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Morphometric and molecular (COX 1) variations of *Asplanchna girodi* clones from Central Mexico

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Abstract

Aim: In the past two decades, studies on cryptic speciation have increased rapidly leading to the need to re-consider cosmopolitanism in Rotifera. Cryptic species complexes are reported in some taxa of rotifers, especially Brachionidae and Epiphanidae. Less is known about Asplanchnidae although several morphotypes are known to exist. In this work, we report the morphometric and molecular aspects of *Asplanchna girodi* (Asplanchnidae).

Methodology: The zooplankton samples were collected at 6 localities in central Mexico. We considered the individuals of *A. girodi* from different zones of a given waterbody as distinct populations and the same species from different geographical regions as distinct clones. For each *A. girodi* population, we obtained morphometric (body size and trophi measurements) and molecular (COX 1) data.

Results: The clones from Xochimilco were larger in size ($707 \pm 10 \mu\text{m}$) while the smallest sized individuals were from Azcapotzalco ($640 \pm 16 \mu\text{m}$). Significant intrapopulation differences in the body size and trophi size were observed for half of the clones considered in this study (Toluca, Xochimilco and Zempoala). A total of 30 sequences were obtained for *A. girodi*; these were composed of 566 residues which 426 were conserved sites and 140 were variables. Comparisons among the interclonal populations of *A. girodi* showed that the clone from Toluca was the most divergent, up to 6.5%; the rest of the interclonal divergence values were < 1.2%.

Interpretation: The molecular marker COX 1 had superior resolution to distinguish to clones within *A. girodi* and to separate cryptic species. Molecular analysis also indicated the existence of a high genetic diversification process ($K2P > 6\%$) within the species. Thus it appears that *A. girodi* is a cryptic species complex. Further this study calls for a more integrative approach using both morphometric and molecular tools to decipher the existence of cryptic speciation in asplanchnids.

Cryptic species in *Asplanchna girodi* group

A. girodi

Species isolation from natural waterbodies

Cultures of :
Alga
Brachionus (prey)

Culture of :
Different strains of
Asplanchna girodi

Cysts

Trophi :
Morphology
Morphometry

DNA
Amplification
Sequencing

To distinguish clones within *A. girodi* to separate cryptic species

Introduction

Origin of sex has been considered as a fundamental topic in evolutionary biology (Stelzer *et al.*, 2010). Traditionally, sexual reproduction has been assumed as a response to environmental heterogeneity and that it is widespread among organisms promoting diversity (Bell, 1982). Even when genetic diversity, divergence and speciation are studied, the type of reproduction of the group in question needs to be considered. Rotifera exhibit three types of reproduction: obligatorily sexual (Seisonidea), obligatorily asexual (Bdelloidea) and cyclic parthenogenesis (Monogononta) (Fontaneto and De Smet, 2015). In monogonont rotifers when sexual reproduction occurs, mate-recognition is important with a glycoprotein (MRP, mate-recognition pheromone) present on the corona of females and responsible for male receptors to initiate mating (Snell, 1998; Gilbert and Walsh, 2005; Snell *et al.*, 2006). It is known that when mate recognition is based on chemoreception, as in the case of rotifers, the morphological changes may remain decoupled from this leading to an increase in the likelihood of cryptic speciation (Knowlton, 1993; Serra *et al.*, 1997; Snell *et al.*, 2006).

