Head morphometry of *Orestias* (Cyprinodontiformes). Response to extreme Southern Altiplano systems?

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Abstract The distribution of *Orestias* (Cypriniforms) species in the southern western Altiplano (17ºS-22ºS) is allopatric; the seven species described inhabit different freshwater systems with extreme climatic characteristics and different ecological conditions, factors that would have enhanced interspecific differentiation. To analyze their head differences we compared jaw morphology of eight species of *Orestias*, seven southern ones and one from Puno Peru, using linear and geometric morphometrics. We found differences among the species with both methods. Nevertheless, none of the external measurements by themselves allows classification of any of the species or populations. The geometric analysis showed differences related to feeding structures such as a protractile jaw, separating the western species in two groups: a northern group with *O. chungarensis, O. parinacotensis, O. piacotensis, O. laucaensis*, *O. puni* and *O.* cf. *agassii* and a southern group, *O. gloriae* and *O. ascotanensis*. The results from this methodology reaffirmed the importance of the extreme environmental conditions of the Altiplano systems to explain the process of adaptation described for the specious genus *Orestias*.

Introduction

Orestias (Cypriniforms), the endemic high Andean South American genus, is distributed from Lacsha Lake Perú, (9°S) to the Ascotán Salt pan in Chile (21º S') (Parenti 1984a, b). Cyprinodontids fish are reported as tolerant to a wide range of temperature and salinity (Davenport and Sayer 1993); their small size is postulated as allowing them to tolerate extreme and unstable environments, permitting the survival of relict populations (Parker and Kornfield 1995; Cruz-Jofré et al. 2016). The evolution of lacustrine systems in the Altiplano to the present Titicaca and Chungará lakes has been strongly controlled by climate, geology and the size of paleo-lakes (Placzek et al. 2006). Their effluents and shallow associated freshwater systems would have contributed to the diversity of physical and chemical characteristics, promoting isolation, diversification and speciation through radiation of *Orestias* in Titicaca Lake and numerous wetlands (bofedales) and salt pans in the southwest Chilean Altiplano (SWA; 17ºS to 22ºS) (Parenti 1984b; Maldonado et al. 2009; Vila et al. 2010, 2013; Arratia et al. 2017). Diversification of *Orestias* in the SWA may be linked to historical vicariant events and contemporary and historical variation in water levels that could have affected the populations from the Plio-Pleistocene until the present (Arratia et al. 2017).

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In the Holocene and late Pleistocene, repeated climatic changes and water levels, intense volcanic activity and major tectonic movements affected the Altiplano freshwater systems (Placzek et al. 2006). As a result of these geological events, large, closed basins were developed on the southwestern side of the Altiplano; with a more arid climate these basins have remained small and unconnected (i.e., Chungará, Cotacotani, Piacota, Lauca, Isluga, Carcote and Ascotán) where *Orestias* maintains small populations that have remained isolated. During all the Quaternary the Altiplano alternated between cold, wet glacial and dry interglacial periods. Due to the repetitive expansions and contractions of the freshwater systems, the populations of *Orestias* would have been subjected to drastic environmental variations, which last until today (Parker and Kornfield 1995; Márquez-García et al. 2009; Vila et al. 2010, 2013; Cruz-Jofré et al. 2016). These systems present high total irradiance above 300 W m^{-2} (Rondanelli et al. 2015), high salinity, reaching conductivities of more than 100 μmhos/cm, pH range between 8.0 and 9.5 and significant diurnal temperature changes (Márquez-García et al. 2009). Consequently, the different populations experienced a wide variety of ecological conditions in their distribution range, probably with local adaptations and particular phenotypic expression as it has been described for *Orestias* from Titicaca Lake (Lauzanne 1982; Maldonado et al. 2009).

The current species distribution in SWA includes *O. chungarensis* (Vila and Pinto 1986) in Chungará Lake (18º15' S, 69º07' W), *Orestias piacotensis* (Vila 2006) in Piacota (18º11'S, 69º15'W), *O. laucaensis* (Arratia 1982) in the Lauca River (18º11' S, 69º16' W) and Cotacotani Lake (18º12' S, 69º14' W) and *O. parinacotensis* (Arratia 1982), in the Parinacota Wetland, (18º10' S, 69º20' W); southern species cited as *O. agassii* (Mann 1954) currently are considered genetically as two different populations, one of these populations is located in Isluga River (19º15'S, 68º42'W) and the other one is in Huasco Salt pan (20º15'S, 68º52'W) (Scott 2010; Vila et al. 2013). The southernmost species are those that live associated with the springs that feed the southern salt pans, *O. gloriae* Vila et al. 2011 in Carcote (21°16'S, 68°19'W) and *O. ascotanensis* Parenti, 1984 in Ascotán Salt pan (21º29'S, 68º19'W). Interestingly, all southern species are allopatric (Vila et al. 2010), adapted to physical and chemical conditions, probably the volume and salinity of presently unconnected different systems such as deeper and shallow lakes rivers and wetlands locally called "bofedales", systems that would have developed different aquatic biodiversity. Lakes sustain abundant zooplankton while rivers and wetlands with abundant macrophytes present diverse aquatic insects, snails and amphipods, characteristics that would have stimulated different adaptations for feeding (Riveros et al. 2012). In general, analyses of the *Orestias* diet have determined their preference for benthonic macroinvertebrates, which are associated with the different substrates and macrophytes of the different Altiplano systems (Guerrero et al. 2015). It still is no consensus on the degree of diet specialization of species (Vila et al. 2006; Guerrero et al. 2015).

