



Original investigation

Geographic variation in cranial morphology of the Water Opossum *Chironectes minimus* (Didelphimorphia, Didelphidae)

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ABSTRACT

The Water Opossum, *Chironectes minimus* (Didelphimorphia, Didelphidae), is the only semi-aquatic marsupial and can be easily identified for its uniquely patterned water resistant pelage and webbed hind-feet. The species is distributed along the Neotropical region (from Southern Mexico to Northwestern Argentina) and is currently divided on four subspecies (*C. m. argyrodytes*, *C. m. panamensis*, *C. m. minimus*, *C. m. paraguensis*), based on its geographical distribution. We evaluated the geographic variation in *C. minimus*, using traditional and geometric morphometrics techniques to capture the size and shape of the skull to identify differences between the populations that could relate to existing subspecies or populations that and would merit particular taxonomic attention. We took 13 skull measurements, and digitized 37, 18, and 23 2D landmarks on the dorsal and lateral view of the cranium and on the lateral view of the mandible, respectively. The sample consisted on 135 individuals from 66 localities. Analyses of both datasets showed that the Southern South America and Northern Central America animals differ from the other populations. Geometric morphometrics shows on the three views that the Amazon animals differ from the others probably due to existing geographical barriers. These morphological differences among populations of the Water Opossum support the need for a proper phylogenetic assessment of these populations, in order to clarify their taxonomic status.

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Introduction

Didelphimorphia is the richest (in species number) of the three orders of marsupials currently occurring in the New World (Gardner, 2008; Wilson and Reeder, 2005), with roughly 110 species in 18 genera, and it is constituted by a single monophyletic family, Didelphidae (Jansa and Voss, 2000). Didelphidae are morphologically conservative marsupials that are presently widely distributed in almost all temperate and tropical habitats of the New World, from Patagonia to southern Canada, and represent the only marsupial radiation outside the Australasian region that is still mostly intact (Astúa, 2015; Voss and Jansa, 2009).

The Yapok or Water Opossum (*Chironectes minimus*, Zimmerman, 1780) is a unique member of the Didelphidae. It represents the basalmost lineage in the monophyletic tribe Didelphini, which includes all big-bodied opossums with a $2n = 22$ karyotype (Flores, 2009; Voss and Jansa, 2009). The Yapok, however, differs from these

and all others New World marsupials because it is the only marsupial with semi-aquatic habits. This unusual habit in marsupials is reflected in several morphological adaptations for underwater foraging such as webbed hind-feet, a pouch with a sphincter that tightly closes its opening when diving, a unique musculature insertion that allows the scrotum to be pulled up and kept in a rudimentary pouch while swimming, large digital pads with a distinctive epidermal structure for perception of stimuli from all directions, and a very dense and wetting resistant pelage (Astúa 2015; Hamrick, 2001; Marshall, 1978; Nogueira et al., 2004).

Even though the Water Opossum is the only species in its genus, four subspecies are currently recognized, based on geographic distributions (Stein and Patton, 2008). *C. minimus minimus* (Zimmerman, 1780) is distributed in Northeastern South America, from southeastern Colombia through the Guianas region up to the delta of the Amazonas river, and to southeastern Peru and adjacent Bolivia; *C. minimus paraguensis* (Kerr, 1792) occurs from Southeastern Brazil to Paraguay and in Argentina; *C. minimus argyrodytes* (Dickey, 1928) occurs in the mountain ranges of El Salvador, in Honduras and in Southern Mexico; and *C. minimus panamensis* Goldman, 1914 occurs from Northwestern Peru to Nicaragua going through Ecuador, Colombia, Venezuela, Panama and Costa Rica (Marshall, 1978; Stein and Patton, 2008). The limits of these

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subspecies, however, are not clear. What appeared to be disjunct populations in the assessment of Marshall (1978) were later found to be more continuously distributed (Stein and Patton, 2008), and with the latest records (Brandão et al., 2015) it appears to have an almost continuous circum-amazonian distribution. As such, proper boundaries of these putative subspecies are presently unknown.

In spite of having a wide geographic distribution, the species range is not continuous at a smaller scale, as its occurrence is linked to the presence of water streams. As a result, the species is broken up into many individual populations that are apparently separated from one another, which could lead to a gradual morphological differentiation among them (Gavrilets, 2003; Queiroz, 2007; Salomon, 2001; Sobel et al., 2010).

The correlation between species geographic range and morphological variation has been well documented ever since Darwin's finches (Darwin, 1859). Geographic isolation is known to be one of the main causes leading to speciation (Gavrilets, 2003; Queiroz, 2007; Salomon, 2001; Sobel et al., 2010). Lately, with the advent of sensitive tools to measure both morphological and genetic changes, even subtle (incipient) speciation processes (such as indicated by subtle geographic variation) can be perceived in several taxa (Cardini et al., 2007; D'Anatro and Lessa, 2006; Reis et al., 2002; Sarà and Vogel, 1996; Sutton and Patterson, 2000).

Previous analyses of morphological geographic variation in other wide ranging Didelphidae species suggest that many of these are possibly composed of several distinct taxa. The Black-eared opossums of the genus *Didelphis*, formerly believed to be a single species, are now divided in two species, just as the White-eared opossums of the same genus, in which a single previously recognized species is currently divided in three species (Cerqueira, 1985; Cerqueira and Lemos, 2000, Lemos and Cerqueira, 2002). More recently, a disjunct population of *Lutreolina crassicaudata* was also considered to constitute a distinct species (Martínez-Lanfranco et al., 2014), and a study investigating geographic variation in *Caluromys lanatus* showed marked differences between populations from Amazonia-Cerrado and Atlantic forest (Fonseca and Astúa, 2015). It is thus possible that a similar situation could be occurring in *C. minimus*. As morphological discontinuities may be interpreted as an indication of interruptions in gene flow (which could lead to populations evolving different morphologies separately), identifying such discontinuities constitutes an important step in the understanding of the current taxonomy and systematics of any biological group. Although traditional linear morphometrics have routinely been used in mammalogy, there has been an increasing use of geometric morphometric tools to quantify and understand morphological variation in mammals and its relation to current taxonomic arrangements in a variety of mammal groups (e.g., Beolchini and Corti, 2004; Evin et al., 2008; Fonseca and Astúa, 2015; Prevosti et al., 2013).

In this study, we used traditional and geometric morphometric methods to quantify geographic variation in cranial morphology among populations of *C. minimus*, in order to assess the existence of possible morphological discontinuities that would support the grouping of populations in subspecies or shed light on the existing diversity in Water Opossums.

Material and methods

Examined material

We examined specimens deposited in the mammal collections of the following institutions: National Museum of Natural History (Washington, DC), American Museum of Natural History (New York, NY), Museum of Vertebrate Zoology (Berkeley, CA), University of Michigan Museum of Zoology (Ann Arbor, MI), Museum

of Southwestern Biology (Albuquerque, NM), University of Kansas (Lawrence, KS), Field Museum of Natural History (Chicago, IL), University of Wisconsin Zoological Museum (Madison, WI), Museu de Zoologia da Universidade de São Paulo (São Paulo, SP), and Museu Nacional (Rio de Janeiro, RJ). A complete list of specimens studied is given in Appendix A.

The juvenile skulls (based on dentition) were excluded from the analysis. Only specimens with complete dentition (upper and lower M4 and P3 fully erupted) were measured (Tyndale-Biscoe and Mackenzie, 1976; van Nievelt and Smith, 2005). Although other didelphids have been shown to continue growing after eruption of the full dentition, all specimens in our sample showed no or only incipient wear on P3, thus corresponding to age class 5 or early class 6 [as defined by Tyndale-Biscoe and Mackenzie (1976) for *Didelphis*], with the exception of only three specimens that could be classified in age classes 6 or 7. As each of these three belonged to three different and distant populations, we believe this did not affect our estimate of geographic variation.

Traditional morphometrics data

Traditional morphometrics employed standard measurements routinely used in analyses of cranial variation in didelphids. This approach was included to allow comparison with other analyses that used the same methodology (Cerqueira and Lemos, 2000; Lemos and Cerqueira, 2002; Martínez-Lanfranco et al., 2014). We took the following distances measured from the skulls (Fig. 1): Condylbasal length (CBL); Length of the nasal (LN); Breadth of the nasal (BN); Infra-orbital breadth (IOB); Post-orbital breadth (POB); Zygomatic breadth (ZB); Length of braincase (LB); Mastoidal breadth (MB); Palatal length (PL); Palatal breadth (from left M4 to right M4) (PB); Tooth row (starting on the canine base) (TR). All measurements were taken with a digital caliper with a 0.01 mm precision.

