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RH: SYSTEMATICS OF HAIRY ARMADILLOS**Systematics of hairy armadillos and the taxonomic status of the Andean hairy armadillo*****(Chaetophractus nationi)***

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Hairy armadillos constitute an ecologically homogeneous and morphologically similar group with currently 5 species classified in the subfamily Euphractinae. Among them, the Andean hairy armadillo *Chaetophractus nationi* (Xenarthra, Cingulata, Dasypodidae) is a small, endangered armadillo that has long been suspected to represent a high-altitude variant of *Chaetophractus vellerosus*. Here we report the 1st phylogenetic systematics assessment of hairy armadillos using morphological and molecular analyses of all described species with focus on the status of the Andean hairy armadillo. Multivariate analyses of shape variation based on 3-dimensional landmark coordinates of skulls allowed a clear differentiation of each species with the exception of *C. vellerosus* and *C. nationi*, within which only a latitudinal and/or altitudinal gradient in size was apparent. Moreover, analyses of mitochondrial DNA control region (D-loop) revealed a single *C. nationi* haplotype that appeared to be identical with a *C. vellerosus* haplotype from Argentina. Identical sequences in *C. vellerosus* and *C. nationi* were also observed for 3 of the 5 non-coding nuclear markers investigated. Based on these data, we propose that *C. nationi* should be considered as a synonym of *C. vellerosus*. However, this taxonomic change should not preclude the protection of the high altitude Bolivian populations that are steadily declining because of their overexploitation for traditional purposes. Finally, phylogenetic analyses of euphractine armadillos based on a combination of 6 non-coding nuclear markers and 2 nuclear exons suggested the paraphyly of the genus *Chaetophractus*, with *Zaedyus pichiy* being more closely related to *C. vellerosus* than to *C. villosus*.

RESUMEN

Los armadillos peludos constituyen un grupo ecológicamente homogéneo y morfológicamente similar que actualmente consiste de 5 especies clasificadas en la subfamilia Euphractinae. Una de ellas, el quirquincho Andino *Chaetophractus nationi* (Xenarthra, Cingulata, Dasypodidae), es un pequeño armadillo amenazado de extinción del cual se ha sospechado durante mucho tiempo que representa una variedad de altitud de *Chaetophractus vellerosus*. Aquí reportamos la primera evaluación de la filogenética sistemática de armadillos peludos utilizando análisis morfológicos y moleculares de todas las especies descritas, poniendo un especial enfoque en el estado del quirquincho Andino. Los análisis multivariados de la forma, basados en landmarks tridimensionales de cráneos, permitieron distinguir claramente las especies, con la excepción de *C. vellerosus* y *C. nationi* en los cuales sólo se halló un gradiente latitudinal y/o altitudinal en el tamaño. Además, los análisis de la región control del ADN mitocondrial (D-loop) mostraron un solo haplotipo de *C. nationi* que fue idéntico a un haplotipo de *C. vellerosus* de Argentina. También se observaron secuencias idénticas para *C. vellerosus* y *C. nationi* en 3 de los 5 marcadores nucleares no codificantes analizados. A partir de estos resultados proponemos que *C. nationi* sea considerado sinónimo de *C. vellerosus*. Sin embargo, este cambio taxonómico no debe excluir la protección de las poblaciones altoandinas de Bolivia que están sufriendo una reducción continua debido a su sobreexplotación para fines tradicionales. Finalmente, los análisis filogenéticos de los armadillos eufractinos basados en una combinación de 6 marcadores nucleares no codificantes y 2 exones nucleares sugieren la parafilia del género *Chaetophractus*, siendo *Zaedyus pichiy* relacionado más estrechamente con *C. vellerosus* que con *C. villosus*.

Key words: hairy armadillos, Euphractinae, *Chaetophractus*, morphometrics, molecular phylogenetics, taxonomy, systematics, conservation

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There are currently 21 described species of armadillos (Xenarthra: Cingulata, Dasypodidae—Gardner 2005). A recent reassessment of their conservation status has shown that virtually all species are affected by hunting and habitat degradation (Abba and Superina 2010). That study, as well as a more recent literature review by Superina et al. (2014), highlighted the scarcity of basic information on the natural history and population dynamics of many armadillo species, and even on the taxonomy of some species.

One clear example is the 5 euphractine armadillos: *Euphractus sexcinctus* (Linnaeus, 1758), *Zaedyus pichiy* (Desmarest, 1804), and 3 species of *Chaetophractus*, which over time have been considered a subfamily called Euphractinae (Frechkop and Yepes 1949), recognized as a tribe (Euphractini, Cabrera 1957), lumped into a single genus (*Euphractus*, Moeller 1968), and reclassified as a tribe by Wetzel (1985b). Subsequently McKenna and Bell (1997) included Wetzel's (1985b) tribes Chlamyphorini (fairy armadillos) and Euphractini into a single subfamily called Euphractinae, and this classification was adopted by Gardner (2005) in his reference taxonomy. However, based on the phylogenetic distinctiveness of fairy armadillos, Delsuc et al. (2012) recently proposed removing them from subfamily Euphractinae and retaining the subfamily Chlamyphorinae. As a consequence, the subfamily Euphractinae would now consist of the 5 species originally included by Frechkop and Yepes (1949), namely *Euphractus sexcinctus*, *Zaedyus pichiy*, *Chaetophractus villosus* (Desmarest, 1804), *Chaetophractus vellerosus* (Gray, 1865), and *Chaetophractus nationi* (Thomas, 1894). Nonetheless, several taxonomic inconsistencies and phylogenetic relationships within this subfamily still need to be elucidated.

The genus *Euphractus* contains a single species: the six-banded armadillo (*E. sexcinctus*). This relatively common species occurs from southern Suriname and adjacent Brazil to Uruguay and northeastern Argentina (Wetzel 1985a; Abba and Superina 2010). It is the largest species of Euphractinae and can be distinguished from the other members of this tribe by its pale yellow or tan carapace and the absence of a movable band at the anterior margin of the scapular shield (Redford and Wetzel 1985). The genus *Zaedyus* is also monospecific: the pichi (*Z. pichiy*) has the southernmost natural range of all armadillos, inhabiting arid and semi-arid environments of central and southern Argentina and Chile (Abba and Superina 2010; Superina and Abba 2014). Unlike other euphractines, the marginal scutes of its carapace bear sharply pointed apices, it has no pelvic glands, and it lacks teeth on the premaxillary (Wetzel 1985a, 1985b; Superina and Abba 2014).

