D) Adhesive disc after dry silver impregnation. (E) Ciliature. (F) Macronucleus with methylene-blue staining. Scale bars: 20 μm.





**Fig. 2.** Diagrammatic drawings of denticles of trichodinids. (A and B) Denticle of *Trichodina bellottii* n. sp. from *Austrolebias bellottii*. (C) *Trichodina hypsilepis* redrawn from Wellborn (1967). (D) *Trichodina heterodentata* redrawn from Duncan (1977). (E) *Trichodina paraheterodentata* redrawn from Tang and Zhao (2013). (F) *Trichodina pseudoheterodentata* redrawn from Tang et al. (2017).



**Fig. 3.** Phylogenetic tree based on 18S rDNA sequences by Bayesian Inference, with the model Trn + I + G applied in MrBayes v.3.2.1. The new sequenced forms are in bold. Numbers given at nodes of branches are the posterior probability value.

shaped, length  $50.83 \pm 0.54$ ; distal ends  $24.8 \pm 0.62$ . Adoral ciliary spiral turns about  $380^{\circ}$ – $410^{\circ}$  around peristomial disc.

### 3.3. Molecular analysis

The topologies of phylogenetic trees inferred by BI and ML analyses are presented in Fig. 3 and Fig. 4 respectively. Two clades, corresponding to the families Trichodinidae and Urceolariidae, are supported by the data analyses of the present study. The new species clustered with *Trichodina hypsilepis* Wellborn, 1967 with full support (100%). The two species were clustered with the remainder of *Trichodina* spp. and *Trichodinella* spp. with support value of 100%.

The genetic distances calculated with MEGA 7, between the new species and *T. hypsilepis*, *T. heterodentata*, *T. paraheterodentata*, and *T. pseudoheterodentata* were 0.023, 0.093, 0.070 and 0.097, respectively.

### 3.4. Geometric morphometric

Denticle silhouettes from each species are exposed in Fig. 5. A total of 174 outline were obtained from each denticle. Fig. 6 shows the power spectrum, were a number of harmonics sufficient to reach approximately 99% of the maximum cumulated was equal to 10. Fig. 7 shows the qualitative analysis of the ability of different numbers of harmonics to recapitulate the shape.

Results of the PCA analysis indicate that over 90 percent of the observed outline shape variation can be represented on eight orthogonal principal component axes. Ordinations of the outline shapes within the two-dimensional subspace conformed by PC1 and PC2 axes are shown in Fig. 8. Table 1 shows the percent (%) of variance contributed by each of the principal components to the morphological variation.

To further assess the variation between the new species and *T. heterodentata*, *T. pseudoheterodentata* and *T. paraheterodentata*, we performed a linear discriminant analysis (LDA) to determine if they, could