Geometric morphometrics data

Geometric morphometrics were applied in addition to the traditional approach, as geometric morphometrics allow for a better visualization of shape variation, as seen in Fonseca and Astúa (2015), for example. 2D landmarks were digitized on two views of the skull and one of the mandible; 37 on the dorsal view; 18 in the lateral view; and 23 on the mandible (Fig. 1). Landmarks definitions are listed in Appendix B. Landmarks were established as to capture overall braincase and rostrum shape, nasal shape, relative lengths of toothrows, and mandible overall shape. Several of these features were used in the description of some of the proposed subspecies as distinctive characters (Marshall, 1978).

Biogeography

The examined material came from 66 localities that were grouped in six groups of localities (for each method), named macro-localities Fig. 2. The geographic coordinates were obtained at the time the skull was photographed, if informed on the specimen label. Other coordinates were obtained through gazetteers from the locality, or city.

The localities were grouped to increase the sample size, and therefore, enhance statistical power of the analysis. The grouping was made based on geographic distance from one locality to another (ignoring political boundaries) and also on preliminary analyses. These preliminary analyses consisted on Canonical Variate Analysis, but with more groups and thus, fewer specimens per group. We were then able to observe overlapping localities, which we grouped in the following CVAs (as long as there was geographic proximity). The localities were grouped as follows: northern

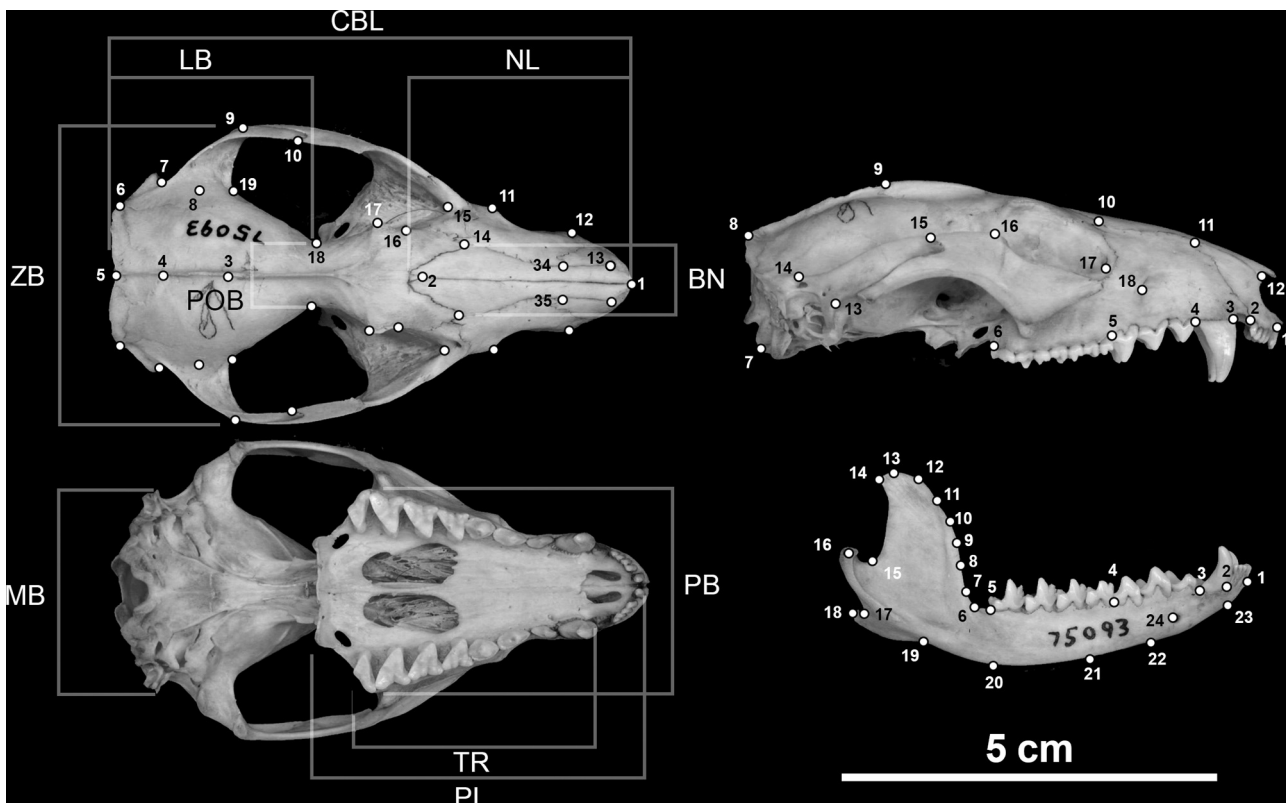


Fig. 1. Linear distances used in the traditional morphometrics analyses and landmarks used in the geometric morphometrics analyses in this study. Refer to the text for the formal definition of distances and landmarks. Landmarks were digitized on both sides but definitions are shown on one side only. Bar = 5 cm.

Central America (Belize, Honduras, Guatemala and Nicaragua); southern Central America (Costa Rica, Panama and individuals from Colombia that are near the border); Ecuador and Colombia (only present in Traditional morphometrics analysis—individuals from Ecuador and Colombia that are south the border with Panama); Peru and Bolivia (only present in geometric morphometrics analysis) (white triangles in Fig. 2); Amazon (northern Brazil and French Guiana) (white circles in Fig. 2); northern South America (North of Colombia and Venezuela) and southern South America (southeastern Brazil and Paraguay) (black squares in Fig. 2). A list of all localities, with their respective macro-localities, is presented in Appendix C.

The four subspecies of *C. minimus* were delimited mostly by geographic distribution (Gardner, 2008; Marshall, 1978). In this study, we divided our sample in six groups, also based on geographic distribution, but not necessarily in the same way the subspecies were defined. Some of our macro-localities coincide with a subspecies range (e.g., the locality South of South America and *C. m. paraguensis*), but others break up subspecies ranges (e.g., *C. m. panamensis* [black circles in Fig. 2] is broken up into N. Central America, S. Central America, N. South America and Peru/Bolivia), or combines different subspecies (e.g., N. Central America combines *C. m. argyrodyctes* [black triangles in Fig. 2] and *C. m. panamensis*). By further subdividing the subspecies ranges into macro-localities we intend to capture differences in skull shape that might exist within subspecies.

Statistical analyses

After digitizing landmarks, we performed a Generalized Procrustes Superimposition to eliminate effects of orientation and position of the images, and to remove isometric size effect, by

scaling all configurations to the same centroid size. Nonetheless, centroid size records are stored for posterior analysis (Zelditch et al., 2004). These procedures were repeated for the raw landmark coordinates of each view.

After effects of orientation, position and size are removed, the resulting landmark configurations retain only shape information (both allometric and allometry-free shape), without isometric size influence. Thus shape and size of the structures can be analyzed separately (Klingenberg et al., 2002; Zelditch et al., 2004). We ran an Univariate Analysis of Variance (ANOVA with Tukey test as post-hoc) with the centroid size extracted from dorsal view and mandible to evaluate size variation between localities. The dorsal view was chosen to represent skull size since it comprises a larger portion of the skull, when compared to the lateral view.

Using the geometric morphometrics data, we analyzed shape differences between localities and between sexes through a Discriminant Analysis followed by a leave-one-out Cross-validation test (Lachenbruch, 1967) and also through a Canonical Variates Analysis (CVA). On the traditional morphometrics data, we assessed the differences between localities through a CVA. Canonical Variates Analysis allows us to evaluate if groups defined a priori can be considered statistically different along axes that are linear combinations of the original variables, orthogonal (i.e., independent) from each other, and ordered so as to maximize inter-group differences relative to within-group variance. Assumptions and tests for those assumptions are described in detail in Webster and Sheets (2010).

The landmarks were digitized with TPSDig program (Rohlf, 2006; Rohlf and Bookstein, 2003), which was also used to create the outlines. Both Discriminant Analysis and Canonical Variates Analysis were performed on MorphoJ (Klingenberg, 2011).