The genus *Chaetophractus* is described as comprising 3 species of hairy armadillos (Wetzel 1985a, 1985b; Wetzel et al. 2008): the larger hairy armadillo (*C. villosus*), the screaming hairy armadillo (*C. vellerosus*), and the Andean hairy armadillo (*C. nationi*). *C. villosus* is considerably larger than the other 2 species (Wetzel 1985a) and has a wider range, occurring from the Chaco region of Bolivia, Paraguay, and Argentina as far south as Santa Cruz, Argentina and Magallanes, Chile (Gardner 2008). The main population of *C. vellerosus* inhabits the Chaco region of Bolivia, Paraguay, and Argentina, while a small, isolated population exists on the coast of Buenos Aires Province, Argentina (Abba and Superina 2010; Abba and Vizcaíno 2011). *C. nationi* was originally described based on a single prepared skin and the fragmentary skull of a young adult from Oruro, Bolivia, housed in the Natural History Museum of London (BMNH 93.9.9.1); there are no other specimens associated with the type specimen. In the only comprehensive account on the taxonomy and distribution of armadillos based on a search of museum collections, Wetzel (1982, 1985b) questioned the validity of *C. nationi* as a separate species from *C. vellerosus*. The only

difference between 2 putative *C. nationi* specimens from Jujuy Province in Argentina and *C. vellerosus* specimens was a broader cephalic shield (part of carapace that covers the head dorsally), a characteristic that Wetzel (1985b) did not consider sufficient to warrant their classification as a separate species and led him to postulate that *C. nationi* might be a high-altitude subspecies of *C. vellerosus*. As a consequence, small hairy armadillos are usually identified based on their relative size and capture site, with larger or more hairy individuals, as well as high-altitude specimens or those originating from western Bolivia, northern Chile and northwestern Argentina being assigned to *C. nationi*. Although the skull of *C. nationi* has not been extensively described, Carrizo et al. (2005) assigned 2 specimens from Tucumán Province in Argentina to *C. nationi*, based on differences of the squamosal-jugal suture and auditory meatus.

The ongoing taxonomic uncertainty about *C. nationi* is reflected in statements made about its distribution. According to Redford and Eisenberg (1992), *C. nationi* is the only armadillo that inhabits high-altitude environments in the Andean Region. Although Gardner (2005, 2008) limited the range of *C. nationi*, the Andean hairy armadillo, to Bolivia and northern Argentina, specimens assigned to this species have also been reported from the Andean Altiplano of Bolivia, Argentina, Chile, and Peru (Abba and Superina 2010). In Bolivia, it has been restricted to the southeastern region in the Oruro, La Paz, and Potosí departments (Anderson 1997). In Argentina, it has been reported from Jujuy, Salta, and Tucumán provinces (Carrizo et al. 2005; Vizcaíno et al. 2006; Abba et al. 2012). It also occurs in the southern regions of Tacna and Puno provinces of Peru (E. López Tejada, Universidad Nacional San Agustín, Arequipa, Perú, pers. comm., October 2012), as well as in the Chilean regions of Arica y Parinacota, Tarapacá, and Antofagasta (Muñoz and Yáñez 2009). The most recent range maps (Abba and Superina 2010) suggest that the distribution areas of *C. nationi* and *C. vellerosus* are separated by at least 80 km.

Finally, from the phylogenetic point of view, early molecular studies including all 3 genera of hairy armadillos failed to resolve the relationships within the subfamily Euphractinae (Delsuc et al. 2002, 2003). Indeed, the concatenation of 3 nuclear protein-coding exons and 2 mitochondrial genes slightly favored a *Chaetophractus* + *Euphractus* clade, but with low statistical support (Delsuc et al. 2003). The grouping of these 2 genera to the exclusion of *Zaedyus* would be congruent with the cladistic study of craniodental characters by Gaudin and Wible (2006). Conversely, a study based on 39 retroposon insertion loci and flanking sequences found multiple evidences for grouping *Chaetophractus* and *Zaedyus* to the exclusion of *Euphractus* (Möller-Krull et al. 2007). Such a relationship was strongly supported by the phylogenetic analysis of more than 12 kilobases of non-coding retroposon flanking sequences, and also by the shared presence of a specific DAS-III3b element insertion, plus a 19-nucleotide diagnostic deletion in the An5 genomic locus. This result is in contradiction with craniodental characters (Gaudin and Wible 2006), but appears to be compatible with relationships obtained from postcranial evidence (Abrantes and Bergqvist 2006). One potential explanation for the discrepancy between these 2 molecular studies may lie in the species used as representatives for the genus *Chaetophractus*. Delsuc et al. (2003) analyzed *C. villosus*, whereas Möller-Krull et al. (2007) relied on *C. vellerosus*. In fact, these 2 species have never been included in the same molecular or morphological phylogenetic data set and may turn out to be only distantly related, in which case such inconsistencies are to be expected.

In the present work, we aimed to investigate the phylogenetic systematics of Euphractinae by including for the first time all extant species in an integrative approach using skull geometric morphometrics and molecular phylogenetic analyses based on mitochondrial and nuclear markers. Within this context we are particularly interested in testing the putative

taxonomic distinctiveness of *C. nationi* and how this may impact the conservation status of Andean hairy armadillos.

MATERIALS AND METHODS

Morphology

We used qualitative and quantitative approaches to evaluate the morphological differences between species. With the qualitative approach we analyzed the variation of the squamosal-jugal suture to assess its specific diagnostic value. Quantitative analyses of form were carried out through classical and geometric morphometrics as described below.

Specimens and landmark data. — We used 2 sets of samples. First, a total of 70 prepared skins assigned to *C. nationi* and *C. vellerosus* were used to measure and evaluate proportions of the cephalic shield (Supporting Information S1). The 2nd set of samples comprised a total of 158 skulls of adult euphractine species and was used to conduct the skull morphogeometric analyses. This latter set included a total of 9 specimens of *C. nationi*, 73 of *C. vellerosus*, 26 of *C. villosus*, 31 of *Z. pichiy*, and 19 of *E. sexcinctus* from mammalogical collections of the Museo Argentino de Ciencias Naturales ‘Bernardino Rivadavia’ (in Buenos Aires), the Colección Fundación Historia Natural Félix de Azara and the Museo de La Plata in Argentina, and the Colección Boliviana de Fauna (in La Paz) in Bolivia (Supporting Information S2). The 9 specimens assigned to *C. nationi* were restricted to an area near the type locality (Oruro, Bolivia). The specimens of *C. vellerosus*, *C. villosus*, *Z. pichiy*, and *E. sexcinctus* covered a broad geographical range. Specimens assigned to *C. nationi* were usually identified based on their site of origin, size, or amount of hair on the carapace (as described in the introduction).