In the past two decades, studies on cryptic speciation, especially in the genus *Brachionus* have increased rapidly leading to the need to re-consider cosmopolitism in Rotifera (Serra *et al.*, 1997; Gómez, 2005; Fontaneto, 2014). These studies were based on different molecular markers, where COX 1 became one of the most widely used because of its absence of introns, its relative easy extraction and amplification, and because it is highly conserved within the different groups of animals (Gissi *et al.*, 2008; Galtier *et al.*, 2009). Today cryptic species complexes are reported in several taxa of rotifers: *B. plicatilis* (Gomez and Snell, 1997; Serra *et al.*, 1997; Gómez, 2005), *Brachionus calyciflorus* (Gilbert and Walsh, 2005), *Epiphanes senta* (Schröder and Walsh, 2007), *Keratella cochlearis* (Derry *et al.*, 2003), *Lecane bulla* (Walsh, *et al.*, 2009). Recent studies suggest an estimate of at least 14 rotifer species from Mexico that could form cryptic species complexes with high genetic divergence (> 3%) among strains from different locations, and even within populations of a given species from the same body of water (García-Morales and Elías-Gutiérrez, 2013). The phenomenon of cryptic speciation in the predatory rotifers of the genus *Asplanchna* has received less attention (Jiménez-Contreras *et al.*, 2013), probably because their identification requires a trophi extraction leading to the dissolution of the entire body (Koste, 1978; José de Paggi, 2002; Wallace *et al.*, 2006). Several rotifers are predators (Fontaneto and De Smet, 2015) and field collected specimens often have more than one taxa in their guts. This makes it indispensable to culture them under laboratory conditions.

The family Asplanchnidae has 3 genera of which *Asplanchna* is the most common and cosmopolitan as well (José de Paggi, 2002). Despite the ease in encountering them in natural

waterbodies, species of *Asplanchna* exhibit some degree of morphological variation within populations of the same species (Gilbert, 1968; Hampton, 1998). Phenotypic plasticity has been recognized as a possible cause of polymorphism in this group (Ge *et al.*, 2012) and it is manifested through changes in the body size due to various factors such as competition, predation and diet type (Gilbert, 1976; Stemberger and Gilbert, 1984; Kennari *et al.*, 2008).

The genus *Asplanchna* has nine morphologically well characterized species. *Asplanchna girodi* is a species often recorded in diverse water bodies around the world including Mexico (Sarma and Elías-Gutiérrez, 1997, Sarma and Elías-Gutiérrez, 2000; Segers, 2007). This species shows conspicuous morphological variations in nature (José de Paggi, 2002) due to several factors, both abiotic and biotic. However, field collected samples often fail to reveal the exact cause of morphological variations due to the effect of several biotic and abiotic factors under natural conditions. Studies with laboratory cultures can minimize these effects to some extent. The objective of this work was to quantify the morphological variations within distinct populations of *Asplanchna girodi* isolated from different geographical regions of Mexico. In addition, we also aimed at understanding if some genetic components play a role in morphological variations of the tested populations of *A. girodi*.

Materials and Methods

Culture of Algae and prey (brachionid) rotifers : The green alga *Chlorella vulgaris* was batch cultured at $22\pm 2^{\circ}\text{C}$ using defined medium (Bold's basal, Borowitzka and Borowitzka, 1988). Algae in the log phase of growth were harvested, centrifuged (at 3000 rpm for 5 min) and resuspended in a small volume of distilled water. The density of the algal concentrate was established using a hemocytometer. *Chlorella vulgaris* was used as the exclusive diet for the brachionid rotifers (*Anuraeopsis fissa*), which in turn became prey for *Asplanchna*. The prey rotifers were separately mass-cultured using moderately hard water (EPA medium: Weber, 1993). The EPA medium was prepared dissolving 96mg NaHCO_3 , 60mg CaSO_4 , 60mg MgSO_4 , and 4mg KCl in 1 L of distilled water.

Culture of *Asplanchna girodi* : Zooplankton was sampled from different waterbodies in Azcapotzalco (Mexico City), Xochimilco (Mexico City), Toluca (State of Mexico), Zumpango (State of Mexico), Zempoala (Morelos) and San Juan del Río (Querétaro) (Table 1). From each water body, we filtered 100 L of lake water using a $50\mu\text{m}$ pore size plankton mesh. Live zooplankton samples were transported to the laboratory where the first 10 adult females of asplanchnids were randomly and individually placed in glass containers with 50ml of moderately hard water EPA (Weber, 1993) and a mixture of brachionids at a density of 5 ind ml^{-1} as food. After the initiation of the clonal cultures, we allowed them to grow to a density of about 5 ind ml^{-1} . From each