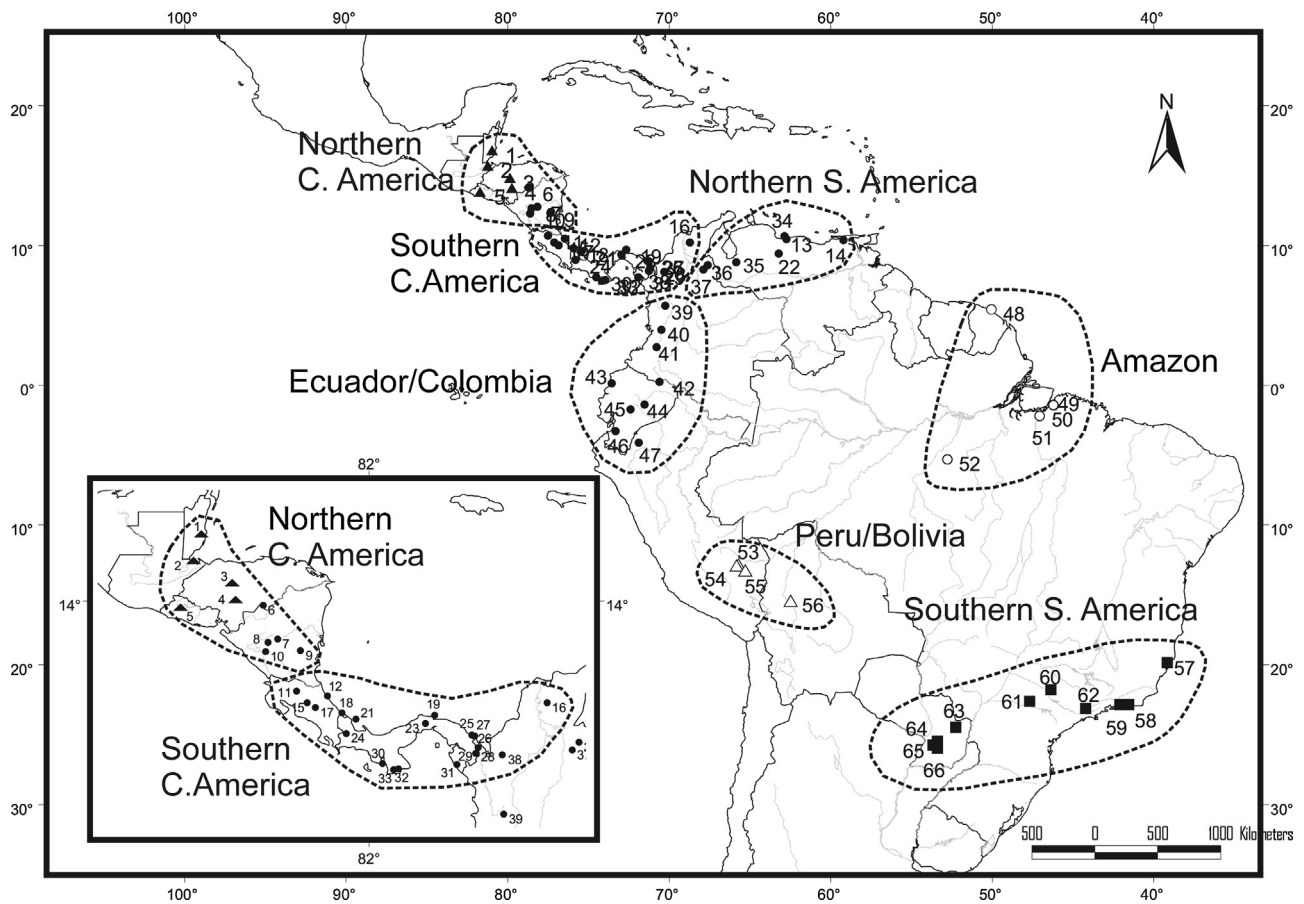


Fig. 2. Distribution of the localities of *Chironectes minimus* with specimens included in this study. Localities were grouped in macro-localities (encircled by dashed-lines) for subsequent analyses, as indicated. The subspecies are represented by different symbols: *C. m. argyrodytes*—black triangles; *C. m. panamensis*—black circles; *C. m. minimus*—white circles; *C. m. paraguensis*—black squares; Peru/Bolivia (unknown subspecies)—white triangles. Numbers indicate localities as listed in Appendix C.

Table 1

Sample sizes for each population used in the linear (LM) and geometric morphometric (GM) analyses (in the latter case, by view or structure analyzed) (total *n* and by sex).

Population	LM (♂/♀/?)	GM (cranium, dorsal) (♂/♀/?)	GM (cranium, lateral) (♂/♀/?)	GM mandible (♂/♀/?)
Northern Central America	29 (17/11/1)	20 (13/6/-)	18 (13/5/-)	18 (13/5/-)
Southern Central America	25 (13/12/-)	45 (22/21/2)	46 (24/21/1)	39 (21/18/-)
Amazon	5 (4/1/-)	23 (12/9/2)	21 (11/8/2)	20 (10/8/2)
Ecuador/Colombia	11 (7/4/-)	-	-	-
Peru/Bolivia	-	5 (4/1/-)	5 (4/1/-)	3 (3/-/-)
Northern South America	7 (4/3/-)	31 (20/9/2)	29 (20/8/1)	22 (14/8/-)
Southern South America	6 (4/2/-)	11 (8/2/1)	10 (8/2/-)	9 (8/1/-)
Total	83 (49/33/1)	134 (79/49/7)	129 (80/45/4)	111 (69/40/2)

Results

Traditional morphometrics (CVA)

We measured a total of 83 skulls, 3 of those were females, 49 males and 1 of unknown sex (Table 1). The Canonical Variates Analysis discriminates Northern Central America (right side of the graphic) and Southern South (left side or bottom) America populations (Fig. 3).

Southern Central America and Northern South America populations overlap, and there is no overlap between Northern South

America and Southern South America. Animals from Ecuador and Colombia and from the Amazon seem to have a skull morphology that is intermediate between those populations.

Geometric morphometrics

We photographed a total of 135 skulls, 43 females, 88 males and 4 specimens of unknown sex (Table 1).

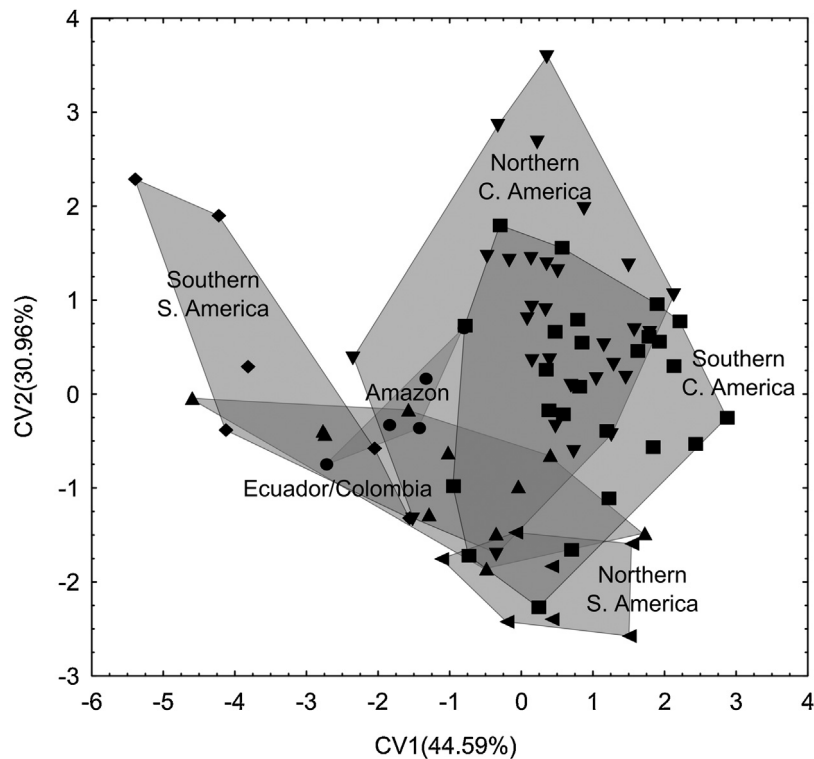


Fig. 3. Canonical Variates Analysis on linear measurements of the skull of *Chironectes minimus*, using macro-localities as grouping factors, and percentage of variance explained by the first two CVs.

