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# Phylogeny explains better than ecology or body size the variation of the first lower molar in didelphid marsupials

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**Abstract:** Tribosphenic molars are considered great innovations in mammals and are related to several structures and variables that can explain adaptation. The aim of this study was to investigate the importance of body size and habitat relation, using a phylogenetic approach, in the first lower molar shape in didelphid marsupials. Geometric morphometric analyses of the lower molar's shape were performed on 261 specimens, 130 females and 131 males, covering 14 genera and 37 species of the Didelphidae family. The molar conformation showed a larger talonid in relation to the trigonid in more arboreal genera, and narrower and longer molars in genera with a larger body size. Phylogeny was the variable with the highest explanation for both females and males (16.17% and 9.02%, respectively). The body size was significant in males, presenting an important influence on molar shape, while the body size in females was not significant when phylogenetic relationship was controlled for. In both sexes, habitat presents a strong effect of phylogeny, with no direct effect on molar shape. Didelphid molar shape is another result of its phylogenetic history and does not respond very much to environmental pressures. Male body size influences molar shape in didelphids, even in the presence of a strong phylogenetic signal.

**Keywords:** body size; dental shape; geometric morphometric; phylogenetic comparative method; variation partitioning.

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## Introduction

Marsupials are a diverse group that presents a peculiar current distribution: a group restricted to the Australasian region and another in the Americas. In the new world, representatives of three extant and two extinct orders of marsupials have been reported to date (Oliveira and Goin 2012), with Didelphimorphia comprising the most diverse order (Eisenberg and Redford 1999). Composed only by the Didelphidae family, this group is monophyletic (Patton et al. 1996, Voss and Jansa 2009), evidenced by morphological characters (e.g. sperm morphology and mammary formula) (Jansa and Voss 2005), postcranial skeletal morphology (Flores 2009), and molecular analyses (e.g. the IRBP gene) (Jansa and Voss 2005, Voss and Jansa 2009). They currently represent 19 genera and about 100 described species (Jansa et al. 2014).

The Didelphidae family in South America is a taxonomically diverse lineage of early Tertiary fauna of South America (Jansa et al. 2014). The most basal didelphids are *Glironia* and Caluromyines, and its clade may have diverged from other didelphids in the late Oligocene and Miocene (Jansa et al. 2014). According to Voss and Jansa (2009), there are four subfamilies: Glironiinae (*Glironia*), Caluromyinae (*Caluromys*, *Caluromysiops*), Hyladelphinae (*Hyladelphys*), and Didelphinae, which is composed of four tribes (Marmosini, Metachirini, Didelphini, and Thylamyini). These tribes and subfamilies represent different lineages of marsupials that evolved in South America, adapting to open or forested niches, with some cases of convergence in this respect (Jansa et al. 2014).

Didelphids present large variations in body size, in which there are genera with representatives that are relatively small and have reduced body mass (lower than 30 g), such as species of *Monodelphis*, as well as larger representatives, which may weigh up to more than 3000 g, such as *Didelphis* (Gardner 2008, Cáceres et al. 2012a). Although with some exceptions, one must pay attention that the didelphids have clear sexual dimorphism, especially related to the body size (Cáceres and Monteiro-Filho 1999), the skull (Astúa 2010, Pavan et al. 2012),

and the dental measures as the upper canine (Pavan et al. 2012). Thus, males show size generally larger than females leading to a necessary attention to this in ecological (Meloro et al. 2014) or evolutionary (Isaac 2005) analyses.

The didelphids occur in several environmental types, especially forests (Vieira and Monteiro-Filho 2003), but also occur extensively in savannas and grasslands (Gardner 2008). Females of didelphids, for example, are closely linked to the environment, giving priority to their offspring and food resources during the reproductive season (Hossler et al. 1994). Overall, these marsupials exhibit omnivorous feeding habitats although tendencies can be found: some species are more carnivorous (consume insects and small vertebrates – e.g. *Lutreolina*) while there is a continuum toward those species that are more frugivorous (e.g. *Caluromys*) (Astúa et al. 2003, Cáceres 2005). Besides these tendencies, a group with a highly specialized diet has not been recorded yet (Vieira and Astúa 2003). Although the diet is a meaningful explanatory variable usually correlated to the teeth morphology, it is very difficult to assert diet in Neotropical marsupials, with quantitative diet being available only for a pool of species in this group (Lessa and Geise 2010). However, other ecological characteristics of didelphids are promptly available and established for the group, such as the substrate use for locomotion (Vieira and Delciellos 2012).

Different food preferences may be related to the availability or palatability of resources (Lessa and Geise 2010) and to distinct uses of strata (ground, understory, and canopy) (Emmons 1995, Vieira and Astúa 2003). Representatives of the most terrestrial habitat species tend toward a more carnivorous and/or omnivorous diet, while those of more arboreal habitats tend to be more frugivorous (Vieira and Delciellos 2012). Field data have corroborated this relationship, with habitat use usually being related to diet, as in the case of terrestrial *Metachirus nudicaudatus*, which are insectivorous and seasonally omnivorous (Cáceres 2004, Lessa and Geise 2014b), and the small-sized, arboreal *Gracilinanus agilis*, which are mainly insectivorous but seasonally frugivorous (Camargo et al. 2011, Lessa and Geise 2014a). Thus, the substrate use of a marsupial species could be a reasonable proxy for its diet: e.g. the more terrestrial is the species, the more it tends to be carnivorous (Vieira and Astúa 2003). If dental adaptation occurs differentially for arboreal and terrestrial marsupials, we expect that it will respond in part to diet requirements.

Subsequently, when diet is mentioned, it is interesting to associate the oral apparatus (formed by the teeth and mandible) with tools that assist in better performance during feeding (Cooke 2011). In general, mammals have a complex variety of tooth shapes (Ungar 2010), which encourages estimation of what could be the "ideal" functional teeth shape (Evans and Sanson 2003). Molars called tribosphenic, for example, form a complex structure with versatile functions and are considered a great innovation in vertebrates, culminating with the marsupial and placental basal diversification (Luo 2007). Thus, marsupial dentition, which includes three premolars and four molars (a configuration that emerged in the early Cretaceous) (Cifelli 1993), shows a great importance for the study of evolution considering shape or size. It is possible to recognize a trigonid in marsupials' tribosphenic lower dentition by its three main cusps: paraconid, protoconid, and metaconid. In the posterior region, there is the talonid, also constituted by three cusps: hypoconid, entoconid, and hypoconulid (Cifelli 1993).

The shape of the teeth may be related to different uses of substrates in some mammal groups (Kay et al. 2001, Williams and Kay 2001), so this relationship could be true for marsupials. For example, there is evidence that crown height is associated with the substrate preference in rodents (Williams and Kay 2001). In addition, the teeth of some ungulates and primates also tend to be associated with habitat preferences (Janis 1988, Kay et al. 2001). Thus, it is important to take into account different habitats, especially because of the lack of comparisons using dental features.