Table 1 : Collecting sites and mean values of temperature, pH and conductivity. Access number is displayed in **Ibold**

Collectionsite	Coordinates	Altitude (m)	Temp (°C)	pH	K	Ibold
Azcapotzalco	19.47, -99.194	2240	22.0	9.0	0.8	IZCON051 - IZCON053
Xochimilco	19.286, -99.102	2240	21.0	8.4	0.7	IZCON079 - IZCON081
Toluca	19.111, -99.758	4200	8.5	6.2	0.3	IZCON073 - IZCON075
Zempoala	19.05, -99.314	2800	12.0	7.4	0.8	IZCON076 - IZCON078
Zumpango	19.782, -99.138	1200	19.0	9.7	0.9	IZCON082 - IZCON084
Querétaro	20.469, -100.013	1900	19.0	8.7	0.8	IZCON097 - IZCON098

jar, we isolated one individual, extracted the trophi and identified it to species level using standard literature (Koste, 1978; Salt, 1978; Jose de Paggi, 2002). The trophi of asplanchnids was extracted by adding 2 drops of sodium hypochlorite 8% (commercial solution). We retained only those jars that contained *A. girodi* and the rest of the jars were discarded. For results description, we considered the individuals of *A. girodi* from different zones of a given waterbody as distinct populations and *A. girodi* individuals from different geographical regions as distinct clones (Jiménez-Contreras *et al.*, 2013). All prey and predator populations were maintain data temperature of $23 \pm 1^\circ\text{C}$, pH 6.8- 7.2, at a continuous but diffused fluorescent lighting and at daily replacement of EPA medium.

Morphometric and morphological analysis : Morphometric data was obtained using individuals from the clonal cultures. We used five monoclonal cultures from each population or cultures. We used five monoclonal cultures from each population or locality; 15 adults from each population were measured using a light microscope (Nikon Model E600) (size of individuals: 4 - 10 $\times$ and trophi: 100 $\times$). The size (length and width) was obtained from living individuals, their bodies were subsequently dissolved using sodium hypochlorite solution at 8% to obtain the trophi. The trophi were oriented in a similar position and were drawn using a calibrated camera lucida; the trophi measurements were obtained using Plan Measure (Scale Master Pro, Model 6025). The measured variables included the length of the inner and outer margin of the ramus, the bulla and the sub-apophysis as shown in Fig. 1. For scanning electron microscopy, trophi obtained from sodium hypochlorite treatment were washed several times using distilled water, dried and mounted on stubs for gold coating following DeSmet (1998). Trophi morphology was observed using scanning electron microscope JEOL, Lv5900 at 10,000-25,000 $\times$. Statistical analysis was carried out using analysis of variance (ANOVA) for intra- and interpopulational differences of *A. girodi*.

DNA amplification and sequencing : For sequencing the DNA, 5-10 individuals of at least five monoclonal cultures of each population were used. In order to avoid interference of prey DNA with the DNA of *Asplanchna*, the asplanchnids were starved for 6-8h, period in which the stomach content was egested. The DNA was extracted by a modified Hot Shot protocol (Montero-Pau *et al.*, 2008): asplanchnids were digested with 30 μl of alkaline lysis buffer (25 mM NaOH, pH 11) in PCR tubes (0.2ml).

Fig. 1 : Details of trophi measurements made for different clones and populations of *A. girodi*. OR= Outer margin of the ramus, IR = Inner margin of the ramus, OB = Outer margin of bulla and S = subapophysis