Orestias morphology measurements and meristic specific characteristics overlap (Parenti 1984a; Vila et al. 2010). To improve morphological information, complementary methods have been developed such as landmark geometric morphometrics, allowing quantitative comparisons (Rohlf and Marcus 1993; Adams et al. 2004). Morphological studies of populations would show possible feeding strategies and morphological adaptations since these represent an important relationship between phenotype and environment (Wainwright 1991; Winemiller 1991; Douglas and Matthews 1992; Motta and Kortsckal 1992; Skúlason and Smith 1995; Winemiller et al. 1995; Svanbäck and Eklö 2002; Langerhans et al. 2003; Higham et al. 2006; Parsons and Robinson 2006, 2007; Maldonado et al. 2009). In fact, the mouth structure especially jaw protraction is an important factor in feeding habits and diversification of Teleostomi (Schaeffer and Rosen 1961; Westneat 2004, 2005; Westneat and Wainwright 1989; Waltzek and Wainwright 2003).

In this study, we compared linear and geometric morphometric of associated head structures, mainly mouth structures. We expected to find differences at the interspecific level within the genus *Orestias*, probably due to historical isolation and particular environmental conditions of each ecosystem in the southern western Altiplano. This information will enhance conservation and allow future comparisons among populations of the species of the genus *Orestias*.

Materials and methods

Specimens from the Limnology Laboratory University of Chile collection (LCUCHILE), included 108 adults

Orestias from eight SWA localities, 13 specimens each of Lauca, Isluga Rivers, Piacota and Chungará Lakes, Parinacota wetland and Huasco, Carcote and Ascotan Salt pans and four specimens of *O. puni* from Titicaca Lake (Fig. 1; Table 1). The detailed collection of specimens were analyzed using linear and geometric morphometrics, firstly with the closed mouth and after that with the opened mouth. We opened the specimens' mouth to the maximum jaw protrusion for the purposes of this study.

Linear morphometric measurements

The following linear measurements were obtained with a digital Vernier (Fig. 2): Specimens with closed mouth: (1) Head length, (2) Snout length, (3) Tip of the snout to pectoral fin ventral insertion. Measurements with open mouth: (4) Snout length, (5) Tip of the snout to pectoral fin ventral insertion, (6) Mouth height, (7) Maxillary length. The morphometric data of the specimens was related to the standard length using the regression of Elliott et al. (1995).

To determine species differences a multivariate analysis of variance (one-way MANOVA) and *a posteriori* Tukey test for unequal samples were performed. With the measurements and matrix obtained, a canonical variation analysis was performed (CVA). A linear discriminant analysis (LDA) was also performed, using locality as the dependent variable. As independent variables each of the standardized variables of the measured specimens were used. This analysis allows to obtain the discriminant level of the set of characters used, as well as to identify the significant morphological variables of populations and species. To establish the morphological pattern among populations and the grouping of the specimens geomorph package was used (Adams et al. 2015) with the software "R" (Team 2014).

Geometric morphometric analysis

For the geometric morphology the left side of each specimen was photographed with the Canon SX 530 (16 Mega Pixel, with 1x optical zoom) camera in two different positions, one with the mouth closed and the other with it opened to maximum protrusion. For each group of specimens, we defined 12 anatomical landmarks corresponding to points or homologous structures that coincide between populations (Dryden and Mardia 2016). With closed and open mouth position with maximum protrusion the landmarks shown in Fig. 3, were: (1) The dorsal margin of head vertical to the center of eye, (2) Posteroventral corner of the maxilla, (3) Antero dorsal point of mouth in fleshy lip, (4) insertion of the operculum on the ventral profile, (5) Opercula width, (6) Supraoccipital, posteromedial tip, (7) Anterior middle eye border, (8) Posterior middle eye border, (9) Middle superior eye border, (10) Beginning of operculum, 11. Tip of the upper jaw, symphysis of the premaxillaries, 12. Tip of lower jaw, symphysis of the dentaries. These landmarks were digitized for all specimens with the software tpsDig2 and tpsRelW (Rohlf 2012).

The software Morpheus was used to analyze the anatomical configurations defined by the landmarks (Slice 1998). As a first step, landmark data from the 108 specimens were superposed with GPA (General Procrustes Analysis) (Rohlf 1990). This method scales all the specimens to one size, moving them to a common location and rotating them so the corresponding landmarks are best aligned, standardizing the landmark configurations for position, orientation, and size (Mitteroecker and Gunz 2009; Rohlf 1990). The analysis considered closed mouth and maximum protrusion independently for each group.

To determine differences among populations or species a PERMANOVA with adjustment of Euclidean distance (Anderson 2005) was developed, with 10000 permutation test using software Primer 6-Permanova 2 (Clarke and Gorley 2006).