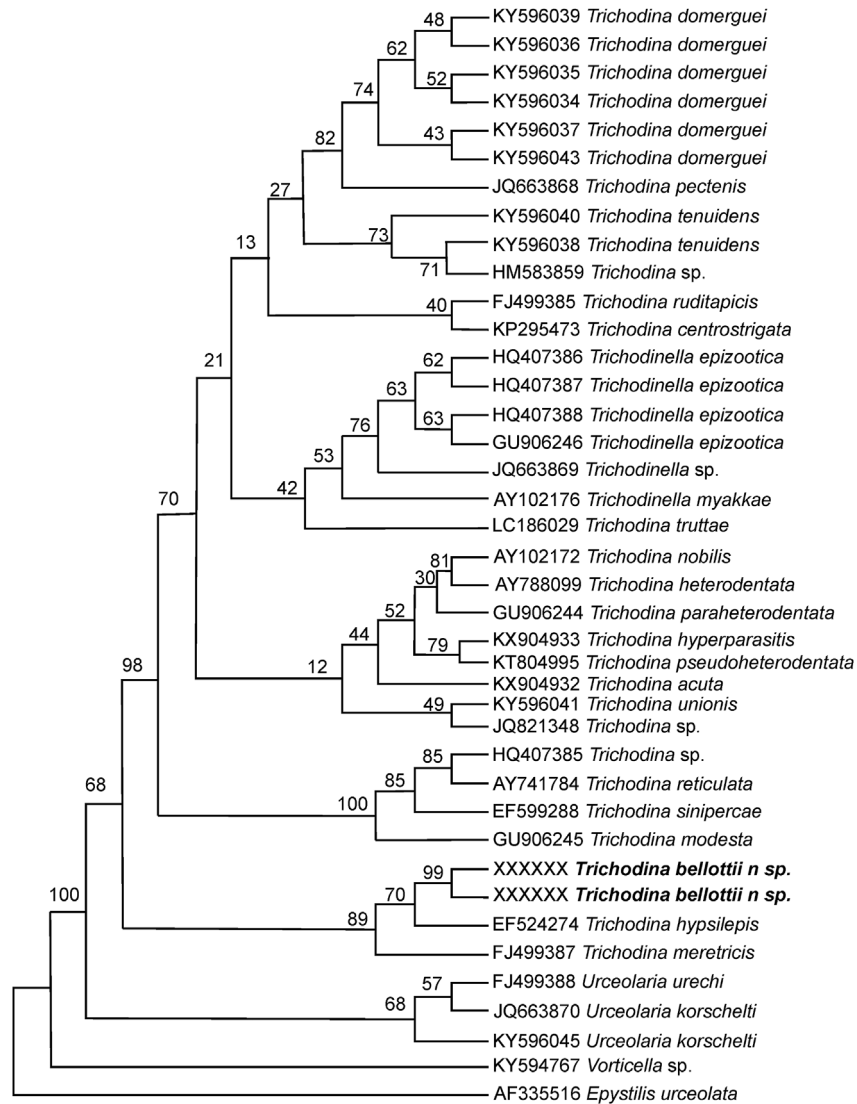


Fig. 4. Tree derived from a Maximum Likelihood (ML) analysis. The bootstrap consensus tree bases on ML inferred from 500 replicates. Bootstrap values for ML are given above nodes.

be quantitatively distinguished based on PCA axes 1 to 8.

The LDA returned the highest correct classifications, successfully distinguishing among species (Wilk's lambda = 0.157,  $p < 0.0001$ ). Based on the LDA, *T. bellottii* n. sp. could be correctly predicted in 71.43%, *T. heterodentata* 68.24%, *T. paraheterodentata* 95.24%, and *T. pseudoheterodentata* 92%. (Table 2, Fig. 9).

PCA scores were also submitted to an analysis of multivariate analysis of variance. Shapes of *T. bellottii*, *T. heterodentata*, *T. paraheterodentata* and *T. pseudoheterodentata* differs significantly (Wilk's lambda = 0.131,  $p < 0.0001$ ). It is nevertheless rewarding to confirm that geometric morphometric methods can easily detect such subtle differences.

#### 4. Remarks

The new species was similar with *Trichodina hypsilepis*, *Trichodina heterodentata*, *Trichodina paraheterodentata*, and *Trichodina pseudoheterodentata* at 96%, 90%, 90%, and 91% homology, respectively.

Recently, *Trichodina cirrhini* Fariya et al., 2017, has been described on *Cirrhinus mrigala* (Cypriniformes: Cyprinidae) from India. The sequence of *T. cirrhini* in Genbank have 206 base-pairs (bp), and after the alignment and the edition of the poorly aligned regions of the rDNA, the

number was significantly reduced to 65 bp. Unfortunately, the morphological comparison was not possible due to the poor quality of the drawings and microphotographs of this species. For this reason, we decided not to include in our analysis.