Moreover, body size can be associated with didelphid habitat as it can be a reasonable prediction for dental shape in mammals (Gordon 2003, Mendoza et al. 2006, Raia et al. 2010). Body size alone has already been emphasized as an influence on molar shape (Caumul and Polly 2005), having an important role on the reconstruction of life history and ecology of fossil species based on teeth (Gingerich et al. 1982). Even in marsupials, there are findings that have evidenced a strong correlation between body size and molar size (Gordon 2003, Hogue and ZiaShakeri 2010). Furthermore, the body size in mammals can be variable, but in general insectivorous species show reduced body size compared to other dietary types, as in marsupials (Hogue and ZiaShakeri 2010), bats (Arita and Fenton 1997), and primates (Fleagle 2013). While this trend is thought to be true, some species of small didelphids, such as *Gracilinanus*, supplement their insectivorous diet with other resources due to seasonality (Lessa and Geise 2014a).

Many studies with mammals show significant results when associating their shape with ecological, functional, and evolutionary features, whether it is with the skull,

mandible, or dentition (Astúa et al. 2000, Caumul and Polly 2005, Raia et al. 2010). When shape is addressed, it appears to be strongly subjected to the action of phylogenetic inertia due to common ancestry, which means that closely related species tend to present similar shape attributes regardless of environmental pressure (Greenacre and Vrba 1984, Caumul and Polly 2005, Klingenberg and Gidaszewski 2010), as it seems to be the case of mandibular development throughout the evolution of mammals (Michaux et al. 2008, Rivals et al. 2008, Prevosti et al. 2011), as well as the evolution of body mass in vertebrates (Abouheif 1999).

If there is a strong phylogenetic signal in these shape structures throughout Didelphidae lineages, as found by Chemisquy et al. (2015), closely related species will exhibit a more similar shape regarding homologous structures than other distantly related species. This will indicate the presence of phylogenetic inertia acting on the evolution of these structures (Raia et al. 2010). If tribosphenic molars, which are inherent to marsupial diversification, assumed versatile functions (Lopatin and Averianov 2006, Luo 2007), allowing a better utilization of food, the variation of their shape could be an important clue in the understanding of feeding evolution in marsupials. The distinct talonid part of the molar is used as a support to crush food, while the trigonid is used to cut (Butler 1992, Luo et al. 2001). If the talonid is large, it may indicate a marsupial with a more frugivorous diet, while a larger trigonid is thought to be related to an insectivorous/carnivorous diet (Voss and Jansa 2009). Even if most posterior molars, as m3, could account for didelphid molar shape variation, they are not sufficient to distinguish them (Chemisquy et al. 2015) just because of its extremely homogeneous diet. Moreover, among the molars, the first molar represents the tooth less variable within species and therefore the most important and safe to be studied (Gingerich and Schoeninger 1979), as seen in studies with fossils (Gingerich 1974, Lazzari et al. 2008) and extant mammals (Polly 2004, White 2009).

Based on these assumptions, the present study aims to verify how the first lower molar (m1) evolved among the Didelphidae family, by testing both sexes independently, and its main predictors of shape variation. As such, the specific aims are 1) verifying the influence of body size on molar shape, evaluating if the increase or decrease of body size in Didelphidae is associated with molar shape variation; 2) testing if the different habitats (use of different substrates in the environment) influence molar shape; and lastly, 3) examining the phylogenetic role on molar shape evolution of Didelphidae. Our hypothesis assumes that there will be a low, but significant, impact of both predictor variables (body size and habitat) on molar shape, as there are a) small-bodied Didelphidae species apparently with diets tending toward insectivory, b) other large-bodied species which are omnivorous or carnivorous, and c) furthermore phylogeny, which should explain more of the molar shape variation, with closely related species, perhaps grouped by lineages (tribes or subfamilies), sharing more molar shape similarities due to relationship rather than the influence of environment or body size. For example, the tribe Didelphini comprises large-bodied species (*Chironectes*, *Didelphis*, *Philander*, and *Lutreolina*; Voss and Jansa 2009) that are variable in habitat occupation but tend to be omnivorous or carnivorous, while the tribe Thylamyini is composed mainly by small-bodied, open-habitat species (Voss and Jansa 2009) occupying mostly grasslands and savannas, with some species or genera going to the forest habitat.

# Materials and methods

## Data collection

A total of 261 adult individuals were photographed, encompassing a total of 14 genera (among the 19 genera of Didelphidae) and 37 species. The number of species which contained sampled females or males was 29 and 36, respectively, with a total of 130 female specimens (mean: 3.51 individuals per species; range: 0–10 individuals) and 131 male specimens (mean: 3.54; range: 0–11) (Table 1).

The specimens were sampled in the collections of the *Museu de Zoologia da Universidade de São Paulo*, São Paulo, Brazil (MZUSP), *Museu Nacional, Universidade Federal do Rio de Janeiro*, Rio de Janeiro, Brazil (MNRJ), *Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"*, Buenos Aires, Argentina (MACN), and from the *Coleção de Mamíferos da Universidade Federal de Santa Maria*, Rio Grande do Sul, Brazil (UFSM). Only adult specimens were examined (complete eruption of the fourth upper molar).

All molar teeth were photographed at standard focal length using a digital camera fixed on a microscope. The lower molars of each specimen were aligned and photographed in occlusal view, with the hypoconid and protoconid in labial view (facing up), so that the occlusal surfaces (tips of cusps) were parallel with the microscope lens (Table 1). The molars in occlusal view were photographed over a checked grid (1 mm), and the photo distortion level was examined by measuring these squares on the sides compared with the center in ten random photos.

**Table 1:** Specimens examined of Didelphidae and respective sample sizes for each sex, species habitat, and focal length for photographs of the molar (m1).