To evaluate the influence of allometry on shape variation, a linear regression analysis was performed in MorphoJ (Klingenberg 2011). This regression included the Log of centroid and the scores of principal components. Centroid was obtained as the square root of the summed squared distances from each landmark to the centroid (Bookstein 1991). A canonical variation analysis (CVA) was performed to establish the morphological variation among species and populations, were grouped using the software Morpho J. (Klingenberg 2011).

We also integrated the matrices obtained from the superposition of the specimens and performed a cluster analysis with Euclidian distances. This summarized the similarities/differences among the analyzed groups. Additionally, we analyzed the partial distortions of the
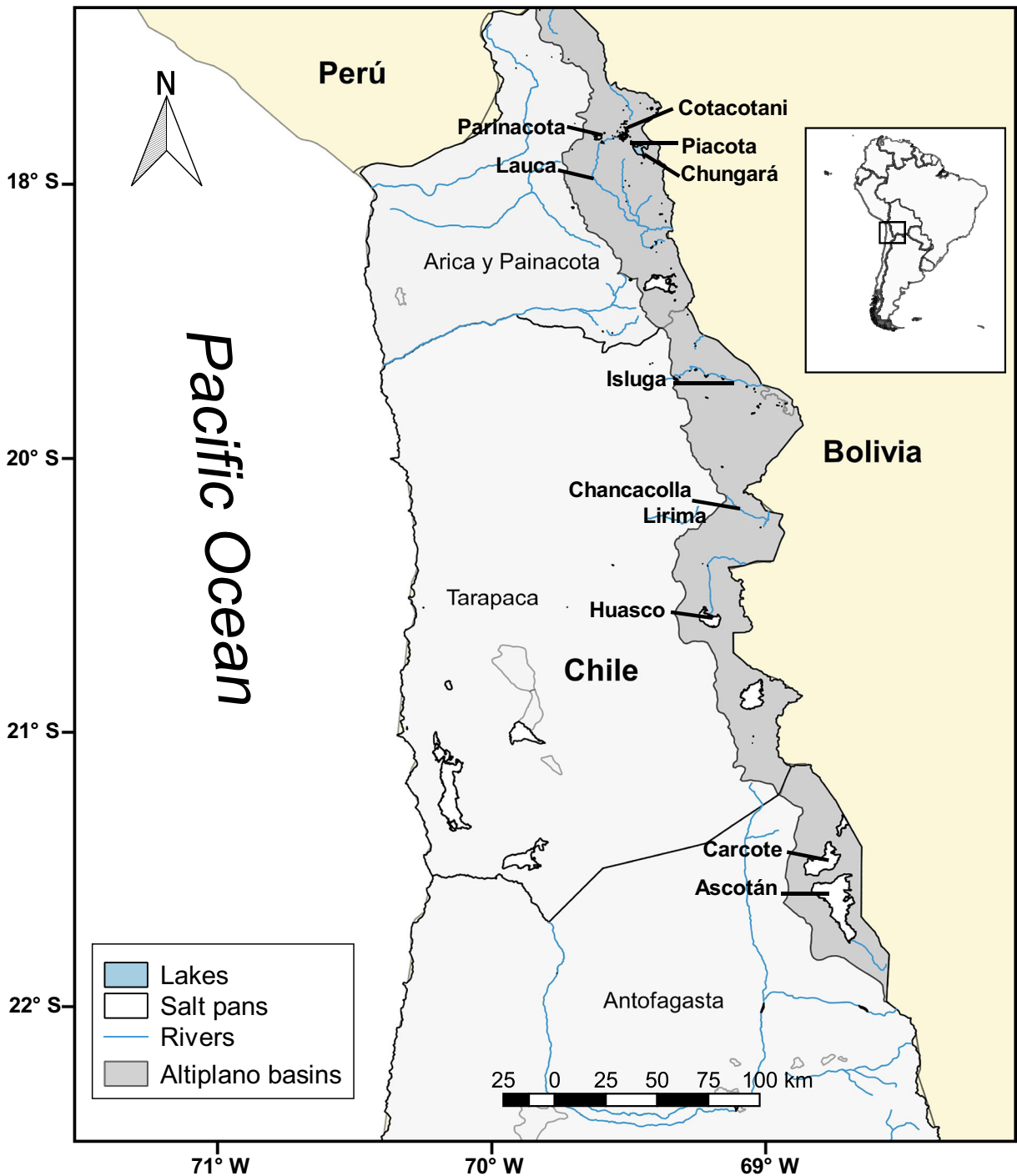
Fig. 1 Location of the Southern Altiplano systems and geographic distribution of *Orestias* in the Southwestern Altiplano (Chile). *O. piacotensis* in Piacota lake, *O. laucaensis* in Lauca River and Cotacotani, *O. parinacotensis* in the Parinacota Wetland, *O. chungarensis* in Chungará Lake, *O.* cf. *agassii* in Isluga River and Huasco Salt pan, *O. ascotanensis* in Ascotán Salt pan, *O. gloriae* in Carcote Salt pan

shape variables using Morpheus software, using the function TPS (thin-plate spline, Bookstein 1991). The