Cranium size

The post-hoc Tukey test found no significant sexual dimorphism of size in either the cranium or the mandible. The centroid size data from the dorsal view show a cline, where the biggest animals are at the highest latitudes (Northern Central America), and they are smaller as the latitude decreases. The specimens from southern South America are slightly larger than the Peruvians/Bolivians, but are still smaller than the specimens from all the other populations (Fig. 4).

The results from the ANOVA show significant differences in size among the populations ($p < .0001$). The post hoc Tukey test found significant differences between the Northern Central America population and all the other populations, except Southern Central America (Table 2). The latter population also differed from Peru/Bolivia and south of South America regions (Table 2).

Mandible size

The ANOVA on centroid size data extracted from the mandible landmark configurations is also significant ($p = 0.0017$), and the results are similar to those from the cranium. Size also decreases with latitude, in the same order as the cranium, and the animals from southern South America are also larger than the ones from Peru/Bolivia, though smaller than the rest of the populations (Fig. 4).

The Tukey post hoc analysis only differ northern Central American animals from Amazonian and southern South American ones (Table 3).

Discriminant analyses

The Discriminant Analysis using Mahalanobis distances is able to differentiate between most of the populations in most of the views (Table 4). The dorsal view seems to be the most informative for significant between-population differences. The Mahalanobis distances increase with geographic distances, with near-by populations such as Southern Central America and Northern South

America differing by 2.1 in the dorsal view and distant populations such as Northern Central America and Southern South America differing by 21.2 mahalanobis (Table 4).

Canonical variates analyses

The CVA on the dorsal view of the cranium clearly distinguishes the Northern Central American from the other populations (Fig. 5). The main morphological features responsible for this differentiation are an elongated snout, with longer and wider (particularly posteriorly) nasals, a narrower braincase and wider zygomatic arches. The Amazonian population, though quite similar to the Peruvian/Bolivian population, is separated from other South American populations for having a wider skull in general as well as a shorter braincase length and longer snout.

The CVA on the lateral view of the cranium shows a differentiation between Southern South America and Peru/Bolivia from all the other populations (Fig. 6). The Amazonian population also appears slightly isolated from the remaining populations. The Amazonian, Southern South American and Peruvian/Bolivian populations have a more robust (less flattened) skull in comparison with the other populations but they differ from each other mainly on the size and position of the premaxilla in relation to the maxilla.

The CVA on the mandible data shows the most differences among groups (Fig. 7). In this analysis we find three populations that are quite isolated from the rest: Southern South America; Amazon; and Northern Central American populations. The main characteristic of Southern South American animals that differs from the rest is shape of the ascending ramus, with a more pointed and posteriorly inflected tip of the coronoid process. The main difference between Amazonian and Northern Central American populations also lay on its ascending ramus, but in this case, on its relative width. Animals from Northern Central America have wider rami whereas Amazonian animals have narrower rami when compared to the other populations, which seem to have intermediate sized rami.

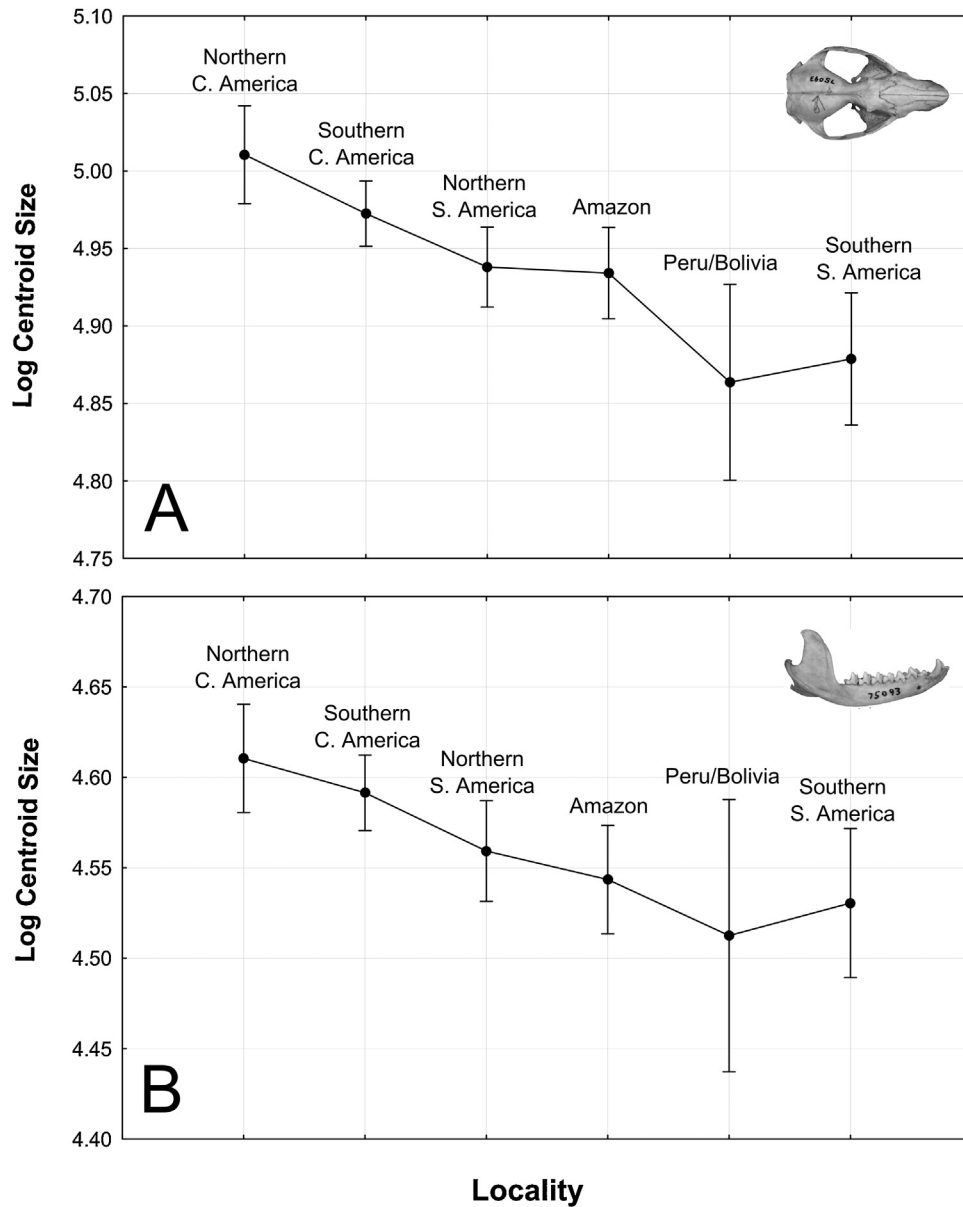


Fig. 4. Variation of the centroid size of the cranium in dorsal view (A) and mandible (B) of *Chironectes minimus* along its N–S distribution. Only results for the centroid size of the cranium in the dorsal view are shown, but results are similar for the lateral view.

Table 2

Results of the Tukey post-hoc tests for cranium size among macrolocalities, following a significant ANOVA ($p < 0.0001$) on those data. Significant pairwise differences are indicated in bold.

	Southern Central America	Northern South America	Amazon	Peru/Bolivia	Southern South America
Northern Central America	0.354	0.006	0.006	<0.001	<0.001
Southern Central America		0.315	0.288	0.016	0.001
Northern South America			1.000	0.259	0.172
Amazon				0.343	0.279
Peru/Bolivia					0.999

Table 3

Results of the Tukey post-hoc tests for mandible size among macrolocalities, following a significant ANOVA ($p = 0.0017$) on those data. Significant pairwise differences are indicated in bold.