| Genera | Species | $N_{females}$ | $N_{males}$ | $N_{total}$ | Habitat | Dm1 |
|---|---|---|---|---|---|---|
| *Caluromys* J. A. Allen 1900 | | | | | | |
| | *Caluromys lanatus* (Olfers 1818) | 5 | 4 | 9 | AR | 1.0× |
| | *Caluromys philander* (Linnaeus 1758) | 7 | 5 | 12 | AR | 1.0× |
| *Philander* Brisson 1762 | | | | | | |
| | *Philander opossum* (Linnaeus 1758) | 6 | 8 | 14 | TA | 1.0× |
| | *Philander frenatus* (Olfers 1818) | 7 | 4 | 11 | TA | 1.0× |
| *Chironectes* Illiger 1811 | | | | | | |
| | *Chironectes minimus* (Zimmermann 1780) | 10 | 5 | 15 | SA | 1.0× |
| *Cryptonanus* Voss, Lunde and Jansa 2005 | | | | | | |
| | *Cryptonanus guahybae* (Tate 1931) | 1 | 2 | 3 | TA | 2.25× |
| | *Cryptonanus chacoensis* (Tate 1931) | 3 | 7 | 10 | TA | 2.25× |
| *Didelphis* Linnaeus 1758 | | | | | | |
| | *Didelphis albiventris* (Lund 1840) | 6 | 6 | 12 | TA | 1.0× |
| | *Didelphis aurita* (Wied-Neuwied 1826) | 4 | 5 | 9 | TA | 1.0× |
| *Glironia* Thomas 1912 | | | | | | |
| | *Glironia venusta* Thomas 1912 | 0 | 1 | 1 | AR | 2.25× |
| *Gracilianus* Gardner and Creighton 1989 | | | | | | |
| | *Gracilinanus agilis* (Burmeister 1854) | 10 | 11 | 21 | AR | 2.25× |
| | *Gracilinanus microtarsus* (Wagner 1842) | 2 | 2 | 4 | AR | 2.25× |
| *Lutreolina* Thomas 1910 | | | | | | |
| | *Lutreolina crassicaudata* (Desmarest 1804) | 9 | 10 | 19 | TE | 1.0× |
| *Marmosa* Gray 1821 | | | | | | |
| | *Marmosa constantiae* (Thomas 1904) | 3 | 1 | 4 | AR | 2.25× |
| | *Marmosa murina* (Linnaeus 1758) | 8 | 8 | 16 | AR | 2.25× |
| | *Marmosa robinsoni* (Bangs 1898) | 1 | 1 | 2 | AR | 2.25× |
| | *Marmosa demerarae* (Thomas 1905) | 7 | 5 | 12 | AR | 2.25× |
| | *Marmosa paraguayana* (Tate 1931) | 2 | 1 | 3 | AR | 2.25× |
| *Marmosops* Matschie 1916 | | | | | | |
| | *Marmosops paulensis* (Tate 1931) | 6 | 3 | 9 | TA | 2.25× |
| | *Marmosops incanus* (Lund 1840) | 1 | 5 | 6 | TA | 2.25× |
| | *Marmosops noctivagus* (Tschudi 1845) | 1 | 1 | 2 | TA | 2.25× |
| *Metachirus* Burmeister 1854 | | | | | | |
| | *Metachirus nudicaudatus* (É. Geoffroy 1803) | 9 | 11 | 20 | TE | 1.6× |
| *Monodelphis* Burnett 1830 | | | | | | |
| | *Monodelphis domestica* (Wagner 1842) | 4 | 4 | 8 | TE | 2.25× |
| | *Monodelphis kunsi* (Pine 1975) | 0 | 1 | 1 | TE | 2.25× |
| | *Monodelphis dimidiata* (Wagner 1847) | 3 | 3 | 6 | TE | 2.25× |
| | *Monodelphis scalops* (Thomas 1888) | 0 | 1 | 1 | TE | 2.25× |
| | *Monodelphis rubida* (Thomas 1899) | 2 | 1 | 3 | TE | 2.25× |
| | *Monodelphis brevicaudata* (Erxleben 1777) | 1 | 1 | 2 | TE | 2.25× |
| | *Monodelphis iheringi* (Thomas 1888) | 1 | 0 | 1 | TE | 2.25× |
| | *Monodelphis americana* (Müller 1776) | 0 | 1 | 1 | TE | 2.25× |
| *Thylamys* Gray 1843 | | | | | | |
| | *Thylamys macrurus* (Olfers 1818) | 0 | 1 | 1 | TA | 2.25× |
| | *Thylamys venustus* (Thomas 1902) | 1 | 1 | 2 | TE | 2.25× |
| | *Thylamys pallidior* (Thomas 1902) | 1 | 1 | 2 | TE | 2.25× |
| | *Thylamys karimii* (Petter 1968) | 9 | 7 | 16 | TE | 2.25× |
| | *Thylamys elegans* (Waterhouse 1839) | 0 | 1 | 1 | TE | 2.25× |
| | *Thylamys pusillus* (Desmarest 1804) | 0 | 1 | 1 | TE | 2.25× |
| *Hyladelphys* Voss, Lunde and Simmons 2001 | | | | | | |
| | *Hyladelphys kalinowskii* (Hershkovitz 1992) | 0 | 1 | 1 | TA | 2.25× |
| TOTAL | | 130 | 131 | 261 | | |

Dm1, molar increase level under the microscope. Habitats: AR, arboreal; TA, terrestrial-arboreal; TE, terrestrial; SA, semiaquatic.

Thus, it was observed that there was no distortion in any part of the photos (lateral related to the center) (t=-0.010; df=18; p=0.992). With this standardization, it is assumed that any error that may have been introduced by this procedure is insignificant or inconsistent in the samples (Mullin and Taylor 2002).

To obtain the average configuration of lower molar cusps based on specimens, six landmarks in the molar (m1) were digitized using tpsDig2 v. 2.16 (Stony Brook, NY, USA, Rohlf 2010a) (Figure 1). All six molar landmarks represent the major cusps (1–5=conids; 6=conulid) (Figure 1) and were chosen because they represent satisfactory and reliable responses to the main dental points related to chewing as only specimens with a low level of cusp wear were utilized (e.g. Polly 2001). The talonid region is evidenced by the links between landmarks 1, 2, 4, 5, and 6, while the region of the trigonid is formed between links 2, 3, and 4, which are thus evidenced regions with different functions. The landmarks were tested for repeatability, following Astúa (2009) as follows. Twenty specimens from one species were selected, and all landmarks were digitized twice (on different days). Estimated repeatability was considered as the intraclass correlation coefficient (ICC). All landmarks presented repeatability of no <0.99 (that is, all landmarks exhibited <2% error) and were thus considered satisfactory and suitable for the subsequent analyses.

## Data processing

We opted to use each specimen here as a sample, as each individual comes from a different location, in order to capture this within-species variability (see Supplementary Appendix 1). From the single, generalized superposition of landmark-estimated points, scaled from the centroid size (CS=square root of the sum of squares of the distances between all points of the setting and their corresponding center), a reference configuration was obtained by rotating these points to minimize their square distances (Procrustes distance), in accordance with the generalized Procrustes analysis (GPA) (Rohlf and Slice 1990, Dryden and Mardia 1998), using the tpsRelw v. 1.49 software (Stony Brook, NY, USA, Rohlf 2010b). This reference configuration corresponds to the tangency points in the tangent space to Kendall's non-Euclidean shape space (Monteiro and Reis 1999, Couette et al. 2005), which can be used in conventional multivariate statistical techniques.

The molar CS, using the natural logarithm (lnCS), was taken as a body size estimator (Raia et al. 2010), once the teeth could be described as a structure directly related to body size (Gordon 2003, Raia and Meiri 2006).

For the predictor habitat (substrate preference of species), we followed the classification of Paglia et al. (2012), with the exceptions of *Cryptonanus* (Vieira and Camargo 2012), *Marmosa murina* (Voss et al. 2001), *Marmosa paraguayana* (Vieira and Monteiro-Filho 2003), *Marmosa robinsoni* (Alvizu and Aguilera 1998, O'Connell 1979), *Thylamys* (Carmignotto and Monfort 2006) and *Thylamys macrurus* (Cáceres et al. 2007), for which more detailed or current information is available. The habitat variable was categorized in a gradient from the semiaquatic/terrestrial species up to the more arboreal species, classified into four levels (Table 1).