We defined 100 cranial landmarks (45 taken on each side and 10 on the midline; Fig. 1; Supporting Information S3). Three-dimensional landmark coordinates were acquired using

a Microscribe G2L digitizer (Immersion Corporation, San José, California). They include landmarks of type I (anatomical), II (mathematical), and III (semi-landmarks) from the lateral side and midline. Each specimen was digitized 3 times and a consensus specimen was used to account for measurement error.

Statistical analyses of size differences.—The morphological analyses were carried out in a morphogeometric framework (Dryden and Mardia 1998). The data were analyzed using MorphoJ (Klingenberg 2011) and Morpho package (Schlager 2013) within R statistical software (R Development Core Team 2012), which supports the use of 3-dimensional sources. The centroid size was used as a proxy for size (Goodall 1991; Dryden and Mardia 1998). According to Hood (2000), centroid size is a geometric measure of size that follows the same mathematical behavior as body mass. Other authors (Frost et al. 2003; Ercoli and Prevosti 2011; Cassini et al. 2012; Meloro and O'Higgins 2012) found that centroid size was highly correlated with body mass and, consequently, an excellent variable for its prediction.

Differences in centroid size among euphractine species, and those between putative *C. nationi* from Oruro (Bolivia, 18°00'S, 67°00'W, 3,700 m above sea level [asl]) and *C. vellerosus* from 3 localities in Argentina (which varied in latitude and altitude) were assessed with the Kruskal-Wallis rank sum test and the Tukey honest significant difference (HSD) multiple comparison test. The 3 localities in Argentina were Catamarca province (northernmost, 27°00'S, and highest in altitude, 3,800 m asl), Córdoba province (intermediate in latitude, 33°00'S, and altitude 500 m asl), and Buenos Aires province (southernmost, 35°10'S, and lowest in altitude, 5 m asl). The sequential Bonferroni correction (Rice 1989) was used to address the problem of multiple statistical tests.

Multivariate shape variation analyses.—The 1st step consisted of landmark configuration superimposition using rotation, translation, and reflection transformations in order to remove spatial variation that did not correspond to form. This was achieved using the

generalized Procrustes analysis (Rohlf 1990) in R. The landmark configurations were projected from the curve Kendall to the tangent Euclidean space (Bookstein 1996), which allows exploration of shape variation using multivariate analyses (e.g., principal component analysis, canonical variate analysis, and discriminant analysis). The Morpho package in R allows 3-dimensional rendering to visualize the change in shape along the principal component of interest. A scanned surface of a putative *C. nationi* cranium (mesh3d object) was then warped using the target landmark configuration by using a thin-plate spline interpolation. A color ramp was incorporated to the mesh material to represent the strength of shape change from the consensus. The patterns of shape change were evaluated in relation to the taxonomy, which allowed defining morphospaces. Canonical variate analysis was used in MorphoJ to find the shape features that best distinguished species. To assess the influence of allometry, the landmark coordinates of aligned specimens were regressed on log₁₀-transformed centroid sizes using MorphoJ software. Then a new canonical variate analysis was performed on the regression residuals (i.e., the shape component not related to size, either geometrical or allometrical).

Discriminant analyses were performed using Procrustes superimposed coordinates (half of crania to avoid colinearity) and repeated using the principal component scores. Correlation coefficients were calculated against the number of components to select the number of principal components to retain. This information was then used to determine how many variables were needed to summarize most shape variation (Fadda and Corti 2000; Cardini et al. 2007).

Discriminant analyses were also performed to evaluate species phenotypic distinctiveness (Cardini et al. 2009). We used this approach in 2 steps, building discriminant functions using subsamples (learning sample) consisting of 5 specimens from, 1st, each euphractine species, and, 2nd, the same specimens of *C. nationi* and *C. vellerosus* that were

used to compare centroid size. These discriminant functions were used to classify the remaining specimens (test sample) that were not included for obtaining the functions. This approach is equivalent to a hold-out sample cross-validation (Hair et al. 1998) for the purpose of assessing the effect of very small sample sizes. Over-fitting is avoided by predicting group affiliation using discriminant functions based on samples that do not include the specimens that are being classified. Instead of taking random subsamples we modified the Cardini et al. (2009) hold-out cross-validation protocol by computing all possible combinations of 5 individuals of each species (126 for putative *C. nationi*; 237,336 for all *C. vellerosus*; 65,780 for *C. villosus*; 169,911 for *Z. pichiy*, and 11,628 for *E. sexcinctus*; combinations for *C. vellerosus* at the 3 localities that varied in latitude and elevation were 237,336 for Buenos Aires; 56 for Córdoba; and 126 for Catamarca and Oruro). The discriminant analysis was repeated 126 times for the euphractine species and 56 times for localities to use all possible combinations of small samples (i.e., putative *C. nationi* and Córdoba locality, respectively). One of all possible combinations of the other categories was also selected randomly (with the combinations eliminated after being used). Thus, the same 5 individuals per species or locality were never used twice. This series of cross-validated discriminant analyses allowed us to explore the robustness of predictions in which sampling error is very large, as may happen when poorly studied populations are included in the analysis.

Molecular analyses

We assessed the phylogenetic relationships of hairy armadillos by collecting both mitochondrial and nuclear markers for all currently described species.

Data acquisition.—Biological samples were collected from 10 Andean hairy armadillos (putative *C. nationi*) kept at the Andean Municipal Zoo in the city of Oruro (Bolivia). The animals came from different localities of the department of Oruro in Bolivia; the zoo staff provided information on capture sites and dates. American Society of

Mammalogists' guidelines (Sikes et al. 2011) were followed for handling of animals, and non-invasive sampling techniques were used to minimize stress. Two buccal swab samples were taken from each animal to harvest epithelial cells by gently rubbing sterile cotton swabs against the inner cheek. These samples were taken before the morning meal in order to prevent any possible contamination in the subsequent DNA extractions. Buccal swabs were dried at room temperature and then wrapped individually in sterile covers. DNA extractions from buccal swabs were performed in La Paz (Bolivia) using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) according to manufacturer instructions.