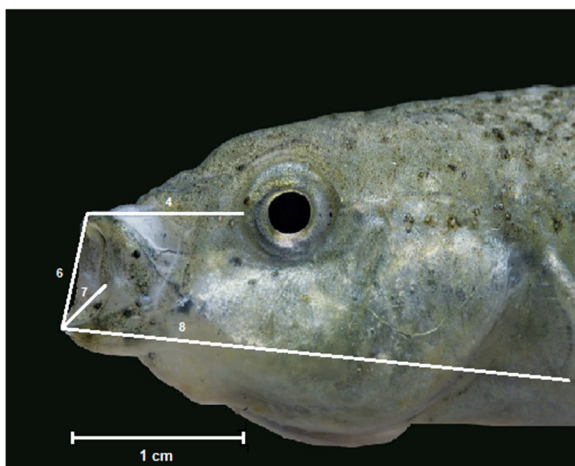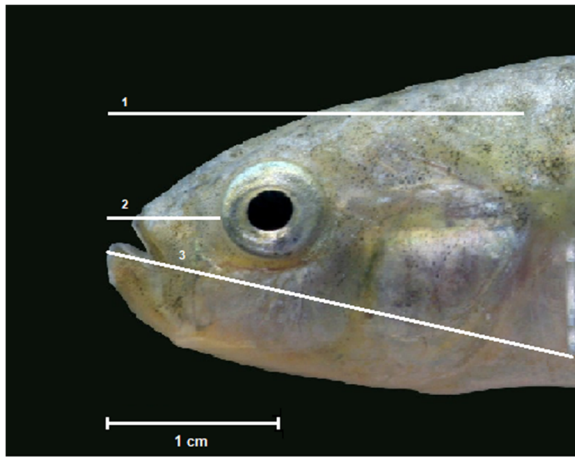specimens were compared using deformation grids as a graphic methodology.

Table 1 Locality of the species of *Orestias* collected and catalog number

Species	Locality	Latitude/ Longitude	Catalog number	N° Ind.	Habitat
O. puni	Puno, Perú	15º 50'S/ 70º 01'W	LCUCHILE 145–148	4	Lake
O. laucaensis	Lauca, Chile	18º 05'S/ 69º15'W'	LCUCHILE 27–39	13	River/Lake
O. piacotensis	Piacota, Chile	18º 11'S/ 69º15''W	LCUCHILE 14–26	13	Lake
O. parinacotensis	Parinacota, Chile	18º 10'S/ 69º20'W	LCUCHILE 40–52	13	Wetland
O. chungarensis	Chungará, Chile	18º 15'/ 69º07'W	LCUCHILE 1–13	13	Lake
O. cf. *agassii*	Isluga, Chile	19º15'S/ 68º10'W	LCUCHILE 53–65	13	River
O. cf. *agassii*	Huasco, Chile	20º 05'S/ 68º 15'W	LCUCHILE 66–78	13	Salt pan
O. gloriae	Carcote, Chile	21º 16′S/ 68º 19'W	LCUCHILE 79–92	13	Salt pan
O. ascotanensis	Ascotán, Chile	21º 29'S/ 68º 15'W	LCUCHILE 93–106	13	Salt pan

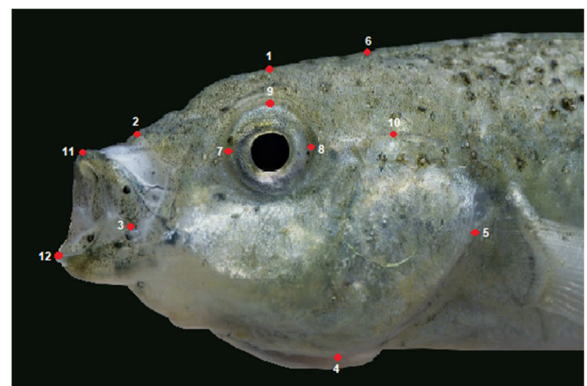**Fig. 2** Measurements of *Orestias* specimens. 1 to 3 closed mouth and 4 to 7 opened mouth

Results

Table 2, shows the linear morphometric measurements and their standard deviations means. The multivariate analysis (MANOVA) indicated significant differences among the species (Wilk's lambda = 0.038; F = 7.52; p < 0.05). The *a posteriori* analysis with the selected variables showed significant differences among groups (p < 0.05). The variables that had the greatest differences among species for both closed and open mouth specimens were snout length (2/4) and distance of the tip of the snout to the pectoral fin ventral insertion (3/5).

The CVA analysis showed that the first two axes explained 85.7% of the variance of the original data, 75.8% for the first axis and 9.9% for the second. For the first axis the variables with most weight among species for both closed and open mouth specimens were the tip of the snout to the pectoral fin ventral insertion (3/5). For

Fig. 3 Landmark locations of *Orestias* collected in the southwestern Altiplano

the second axis, the variables with highest weight for both closed and open mouth were length of head and maxillary (1/7). The grouping of the specimens determined by the two first axes of the CVA analysis showed a separation of the *O. gloriae* (Carcote) specimens from all other specimens throughout the axis 1 and little gaps in the axis 2 (Fig. 4).

The discriminant analysis (LDA) showed differences among the studied species with the characters analyzed (Wilk's λ: 0.03833, p < 0.05), correctly assigning a high percentage of the specimens in the classification matrix (Table 3). *O. puni*, *O. chungarensis*, *O. laucaensis* and *O.* cf. *agassii* (Huasco Salt pan) had more than 75% correct assignment and *O. gloriae* from Carcote Salt pan showed 100% correct assignment. Only *O. parinacotensis* showed less than 50% correct assignment; the specimens were assigned to five different groups although the majority was assigned to three species (Table 3).