	Southern Central America	Northern South America	Amazon	Peru/Bolivia	Southern South America
Northern Central America	0.906	0.138	0.026	0.167	0.028
Southern Central America		0.448	0.104	0.347	0.102
Northern South America			0.972	0.857	0.860
Amazon				0.974	0.996
Peru/Bolivia					0.998

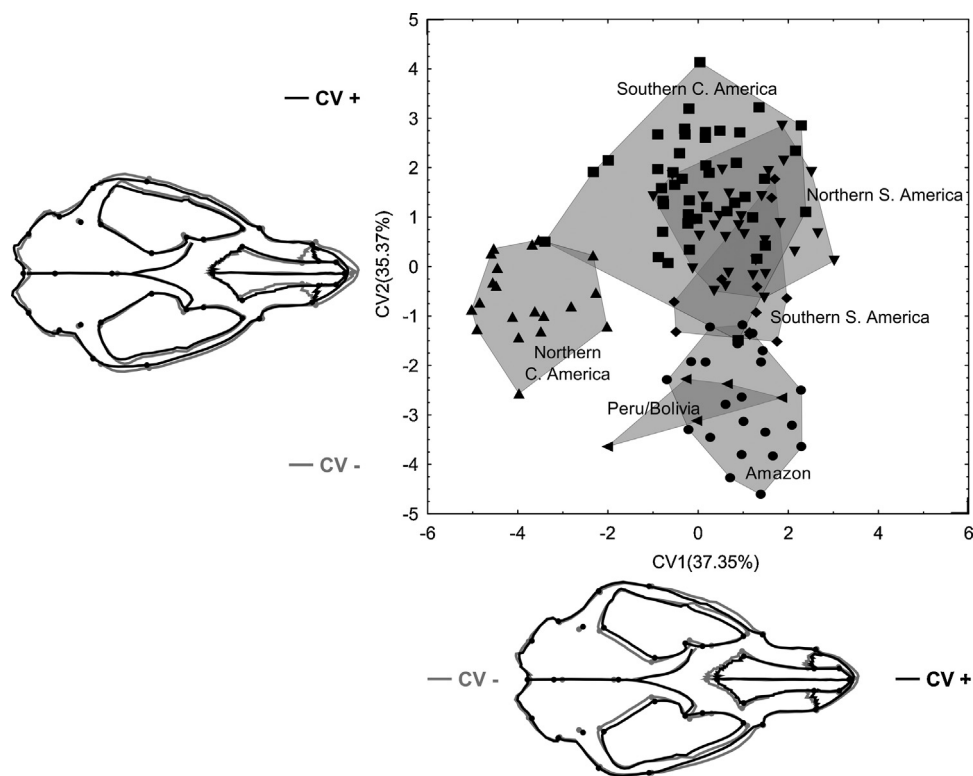


Fig. 5. Canonical Variates Analysis on shape Variates of the cranium in dorsal view of *Chironectes minimus*, using macro-localities as grouping factors, and percentage of variance explained by the first two CVs. Shapes indicate difference from shape on the negative end of each CV (gray landmarks and outlines) to shapes on its positive end (black landmarks and outlines).

Discussion

Populations of the Water Opossum throughout its range are not uniform in cranial size and shape. Although results from traditional and geometric morphometrics are not identical, they clearly indicate that some populations are distinct. These morphological discontinuities are consistent with present or past ecological or geological patterns or events, and are similar to those found in other taxa. They indicate that the actual taxonomic status of these populations needs to be adequately assessed with additional tools that could reveal their phylogenetic distinctiveness, ultimately indicating whether Water Opossums constitute a single species or not.

Size variation

A plethora of environmental and ecological variables have been proposed to affect body size in mammals (Damuth, 1981; Damuth and MacFadden, 1990; Peters, 1983). For instance, in vervet monkeys, body size increased with proximity to human settlements, where the animals exploited the easier access to human food (Turner et al., 1997). Preliminary observation suggest that the Brazilian White-eared opossum, *Didelphis albiventris*, also reaches

larger body sizes in more urban areas, possibly by exploiting human leftovers from garbage (D. Flores, pers. comm.). Seasonality, on the other hand, affects body weight in mammals by increasing the need of fat storage during periods of food scarcity (Lindstedt and Boyce, 1985). In opossums, large variations of body weight due to increase in storage of body fat seemingly occurs only in the genus *Didelphis*, and particularly in *Didelphis virginiana* populations from higher latitudes (Gardner and Sunquist, 2003). Variations in body weight, however, are more necessarily reflected in overall body (or cranial) size, as fat storage (and usage) can vary from season to season, yet are not necessarily reflected in changes (=increase) in body structures. A geographical pattern in body size variation can also be related to Bergmann's rule, which states that low temperatures are associated with larger bodies in mammals due to thermoregulation, where the body volume/surface ratio must be smaller, to reduce heat-loss (Clausen et al., 2013; Martinez et al., 2013). None of these environmental factors, however, seem to apply to the Water Opossum. Within its range, those regions with lowest temperatures and better-defined seasons are those where the animals are smallest, in southernmost localities. The exploitation of human settlements by the Water Opossum is also extremely unlikely, as they are only found in secluded, wild habitats, and their diet consists of fish and

Table 4
Mahalanobis distances between macrolocalities as given by the Pairwise discriminant analyses for shape. Letters indicate view of the cranium (D for dorsal and L for lateral) and lateral view of the mandible (M). Significant ($p < 0.05$) pairwise differences are indicated in bold.

Mahalanobis distances	Southern Central America			Northern South America			Amazon			Peru/Bolivia			Southern South America		
	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M
Northern Central America	5.4	4.3	8.3	10.9	5.5	8.5	8.6	9.9	7.0	6.4	10.6	4.3	21.2	16.5	7.5
Southern Central America				2.1	2.3	3.6	5.1	6.4	8.0	9.6	9.8	18.1	7.0	4.8	11
Northern South America							5.6	6.6	11.9	19.7	15.6	4	10	8.2	5.6
Amazon										8.2	10.1	4.5	10	7.6	3.8
Peru/Bolivia													2.7	3.8	2.9

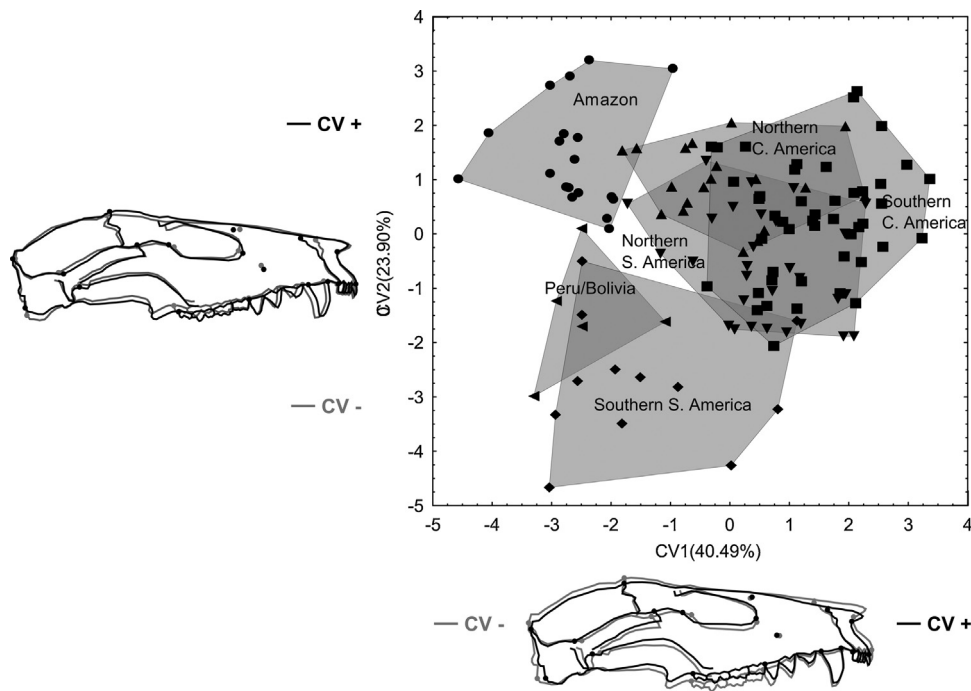


Fig. 6. Canonical Variates Analysis on shape Variates of the cranium in lateral view of *Chironectes minimus*, using macro-localities as grouping factors, and percentage of variance explained by the first two CVs. Shapes indicate difference from shape on the negative end of each CV (gray landmarks and outlines) to shapes on its positive end (black landmarks and outlines).