Wellborn (1967) described *Trichodina hypsilepis* Wellborn, 1967 on *Notropis hypsilepis* (Cypriniformes: Cyprinidae) from Alabama, USA. This species was later redescribed by Arthur and Lom (1984) from unidentified tadpoles of various species of frogs. Morphologically the new species can be easily differentiable from *T. hypsilepis* by (Table 3, Fig. 2C): (1) the central part in the new species is more robust than in *T. hypsilepis*, (2) the new species has larger denticle span, (3) the new species has smaller denticle length., (4) in *T. hypsilepis* the ray is relatively thin and with a sharp point, whereas in the new species the ray is robust with a rounded point.

In terms of denticle morphology, our material most closely resembles *T. pseudoheterodentata*, described on *Ictalurus punctatus* (Siluriformes: Ictaluridae) from China (Tang et al., 2017). Nevertheless, the new species can be easily differentiable by: (1) the body of the new species has medium diameter, whereas *T. pseudoheterodentata* is a large size trichodinid (Table 3); (2) the denticle length and the central part width in the new species are smaller than *T. pseudoheterodentata* (Table 3, Fig. 2F) and (3) in some specimens of the new species, the

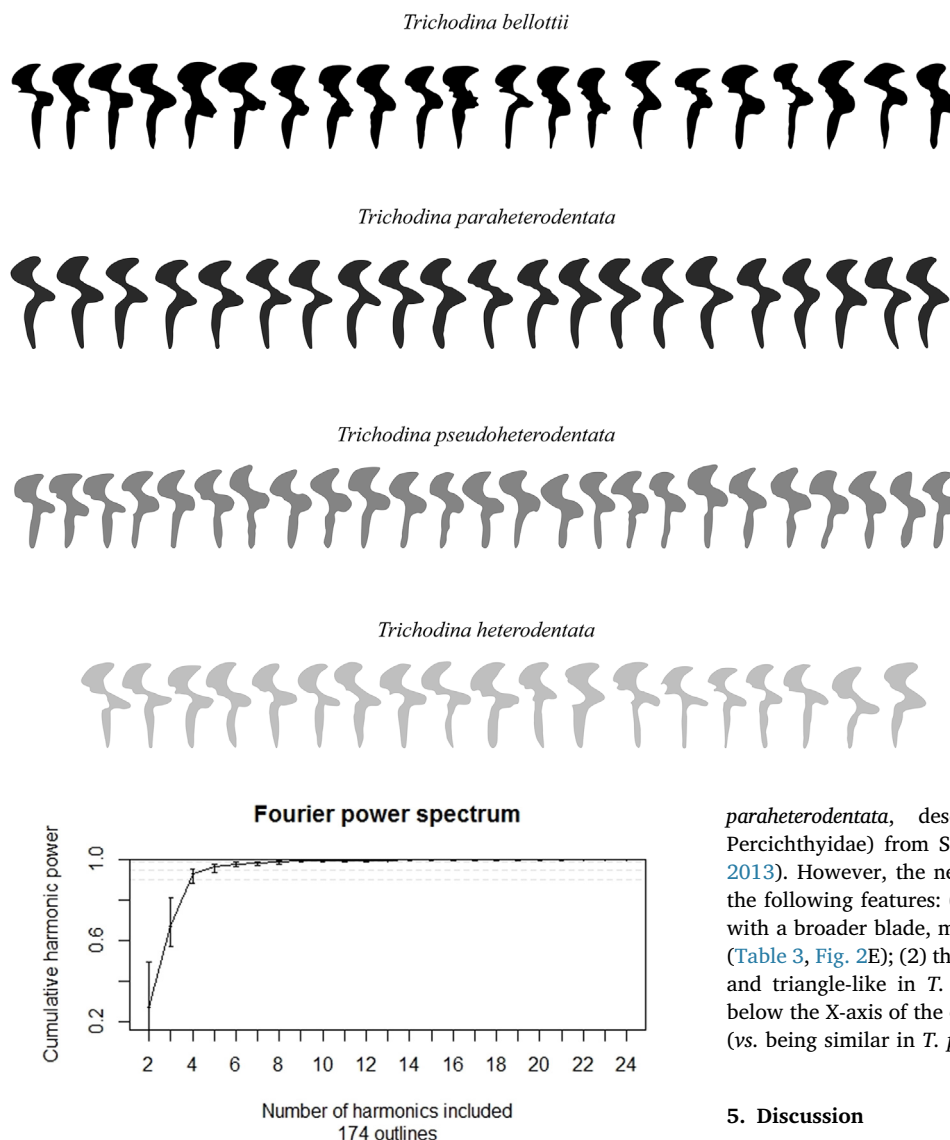


Fig. 5. Denticles silhouettes utilized on Fourier analysis. *Trichodina bellottii* n. sp., *Trichodina heterodontata* redrawn from Duncan (1977); Albaladejo and Arthur, 1989; Bondad-Reantaso and Arthur, 1989; Van As and Basson, 1989; Basson and Van As, 1994; Al Rasheid et al., 2000; Asmat, 2004; Dove and O'Donoghue, 2005; Dias et al., 2009; Martins et al., 2010; Benites de Pádua et al., 2012; Miranda et al., 2012; Valladão et al., 2014. *Trichodina paraheterodontata* redrawn from Tang and Zhao (2013). *Trichodina pseudoheterodontata* redrawn from Tang et al. (2017).

Fig. 6. Fourier harmonic power spectrum based on Elliptical Fourier analysis.

connection of the central part with preceding denticle is irregular, this was not reported by author of *T. pseudoheterodontata*.