According to the linear discriminant analysis, the significant variables among species for both open and closed mouth specimens were the snout length (4/2), mouth height (6) and maxillary length (7) with the head length (1).

Geometric morphometric analyses found significant difference among species morphology for the closed mouth group (PERMANOVA Pseudo-F = 5.5458, p = 0.001). The *a posteriori* analysis showed differences among all pairs (p < 0.05), with exception of the *O. chungarensis – O. puni* pair (p = 0.095).

The open mouth group showed significant differences among species (PERMANOVA pseudo-F = 6.872; p = 0.001). The *a posteriori* analysis showed differences among all populations (p < 0.05) except for four pairs: *O. ascotanensis - O. gloriae* (p = 0.077), *O. chungarensis - O. laucaensis* (p = 0.354), *O. chungarensis - O. puni* (p = 0.139) and *O.* cf. *agassii* (Huasco) - *O. parinacotensis* (p = 0.062).

The CVA analysis was performed with the coordinates obtained after the superimposition by GPA. As a result, the two first axes explained 52.5% of the original data variance for the group with closed mouth, 28.2% for the first axis and 24.3% for the second axis. The variables with most weight in the first axis were; insertion of the operculum on the ventral profile (4), Opercula width (5) and for the second axis were; anterior middle eye border (7) and posterior middle eye border

(8). For the group open mouth, the first two axes explained 65.4% of the original data variance. The first axis explained 43.7% and the second 21.7%. The variables with most weight in the first axis were; tip of the upper jaw, symphysis of the premaxillaries (11) and tip of lower jaw, symphysis of the dentaries (12), instead of the second axis were; posterior middle eye border (9) and middle superior eye border (10).

The cluster analysis with the matrix of the integrated superposition of the distance data from both the closed mouth and opened mouth individuals (Fig. 5), showed a basal separation between *O. puni* from Lake Titicaca and the rest of the *Orestias* species studied. Among the SWA species, the separation was similar to that observed geographically (Fig. 1). The first division separated northern and southern groups: *O. ascotanensis* (Ascotán), *O. gloriae* (Carcote) and *O.* cf. *agassii* (Huasco); in the northern region, the most basal division separated *O. piacotensis* (Piacota), *O. parinacotensis* (Parinacota) and *O.* cf. *agassii* (Isluga) with *O. laucaensis* (Lauca) and *O. chungarensis* (Chungará) together.

The analysis of geometric morphometric differences may also be seen graphically with the thin plate spine function (TPS). This technique shows the change that the shape of a species must undergo to resemble the morphological shape of another species (Bookstein 1991; Toro et al. 2010). The greater differences were found among the more geographically distant groups. For the group closed mouth were *O. gloriae - O. puni*, *O. gloriae - O. piacotensis* and maximum protrusion were *O. ascotanensis - O. chungarensis*, *O. chungarensis - O. piacotensis* (Fig. 6). The evaluation of the Procrustes analysis of variance (ANOVA) showed that errors were negligible for the *Orestias* (p was always < 0.001).

Discussion

Orestias has been described as a specious genus (Parenti 1984a, b; Parker and Kornfield 1995), a characteristic that could have been enhanced by the climatic and volcanic variability occurring in central southern Altiplano (SWA) since the Pliocene. The fluctuations of the hydrological levels of basins due to a succession of dry and wet periods, in addition to the change in geology for

Table 2 Main morphometric measurements of *Orestias* collected. Mean standardized length and standard deviation (mm). Standard Length (SL). Specimens with closed mouth: 1.Head length (HL), 2 Snout length (St. L); 3. Tip of the snout to pectoral fin ventral insertion (TS − PCVI). Specimen with open mouth: 4. Snout length (St. L); 5.Tip of the snout to pectoral fin ventral insertion (TS − PCVI); 6. Open mouth height (OMH); 7 Maxillary length (ML)