Following Poljak (2009), who previously conducted phylogeographic studies of *C. villosus* and *C. vellerosus* based on a 485 bp fragment of the 5'-end of the mitochondrial control region (D-loop), we collected comparative data of the same marker for the 10 putative *C. nationi* individuals using the universal primers Thr-L15926: 5'-CAATTCCCCGGTCTTGTAACC-3' and DL-H16340: 5'-CCTGAAGTAGGAACCAGATG-3' (Vilà et al. 1999). We additionally considered 10 individuals of *Z. pichiy* from Mendoza province in Argentina and 1 of *C. villosus* for which tissue samples were available in the Mammalian Tissue Collection of the Institut des Sciences de l'Evolution de Montpellier, France (Catzefflis 1991). For these samples, total genomic DNA was extracted from tissues preserved in 95% ethanol, using the QIAamp DNA extraction kit (QIAGEN GmbH, Hilden, Germany). Amplification by polymerase chain reaction of the mitochondrial D-loop from these extracts was conducted using newly designed primers: DLP_Eup_fwd (direct): 5'-GGTCTTGTAACCATAAATG-3' and DLP_Eup_rev (reverse): 5'-CCTGAAGAAAGAACCAGATG-3'. The following polymerase chain reaction conditions were used: 95°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 30 s (denaturation), 55°C for 30 s (hybridization), 72°C for 45 s (extension), and a final extension step at 72°C for 10 min.

We also selected 6 non-coding nuclear markers (M133, M161, M219, M255, NC75, and An5) from the 39 genomic fragments used by Möller-Krull et al. (2007). These markers were chosen in order to maximize nucleotide variability among the available sequences for euphractine armadillos: a screaming hairy armadillo (*C. vellerosus*), a six-banded armadillo (*E. sexcinctus*), and a pichi (*Z. pichiy*). For each non-coding locus, the sequences from these 3 species were aligned using MAFFT (Katoh et al. 2005), and potential polymerase chain reaction primer pairs were designed automatically within Geneious Pro (Kearse et al. 2012) using Primer3 default parameters (Rozen and Skaletsky 2000). The newly defined primers (Supporting Information S4) were then used to amplify the 6 non-coding nuclear fragments in an Andean hairy armadillo (putative *C. nationi*) from Oruro (Bolivia), a large hairy armadillo (*C. villosus*), and a new screaming hairy armadillo individual (*C. vellerosus*) from Mendoza (Argentina). The following polymerase chain reaction conditions were used for all 6 non-coding nuclear markers: 95°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 30 s (denaturation), optimal annealing temperature (Supporting Information S4) for 30 s (hybridization), 72°C for 45 s (extension), and a final extension step at 72°C for 10 min.

Finally, we targeted 2 protein-coding nuclear exons (BRCA1 exon 11 and VWF exon 28) that previously have been successfully used for xenarthran phylogenetics (Delsuc et al. 2002, 2003, 2012). Partial sequences of these exons were amplified for the same *C. vellerosus* individual as for the non-coding nuclear markers, in the following overlapping fragments, using primers defined in Delsuc et al. (2012): B21-C788 (760 bp), B608-C1250 (650 bp), and B1204-C1843 (650 bp) for BRCA1, and V161-W649 (490 bp) and V431-W980 (550 bp) for VWF. Polymerase chain reaction conditions for the 2 protein-coding nuclear markers were: 95°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 30 s (denaturation), 55°C for 30 s (hybridization), 72°C for 45 s (extension), and a final extension step at 72°C for 10 min.

All mitochondrial and nuclear polymerase chain reaction products were then purified from 1% agarose gels using Amicon Ultrafree-DA columns (Millipore Corporation, Bedford, Massachusetts) and sequenced on both strands using the polymerase chain reaction primers with the Big Dye Terminator cycle sequencing kit on an Applied ABI Prism 3130XL automated sequencer. The newly obtained sequences have been deposited in the European Nucleotide Archive under accession numbers HG514997 to HG515006 and LN613154 to LN613182.

Phylogenetic analyses.—The new mitochondrial D-loop sequences, including 10 obtained for *C. nationi* from Bolivia, 10 for *Z. pichiy* from Argentina, and 1 for a *C. villosus* from Argentina, were combined with available sequences of the same fragment for 30 *C. vellerosus* (Poljak 2009) and 76 *C. villosus* (Poljak et al. 2010), all coming from individuals sampled in Argentina. The 127 sequences were aligned using MAFFT within Geneious Pro, and removing 2 sites containing gaps for less than half of the sequences led to a final alignment containing a total of 483 nucleotide sites.

The new euphractine sequences obtained for each of the 6 non-coding nuclear markers were combined with the armadillo sequences previously obtained by Möller-Krull et al. (2007). Each individual data set was then aligned using MAFFT and manually trimmed to the region amplified with the new primers. Ambiguously aligned sites were then filtered by using the program Gblocks (Castresana 2000) with default relaxed parameters. The subsequent concatenation of the 5 non-coding data sets yielded an alignment totaling 2,131 nucleotide sites for 11 taxa.

The new BRCA1 and VWF sequences obtained for *C. vellerosus* were added to the armadillo sequences previously obtained by Delsuc et al. (2012). Each nuclear protein-coding data set was then aligned using MACSE (Ranwez et al. 2011), which allows conservation of the coding-frame. Ambiguously aligned codons were then filtered using Gblocks (Castresana

2000) with default relaxed parameters. This resulted in final alignments of 2,793 sites for BRCA1 and 1,221 sites for VWF. The combination of the 2 nuclear protein-coding data sets led to an alignment of 4,014 nucleotide sites for 10 taxa. The final nuclear concatenation of non-coding and protein-coding data sets represents an alignment of 6,145 nucleotide sites for 10 taxa with 6% missing data. All data sets are available upon request.