The tubes with the individuals were maintained at 94°C for 30min and then quickly transferred to ice and maintained for 3- 4min. Later, they were added 30 μl of neutralizing buffer (1 M Tris-HCl, pH 7). Extracted DNA was used to amplify the fragment of the gene encoding the Cytochrome Oxidase subunit IHC02198_t1 using primers (5'CAGGAAACAGCTATGACTAAACCTTCAGGGTGACCAAAAAATCA3 ') and LCO1490_t1 (5'TGTAAACGACGCGCCAGTGGTCAACAAATCATAAAGATATGG3') (Footitt *et al.*, 2009). The PCR reaction (final volume 50 μl) was performed with 45 μl of Taq DNA Polymerase Ampliqon® Master Mix RED1.1x (Tris-HCl, pH 8.5, $(\text{NH}_4)_2\text{SO}_4$, 0.11% Tween20, 0.22m of each dNTP, 0.11units / μl Taq polymerase Ampliqon; Inert red dye and stabilizer), 0.5 μl of each primer (10

mM) and 4 µl of DNA template. The amplification program consisted of an initial denaturing cycle at 94 °C for 3min, followed by 30 cycles consisting of a denaturation period at 94 °C for 40s, an annealing at 45°C for 40s, and an extension at 72° C for 40s. Finally, an extension cycle at 72°C for 2min.

An aliquot of the amplification product was used to verify the size of the amplicon with an agarose gel electrophoresis (1%). The amplified product was purified using an illustra purification kit GFX PCR DNA and a Gel Band Purification Kit GE®. Finally, the purified amplicon was subjected to a sequencing reaction in the Molecular Biochemistry Laboratory at the Biotechnology and Prototypes Unit (UBIPRO) FESI UNAM, using an automated sequencer 3100 Genetic Analyzer (Applied Biosystems). The obtained electropherograms were reviewed and edited using the Chromas Lite 2.0. The sequences were aligned with Clustal W (Thompson *et al.*, 1997) with default settings, the genetic divergence was calculated (Kimura two parameters) and a dendrogram was constructed using Neighbor-joining with 5 Mega program (Tamura *et al.*, 2011).

As a standard procedure, the sequences with the accepted numbers shown in Table 1 are in process of being uploaded to the global database Ibold Systems.

Results and Discussion

Morphological markers : The clones from Xochimilco were larger in size ($707 \pm 10 \mu\text{m}$) while the smallest sized individuals were from Azcapotzalco ($640 \pm 16 \mu\text{m}$). For the rest of the clones, the body size was within this range (Toluca: 698 ± 15 , Zempoala: 671 ± 12 and Zumpango: $648 \pm 15 \mu\text{m}$) (Table 2). Trophi size (Fig. 2), specifically the ramus was not directly related to body size. Thus, the largest sized rami were observed in Zempoala and Zumpango populations (73 ± 0.4 in both cases); the smallest size was present in Xochimilco ($61 \pm 0.4 \mu\text{m}$) whose clonal populations had a larger body size. Significant intrapopulation differences in the body size and trophi size were observed for half of the clones considered in this study (Toluca, Xochimilco and Zempoala) ($p > 0.05$, F-test, Table 4). The population from Toluca was significantly different from the rest of the clones in most of the morphometric variables (body length, bulla and sub-apophysis) while the lowest difference was from Xochimilco (ramus) (Fig. 3). The body size of most predatory rotifers including *Asplanchna* measures about $400 \mu\text{m}$ (Koste, 1978). In this regard, the sizes of asplanchnids collected in the localities were within the range reported by José de Paggi (2002).

Table 2. Morphometric *A. girodi* monoclonal cultures of all data selected populations. Body size and the diameter of the cysts are shown. All measurements are given in µm. The mean $\pm$

Table 2 : Morphometric *A. girodi* monoclonal cultures of all data selected populations