| Species | Morphometric measurement and body proportion (mm) | | | | | | | | | |
| | SL | | | Closed mouth | | | Open mouth | | | |
	MIN	MAX	Mean ± SE	1. HL Mean ± SE	2. St.L Mean ± SE	3. TS - PCVI Mean ± SE	4. St.L Mean ± SE	5. TS - PCVI Mean ± SE	6. OMH Mean ± SE	7. ML Mean ± SE
O. gloriae	24	51	46.62 ± 8.17	13.91 ± 2.51	3.29 ± 0.91	15.48 ± 2.95	4.72 ± 1.4	16.30 ± 3.14	3.82 ± 0.99	3.06 ± 0.77
O.chungarensis	28	64	62.74 ± 14.39	17.09 ± 3.80	3.61 ± 1.22	17.96 ± 4.20	3.72 ± 1.23	18.79 ± 4.41	2.63 ± 0.56	4.41 ± 0.53
O.piacotensis	31	55	46.53 ± 4.82	13.58 ± 1.18	2.75 ± 0.56	14.84 ± 1.97	3.18 ± 0.54	15.43 ± 2.06	2.48 ± 0.26	3.19 ± 0.24
O. cf. agassii (Huasco)	28	58	47.42 ± 8.33	14.09 ± 1.82	3.42 ± 0.69	14.01 ± 2.45	4.16 ± 0.81	14.75 ± 2.64	2.12 ± 0.51	2.85 ± 0.41
O. ascotanensis	29	60	51.83 ± 11.82	16.17 ± 3.10	3.76 ± 1.09	17.01 ± 3.64	4.36 ± 1.10	18.23 ± 4.13	3.23 ± 0.54	3.75 ± 0.53
O. cf. agassii (Isluga)	26	62	54.63 ± 8.61	15.56 ± 2.41	3.58 ± 0.56	16.60 ± 2.57	3.74 ± 0.53	17.48 ± 2.74	2.67 ± 0.44	3.50 ± 0.69
O. laucaensis	25	60	55.89 ± 11.02	14.94 ± 2.42	3.18 ± 0.80	15.58 ± 2.25	3.25 ± 0.82	16.20 ± 2.32	2.44 ± 0.41	3.30 ± 0.55
O. parinacotensis	25	54	48.78 ± 13.06	14.60 ± 3.38	3.08 ± 0.87	14.64 ± 3.81	3.56 ± 1.14	15.39 ± 4.10	2.48 ± 0.74	2.96 ± 0.78
O. puni	35	110	94.68 ± 9.14	27.19 ± 0.89	6.41 ± 0.57	28.56 ± 0.92	6.64 ± 0.59	29.84 ± 1.25	3.03 ± 0.57	5.11 ± 0.74

Fig. 4 Canonical variation analysis of *Orestias*
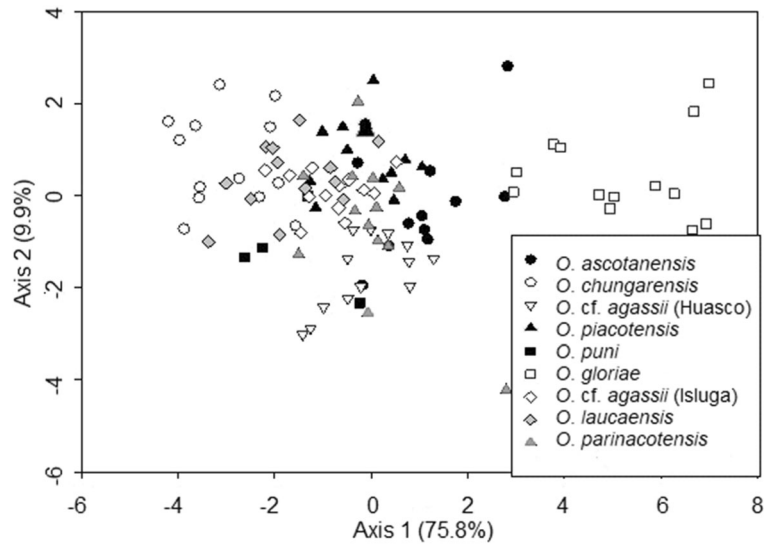
volcanic activity have generated fractionated freshwater systems, which vary from shallow lakes to rivers and wetlands (Placzek et al. 2006) with different ionic contents varying from Ca-Mg-HCO3 to Na-Cl (Risacher et al. 2003a, b).
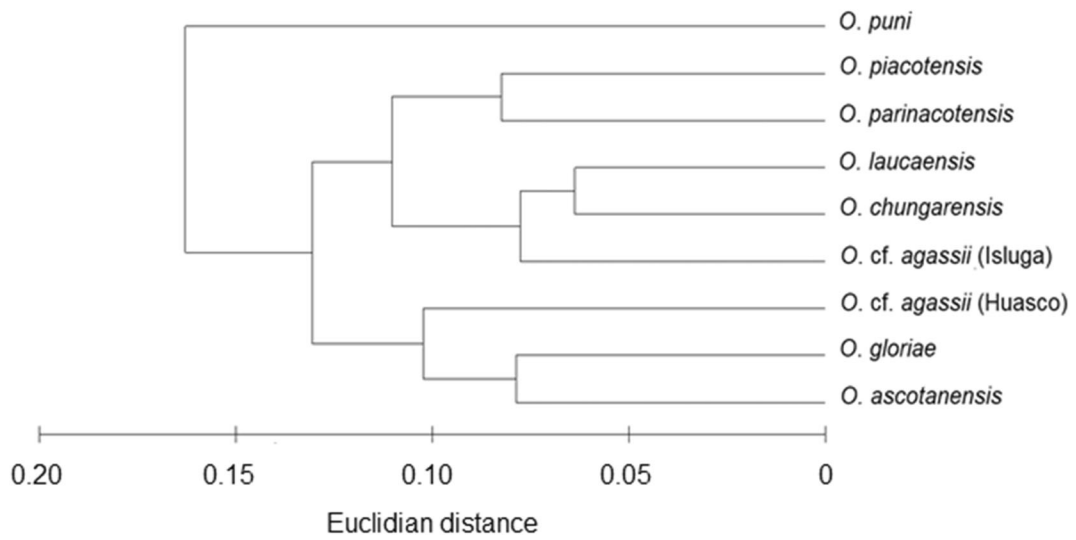
Studies have described the speciation of *Orestias* as the result of a process of fragmentation of one or more ancestral populations (Vila et al. 2013). The presence of