For a variance description of the points (whose originals are the landmarks in the molars) in tangent space, a principal component analysis (PCA) using the covariance matrix of the coordinates of the landmarks was performed in order to reduce the variance in main axes where the data are represented (Klingenberg and Gidaszewski 2010) using tpsRelw v. 1.49 (Stony Brook, NY, USA, Rohlf 2010b). The principal component axes (here called "relative warps" – RWs) describe molar shape where the first axes better explain the molar variation.

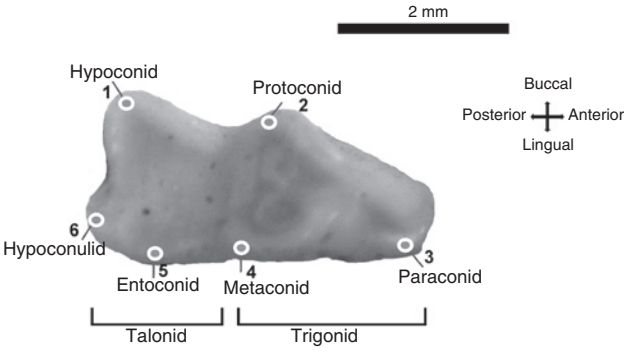


**Figure 1:** Position of the six landmarks on the occlusal view of the first lower molar (m1) in a specimen of *Metachirus nudicaudatus*, and tooth nomenclature used in the study.

## Statistical analysis

First, the existence of sexual dimorphism was evaluated using a two-way analysis of variance (ANOVA), with sex and species as factors generating an interaction (sex×species) term using lnCS data. A two-way multivariate analysis of variance (MANOVA) was also applied but now on molar shape data with the same factors, sex, species, and its interaction term (Astúa et al. 2000, Caumul and Polly 2005). As expected, sexual dimorphism was detected, and males and females were treated separately in this study (see later).

In order to test the hypothesis that species differ in molar shape, a MANOVA was employed, using values of the first two RWs, including species with at least two specimens.

Simple and multiple linear regressions were used to test if body size (lnCS) and habitat influence molar shape variation (the dependent variable). Both regressions were performed using the tpsRegr v. 1.38 statistical software (Stony Brook, NY, USA, Rohlf 2011).

For analyses involving phylogeny, controlling the phylogenetic effect on shape, a phylogenetic hypothesis was constructed based on the literature. Articles by Voss and Jansa (2009) and Cardillo et al. (2004) were used as a base to manually assemble a tree including all taxa, in addition to Pine et al. (2013) for *Monodelphis* (Figure 2). The tree was constructed with the help of the Mesquite 2.75 software (Maddison and Maddison 2011). Node data were mainly extracted from Steiner et al. (2005), Bininda-Emonds et al. (2007), Drummond et al. (2006), and Palma et al. (2002) [compilation of Hedges et al. (2006) and Kumar and Hedges (2011)]. Branch lengths were based on the estimated minimum ages. Undated nodes (which were rare in our data) were evenly spaced between the dated nodes using the BLADJ algorithm (branch length adjustment; Webb et al. 2008) in the Phylocom software. As specimens were used as a reference base for phylogeny, polytomies within each species were employed when more than one specimen was in the tree. Next, a phylogenetic distance matrix was created between all pairs of analyzed specimens, from which orthogonal eigenvectors were extracted. The software used was the R Development Core Team (Vienna, Austria, 2012), with the PVR package (Santos et al. 2013).

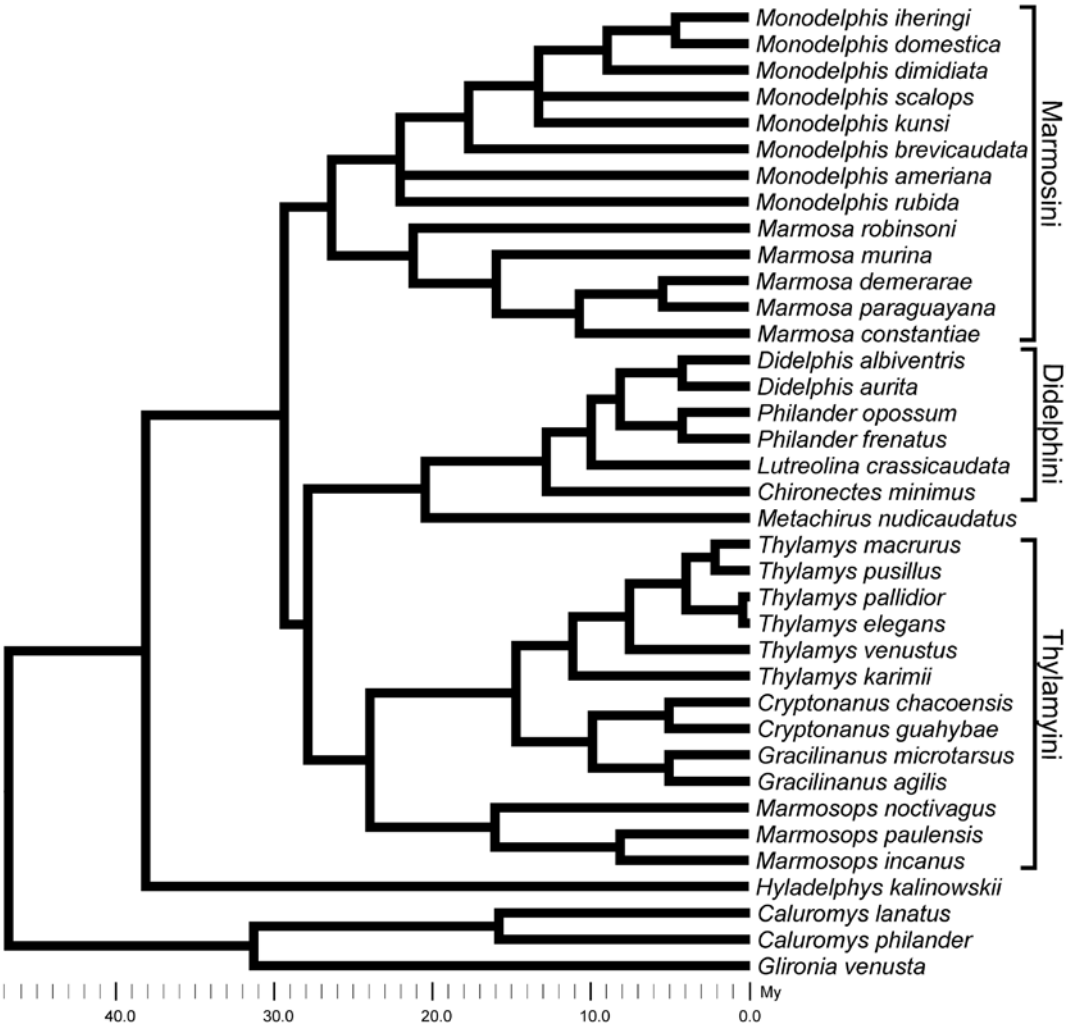


**Figure 2:** Phylogenetic hypothesis of species used (Didelphidae) constructed from articles by Voss and Jansa (2009) and secondarily Cardillo et al. (2004), in addition to Pine et al. (2013) for *Monodelphis*.
Main tribes are according to Voss and Jansa (2009). Time is in millions of years (My).