Site	Morphometric variables				
	Lenght	Width	Ramus	Bulla	Sub-apophysis
Azcapotzalco					
Azc1	603 ± 25^a	283 ± 9^a	61 ± 0.8^a	16 ± 0.4^a	3.5 ± 0.1^a
Azc2	677 ± 28^a	324 ± 16^a	62 ± 0.7^a	17 ± 0.5^a	3.3 ± 0.1^a
Azc3	641 ± 29^a	316 ± 16^a	62 ± 0.8^a	17 ± 0.5^a	3.3 ± 0.1^a
Xochimilco					
Xoc1	689 ± 21^a	284 ± 11^a	54 ± 1.2^a	16 ± 0.4^a	3.9 ± 0.1^a
Xoc2	700 ± 17^a	288 ± 7^a	55 ± 1^a	15 ± 0.4^a	3.6 ± 0.1^a
Xoc3	731 ± 12^a	301 ± 3^a	66 ± 8^b	17 ± 0.4^a	3.9 ± 0.1^a
Nevado de Toluca					
NT1	761 ± 25^a	352 ± 12^a	67 ± 1^a	$18 \pm 0.3^{a,b}$	4.3 ± 0.1^a
NT2	644 ± 15^b	345 ± 12^a	64 ± 0.8^a	18 ± 0.5^a	$4.3 \pm 0.1^{a,b}$
NT3	$689 \pm 26^{a,b}$	347 ± 18^a	64 ± 0.9^a	20 ± 0.5^b	3.8 ± 0.1^b
Zempoala					
Zem1	627 ± 22^a	287 ± 16^a	71 ± 0.7^a	19 ± 0.4^a	4.2 ± 0.2^a
Zem2	$677 \pm 15^{a,b}$	$297 \pm 6^{a,b}$	73 ± 0.9^a	20 ± 0.7^a	4.5 ± 0.1^a
Zem3	709 ± 21^b	331 ± 11^b	73 ± 0.5^a	19 ± 0.6^a	4.2 ± 0.2^a
Zumpango					
Zum1	641 ± 24^a	329 ± 13^a	72 ± 0.7^a	19 ± 0.3^a	4.2 ± 0.2^a
Zum2	660 ± 30^a	337 ± 14^a	73 ± 0.9^a	20 ± 0.7^a	4.5 ± 0.1^a
Zum3	641 ± 25^a	327 ± 15^a	73 ± 0.5^a	19 ± 0.5^a	4.2 ± 0.2^a
Querétaro					
Qro1	714 ± 22	355 ± 15	76 ± 0.7	21 ± 0.4	4.2 ± 0.1

Body size and the diameter of the cysts are shown. All measurements are given in microns. The mean $\pm$ standard error based on 15 individuals of each monoclonal culture is shown. Legends (Azc, Xoc, NT, Zem, Zum and Qro) placed within each clone, show the three populations characterized. The intrapopulation differences were analyzed with 1-way ANOVA, similar letters represent no significant differences ($p > 0.05$)

Table 3 : Interpopulational divergence of *A. girodi*, in addition the table shows the sequences of *A. sieboldii*, *A. silvestrii*, *A. tropica* and *A. sp. AMD*

Clone	Azc	Xoc	NT	Zem	Zum	Qro	<i>A. sieboldii</i>	<i>A. silvestrii</i>	<i>A. tropica</i>	<i>A. sp. MD</i>
Azc	-									
Xoc	0.7	-								
NT	6.4	6.4	-							
Zem	0.8	1.2	6.5	-						
Zum	0.1	0.6	6.2	0.6	-					
Qro	0.1	0.6	6.2	0.6	0	-				
<i>A. sieboldii</i>	17.7	17.3	17.2	17.7	17.5	17.5	-			
<i>A. silvestrii</i>	16.8	16.7	16.9	16.7	16.6	16.6	12.1	-		
<i>A. tropica</i>	19.8	19.4	19.5	20.1	19.6	19.6	17.8	17.4	-	
<i>A. sp. AMD</i>	7.2	7	6	7.1	7	7	15.8	15.8	18.5	-

standard error based on 15 individuals of each monoclonal culture is shown. Legends (Azc, Xoc, NT, Zem, Zum and Qro) placed within each clone, show the three populations characterized. The intrapopulational differences were analyzed with 1-way ANOVA, similar letters represent no significant differences ($p > 0.05$).