Orestias in the isolated aquatic systems of the SWA region could be explained by the dispersion of the fish, which could have occurred with the formation of Paleolakes (Keller and Soto 1998). In this case, the morphological diversification of the groups could occur through allopatric speciation caused by habitat instability and very heterogeneous environmental conditions described for these systems, where the structure and

Table 3 Matrix from linear discriminant analysis (LDA) obtained from the results of linear morphometric measurements

Species	% Correct	*O. puni*	*O. laucaensis*	*O. piacotensis*	*O. parinacotensis*	*O. chungarensis*	*O.* cf. *agassii* (Isluga)	*O.* cf. *agassii* (Huasco)	*O. gloriae*	*O. ascotanensis*
O. puni	75.00	3	1	0	0	0	0	0	0	0
O. laucaensis	76.92	0	10	2	0	1	0	0	0	0
O. piacotensis	69.23	0	0	9	1	0	2	0	0	1
O. parinacotensis	30.77	1	0	1	4	0	4	3	0	0
O. chungarensis	76.92	0	3	0	0	10	0	0	0	0
O. cf. *agassii* (Isluga)	69.23	0	1	1	0	2	9	0	0	0
O. cf. *agassii* (Huasco)	84.62	0	0	0	1	0	1	11	0	0
O. gloriae	100	0	0	0	0	0	0	0	13	0
O. ascotanensis	53.85	0	0	1	2	0	1	1	1	7

Fig. 5 Cluster analysis Superimposed of the integrated matrix
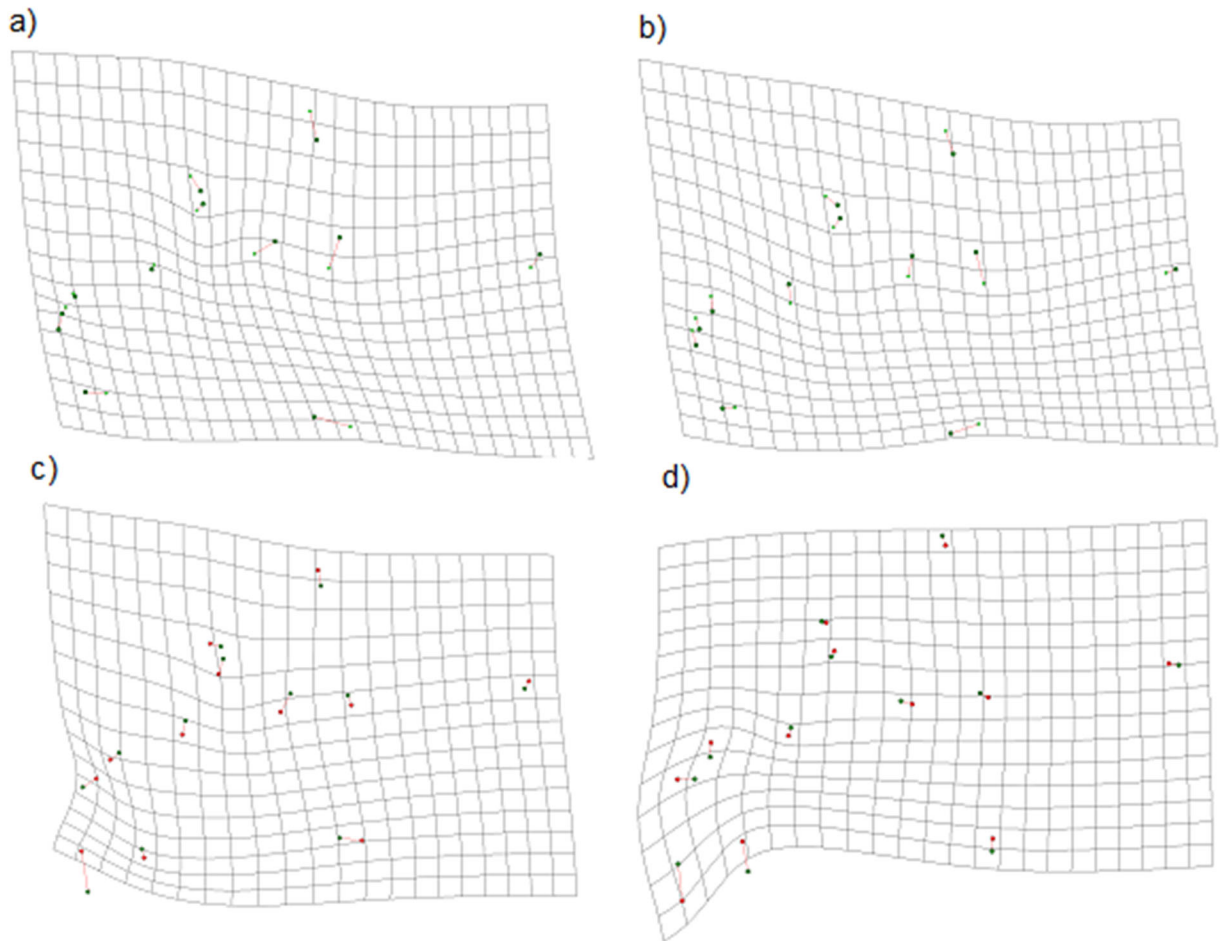
Fig. 6 Deformation grids of species showing the greatest differentiation between consensus individuals, for the two study groups. Closed mouth; (a) *O. gloriae - O. puni* and (b) *O. gloriae - O. piacotensis*. Maximum protrusion; (c) *O. ascotanensis - O. chungarnsis* and *O. chungarensis - O. piacotensis*

composition of the aquatic biota would be determined by the physical and chemical variables of the water (Northcote 2000; Márquez-García et al. 2009).