A variance partitioning analysis (Desdevises et al. 2003) was applied to quantify how much each predictor variable explained the response variable (molar shape) when all predictors were analyzed simultaneously. The variance partitioning computes multiple regressions of a number of independent variables, involving a series of linear regressions followed by subtractions (Raia et al. 2010). Thus, the molar shape variable (in fact, eight shape variables as RWs, representing 100% of the shape variation) was used as a dependent variable, and body size (lnCS), habitat, and phylogeny (first four orthogonal eigenvectors of a PCA made from original data) (Carrascal et al. 2008) as independent variables. The variance partitioning was performed using the vegan package (Oksanen et al. 2011) through the R Development Core Team software (Vienna, Austria, 2012).

The method chosen to investigate the significance of the relationship between body size (lnCS) or habitat and molar shape by considering (or controlling) the phylogenetic relationship among species was the phylogenetic generalized least squares (PGLS). For which the covariance matrices were extracted from each phylogenetic topology using the PhyloCov module, with the help of the NTSYSpc 2.21f software (Exeter Publication, NY, USA, Rohlf 2009). PGLS analysis incorporates the phylogenetic matrix as an error term for the regression. This test is equivalent to computing independent contrasts in an independent manner for shape-variable blocks and re-performing a partial least square (PLS) analysis (Meloro et al. 2011). With this, and different from the variation partitioning analysis, we focus here on the independent significance of each predictor (body size or habitat) on molar shape, without relationship effects among species.

## Results

For sexual dimorphism, the two-way ANOVA was significant for the first molar size data for both factors (sex and species) and its interaction (sex: $F=45.650$, $p<0.001$; species: $F=282.229$, $p<0.001$; interaction: $F=3.141$, $p<0.001$). However, in the two-way MANOVA with molar shape data, the factor sex was not significant ($F_{8, 189}=0.323$; $p=0.956$), but for the factor species ($F_{288, 1568}=2.638$; $p<0.001$) and for the two factor (sex vs. species) interaction, the results were significant ($F_{216, 1568}=1.716$; $p<0.001$). Thus, because the interactions between sex and species are significant, we treat the sexes here as independent units of work, following Astúa et al. (2000).

The first seven RWs from the PCA performed with molar data explained cumulatively a total variation of 97.31% in females, and 96.82% in males. The first two RWs together explained 53.91% of total molar variance in females, and 56.29% in males. The first RW (29.64% females, 35.45% males) compared with the second RW (24.26% females, 20.84% males) showed important differences related to molar shape in didelphid species (Figure 3A–F).

Differences in talonid and trigonid proportions were perceived between males and females despite the partial overlap in the plots (Figure 3). Males were more distinguished among the size categories than females. Observing both axes 1 and 2, intermediate-sized species were distinguished as having more developed talonids, independent of sex. In this sense, some intermediate-sized species have a large talonid compared with the trigonid (e.g. *Caluromys* spp.) or a very elongated talonid compared with the trigonid (e.g. *Glironia*). Species with large body sizes, particularly males, show an elongated and narrow talonid and trigonid in the same proportion, given a general elongated format to the molar. Small-sized species tend to show a proportional talonid and trigonid, both parts being enlarged but not elongated (Figure 3A and B). In terms of habitat, males were more differentiated among the habitat categories than females, as arboreal females were well-overlapped with other categories. Axis 2 splits these categories better for both sexes, with arboreal species (such as *Caluromys*) showing larger talonid areas in comparison with terrestrial species (e.g. *Monodelphis*, *Chironectes*, and *Lutreolina*) which exhibited larger trigonid areas and a more elongated molar (Figure 3C and D). For the lineages of Didelphidae, the more separated groups were the more primitive ones, i.e. Caluromyinae and Glironiinae, with the first group presenting a wide talonid and a small trigonid area, and the second group with a narrow molar, given by the significant elongation of the talonid. Other lineages exhibit large overlapping, mainly for males; Marmosini and Thylamyini show a similar range of molar deformations, with some discrimination for females according to axis 2. Male Didelphini shows considerably less variation in molar shape with regard to other lineages, and female Marmosini tends to have a proportional talonid and trigonid, both slightly elongated. Representative genera of different tribes (Marmosini or Thylamyini) present large molars both for talonid and trigonid (such as *Monodelphis* and *Thylamys*), oriented to the left in axis 1 (Figure 3E and F).

The MANOVA performed on females showed significant differences in shape between species (Wilks's $\lambda=0.203$, $F_{40, 200}=6.092$, $p<0.001$), as well as in males (Wilks's $\lambda=0.224$, $F_{38, 188}=5.501$, $p<0.001$). Thus, it is possible to differentiate shape divergence especially between genera for both sexes (see Supplementary Appendices 2 and 3).