A phylogenetic network was first constructed from Kimura-2-parameter distances inferred from the complete D-loop mitochondrial data set using the neighbor-net algorithm (Bryant and Moulton 2004) within SplitsTree4 (Huson and Bryant 2006). Maximum likelihood phylogenetic reconstructions were then conducted for the 3 nuclear data sets (nuclear non-coding, nuclear protein-coding, and nuclear concatenation) with RAxML (Stamatakis 2006) using separate GTRGAMMA models for accommodating heterogeneity in substitution patterns among partitions: 6 for the nuclear non-coding data set, 2 for the nuclear protein-coding data set, and 8 for the nuclear concatenation. Maximum likelihood bootstrap values (BP_{ML}) were computed by using 1,000 pseudo-replicates of the same maximum likelihood heuristic search. Bayesian phylogenetic reconstructions were conducted with PhyloBayes 3.3f (Lartillot et al. 2009) using the CAT-GTR-G4 site-heterogeneous mixture model (Lartillot and Philippe 2004). Two independent Monte Carlo Markov Chains (MCMCs) starting from random trees were run for 100,000 cycles with trees and associated model parameters being sampled every 10 cycles. The initial 1,000 trees (10%) sampled in each MCMC analysis were discarded as burn-in as determined by monitoring both the log-likelihood value across generations and the maximum difference in clade posterior probabilities between the 2 chains. The 50% majority-rule Bayesian consensus tree and the associated posterior probabilities (PP_{CAT}) were computed from the remaining 18,000 (2x9,000) combined trees.

RESULTS

In this section, we first present the results of the morphometric analyses aiming at revealing the morphological differentiation among hairy armadillo species in terms of both size and shape. Then, we report on the molecular phylogenetic results obtained from the analysis of mitochondrial and nuclear markers.

Morphology

Squamosal-jugal suture.—A close examination of the squamosal-jugal suture in specimens from a population of *C. vellerosus* in its easternmost distribution in Argentina (Magdalena, Buenos Aires) and putative *C. nationi* from Oruro in Bolivia revealed substantial variability, with rounded and angular sutures found in both populations (Fig. 2).

Cephalic shield and skull variations.—Descriptive statistics (median, quartile, and range) of width:length ratio of the cephalic shield of the subsample of *C. vellerosus* and putative *C. nationi* grouped for localities are shown in Fig. 3a. There is an apparent general pattern with specimens from the northern end of the distribution having higher median ratios than those from the southeastern end. There is, however, much overlap among the specimens from high and low altitudes independent of latitude. For example, compare Catamarca (300 to 3,700 m asl) in the north, to Buenos Aires (5 to 150 m asl), San Luis (around 400 m asl), and Mendoza (500 to 1,900 m asl) in the south. The specimens from the northernmost end of the distribution in Santa Catalina (Jujuy, Argentina, 3,800 m asl), Oruro (3,700 m asl), and Nuestra Señora de La Paz (Bolivia, around 4,000 m asl) show higher means and quartiles, but their lower range end overlaps with the upper ends of all other populations.

The mean centroid size \pm standard deviation values in euphractine species are compared in Fig. 3b. The centroid size values for each species followed a normal distribution (all Shapiro-Wilk tests were non-significant; $W \approx 0.97$ for each species). In addition we found

significant differences among species with both parametric and non-parametric tests (ANOVA $F_{(4, 153)} = 1745.2$; Kruskal-Wallis $X^2 = 123.797$, $d.f. = 4$; $P < 0.0001$ for both tests). The post-hoc Tukey Honest Significant Difference test indicated that all pair-group comparisons were significant at $P < 0.0001$ after Bonferroni correction.

In the latitudinal and altitudinal analysis (Fig. 3c), centroid size values of the 4 localities were normally distributed (all Shapiro-Wilk tests were non-significant; $W \sim 0.92$), and there were significant differences among localities with both parametric and non-parametric tests ($F_{(3, 55)} = 18.503$; Kruskal-Wallis $X^2 = 29.46$, $d.f. = 3$; $P < 0.0001$ for both). However, the post-hoc Tukey Honest Significant Difference test indicated that only 3 of 6 pair-wise comparisons were significantly different. *C. nationi* from Oruro had significantly larger centroid size than *C. vellerosus* from Córdoba and Buenos Aires ($P < 0.001$ after Bonferroni correction), and *C. vellerosus* specimens from Catamarca (the northernmost and highest locality) were significantly larger than those from Buenos Aires ($P = 0.00046$ after Bonferroni correction), but not significantly different from Bolivian putative *C. nationi* specimens ($P = 0.194$ after Bonferroni correction). These results indicate that there were no differences in centroid size between adjacent localities, but suggest a latitudinal and/or altitudinal gradient in size.

The principal component analysis of tangent space coordinates of euphractine crania resulted in the first 15 principal components accounting for about 75% of the total variation; a total of 29 principal components were necessary to reach 85%. Only the morphospace depicted by principal coordinates 1 and 2 (~46% cumulative variance) showed a clear taxonomic differentiation between all species, except between putative *C. nationi* and *C. vellerosus* (Fig. 4a). *Z. pichiy*, *C. nationi*, and *C. vellerosus* lie on the negative side of principal component axis 1, while *C. villosus* and *E. sexcinctus* are located on the positive side. On principal component axis 2, *Z. pichiy* and *E. sexcinctus* lie on the negative side, *C.*

villosus is around zero, and putative *C. nationi* and *C. vellerosus* are on the positive side. The morphospaces depicted by the remaining principal components are uninformative, with the exception of the 3rd principal component (accounting for 6% of variation) that separates *C. villosus* on negative values from all other species.

The variation in shape associated with principal component axis 1 (24.73% of variation) involves almost all landmarks except those of the palate. On the negative values the rostrum is low, with the nasal extending caudally. The zygomatic arch is laterally expanded, and the zygomatic process of the jugal is ventrally expanded. The orbits are large and displaced dorsally and posteriorly. The cranial vault is high and wide, and the nuchal crest is anteriorly displaced. The foramen magnum is larger and displaced ventrally, as are the condyles. Positive values show opposite tendencies (Fig. 4b). This morphology represents a slender but high rostrum with a more globular calvarium on the negative side, while on the positive side the cranium is lower and larger. The variation in shape associated with principal component axis 2 (21.83%) also involves all landmarks. On the negative side, the premaxillary, along with the jugal teeth and palate, extend and narrow anteriorly. The orbits are posteriorly displaced and the descending process of the jugal is displaced medially and dorsally. While the nuchal crest displaces posteriorly, the foramen magnum does so anteriorly. The opposite tendency is shown on the positive side (Fig. 4c). This morphology represents a long snout on the negative side, and a short rostrum with an expanded jugal descending process on the positive.