In this context, linear morphometric analysis got a high assignment rate for the SWA specimens compared to the original description of these species showing that the measurements used have had good discriminant taxonomy for *Orestias*. The geometric morphometry analysis showed significant differences among the studied species. The interspecific and inter-population differences were supported by the linear classification, which designated a high percentage of the specimens to their corresponding species. The Tukey tests and the CVA analysis coincided in that the measurements evaluated that best described the interspecific differences were tip of the snout to pectoral fin ventral insertion closed mouth and open mouth height at maximum protrusion; Snout length and the height of the oral region. The importance of these characteristics would explain the different foraging strategies described for the genus (Maldonado et al. 2009; Riveros et al. 2012; Guerrero et al. 2015).

The biotic components of the SWA systems sustain abundant zooplankton, coastal macrophytes and macro-invertebrates that grow in shallower systems as aquatic insects, snails and crustaceans. Many authors have suggested that *Orestias* differentiated in habitat and feeding from small planktonic to bigger prey such as insects and mollusks, according to this their morphology would have evolved in accordance with their feeding habits (Lauzanne 1982; Parker and Kornfield 1995; Maldonado et al. 2009; Riveros et al. 2012; Guerrero et al. 2015). Along with their extreme physical and chemical characteristics, SWA systems have been fractionated through time and they have been described as each sustaining a small population of specific *Orestias* (Vila et al. 2010; Arratia et al. 2017). Seven species have been currently described as evolving in allopatry, however their validity has been argued, since morphological and meristic measurements overlap (Parenti 1984a; Vila et al. 2011). As it has been postulated, in allopatry morphological variation would be the result of adaptation to different habitat characteristics (Vila et al. 2010, 2013).

Using geometric morphometric was possible to detect interspecific differences among the studied groups. The group formed by specimens with closed mouth showed differentiation for the majority of the pairs of species, with the exception of *O. chungarensis* and *O.*

puni. The landmarks that most contributed to differentiation in this group according to the CVA analysis were those related to the operculum (landmarks 10, 4 and 5). In the group with the maximum maxillary protrusion, there were more pairs of these species that could not be differentiated by the PERMANOVA. However, *O. gloriae* and *O. chungarensis* differed from the other species and populations being the capacity of protrusion important in the variation according to the CVA analysis (landmarks 11, 12 and 3).

In fact, the feeding of *Orestias* implies sucking a water flow generated by the operculum (Higham et al. 2006; Parsons and Robinson 2007). There is a compromise between maximizing the incoming water velocity and its volume, and this is reflected in the morphology of the oral structures. The optimum head shape for large and evasive prey should include a large oral region that maximizes the water volume, while for small and non-evasive prey the predator should have a small mouth that allows high frequency rapid movements (Maldonado et al. 2009). Thus, the relative head size, the protrusion capacity and in general the mouth structure should correlate with the diet of a species. This process has been amply described for cichlids; their adaptive radiation in Lake Malawi is one of the most studied and cited examples (Motta 1984; Albertson et al. 2003, 2005). There is still a lack of more detailed studies on SWA *Orestias* feeding, since there is not a consensus if they are specialists or generalists in feeding, even so, the major mouth protractibility of *O. gloriae* and *O. chungarensis* would show coincidence with the feeding of macroinvertebrate benthics such as larvae of aquatic insects, amphipods and mollusks (Vila et al. 2011). Both species live in lacustric habitat (Vila et al. 2006). Besides, springs and running water species such as *O. ascotanensis* has shown a larger head. This is of interest to mention that the detailed internal morphology description of one southern *Orestias* led to the recent description of a new genus and species *Pseudorestias lirimensis* (Arratia et al. 2017).

In this sense, the cluster analysis integrating the results of the closed mouth and maximum protrusion of specimens separated two groups of *Orestias*, those of the extreme north including *O. chungarensis*, *O. parinacotensis*, *O. piacotensis* and *O. laucaensis* from Lauca National Park and *O. puni* of Puno, Peru and *O. cf. agassii* from Isluga, and a group of more southern species, *O. cf. agassii* from Huasco, *O. gloriae* and *O. ascotanensis* from Carcote and Ascotán. This separation

of northern and southern groups is similar to that described by Lüssen et al. (2003), who found a division between *Orestias* species using molecular tools (mitochondrial markers) and to the results of Scott (2010).

The results of morphologic measurements and geometric explain the particular morphological divergence that has occurred in each of the studied localities. from 17º to 22º S in Chile, where seven species have been described and up to now with some populations still not specifically classified (Scott 2010; Vila et al. 2011, 2013; Arratia et al. 2017), this study has been of importance to corroborate the SWA *Orestias* species descriptions validity. This is also of present importance in considering that all these species are in danger of extinction according to the country regulations of the Environmental Ministry.

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