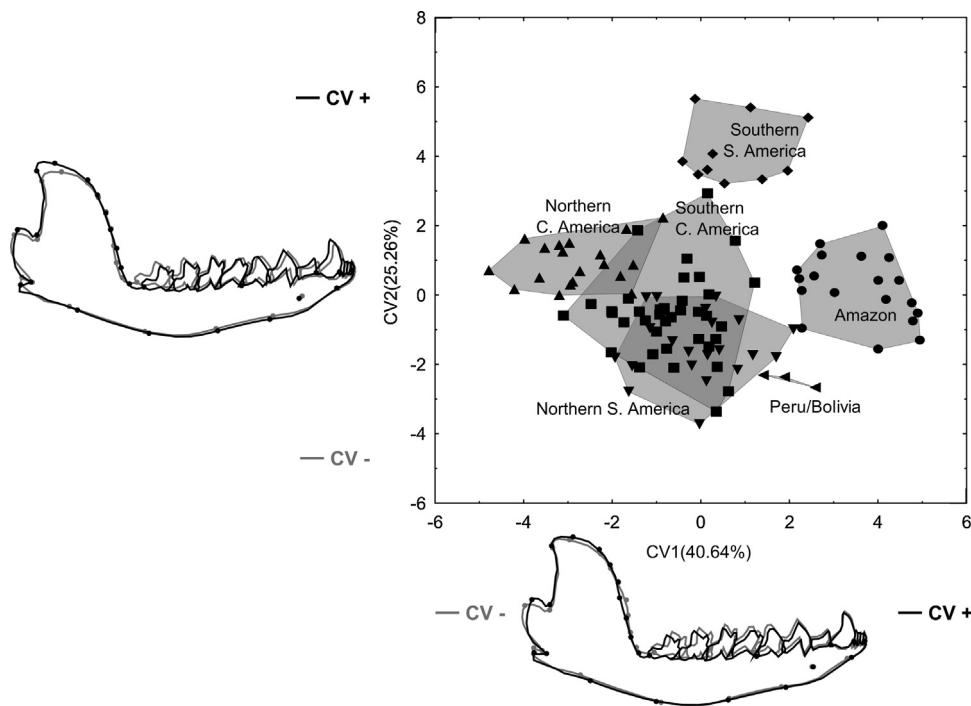


Fig. 7. Canonical Variates Analysis on shape variables of the mandible of *Chironectes minimus*, using macro-localities as grouping factors, and percentage of variance explained by the first two CVs. Shapes indicate difference from shape on the negative end of each CV (gray landmarks and outlines) to shapes on its positive end (black landmarks and outlines).

crustaceans only. Nevertheless, there are other, non-environmental factors that can produce a cline, such as drift in combination with spatially restricted gene-flow (Storz, 2002), which could cause the pattern seen in *C. minimus*. Other Didelphidae seem to contradict Bergmann's rule: in *C. lanatus*, for instance, populations from South-eastern Brazil, Argentina and Paraguay had the smallest skull sizes (Fonseca and Astúa, 2015).

Northern Central America population

Specimens from Northern Central America differ from the other populations, particularly in dorsal cranium shape (they have clearly narrower braincases and longer nasals), but also, to a lesser extent, in mandible shape (Figs. 5 and 7). They are also significantly

larger than all other populations except Southern Central America (Table 2).

Recent works show evidence of several marine incursions in the same region, isolating populations from northern Costa Rica and southern Nicaragua (Castoe et al., 2009; Jones and Johnson, 2009). The marine gap known as the Nicaraguan depression, matches the area of morphological discontinuity revealed in our data. The Nicaraguan depression separates two highland masses, the Chorotis block highlands (Honduras and Nicaragua) to the north, and the Lower Central American highlands during the Miocene and the majority of Pliocene (Iturralde-Vinent, 2006; Iturralde-Vinent and MacPhee, 1999), from which the oldest fossils from *C. minimus* were found (Gardner, 2008; Marshall, 1977, 1978; Marshall, 1982). The Nicaraguan depression has been identified as a major phylogeographical break for many taxa, including frogs (Savage, 1987), salamanders (Parra-Olea et al., 2004), snakes (Savage, 1982), birds (Pérez-Emán, 2005), plants, insects, and fish (Halas et al., 2005; Marshall and Liebherr, 2000).

Specimens from Nicaragua were grouped in the Northern Central America population due to previous analyses (unpublished data) that showed that, despite being classified amongst *C. m. panamensis* they resemble the subspecies *C. m. argyrodytes*. Our results suggest that animals from Nicaragua should be considered as belonging to *C. m. argyrodytes* instead of as *C. m. panamensis*, as they have been until present (Marshall, 1978), and that the boundary between the two groups may be located at the Nicaraguan depression. The conspicuous morphological differences between *C. m. argyrodytes* (including animals from Nicaragua) and all other populations suggest it could represent a distinct species. Some of the morphological differences found here had been reported previously (Marshall, 1978), although only differences visible on the dorsal view of the cranium were confirmed by our results. Others, such as those regarding differences in maxillary tooththrow, were not confirmed by our analyses. Other morphological differences between populations of Northern Central America and those traditionally considered as *C. m. panamensis* are related to fur color or pattern, which were not assessed here (Marshall, 1978). In fact, morphological discontinuities, interpreted as a proxy for a break in gene flow, have been used to separate other Didelphidae taxa with wide distribution ranges in two or more species, which were later confirmed by phylogenetic analyses, such as in opossums of the genus *Didelphis* (Cerqueira and Lemos, 2000; Lemos and Cerqueira, 2002). Nevertheless, clearly phenotypic divergent populations of *Monodelphis touan* (Pavan et al., 2012) from North and South of the Amazon River were not interpreted as distinct species, as molecular data failed to clearly recover them as two distinct and reciprocally monophyletic units. Likewise, in *Gracilinanus microtarsus*, distinct phenotypes did not match molecular phylogenies, and as a consequence, these morphologically distinct populations were considered a single species (Lóss et al., 2011). On the other hand, a morphologically distinct and disjunct population of the genus *Lutreolina* with clear phylogenetic divergence led to its recognition as a distinct species (Martínez-Lanfranco et al., 2014). As a consequence, pending additional morphological analyses including external morphological characters such as coat color and qualitative characters, and adequate assessment of the phylogenetic relationships of these different populations, using several molecular markers, we prefer to treat them as morphologically distinct populations of a single species.

Amazonian population

The distinct skull shape of this population, when compared to the other South American populations is clearly noticeable in the geometric morphometrics analyses (Figs. 5–7). Several analyses of geographic variation of small mammals show a consistent pattern,

in which Amazonian populations diverge phylogenetically from the Atlantic forest populations (Costa, 2003; Fonseca and Astúa, 2015; Patton and Costa, 2003; Patton et al., 1997). Amazonian populations of *C. minimus* were long considered to be disjunct from Atlantic Forest populations. However, recent records of *C. minimus* in the Brazilian Cerrado and Eastern Amazonia (Ardente et al., 2013; Brandão et al., 2015) suggest that such pattern was merely the result of inadequate sampling. As those specimens were not included in this analysis, we cannot ascertain that the differences observed in our results are not an extreme of a gradual variation or if the Eastern Amazon population is really distinct. The same could be occurring between the Eastern Amazon population and that from Venezuela and Colombia.

Southern South America population

This population differed from the others in the CVA of the lateral view, the mandible, and that of the linear measurements. The analysis using linear measurements shows some overlap between the individuals from southern South America, Amazon and Ecuador/Colombia, but clearly distinguishes the Northern and Southern South American population.

Even with the new records from Central Brazil, there is a break on the continuity of the distribution of *C. minimus* in Bolivia. This discontinuity might be explained by the geographical characteristics of the dry Chaco. The dry Chaco has a dry climate and a dominant xerophytes vegetation that may act as effective barriers to forest animals (Vanzolini, 1970; Vivo, 1997; Chemisquy and Flores, 2012), especially those which possess a semi-aquatic habit.

Among the Didelphidae, several genera and species present phylogenetic divergence between Amazon and Atlantic forest populations, such as *Marmosops*, *Philander*, *Didelphis*, *Caluromys*, *Metachirus*, *Marmosa demerarae* and *Marmosa murina* (Costa, 2003; Patton and Costa, 2003). This pattern also occurs in rodents such as *Rhipidomys*, *Oecomys trinitatis* and *Hylaeamys megacephalus* (Costa, 2003). Yet, as the distributional gap previously reported for *C. minimus* (Stein and Patton, 2008) seems to be resulting from insufficient sampling (Ardente et al., 2013; Brandão et al., 2015), a proper assessment of the morphometric discontinuity between Southern South America populations and all others depends on the inclusion of specimens from Central Brazil. It should be noted, that a divergence of a Southern population from all others has been observed in other Didelphidae taxa, such as *Caluromys lanatus* (Fonseca and Astúa, 2015).