Additionally, one species related with *T. pseudoheterodontata* is *T. heterodontata*. Likewise, in our species, the variation in denticle morphology was observed in *T. heterodontata* by several authors (Duncan, 1977; Albaladejo and Arthur, 1989; Bondad-Reantaso and Arthur, 1989; Van As and Basson, 1989; Basson and Van As, 1994; Al Rasheid et al., 2000; Asmat, 2004; Dove and O'Donoghue, 2005; Dias et al., 2009; Martins et al., 2010; Benites de Pádua et al., 2012; Miranda et al., 2012; Valladão et al., 2014). Despite this, our material can be differentiated from the original population of *T. heterodontata* (Table 3, Fig. 2D) by: (1) the new species has a more robust denticle with a broader blade, more developed blade connection, and thick ray (vs. relatively narrow blade and undeveloped blade connection in *T. heterodontata*); (2) blade with prominent posterior projection in the new species (vs. not present in *T. heterodontata*); (3) the central part of the new species is broader than that of *T. heterodontata*, and the shapes above and below the X-axis of the central part in the new species were not similar (vs. being similar in *T. heterodontata*). (4) The ray are forward directed in *T. heterodontata*, whereas is backward directed in the new species.

Another species with similar denticle morphology is *T.*

*paraheterodontata*, described on *Siniperca chuatsi* (Perciformes: Percichthyidae) from Shapingba, Chongqing, China (Tang and Zhao, 2013). However, the new species differs from *T. paraheterodontata* by the following features: (1) the new species has a more robust denticle with a broader blade, more developed blade connection, and thick ray (Table 3, Fig. 2E); (2) the new species has sickle-shaped blade (vs. acute and triangle-like in *T. paraheterodontata*); (3) the shapes above and below the X-axis of the central part in the new species were not similar (vs. being similar in *T. paraheterodontata*).

### 5. Discussion

Traditional morphometry has been a fundamental tool to distinguish between trichodinids species. However, in many cases, this is hampered by the high degree of variation that denticles present within a population or even within the same individual. In recent years, molecular identification has helped to distinguish between very similar species. However, in many cases multiple infections occur in the same host, and often the different species cannot be distinguished from each other for proper isolation. Geometric morphometry provides information regarding changes in shape independently of the size of the individual, and on the other hand can also be used in cases with multiple infections.