Asplanchna is a widely distributed rotifer genus in fresh waterbodies and the species *A. brightwellii*, *A. girodi*, *A. sieboldii* and *A. priodonta* are often encountered in plankton collections (José de Paggi, 2002). In this work, we were able to obtain *A. girodi* in 6 water bodies in Central Mexico. This coincides with several authors (Sarma and Elías-Gutiérrez, 1997, Sarma and Elías-Gutiérrez, 2000) who have mentioned that in the central part of Mexico *A. girodi* is very common.

It is known that the body size of rotifers can be affected by various factors including the availability and quality of the food (Nandini and Rao, 1998; Hu *et al.*, 2002; Geng *et al.*, 2003), temperature (Carlin, 1943; Pejler, 1962) and predation (Gilbert, 1967; Halbach, 1970; Pourriot, 1973). However, in this work rotifers were cultured under similar conditions, so the body size variations of *A. girodi* reflect the clonal history rather than the influence of environmental factors. Based on morphometric data there was no clear separation among the populations of the same waterbody. There were significant differences in the morphological characteristics among the clones (Table 5). However, the clones from Zumpango and Zempolala showed no significant differences in any of the morphometric characters.

Some studies (Walsh and Zhang, 1992; Walsh, 1995; King and Serra, 1998) suggest a clear relationship between genetic variations and morphological features, the last one acting as a reproductive barrier. In this work, we did not study the cross mating among the clones because unlike *Brachionus* (Rico-Martínez *et al.*, 1997), *Asplanchna* females can be cannibalistic and may feed on their own males (Gilbert, 1980) because males are much smaller than the females (body length 500 vs 1000 μm) (Serra and Snell, 1998). Our morphometric data on the trophi

morphology of *A. girodi* suggest an allometric growth pattern where the body size was not correlated with trophi size for most of the clones. This allometric growth pattern occurs in several species of rotifers where a significant relationship between the size of the lorica and size of the trophi is often, but not always, evident (Sarma and Dumont, 1993; Fontaneto and Melone, 2006). Brachionid and euchlanid rotifers increase their body size up to 85% beyond age at first reproduction (*B. plicatilis*: Snell and Carrillo, 1984; *Euchlanis dilatata*: Gulati *et al.*, 1993). However, unlike the lorica, the trophi does not grow once the neonate is born or hatched (Fontaneto and Melone 2005) and therefore it is unlikely that a positive relationship exists between the rotifer size and trophi size.

Molecular marker : A total of 30 sequences was obtained for *A. girodi*; these were composed of 566 residues which 426 were conserved sites and 140 were variables. The nucleotide diversity varied between 0.0 and 0.03 within the populations tested here. Recent studies suggest the occurrence of saturation of certain genes and their effect on the phylogenetic information (Xia *et al.*, 2003; Xia and Lemey, 2009). However, in this work we used only one species and the number of gene sequences was also low and hence these data were not sufficient to test the occurrence of gene saturation in *A. girodi*.

Interspecific divergence of *A. girodi* and the species used as out group was, on average, 13.8% for all populations. Among the interclonal populations of *A. girodi*, the clone of Toluca was the most divergent, up to 6.5%; the rest of interclonal divergence values were $< 1.2\%$. The maximum value of interclonal divergence observed here (6.5%) can be classified as high; however, this is within the range known for cryptic species (Avisé, 1994; Moriyama and Powell, 1996; Avisé and Walker, 1999) including rotifers *B. plicatilis*; (Gomez *et al.*, 2000; Gómez *et al.*, 2002; Gómez, 2005), *Keratella cochlearis* (Derry *et al.*, 2003) and *B. calyciflorus* (Gilbert and Walsh, 2005). Our results suggest the presence of cryptic species complexes in *A. girodi*. Additionally, in a recent work García-Morales and Elías-Gutiérrez (2013) account for at least 14 taxa, located in Mexico,

Fig. 2 : Trophi comparison of *A. girodi*: Azcapotzalco (Mexico City), Xochimilco (Mexico City), Nevado de Toluca (State of Mexico), Zumpango (State of Mexico), Zempoala (Morelos) and San Juan del Río (Querétaro) based on laboratory cultures

where the cryptic speciation appears to be a nodal factor for their populations, highlighting the fact that these differences are observed greater than 3% and is mainly observed among populations from different locations. However, it is necessary to complement these results with ecological and cross-mating experiments to confirm the possible ecological differentiation and the existence of reproductive isolation (Schröder and Walsh, 2007).