C. minimus nominal taxa

Overall, the subspecies boundaries mostly coincide with the morphological affinities we found in this study. Animals from the Amazon seem to differ from the others, thus corroborating the existence of *C. m. minimus* as a distinct population, and the same observation can be applied to animals from Southern South America and the taxon *C. m. paraguensis*. Yet, our results suggest some changes on the classification of some of the populations. First, we suggest that the geographic distribution of *C. m. argyrodytes* be expanded to contain part of Nicaragua, with its southernmost boundary as the Nicaraguan depression, as shown in Fig. 2. Second, animals from Peru/Bolivia seem to have an intermediate skull shape between *C. m. minimus* (Amazon) and *C. m. paraguensis* (south of South America) and resemble these two subspecies more than *C. m. panamensis*, as they were considered to be until the present (Gardner, 2008). The proper affinities of the Peruvian and Bolivian specimens, however, remain to be assessed with morphometric analyses of specimens from Central Brazil.

Chironectes distribution and current knowledge

An adequate appraisal of the geographic variation of any taxon, as well as a comprehensive phylogeographic analysis of its populations, depends primarily on a good knowledge of its distribution and on an good amount of samples in museums. The distribution of *C. minimus* showed, until recently, a disjunct distribution of two populations (Stein and Patton, 2008), yet, with the recent records reported (Ardenete et al., 2013; Brandão et al., 2015), it remains absent from Northeastern Brazil and Central Amazonia. While its ecology has been more thoroughly studied in the last decade (Fernandez et al., 2015; Galliez and Fernandez, 2012; Galliez et al., 2009; Leite et al., 2013; Palmeirim et al., 2014), those studies are concentrated on populations from Southeastern Brazil, and it is still unclear how much these distribution gaps are the consequence of a lack of proper surveys. Although adequate surveys in the Caatinga are still incipient (Carmignotto et al., 2012), recent surveys on the Atlantic Forest portion located North of the São Francisco River (ca. 10°S) failed to find any specimens of *C. minimus*, yet recorded other semi-aquatic river mammals, such as water rats, *Nectomys squamipes* and *Nectomys rattus* (Oliveira and Langguth, 2004), and the Neotropical otter, *Lontra longicaudus* (Astúa et al., 2010), indicating that suitable habitat exists in this region. Likewise, some intensive surveys also failed to record *C. minimus* in certain regions of the Amazon (e.g., Patton et al., 2000). If these gaps are not the results of collection bias, then climatic or biological factors could explain their absence from those areas, and thus help us better understand its distribution, biogeography and evolution.

Our results show an evident variation in both shape and size of the skull of *C. minimus* throughout its distribution. Climatic and geographic factors probably work (or worked) as geographic barriers that contribute to the isolation of some populations. Some of the populations depicted here have measurable morphological distinction and could, therefore, with proper phylogenetic assessment, be considered as different species.

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Appendix A. List of specimens examined and included in the analyses.

Acronyms institutions: AMNH: American Museum of Natural History; FMNH: Field Museum of Natural History; KU: Museum of Natural History, University of Kansas; MN: Museu Nacional, Rio de Janeiro; MNHN: Muséum National d'Histoire Naturelle; MSB: Museum of Southwestern Biology, University of New Mexico; MVZ: Museum of Vertebrate Zoology, University of California, Berkeley; MZUSP: Museu de Zoologia da Universidade de São Paulo; USNM: National Museum of Natural History, Smithsonian Institution; UMMZ: University of Michigan Museum of Zoology; UWZM: University of Wisconsin Zoological Museum.

Specimens included in both analyses: AMNH: 123288; 129703; 129704; 133198; 141918; 148720; 164494; 182939; 264571; 264572; 30752; 33027; 34121; 37482; 37483; 47190; 62365; 72019; 72020; 96758; 96759; 96760; 97319. KU: 114474; 114475; 114481; 114482; 114483; 114484; 114485. MNHN: 1195202; 200725. MVZ: 130323; 130324; 130325; 130326; 130327; 144314. UMMZ: 124678; 124679; 124680; 124681; 134022; 134023. USNM: 280910; 280912; 280914; 280916; 280917; 282605; 292169; 305166; 305167; 306465; 306466; 309335; 309338; 309339; 309343; 309344; 309345; 309348; 315093; 315094; 322989; 322990; 322991; 322993; 334488; 334489; 335045; 335048; 335049; 335050; 335051; 335052; 335053; 335055; 335056; 335057; 336194; 337965; 360139; 360141; 362361; 362364; 362365; 362369; 362370; 362371; 362372; 385097; 396418; 396513; 406972; 418562; 516614; 517236; 517238; 517239; 517240; 517241; 541020; 564613.

Specimens included only in geometric morphometrics analyses: AMNH: 212909; 266477. FMNH: 44047; 50908; 53527; 58807; 60094; 60502; 60576; 60577; 69224; 69329; 75092; 75093; 89360; 90066; 90088; 90089; 90092; 90094; 94292. KU: 110652; 110653; 110656; 26928; 29302; 70194. MN: 10507; 1281; 23752; 25711; 25738; 30373. MSB: 68330. MVZ: 153307; 166507. MZUSP: 17063; 17081; 4644; 4651; 4654; 4658; 4659; 4669; 4673; 4755; 4762; 4837; 6701; 7793. USNM: 306468; 464516; 583002. UWZM: 31293.

Appendix B. Landmark definitions.

Dorsal view—the landmarks on the dorsal view were placed on: (1) tip of the nasal; (2) posterior end of left and right junction of nasal; (3) junction between right and left suture of frontal and parietal bones; (4) intersection between right and left parietal bones and interparietal bone sutures; (5) posterior end of interparietal bone, on the intersection of the sagittal line with the nuchal crest; (6/20) intersection between interparietal-parietal suture and the braincase contour on the nuchal crest; (7/21) squamosal curving point at the beginning of the nuchal crest anterior to the post-tympanic process; (8/22) point where the braincase is larger; (9/23) most curvy point of the zygomatic arch (posterior-laterally); (10/24) intersection between squamosal and jugal bone suture, on the interior side of the zygomatic arch; (11/25) prominence formed by the infraorbital foramen on the maxilla; (12/26) prominence formed by the canine root, on the maxilla; (13/27) anterior end of the suture between the nasal bone and the premaxilla; (14/28) intersection of sutures between nasal and frontal bones and maxilla; (15/29) most anterior point of the orbit; (16/30) the narrowest point of the skull on the frontal bone, anterior to the orbital process (inter-orbital breadth); (17/31) tip of the orbital process of the frontal bone; (18/32) narrowest point of the skull on the frontal bone, posterior to the orbital process (post-orbital breadth); (19/33) intersection between the anterior rim of the squamosal process that forms the zygomatic arch and the rim of the braincase;

(34/35) intersection between nasal, frontal bones and premaxilla; (36/37) tip of orbital process of the jugal bone, on the zygomatic arch.

Lateral view—on the lateral view the landmarks were placed on: (1) anterior base of the first superior incisive; (2) posterior base of the fifth superior incisive; (3) anterior base of the superior canine, on the maxillary bone junction with the tooth; (4) posterior base of the superior canine, on the maxillary bone junction with the tooth; (5) base of the third premolar and the first superior molar; (6) posterior base of the fourth superior molar, where the tooth meets the maxillary bone (posterior end of the molar series); (7) most posteroventral point of the occipital condyle; (8) most posterodorsal point of the braincase (posterior end of the sagittal line, junction with the nuchal crest); (9) top of the sagittal crest, where the frontal and parietal bones meet; (10) intersection between frontal, maxillary and nasal bones sutures; (11) junction of the nasal, premaxillary and maxillary bones sutures; (12) anterior end of the sutures between the nasal and premaxillary bones; (13) most internal point (posterodorsally) of the glenoid fossa; (14) most posterior point of the zygomatic arch; (15) suture between the jugal and squamosal bone, on the dorsal edge of the zygomatic arch; (16) jugal bone's frontal process end, on the zygomatic arch; (17) most anterior point of the orbit; (18) Lacrimal canal.