The outline-based method for specimens from different trichodinids species, can be described quantitatively using the changes of shape of the denticles. Fourier analysis results in a useful tool to distinguish these trichodinids species (82.56%). The high morphological variability present in *T. heterodontata* is also exposed in discriminant analysis through its relatively lower percentage of specimens correctly classified, compared to the rest of the analyzed species (*T. bellottii* 71.43%, *T. paraheterodontata* 95.24%, *T. pseudoheterodontata* 92%). Future studies of different species and populations will allow to determine the validity of the Fourier analysis as a method to discriminate trichodinids species and populations.

Based on traditional morphological features, geometric morphometry, and molecular comparison among the related species, *Trichodina bellottii* n. sp. is identified as a new member of the genus *Trichodina*.

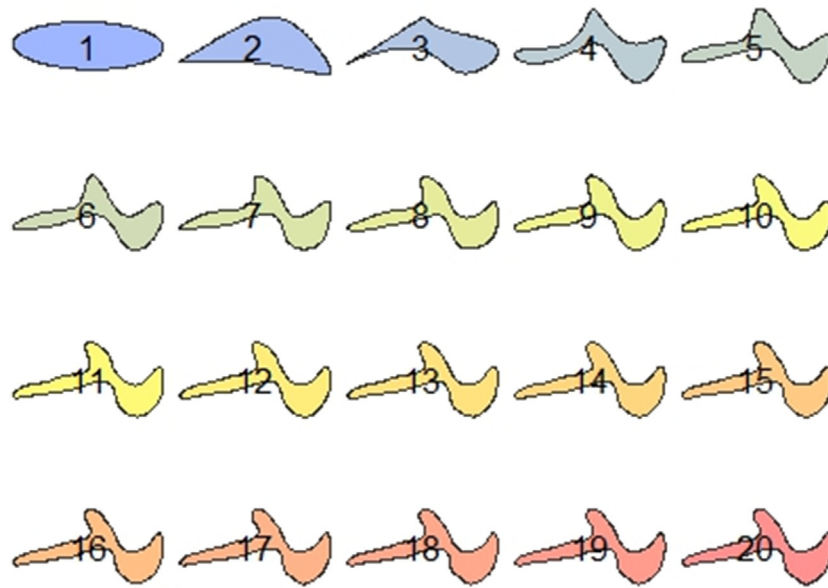


Fig. 7. Reconstructed denticles shapes using a range of 20 harmonic.