The graphical representation of the distances among the interclonal *A. girodi* is shown in Figure 4. The dendrogram of *A. girodi* showed the formation of three groups, the base of these is not equivalent to each population. In this tree, it is shown a group of five clones among those found in Azcapotzalco, Xochimilco, Zempoala, Zumpango and Querétaro, a second group of a population from Toluca and the third group consisting of the four remaining populations from Toluca (Fig. 5). The divergence within

Fig. 3 : Morphometric markers values of *A. girodi* populations from different localities, based on the average of 45 individuals per location. For each of the variables, similar letters on the bars indicate no significant differences between populations ($p > 0.05$)

the groups was always $< 1\%$. Higher divergence value (7.2%) was present between groups 1 and 3, while a low (2.5%) divergence was between groups 1 and 2. The average divergence among the groups formed in the dendrogram was 6.3%. Kawecki and Ebert (2004) mention that to meet local adaptation, at least three conditions must be fulfilled: 1) the

pressure of divergent selection in different habitats 2) the existence of the interaction between a genotype and a habitat and 3) the existence of a genotype $\times$ environment that leads to a better adaptation over foreign genotypes. In the case of *A. girodi* all locations had elevated values of divergence (7.2%) relative to the population of Toluca.

Table 4 : Results of one way-ANOVA performed on the selected morphological characters: length, width, ramus, bulla and sub-apophysis of *A. girodi* within the five clones. df = freedom degrees, SS = sum of squares, MS = mean squares, F = F-ratio, *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$

Source of variation	df	SS	MS	F	Source of variation	df	SS	MS	F
Clone - Azcapotzalco					Ramus				
Length					Between populations	2	83.197	41.598	3.419 ^{ns}
Populations	2	41831.1	20915.6	1.8 ^{ns}	Error	42	511.031	12.167	-
Error	42	475360	11318.1	-	Bulla				
Width					Between populations	2	29.571	14.786	4.518 ^{ns}
Populations	2	14417.8	7208.9	2.4 ^{ns}	Error	42	137.441	3.272	-
Error	42	125813	2995.5	-	Subapophysis				
Ramus					Between populations	2	2.807	1.404	5.474**
Between populations	2	12.8	6.4	0.7 ^{ns}	Error	42	10.769	0.256	-
Error	42	385.5	9.2	-	Clone - Zempoala				
Bulla					Length				
Between populations	2	17.9	8.9	2.6 ^{ns}	Between populations	2	52124.4	26062	4.6*
Error	42	146.1	3.5	-	Error	42	237920	5664.7	-
Subapophysis					Width				
Between populations	2	0.342	0.171	0.758 ^{ns}	Between populations	2	15804.4	7902.2	3.9*
Error	42	9.467	0.225	-	Error	42	84320	2007.6	-
Clone - Xochimilco					Ramus				
Length					Between populations	2	21.9	10.9	1.5 ^{ns}
Between populations	2	13813.3	6906.67	1.592 ^{ns}	Error	42	314	7.4	-
Error	42	182187	4337.8	-	Bulla				
Width					Between populations	2	8	4	0.9 ^{ns}
Between populations	2	2471.1	1235.6	1.4 ^{ns}	Error	42	194.1	4.6	-
Error	42	37173.3	885.1	-	Sub-apophysis				
Ramus					Between populations	2	0.815	0.408	0.613 ^{ns}
Between populations	2	1351.5	675.8	1.9 ^{ns}	Error	42	27.9	0.7	-
Error	42	15160.5	361	-	Clone - Zumpango				
Bulla					Length				
Between populations	2	22.7	11.4	4.9*	Between populations	2	3484.4	1742.2	0.2 ^{ns}
Error	42	95.5	2.3	-	Error	42	446347	10627	-
Subapophysis					Width				
Between populations	2	1.1	0.5	2.1 ^{ns}	Between populations	2	924.4	462.2	0.1 ^{ns}
Error	42	10.7	0.3	-	Error	42	126720	3017.1	-
Clone - Toluca					Ramus				
Length					Between populations	2	21.987	10.994	1.470 ^{ns}
Between populations	2	105031	52515.6	6.8**	Error	42	314.004	7.476	-
Error	42	323627	7705.4	-	Bulla				
Width					Between populations	2	8	4	0.9 ^{ns}
Between populations	2	373.3	186.7	0.06 ^{ns}	Error	42	194.1	4.6	-
Error	42	127947	3046.3	-	Sub-apophysis				
					Between populations	2	0.815	0.408	0.613 ^{ns}
					Error	42	27.929	0.665	-