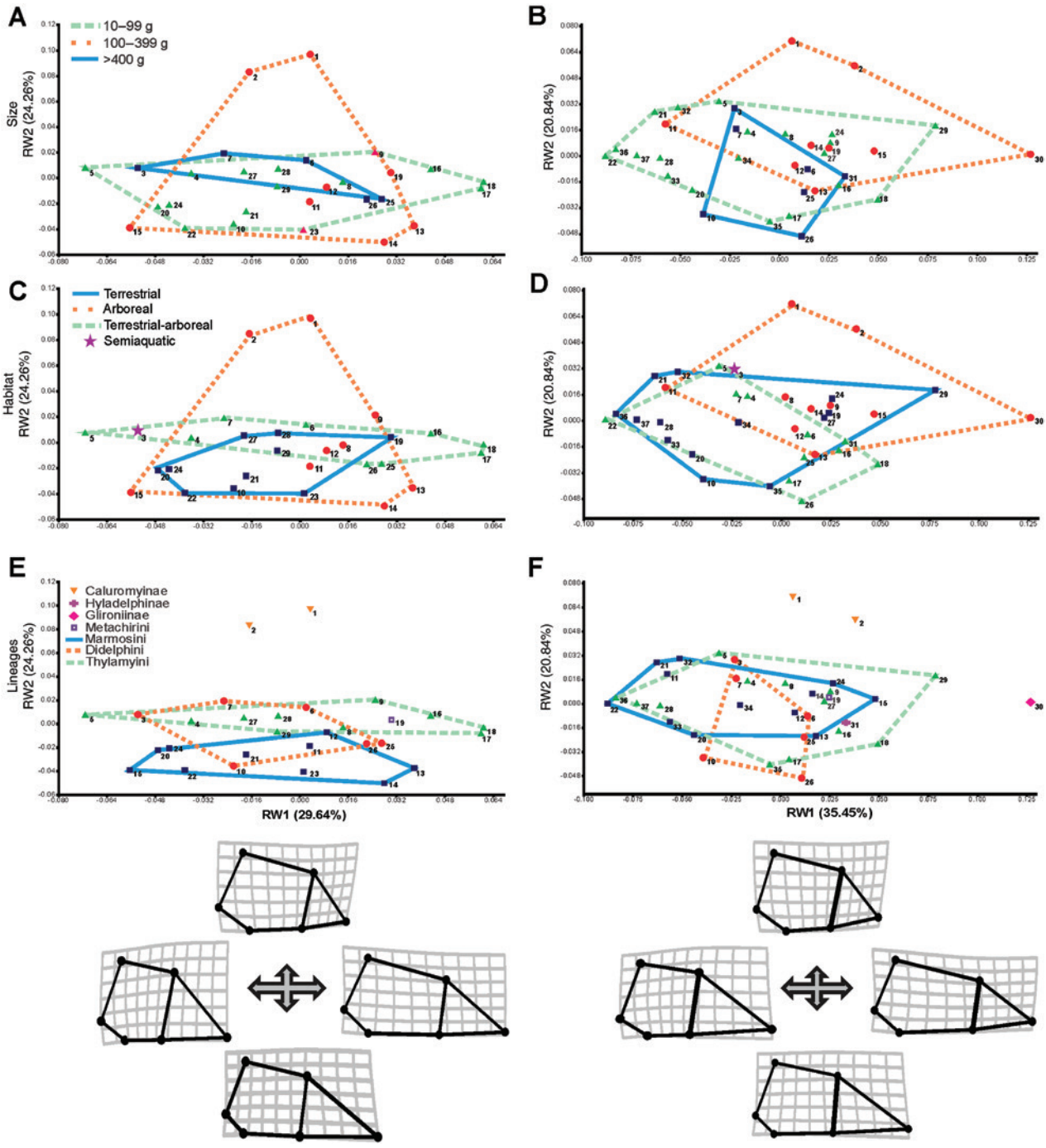


**Figure 3:** Scatter plot of relative warp (RW1 and RW2). Transformation grids visualize shape deformations relative to the mean at the positive and negative extremes of RW axes.
(A) body size of females; (B) body size of males; (C) habitat of females; (D) habitat of males; (E) lineages of females; (F) lineages of males. Body sizes classified according to Paglia et al. (2012) and subfamilies/tribes according to Voss and Jansa (2009). Subtitles: see in Supplementary Appendix 1.

For females, regression analyses showed a significant influence of body size, explaining 2.51% of total variance in shape (Wilks's $\lambda$=0.726, $F_{8,\ 121}$=5.695, p<0.001), whereas habitat explained 9.69% of total variance in molar shape (Wilks's $\lambda$=0.490, $F_{32,\ 436.8}$=2.909, p<0.001) (Table 2). Similar results were noticed for males, with body size explaining 1.91% (Wilks's $\lambda$=0.748, $F_{8,\ 122}$=5.127, p<0.001) and habitat explaining 8.01% of total shape variance (Wilks's $\lambda$=0.545, $F_{32,\ 440.4}$=2.451, p<0.001) (Table 2). Both for females and males, deformation grids of body

**Table 2:** Regression (lm) and PGLS analyses performed for females and males of Didelphidae, with PGLS using phylogenetic covariance matrices to test the association between molar shape and body size (lnCS) or habitat.

| | Wilks's $\lambda$ | $F_s$ | df1 | df2 | p-Value |
|---|---|---|---|---|---|
| Females | | | | | |
| Body size (lm) | 0.726 | 5.695 | 8 | 121 | **<0.001** |
| Habitat (lm) | 0.490 | 2.909 | 32 | 436.8 | **<0.001** |
| Body size (PGLS) | 0.971 | 0.442 | 8 | 121 | 0.894 |
| Habitat (PGLS) | 0.983 | 0.260 | 8 | 121 | 0.977 |
| Males | | | | | |
| Body size (lm) | 0.748 | 5.127 | 8 | 122 | **<0.001** |
| Habitat (lm) | 0.545 | 2.451 | 32 | 440.4 | **<0.001** |
| Body size (PGLS) | 0.683 | 7.059 | 8 | 122 | **<0.001** |
| Habitat (PGLS) | 0.946 | 0.877 | 8 | 122 | 0.538 |

Values of $p<0.05$ are indicated in bold.

size values (lnCS) showed slight opposite deformations, with molar enlargement in smaller females, especially regarding the talonid; the same is seen for larger males, which presented a generally larger talonid (Figure 4A and B). The molar deformation related to habitat suggests that more arboreal species tend to have slight enlargement of molar shape and terrestrial species tend toward a molar narrowing, but this is hardly noticeable in both sexes; arboreal males tend to display a larger talonid than terrestrial ones (Figure 4C and D).

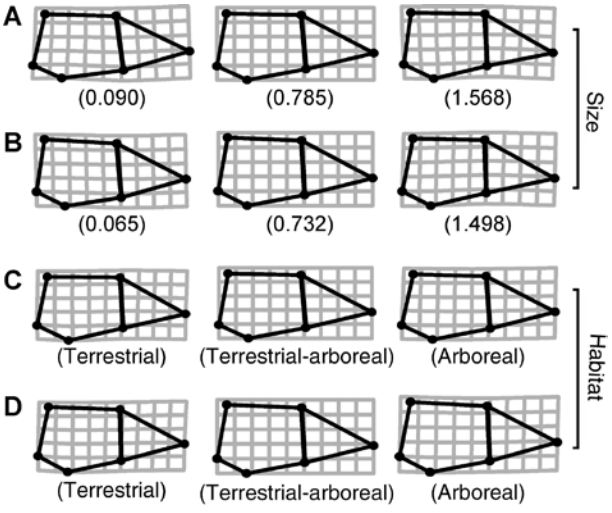


**Figure 4:** Shape deformations related to the first molar (m1). Deformation grids of the predicted shape of m1 for females (A) from the minimum (left, 0.090), medium (center, 0.785), and maximum (right, 1.568); males (B) from the minimum (left, 0.065), medium (center, 0.732), and maximum (right, 1.498) values of natural log-transformed centroid size (body size). Deformation grids related to habitat for females (C) and males (D) from the most terrestrial/semi-aquatic (left) to the most arboreal (right).

In variance partitioning analysis, the results for both sexes were similar (Table 3A and B). Phylogeny has the highest explanation of shape variance (females: Adj. $R^2$=16.17%; males: Adj. $R^2$=9.02%). Habitat was the predictor with the second highest explanation (females: Adj. $R^2$=7.46%; males: Adj. $R^2$=5.38%). In both sexes, the effect of body size had a low percentage of explanation (females: Adj. $R^2$=1.16%; males: Adj. $R^2$=1.47%). Although interactions between predictors were practically nonexistent,