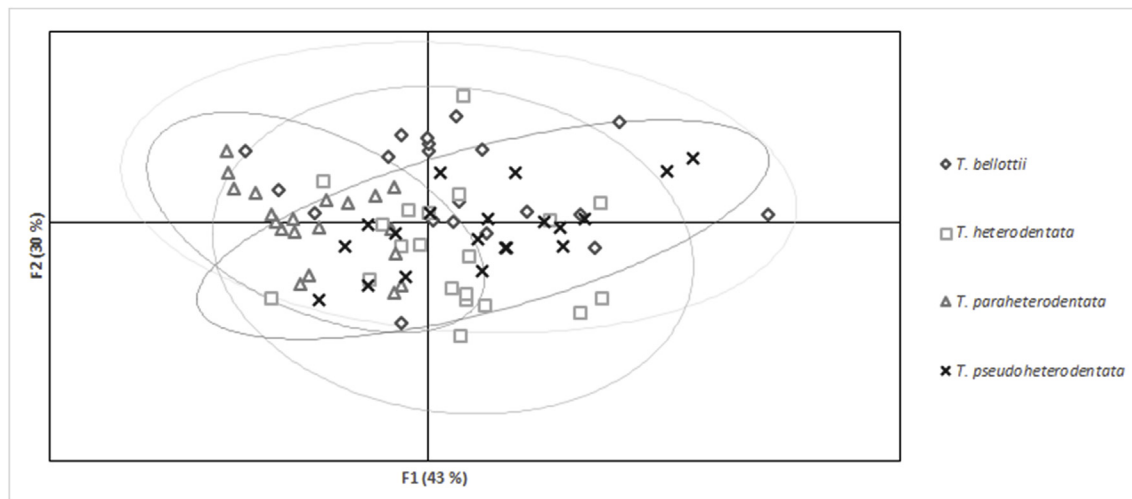


Fig. 8. PCA. Principal component scatter plot (PCA) conducted on the elliptic Fourier descriptions of denticles shapes using the first 10 harmonics; this figure shows the first two principal components (PC1 and PC2 are on the x and y-axes, respectively).

**Table 1**  
Principal components analysis scores based on Fourier coefficients from 20 harmonics.

	Eigenvalue	Proportion (%)	Cumulative (%)
Prin1	3,19E-03	31,7826	31,7826
Prin2	1,93E-03	19,2431	51,0258
Prin3	1,68E-03	16,7156	67,7414
Prin4	8,32E-04	8,2921	76,0335
Prin5	6,64E-04	6,6146	82,6481
Prin6	3,97E-04	3,9556	86,6038
Prin7	2,61E-04	2,6058	89,2096
Prin8	1,79E-04	1,7875	90,9971

During this research, the Killifish *Austrolebias bellottii* were captured from a temporary pond in the Reserva Natural Punta Lara. This is an annual fish able to maintain permanent populations in temporary habitats by combining rapid growth and development with diapause eggs that survive the dry season buried in the mud (Mills and Vevers, 1989). Recently, it was postulate that some form of dormant stage probably

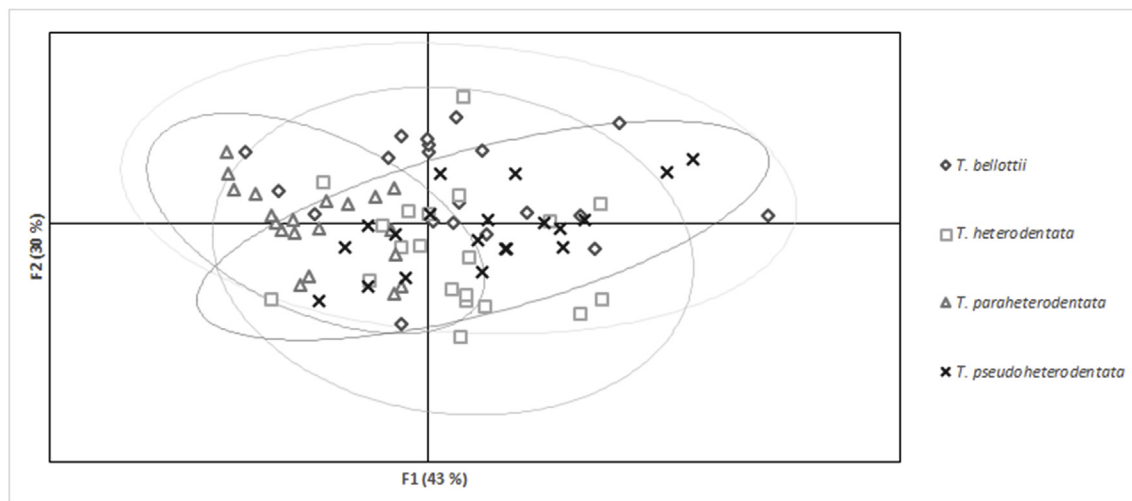
exists for *Trichodina diaptomi*, a parasite of copepod *Metadiaptomus transvaalensis* from South Africa (West et al., 2016). *T. diaptomi*, is a cosmopolitan trichodinid who successfully occurs on hosts that are not available year-round. It also appears to be able to remain alive during unfavorable conditions such as the complete dry period of their aquatic habitat along with the disappearance of their hosts.