The canonical variate analysis of euphractine species supports differentiation. Four canonical variate axes were obtained ($n-1$ categories); the 1st accounted for ~95% of the variance and showed a clear separation of *C. villosus* from *E. sexcinctus* and a group formed of *Z. pichiy*, putative *C. nationi* plus *C. vellerosus*, which have the same canonical variate analysis values on axis 1. These latter 3 species were differentiated on canonical variate

analysis axis 2, which only accounted for 2.5% of total variance. All possible pairs of Procrustes distances were significant ($P < 0.0001$) after 10,000 rounds of permutation tests (Table 1; values above of the diagonal).

The regressions of shape on log₁₀-transformed centroid size were significant (P -value < 0.0001) after 10,000 rounds of permutation tests (Fig. 5a). The allometric scaling of overall cranial shape variation was about 24.65%. The main shape change from small forms to larger ones involved a longer palate, short nasal length on the midline, larger frontal, lower height of the calvaria and occiput, broader muzzle, and a smaller orbit (Fig. 5b). *Z. pichiy*, *C. vellerosus*, and putative *C. nationi* formed a compact and largely overlapping point cloud in the regression shape space (Fig. 5a). The canonical variate analysis on the regression residuals supports species differentiation. Three canonical variate axes, accounting for over 99% of cumulative variance, were obtained. The 1st one accounted for ~57% of variance and the 2nd for ~29%. These 2 canonical variate analysis axes showed a clear separation between all species. All possible pairs of Procrustes distances were significant ($P < 0.0001$) after 10,000 rounds of permutation tests (Table 1; values below the diagonal).

The mean percentages of individuals classified by discriminant function analyses according to species are shown in Table 2 (hold-out cross-validation). For the hold-out samples, results were averaged across the 126 repetitions. The upper part of Table 2 is based on half cranium using shape coordinates and the lower part used the first 25 principal component scores (82.34 % of cumulative shape variation and 99.37% of correlation with Procrustes chord distances). The specimens not included in the model were correctly classified, on average, over 90% of the time when shape coordinates were used. With principal component scores, the mean percentage of correct classifications was lower in almost all species but principally in putative *C. nationi* and *C. vellerosus*. These results indicate that Procrustes chord distances show higher phenotypical distinctiveness than

principal component scores. All species had high phenotypical distinctiveness; although *C. vellerosus* and putative *C. nationi* had the lowest values, they were still extremely high (> 90%), even when specimens at the 2 extremes of the geographical distribution were assessed.

The mean percentages of individuals classified by discriminant function analysis based on half cranium using shape coordinates, according to localities are shown in Table 3 (hold-out cross-validation). For the hold-out samples, results were averaged across the 56 repetitions. Except for the Oruro population, the specimens not included in the model were not correctly classified the majority of the time. Interestingly, the percentage of specimens that were confounded with Oruro specimens increased with the elevation of the other localities (from 2 to 11% of the time).

Molecular phylogenetics results

The phylogenetic network analysis of mitochondrial data showed 3 distinct groups: *C. villosus* and *Z. pichiy* each form distinct sequence clusters, whereas the sequences of putative *C. nationi* fall inside the haplotype diversity of *C. vellerosus* in a single group (Fig. 6). The distance network also illustrates the distinctiveness of *C. villosus*, which appears quite distant from the *Z. pichiy* and *C. vellerosus* + *C. nationi* clade. Additionally, D-loop sequences of *C. nationi* and *C. vellerosus* share a diagnostic insertion of a single nucleotide (adenine) at position 324 of the alignment relative to *Z. pichiy* and *C. villosus*. In fact, only a single mitochondrial haplotype was detected among the 10 D-loop sequences obtained for *C. nationi* despite the fact that the animals were collected at different localities within the department of Oruro in Bolivia. Moreover, this single putative *C. nationi* haplotype is identical to *C. vellerosus* haplotype C, represented by 3 sequences, which all originate from Taco Ralo (Tucumán, Argentina—Poljak 2009). These results demonstrate that *C. vellerosus* and *C. nationi* share common haplotypes and can therefore not be distinguished on the basis of sequence diversity of their mitochondrial control region. They also show that

mitochondrial genetic diversity is reduced in *C. nationi*, with a single haplotype shared by 10 individuals found at the scale of the Oruro department.

The concatenation of the 6 nuclear non-coding loci represented a total of 2,131 sites of which 475 were variable (22%) and 216 parsimony informative (10%) for the 11 armadillo species considered. These figures dropped to 55 variable (2.5%) and 13 parsimony informative (0.6%) sites when considering only euphractine taxa. The maximum likelihood tree inferred from the concatenation (Fig. 7a) revealed relationships relatively congruent with the results obtained by Möller-Krull et al. (2007) on the basis of an extended set of 39 nuclear genomic fragments of the same type. This phylogenetic tree, rooted with Dasypodinae (*Dasypus*), supports the monophyly of the subfamilies Tolypeutinae (*Priodontes*, *Cabassous* and *Tolypeutes*), and Euphractinae (*Euphractus*, *Zaedyus* and *Chaetophractus*) in agreement with previous studies based on mitochondrial and nuclear markers (Delsuc et al. 2002, 2003). However, the pink fairy armadillo (*Chlamyphorus truncatus*; Chlamyphorinae) appeared as the sister-group of Euphractinae rather than with Tolypeutinae as in Möller-Krull et al. (2007; see also Delsuc et al. 2012), but this alternative position received only limited support.

The biggest surprise in this tree was perhaps the paraphyly of the genus *Chaetophractus*. *C. villosus* clustered with *E. sexcinctus* with moderate support ($BP_{ML} = 74$; $PP_{CAT} = 0.79$) on the one side, and *Z. pichiy* unambiguously grouped with *C. vellerosus* and *C. nationi* ($BP_{ML} = 100$; $PP_{CAT} = 1.0$) on the other side. The regrouping of *E. sexcinctus* and *C. villosus* is nevertheless incompatible with the 19-nucleotide shared deletion occurring in An5 for *C. villosus*, *Z. pichiy*, and *C. vellerosus*. Finally, *C. vellerosus* and *C. nationi* appear strongly monophyletic and have very similar sequences, as illustrated by the very short branches separating the 2 species in the tree. Indeed, for the 5 non-coding nuclear fragments that can be compared, the 2 species have identical sequences for M133 (334 bp), M161 (641

bp), and NC75 (142 bp), with differences only occurring in M219 (1 A-G transition in 321 bp) and mostly in M255 (2 C-T transitions and two 2-bp deletions in 259 bp).