**Table 3:** Variation partitioning results for South American Didelphidae.

| | Df | $R^2$ | Adj. $R^2$ | F | p-Value |
|---|---|---|---|---|---|
| A | | | | | |
| Size | 1 | 0.02676 | 0.01915 | 3.5192 | **<0.005** |
| Habitat | 3 | 0.09672 | 0.07522 | 4.4974 | **<0.005** |
| Phylogeny | 4 | 0.18823 | 0.16226 | 7.2463 | **<0.005** |
| Size+habitat | 4 | 0.11743 | 0.08919 | 4.1580 | **<0.005** |
| Size+phylogeny | 5 | 0.20821 | 0.17628 | 6.5213 | **<0.005** |
| Phylogeny+habitat | 7 | 0.28058 | 0.23930 | 6.7972 | **<0.005** |
| All factors | 8 | 0.29731 | 0.25085 | 6.3995 | **<0.005** |
| Individual fractions | | | | | |
| Size \| phylogeny+habitat | 1 | | 0.01156 | 2.8818 | **<0.005** |
| Habitat \| size+phylogeny | 3 | | 0.07458 | 5.1146 | **<0.005** |
| Phylogeny \| size +habitat | 4 | | 0.16166 | 7.7437 | **<0.005** |
| Size \| habitat | 1 | | 0.01397 | 2.9329 | **<0.005** |
| Size \| phylogeny | 1 | | 0.01402 | 3.1277 | **<0.005** |
| Habitat \| size | 3 | | 0.07004 | 4.2808 | **<0.005** |
| Habitat \| phylogeny | 3 | | 0.07704 | 5.2199 | **<0.005** |
| Phylogeny \| size | 4 | | 0.15712 | 7.1040 | **<0.005** |
| Phylogeny \| habitat | 4 | | 0.16408 | 7.7945 | **<0.005** |
| B | | | | | |
| Size | 1 | 0.01673 | 0.00911 | 2.1950 | **<0.005** |
| Habitat | 3 | 0.08001 | 0.05828 | 3.6819 | **<0.005** |
| Phylogeny | 4 | 0.11677 | 0.08873 | 4.1645 | **<0.005** |
| Size+habitat | 4 | 0.09674 | 0.06806 | 3.3737 | **<0.005** |
| Size+phylogeny | 5 | 0.13888 | 0.10444 | 4.0321 | **<0.005** |
| Phylogeny+habitat | 7 | 0.18960 | 0.14348 | 4.1110 | **<0.005** |
| All factors | 8 | 0.21003 | 0.15823 | 4.0545 | **<0.005** |
| Individual fractions | | | | | |
| Size \| phylogeny+habitat | 1 | | 0.01475 | 3.1548 | **<0.005** |
| Habitat \| size+phylogeny | 3 | | 0.05379 | 3.6624 | **<0.005** |
| Phylogeny \| size+habitat | 4 | | 0.09016 | 4.3740 | **<0.005** |
| Size \| habitat | 1 | | 0.00978 | 2.3330 | **<0.005** |
| Size \| phylogeny | 1 | | 0.01571 | 3.2103 | **<0.005** |
| Habitat \| size | 3 | | 0.05896 | 3.7203 | **<0.005** |
| Habitat \| phylogeny | 3 | | 0.05475 | 3.6848 | **<0.005** |
| Phylogeny \| size | 4 | | 0.09533 | 4.4330 | **<0.005** |
| Phylogeny \| habitat | 4 | | 0.08520 | 4.1582 | **<0.005** |

Molar shape represents the dependent variable. Body size (size), habitat, and phylogeny represent independent variables. p-Value test for the significance of F after 1000 permutations. Significance is highlighted in bold. The symbol | refers to the residual effect of the factor to its left, once the effect of the factor(s) to the right is accounted for. (A) females; (B) males.

there was a low level of interaction between phylogeny and habitat for males (Figure 5A and B).

PGLS analysis showed that the relationship between molar shape and body size in males is significant even for controlling phylogeny. For females, body size did not remain significant after controlling phylogeny. For both sexes, habitat did not remain significant when controlled for phylogenetic effects. This suggests that most molar variation in didelphids is influenced mainly by phylogeny and that only body size of males significantly influences a low percentage of this variation (Table 2). The interaction of phylogeny and habitat, although low, is evidence that the environment can contribute to molar adaptation.

## Discussion

One of the greatest evolutionary innovations in mammals was the development of tribosphenic molars, which are the basis of many dental adaptations we see today in the group (Ungar 2010). The shape variation of such molars in didelphids showed us that they are under the strong influence of phylogenetic relatedness rather than body size or habitat, confirming the findings of Chemisquy et al. (2015) on didelphid molars. Thus, body size assumes a secondary role when influencing molar shape adaptation in didelphids, and habitat is apparently not meaningful in such a role. However, when habitat is considered with phylogeny, this should play an important role in the adaptive radiation of didelphid lineages. Our large sample size allowed us to detect some differences about each sex that are important, such as regions of trigonid and talonid (as described above), as the sexual dimorphism is present in the group. This reinforces the importance of examining a large sample of specimens to detect differences that are not seen otherwise.

Didelphid marsupials are evidenced in the literature as animals which display sexual dimorphism in various features, whether it is related to skull or body size, with males being larger than females (Cáceres and Monteiro-Filho 1999, Astúa et al. 2000, Astúa 2010). Unlike the result that Chemisquy et al. (2015) reported, that is, no effect of size itself using both sexes together, the results found here corroborate the distinction of sexes in didelphids. For some species, molar shape is also different between males and females, supporting the importance of allometry already found in several mammal species (Isaac 2005, Lindenfors et al. 2007). Such differentiation could be driven by the influence of body size itself, but rather by different priorities regarding each sex strategy (Cáceres et al. 2012b). This occurs both within species and overall for the family Didelphidae (Gentile et al. 2012). As they are larger than females and have bigger home ranges, males tend to search for mates, setting foraging for food as a secondary priority (in opposition to females) (Ryser 1992, Cáceres 2003). Females, however, are much more related to food resource availability, ensuring food resources most of the time, with higher fidelity to specific parts of their home range (Hossler et al. 1994, Cáceres 2003, Cáceres and Machado 2013). These constraints between male and female life strategies could be driving by the molar shape differences seen in didelphids here, particularly those related to body size.

Chemisquy et al. (2015) used a relatively small sample (half the sample size used in this study), and often statistical tests for sexual dimorphism are only

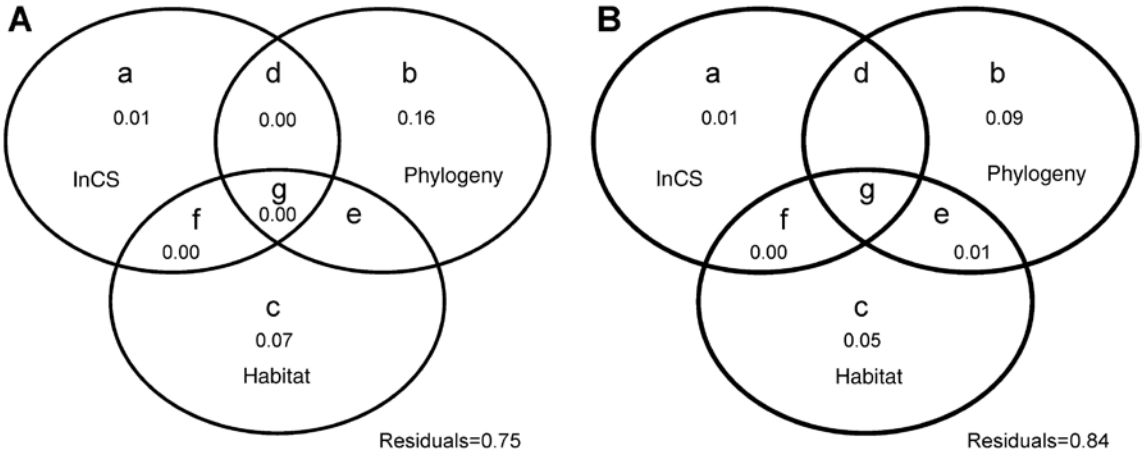


**Figure 5:** Graphics of the three factors analyzed in variation partitioning analyses to illustrate both their individual contribution for explaining shape variance.
(a) lnCS; (b) phylogeny; and (c) habitat and their interacting components (d, e, f, g). (A) females; (B) males.