The Toluca system is a crater lake, located at an altitude of 4200 m above sea level, for about 3,300 years without activity and quite isolated from all surrounding water bodies (Macías *et al.*, 1997; Armienta *et al.*, 2008). These conditions favor the existence of reproductive isolation and the extreme environmental conditions in the system, such as temperature or U.V. radiation, as strong selection force, may have generated the existence of cryptic species. In other studies of high altitude ecosystems and contrasting environmental conditions, it has been documented environmental factors influence in creating divergence between populations of rotifers (Alcántara-Rodríguez *et al.*, 2012).

Lake Zempoala shares three characteristics with Nevado de Toluca: volcanic origin, high altitude and significant variations in temperature (García-Rodríguez and Tavera, 2002; Armienta *et al.*, 2008). The remaining of the populations studied are also from high altitude regions (near or over 2000 masl) and along the Trans-Mexican volcanic belt between the Nearctic and Neotropical regions, which can be related to the presence of typical species from both areas (García-Rodríguez and Tavera, 2002; Alcocer and Bernal-Brooks, 2010). These features can help us to understand the little divergence between the populations of

Table 5 : Results of one way-ANOVA performed on the selected morphological characters: length, width, ramus, bulla and sub-apophysis of *A. girodi* among the five clones. df = freedom degrees, SS = sum of squares, MS = mean squares, F = F-ratio, *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$

Source	df	SS	MS	F
Length				
Between clones	4	156611.6	39152.9	4.6***
Error	220	1881724.4	8553.2	-
Width				
Between clones	4	92931.6	23232.9	9.5***
Error	220	535964.4	2436.2	-
Ramus				
Between clones	4	7545.5	1886.4	22.8***
Error	220	18176.6	82.6	-
Bulla				
Between clones	4	364.5	91.1	23.5***
Error	220	853.6	3.9	-
Sub-apophysis				
Between clones	4	29.8	7.4	17.7***
Error	220	92.7	0.4	-

Fig. 4 : NJ tree realized with 30 sequences of asplanchnids from 5 geographically different populations, based on Kimura 2 Parameter (K2P) distances. The outgroups were obtained from the genebank

group I. On the other hand, the intermediate position of the population NT1 is possibly due to the geographical components and relatively recent divergence processes (Schröder and Walsh, 2007), considering that the volcano has been inactive for about 3,000 years (Macias *et al.*, 1997; Armienta *et al.*, 2008). In addition to this, it has been observed that some cryptic species may occur by sympatric speciation with specific ecological roles in the same geographical areas (Ortells *et al.*, 2003; Schröder and Walsh, 2007).

The two approaches used for the specific delimitation of *A. girodi* are essential and are not mutually exclusive. However, the molecular marker COX 1 had superior resolution to distinguish to clones within *A. girodi* and to separate cryptic species. Molecular analysis indicates the existence of a high genetic diversification process ($K2P > 6\%$) within the species. *A. girodi* is a cryptic species complex and this study calls for a more integrative approach using both morphometric and molecular tools to decipher the existence of cryptic speciation in asplanchnids.

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