The combination of the 2 nuclear exons BRCA1 and VWF yielded a total of 4,014 nucleotide sites, of which 526 were variable (13%) and 250 parsimony informative (6%) for the 10 armadillo species included. Again, these numbers were highly reduced when focusing solely on Euphractinae, with only 55 variable (1.4%) and 5 parsimony-informative (0.1%) sites. The maximum likelihood phylogram obtained from these nuclear genes (Fig. 7b) strongly supported the respective monophyly of Tolypeutinae and Euphractinae as previously reported (Delsuc et al. 2003). The sister-group relationship between Chlamyphorinae and Tolypeutinae was recovered, but with moderate to low statistical support. However, this protein-coding nuclear data set left unresolved the relationships within both Tolypeutinae and Euphractinae, where internal nodes received low statistical support, especially from the Bayesian analysis. In the maximum likelihood phylogram, the genus *Chaetophractus* appears monophyletic within Euphractinae, *C. villosus* grouping with *C. vellerosus* with moderate support ($BP_{ML} = 75$). However, the Bayesian analysis did not support such a node. Finally, *Z. pichiy* appeared as the sister-group to the remaining euphractine species, but again with only moderate to low statistical support.

The phylogenetic trees inferred from the concatenation of all non-coding markers and exons totaling 6,145 nucleotide sites were fully congruent between maximum likelihood and Bayesian approaches. The maximum likelihood phylogram (Fig. 7c) was in accordance with previously obtained phylogenies (Möller-Krull et al. 2007; Delsuc et al. 2012) concerning the sister-group relationship between Chlamyphorinae and Tolypeutinae and the grouping of *Cabassous* and *Tolypeutes* within Tolypeutinae. Within Euphractinae, the maximum likelihood topology obtained from the concatenation was fully congruent with the phylogeny obtained from the most informative non-coding partition in supporting the paraphyly of the

genus *Chaetophractus* (Fig. 7a). The only differences are in statistical support values, which slightly increased for joining *E. sexcinctus* and *C. villosus*, and slightly decreased for grouping *Z. pichiy* with *C. vellerosus*.

DISCUSSION

Taxonomic status of the Andean hairy armadillo.—The taxonomic status of *C. nationi* and its distinction from *C. vellerosus* have long been a matter of contention (Wetzel 1985b; Carrizo et al. 2005). This study assessed for the first time the taxonomic status of the Andean hairy armadillo, *C. nationi*, using geometric morphometrics and molecular analyses. The morphological data collected for Bolivian specimens of this species complete preliminary information obtained for individuals from Argentina (Wetzel 1985b; Carrizo et al. 2005), and the mitochondrial and non-coding nuclear sequences presented here represent the first molecular genetic data ever retrieved for putative *C. nationi* individuals.

The broader cephalic shield of *C. nationi* did not convince Wetzel (1985b) to support the species distinction. The *C. nationi* type specimen has a cephalic shield of 71 mm width and 60 mm length, and a width to length ratio of 1.183. In our sample, only individuals from Santa Catalina (Jujuy, Argentina) and Oruro and Nuestra Señora de La Paz (Bolivia) showed a ratio > 1.10 , which were all lower than that of the type specimen. Among these, only the specimens from La Paz would potentially include *C. nationi* within their geographic range. However, this range is very wide, and its lower values largely overlap the range of values of virtually all other populations. Actually, several populations show large variation, independently of the number of specimens or diversity of environments represented in the sample. This large variation ultimately precludes the use of the cephalic shield width as a diagnostic character. Incidentally, intraspecific differences in the proportions of the head and the breadth of the cephalic shield have also been observed in *Z. pichiy*, with individuals at

higher latitudes being smaller and having more slender heads than pichis at lower latitudes (Superina 2008).

In a comparison of a specimen from Tucumán province, Argentina, with 2 skulls of *C. vellerosus* and 1 of *C. villosus*, Carrizo et al. (2005) assigned the Tucumán specimen to *C. nationi*, based principally on differences of the squamosal-jugal suture. This character was originally used by Wetzel (1985b) to discriminate among euphractine species. However, as demonstrated above, this character shows substantial variability in populations of *C. vellerosus* and putative *C. nationi*, precluding the use of this feature as a diagnostic. Moreover, the holotype skull used by Thomas (1894) in his original description of the species, consisted of only the rostral portion, so a comparison of squamosal-jugal suture is not possible. In summary, if the morphological differences observed by Carrizo et al. (2005) had a taxonomic consequence, that implies that the specimen they examined belongs to a different taxon than *C. vellerosus* and its assignment to *C. nationi* should be considered tentative.

Our geometric morphometric analyses revealed only subtle differences in skull shape between specimens from the type locality of *C. nationi* and specimens of *C. vellerosus* based on canonical analysis, whereas they were clearly distinct from *C. villosus*, *Z. pichiy*, and *E. sexcinctus* in all analyses. The only significant difference we identified between putative specimens of *C. nationi* and *C. vellerosus* was related to size, with putative *C. nationi* specimens being significantly larger than *C. vellerosus* individuals from Buenos Aires and Córdoba provinces in Argentina, an observation already made by Thomas (1894) in his original description of *C. nationi*. Small hairy armadillos are found from near sea level on the coast of Buenos Aires province (Carlini and Vizcaíno 1987; Abba et al. 2011) to Andalgalá in Catamarca province (1,080 m asl—Gregor 1985) and as high as the Puna desert at 3,500 to 4,000 m asl (Thomas 1919). According to our results, which revealed no significant

difference among adjacent sampling localities, the difference in size may be related to a latitudinal and/or altitudinal gradient, with specimens of *C. vellerosus* and putative *C. nationi* living at higher latitudes and/or altitudes being significantly larger. This observation is compatible with predictions from Bergmann's rule, which postulates an increase in body size in individuals within a species living at higher altitudes, possibly because of a temperature effect on mammalian physiology (Ashton et al. 2000). The difference in size observed between *C. vellerosus* and putative *C. nationi* might thus be explained by the occurrence of phenotypic plasticity within a single species that is widely distributed across an altitudinal gradient.