Some snails (eg. *Biomphalaria* sp.) are able to withstand desiccation for months while buried in the mud bottom by sealing their shell opening with a layer of mucus (Rozenaal, 1997). During this period, the humidity conditions are maintained inside the snail, so it is an environment that supports the maintenance of thichodinids population.

The discovery of the new species in such particular environment made it necessary to determinate if this trichodinid can survive the environment desiccation during the dry season or if it is eventually infected from another infected host present in the environment in the rainy season.

**Table 2**  
Confusion matrix from linear discriminant analysis (LDA) based on PC1-PC8 axes.

From \ To	<i>T. bellottii</i>	<i>T. paraheterodontata</i>	<i>T. pseudoheterodontata</i>	<i>T. heterodontata</i>	Total	% Correct
<i>T. bellottii</i>	15	2	2	2	21	71,43%
<i>T. paraheterodontata</i>	0	20	1	0	21	95,24%
<i>T. pseudoheterodontata</i>	1	0	23	1	25	92,00%
<i>T. heterodontata</i>	3	1	2	13	19	68,42%
Total	19	23	28	16	86	82,56%



**Fig. 9.** Linear discriminant analysis (LDA) of *Trichodina* spp. using normalized elliptical Fourier descriptors. Percentages indicate the proportion of the trace captured in each LD component.

**Table 3**  
Morphological data (in  $\mu\text{m}$ ) of *Trichodina hypsilepis*, *Trichodina heterodontata*, *Trichodina paraheterodontata*, and *Trichodina pseudoheterodontata* and *Trichodina bellottii* n sp.

	<i>T. hypsilepis</i>	<i>T. hypsilepis</i>	<i>T. heterodontata</i>	<i>T. paraheterodontata</i>	<i>T. pseudoheterodontata</i>	<i>T. bellottii</i> n sp.
Author	Wellborn, 1967	Arthur and Lom, 1984	Duncan, 1977	Tang and Zhao, 2013	Tang et al., 2017	This study
Locality	Alabama, Unites States	Havana, Cuba	Philippines	Shapingba, Chongqing, China	Changshou Lake in Chongqing, China	Reserva Natural Punta Lara, Buenos Aires, Argentina
Host	<i>Notropis hypsilepis</i>	Unidentified tadpoles	<i>Tilapia zillii</i> , <i>Oreochromis mossambicus</i> , <i>Trichogaster trichopterus</i>	<i>Siniperca chuatsi</i>	<i>Ictalurus punctatus</i>	<i>Austrolebias bellotti</i>
Site of infection	Body and Fins	Skin	Skin	Skin	Gills	Skin and fins
Body diameter	63–80	55.1–85.7	58–108	45–64	73–82.5	61.21–75.31
Adhesive disc diameter	46–57	39.8–56.1	45–74	37–55	61.5–74	50.07–64.10
Border membrane width	4–4.5	4.1–6.1	3.4–5.5	3–6	4–6	3.74–6.97
Diameter of denticulated ring	27–35	25.5–34.2	29–45	22–34	39–47.5	30.79–42.19
Denticle number	21–24	20–23	20–22	20–22	23–25	21–26
Radial pins per denticle	10	9–12	6–14	10–12	10–12	11–12
Denticle span		14.3–19.4		12–19	19–23	15.10–21.05
Denticle length	11–13	10.2–14.8	7.5–11	5.5–12	9.5–11.5	6.72–9.27
Blade length	5–6	4.6–5.6	4.7–7.1	4.5–7	5.5–9	3.98–6.72
Central part width	2–3	2–3.1	1.4–3.4	2.5–5	4.5–5	3.19–4.68
Ray length	7–9	6.6–11.2	3–12	6–9	8.5–10	7.27–10.98
Adoral ciliary spiral	370°–380°	about 390°		400°–420°	390°–410°	380°–410°

**Declarations of interest**

None.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jppaw.2018.06.004>.

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