significant when samples are large (Rossi et al. 2010; type II error). Therefore, this demonstrates the importance of treating the two groups separately, as the vast majority of studies have found important results when conveniently dividing the sexes. As already emphasized, sexual dimorphism is quite common in didelphids (Pine et al. 1985, Maunz and German 1996, Costa et al. 2003, Galliez et al. 2009, Rossi et al. 2010). The differences we observed between males and females (as the isolated effect of body size) corroborate mainly Astúa (2010), who found that sexual dimorphism is not homogeneous in all Didelphidae but at least in a large part of the group (50%–60% of the species with significant sexual dimorphism in cranial size or shape).

The apparent significance of the variable habitat throughout the analyses proved to be false when correcting by phylogeny, showing that both diet (following Chemisquy et al. 2015) and habitat (vertical use of trees) as the most likely ecological indicators of molar shape have no direct influence on them, at least when considering them alone. This occurs due to the fact that closely related species tend to be more similar than those that are more distant (Pagel 1999). That is, species relatedness often masks the influence of environment, which ultimately has less importance in shaping dental morphology than previously thought. Therefore, the phylogenetic signal in didelphid molar shape is high, implying that any ancestor more heavily dictates the molar shape of its descendant species, independent of environmental pressure (Polly 2001, Meiri et al. 2005, Alvarez et al. 2011).

Morphological changes in mammalian traits can be strongly dependent on phylogeny, especially structures such as teeth where most aspects of shape have the potential to be phylogenetically structured (Kangas et al. 2004). This has been seen by Hogue and ZiaShakeri (2010) in molar crests of marsupials, for example. *Caluromys* and *Glironia* (both belonging to Caluromyinae) are the most basal taxa in Didelphidae (Flores et al. 2010, Jansa et al. 2014) and retain a particular first molar shape (mostly with an expanded, elongated talonid area), well differentiated from the pattern seen in more advanced didelphids. This first molar pattern is lost with the subsequent evolution of didelphid lineages, where a major development of the trigonid took place, irrespective of habitat colonization. Maybe the possible mass extinction event that didelphids suffered during the early middle Miocene may have strongly reflected on their conservative phylogeny, especially when we know that most modern didelphid clades diversified relatively recently (Jansa et al. 2014).

In fact, major lineages of Didelphidae, such as Marmosini and Thylamyini, evolved to become similar in this molar trait, except perhaps for female Marmosini in some molar features (Figure 3E). Even the large-sized, derived lineage Didelphini shares similar molar shape configurations with these latter and advanced lineages. The ancestral didelphid was an arboreal, frugivorous form (Jansa et al. 2014) that now has a more developed talonid than trigonid, according to our data, which is related to a diet in which fruits are crushed using enlarged or elongated talonids (Voss and Jansa 2009). After that, lineages successively became more terrestrial, and, with time, the molar shape assumed a different configuration (now emphasizing the trigonid), mostly independent of habitat, facilitating insectivory throughout the elongated, cutting trigonids (Voss and Jansa 2009).

Despite the phylogenetic factor *per se*, slight differences due to habitat and body size can be perceived in molar shape variation. For habitat, this was seen through the interaction with phylogeny, meaning that habitat was somewhat important in the adaptive radiation of didelphid lineages, like in Thylamyini, which has radiated mostly to open, dry vegetation (Jansa et al. 2014). So, the adaptive radiation of didelphids is accompanied by a general change of habitat, from the more primitive and arboreal habitat to the more derived and terrestrial/scansorial habitat, linking to the molar shape configuration that evolved from a relatively small to a large trigonid. Such trend is however modulated by phylogeny. Following the probable Miocene mass extinction, the reduction of the forest environments allowed the development of open habitats giving more chances for diversification of the surviving Miocene terrestrial marsupials via a rapid cladogenesis (or adaptive radiation) (Jansa et al. 2014). This also had other evolutionary consequences by inducing a major shift in feeding habits of the family (from frugivory related to a larger talonid, to insectivory related to a larger trigonid; Voss and Jansa 2009), with lineages becoming more insectivorous with time. This may be related to the tendency in the family for descending from the trees and becoming more terrestrial in its adaptive radiation, which happened independently in different clades such as *Monodelphis* in Marmosini, and *Thylamys* and *Cryptonanus* in Thylamyini (Jansa et al. 2014). This is based on the fact that the talonid is configured to crush soft food, while the trigonid supports cutting hard material (Butler 1992, Luo et al. 2001).

Controlling for phylogeny, body size, particularly in males, had an important effect on molar shape. In this sense, intermediate-sized species in part matched with the most primitive taxa regarding molar shape, whereas the larger ones (e.g. Didelphini) tended to show elongated molars, and small taxa tended to show larger and wider

molars, both with large talonid and developed trigonid. These tendencies are relevant because they match with the expectation that large didelphid species will be generalist, mostly omnivorous, and that small species will tend to be more insectivorous (Arita and Fenton 1997, Fleagle 2013). Smaller species of Didelphidae apparently are well adapted to feed on insects, which are highly available for them (Case 1979, Paglia et al. 2012), mainly in terrestrial habitats (Santori et al. 1995, Astúa 2009). Despite their diet is primarily insectivorous, the fact that they have proportional equal parts of talonid and trigonid is possibly related to the resource seasonality, corroborating their omnivory for periods of food scarcity (Lessa and Geise 2014a). The absence of the relationship of size with molar shape for females, alternatively, could be an artifact of sampling, because there was a lack of two important clades for them in the analyses: *Glironia* and *Hyladelphys*.

The strong association between shape variation and phylogenetic relationships in Didelphidae did not suggest that the group is morphologically conservative, at least with regard to the first molar shape, which is in accordance to other authors that have been worked with different traits (e.g. Astúa et al. 2000, Astúa 2009). Thus, when considering molar dentition, didelphids did not conserve primitive characteristics, as molar shape configuration has changed with the evolution of derived lineages. The molar shape configuration evolved and changed throughout time and lineages, maintaining a strong phylogenetic signal as observed specifically in didelphids (our study; Chemisquy et al. 2015) and elsewhere in other mammals (Raia et al. 2010, Alvarez et al. 2011). The environment (habitat) presents a minor role in didelphid molar shape evolution, but has an intrinsic role in the adaptive radiation of lineages, such as those that happened in the late Miocene when open, dry habitats became available (Jansa et al. 2014). As already emphasized (Ungar 2010), molar adaptation also involves adaptive responses to dietary changes (which are habitat-dependent), as they are indicators of what food is being processed, and therefore should not be ignored in future studies (especially if fossil taxa are included).

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