Lateral view of Mandible—on the mandible: (1) base of the first inferior incisive; (2) base of the fourth inferior incisive; (3) posterior end of the inferior canine alveoli; (4) base of the third premolar and the first molar where the teeth encounters the dentary bone; (5) posterior base of the inferior fourth molar, where the teeth encounters the dentary bone; (6) where the horizontal ramus of the mandible and beginning of the coronoid process meets (base of the coronoid process); (7–12) contour of the coronoid process, as equally distant points; (13) Highest point of the coronoid process; (14) posterior end of the coronoid process (beginning of the posterior edge of the coronoid process); (15) point of biggest inflexion of the curvature between the articular process and the posterior edge of the coronoid process (base of the posterior edge); (16) lateral end of the articular condyle; (17) posterior base of the angular process; (18) tip of the angular process; (19) anterior base of the angular process; (20) projection of point 5 on the ventral edge of the mandible, perpendicular to the line formed by point 4 and 5; (21) projection of the place where the first and the second molars meet on the ventral edge of the mandible perpendicular to the line formed by point 4 and 5; (22) projection of the place where the second and the third premolars meet on the ventral edge of the mandible perpendicular to the line formed by point 3 and 4; (23) projection of point 2 on the ventral edge of the mandible, perpendicular to the line formed by point 1 and 2; (24) antero ventral end of masseteric fossa; (25) anterior mental foramen.

Appendix C. List of localities (organized by macro-localities, as indicated in Fig. 2). Numbers refer to localities indicated in Fig. 2.

Northern Central America: 1.—Cockscomb Basin Wildlife Sanctuary; Stann Creek; Belize (16°46'N; 89°31'W). 2.—Finca Rio Frio; Morales; Izabal; Guatemala (15°40'59"N; 89°05'W). 3.—Muras de Oro; Honduras (14°48'N; 88°39'W). 4.—Tegucigalpa; Francisco Morazan; Honduras (14°06'N; 88°47'W). 5.—Zapotitan; Libertad; El Salvador (13°48'N; 90°33'W). 6.—Jalapa; Nueva Segovia; Nicaragua (13°57'N; 86°W). 7.—17 km North and 15 km East of Santa Rosa; Boaco; Nicaragua (12°35'N; 86°35'W). 8.—6 km West of Teustepe; Teustepe; Boaco; Nicaragua (12°27'N; 86°12'W). 9.—Cara de Mono; Zelaya; Nicaragua (12°07'0.12"N; 85°32'W). 10.—14 km South of Los cocos; Boaco; Nicaragua (12°05'N; 86°06'W). Southern Central America: 11.—Vilagual; Alajuela; Costa Rica

(10°29'N; 85°21'W). 12.—Turrialba; Cartago; Costa Rica (10°17'N; 84°36'W). 15.—Alajuela; San Carlos; Costa Rica (10°01'0.12"N; 85°47'W). 16.—Colonia Agrícola de Caracolicito; Magdalena; Colombia (10°N; 75°30'W). 17.—Agua Caliente; Cartago; Costa Rica (9°49'0.12"N; 84°07'0.12"W). 18.—Sibube; Bocas del Toro; Panama (9°36'N; 83°11'W). 19.—Mandinga; San Blas; Panama (9°29'30"N; 80°57'W). 20.—7 km Southwest of Changuinola; Bocas del Toro; Panama (9°26'N; 83°29'W). 21.—Bocas del toro; Panama (9°21'N; 83°47'W). 23.—Canal zone; Cerro Azul; Panamá (9°10'0.12"N; 80°35'W). 24.—Finca Santa Clara 14.5 Km Northwest of El Volcan; Chiquiri; Panama (8°46'0.12"N; 83°22'0.12"W). 25.—Rio Pito; San Blas; Panama (8°42'N; 78°28'0.12"W). 26.—Quebrada Venado; Armila; San Blas; Panama (8°40'0.12"N; 78°32'W). 27.—Pto. Obaldia; San Blas; Panama (8°40'0.12"N; 78°35'W). 28.—Tarcuna Village Camp; Darien; Panama (8°11'N; 78°43'W). 29.—Head of Rio Paya; Darien; Panama (7°57'48"N; 78°36'02"W). 30.—Isla Cebaco; Veraguas; Panama (7°32'25"N; 82°50'40"W). 31.—Rio Immamado; Jaque; Darien; Panama (7°31'0.12"N; 79°50'W). 32.—Guanico Arriba; Los Santos; Panama (7°20'N; 81°30'W). 33.—Cerro Hoya; Los Santos; Panama (7°18'N; 81°18'W). 38.—Bolívar; Socorré; Colombia (7°54'N; 77°42'W). Northern South America: 13.—El Limón 4 km Northwest of Maracay; Guamita; Aragua (10°14'21"N; 68°25'52"W). 14.—Caripe, 5 km Northwest of San Agustín; Monagas; Venezuela (10°12'N; 64°31'0.12"W). 22.—Aragua; Maracay; Guamita; Venezuela (9°12'59"N; 59°53'0.24"W). 34.—Cuyagua; Aragua; Venezuela (10°28'35"N; 68°17'56"W). 35.—1 km North and 2 km West of Caracas; Merida; Venezuela (8°35'53"N; 72°51'18"W). 36.—Rio Tarra; San Calixto; Santander; Colombia (8°24'05"N; 74°47'30"W). 37.—Villanueva; Vallecupar; Magdalena (8°05'26"N; 74°30'13"W). Ecuador/Colombia: 39.—Bagadó; Chocó; Colombia (5°30'N; 77°45'W). 40.—Rio Frio; Valle del Cauca; Cauca; Colombia (3°45'N; 77°30'W). 41.—Sabanetas; Cauca; Colombia (2°32'N; 77°07'0.12"W). 42.—Head of Catapino River; Cordillera de Galeras; Napo; Ecuador (0°03'N; 77°20'W). 43.—Manabi; San Jose; Ecuador (1°57'S; 81°56'W). 44.—Bobonasa River; Pastaza; Ecuador (2°25'S; 78°15'W). 45.—Meeting of rivers Chimbo and Coco; Chimborazo; Ecuador (2°05'S; 79°15'W). 46.—Rio Pindo; El Oro; Ecuador (4°30'S; 80°10'0.12"W). 47.—Head of Rio Huampami, East of Huampami, Cenepa River; Amazon; Peru (5°41'13"S; 79°52'19"W). Amazon: 48.—Near Sinnamary; Paracou; French Guiana (5°23'N; 53°03'W). 49.—Otinga; Pará; Brazil (2°33'S; 49°30'W). 50.—Ilha das Onças; Belém; Pará; Brazil (2°31'48"S; 49°31'12"W). 51.—Rio Tocantins; Cametá; Pará; Brazil (3°45'36"S; 50°30'W). 52.—Rio Tocantins; Ilha do Taluna; Pará; Brazil (6°39'S; 57°53'W). Peru/Bolivia: 53.—Hacienda Cadena; Quince Mil; Cuzco; Peru (13°10'1.2"S; 71°W). 54.—Rio Alto; Hacienda Erika; Madre de Dios; Peru (13°06'S; 72°47'W). 55.—Rio Silvera; Huajyumbé; Quince Mil; Cuzco; Peru (14°45'S; 70°35'W). 56.—La Reserva; La Paz; Bolivia (16°33'36"S; 68°41'24"W). Southern South America: 57.—Santa Tereza; Espírito Santo; Brazil (20°04'12"S; 41°24'W). 58.—Jacarepaguá; Rio de Janeiro; Rio de Janeiro; Brazil (23°04'48"S; 44°39'W). 59.—Muriqui; Mangaratiba; Rio de Janeiro; Brazil (23°04'48"S; 44°03'W). 60.—Jacaré-guaçu; Boa Esperança; São Paulo; Brazil (22°10'0.12"S; 49°04'0.12"W). 61.—Assis; São Paulo; Brazil (23°20'24"S; 51°35'24"W). 62.—Rocha; São Paulo; Brazil (24°48'30"S; 47°33'58"W). 63.—Northeast Curugaty; Canendeyu; Paraguay (25°27'S; 56°18'W). 64.—10 km South Salto de Pirareta; Pirebebuy; Cordillera; Paraguay (26°27'S; 58°57'W). 65.—Southeast Pirebebuy; Paráguari; Paraguay (26°11'39"S; 58°40'35"W). 66.—Parque Nacional Ybycui; Paráguari; Paraguay (27°58'59"S; 58°57'01"W).

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