From the molecular point of view, our results reinforce the morphological observation that individuals assigned to *C. nationi* could not be distinguished from *C. vellerosus*. The 2 groups shared common mitochondrial haplotypes, with the unique mitochondrial haplotype detected in putative *C. nationi* being identical to *C. vellerosus* individuals from Taco Ralo (Tucumán, Argentina). In addition, the nuclear divergence between them based on non-coding regions flanking retroposon elements was extremely low, with no difference detected in 3 out of the 5 markers investigated. Despite their distinct geographic distributions, there is thus evidence of gene flow between *C. vellerosus* and the hairy armadillos from near the type locality of *C. nationi*. The most recent range maps (Abba and Superina 2010) show that their distributions are almost parapatric, with a gap of only 80 km occurring in northern Argentina, most probably reflecting the scarcity of field studies and the corresponding lack of georeferenced localities from the potential area of contact. Indeed, one of us (AMA) recently confirmed the presence of small hairy armadillos in this gap. There is thus no obvious geographic barrier that would prevent contact between individuals of these 2 populations.

The populations of *C. vellerosus* from eastern Bolivia, the Chaco of Paraguay, and northwestern Argentina, have been considered as a subspecies, *C. v. vellerosus* Gray, 1865,

different from those from central Argentina and Mendoza Province towards the south that were classified as *C. v. pannosus* Thomas, 1902 (Wetzel et al. 2008). Despite subtle morphological differences in skull shape, only evidenced on the 2nd axis of the canonical variate analysis, Andean hairy armadillos are similar in size to specimens of *C. v. vellerosus*, with which they also share mitochondrial haplotypes. They might thus be viewed as being part of this subspecies. Nevertheless, the reality of the subspecies definition within *C. vellerosus* should be further explored in the future by conducting more detailed phylogeographic and population genetics studies encompassing individuals more densely sampled across the species range.

In summary, our results indicate that the screaming hairy armadillo *C. vellerosus* and the Andean hairy armadillo *C. nationi* most likely represent a single, widely distributed species, in agreement with early observations (Wetzel 1985b). In line with this conclusion, Luaces (2011) recently confirmed that the karyotype of *C. vellerosus* ($2n = 62$) was identical to that of a *C. nationi* from Oruro originally analyzed by Cook et al. (1991), but it was different from *C. villosus* ($2n = 60$ —Jorge et al. 1977). We therefore propose to synonymize *Chaetophractus nationi* (Thomas, 1894) with *Chaetophractus vellerosus* (Gray, 1865) following on the taxonomic principle of priority.

Conservation implications.—Synonymizing *C. nationi* with *C. vellerosus* implies a reassessment of the conservation status of hairy armadillos for the International Union for Conservation of Nature Red List of Threatened Species. *C. vellerosus* is currently listed as Least Concern (LC) because it is widespread and the harvest rates by hunting are not at a level that would warrant concern. The Andean hairy armadillo *C. nationi* is the most threatened of all 3 *Chaetophractus* species. It is currently listed as Vulnerable (VU A2acd) by the International Union for the Conservation of Nature Red List of Threatened Species due to an estimated population decline of over 30% (International Union for the Conservation of

Nature 2014). It is considered Endangered (EN) in Bolivia (Pérez-Zubieta et al. 2009) and Chile (Servicio Agrícola y Ganadero 1998), Vulnerable (VU) in Peru (Supreme Decree 034-2004-AG), and Data Deficient (DD) in Argentina (Superina et al. 2012). In addition, *C. nationi* is listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (2013), which has established a zero annual exportation quota for this species because it is threatened primarily by intensive hunting for traditional purposes (Peredo 1999). Locally known as ‘quirquincho,’ it is considered an emblematic animal of the Bolivian highlands, particularly from the Oruro department (Cáceres 2004), where it is closely linked to cultural aspects. There is a huge market for taxidermy in Bolivia, and *C. nationi* carapaces are used for crafting musical instruments called ‘charangos’ and to build the so-called ‘matracas’ used in traditional dances, alongside with amulets and souvenirs (Romero-Muñoz and Pérez-Zubieta 2008; Pérez-Zubieta et al. 2009). *C. nationi* is also threatened by continuing habitat degradation due to agricultural activities (Ríos and Rocha 2002; Pérez-Zubieta 2011; International Union for Conservation of Nature 2014).

After incorporating the populations formerly classified as *C. nationi* into *C. vellerosus*, the species as a whole should be listed as Least Concern (LC) at the international level as it does not reach any of the established thresholds for listing species in a threatened category (International Union for Conservation of Nature 2001). Nevertheless, it should be taken into account that the International Union for Conservation of Nature Red List of Threatened Species assesses conservation status at the global level, and a species globally listed as of Least Concern may well be endangered in part of its range. Roughly 60% of the Bolivian range corresponds to the range of the high-altitude population previously known as *C. nationi*. Peredo (1999) reported almost 15 years ago that increasing difficulties in finding *C. nationi* in the wild had led artisans from the region of Oruro and La Paz to start using the Near Threatened *C. vellerosus* (Tarifa and Romero-Muñoz 2009) from the south of Bolivia to

manufacture ‘charangos’ and ‘matracas.’ As Municipal Bylaw 31/99 in Oruro did not succeed in reducing the use of *Chaetophractus* carapaces in traditional dances (Pérez-Zubieta et al. 2009), it is probable that the impact of hunting is increasingly affecting both the high-altitude (formerly *C. nationi*) and southern (*C. vellerosus*) populations of Bolivia.

Our genetic data indicate that *C. nationi* specimens from Bolivia share a unique mitochondrial haplotype, which is also found in a *C. vellerosus* specimen from Argentina. This absence of genetic diversity observed in the 10 individuals sampled at the scale of the Oruro region suggests a local reduction of effective population size. There is therefore no doubt that the synonymized *C. vellerosus* would have to be listed in a threatened category—either Vulnerable or Endangered—within Bolivia and that conservation measures should be implemented as soon as possible to save the species from regional extinction.

Phylogeny of hairy armadillos.—This study is the first to assess the phylogenetic systematics of hairy armadillos, combining morphological and molecular analyses of all described extant species. As originally reported by Wetzel (1985b) based on simple cranial measurements, our 3-D geometric morphometrics analyses revealed marked differences in skull size and shape among Euphractinae, except for *C. vellerosus* and *C. nationi*, which completely overlapped in principal component analysis (Figs. 3, 4, and 5). These data provide evidence for 4 distinct morphological entities within euphractine armadillos that correspond to each species (assuming that *C. nationi* is part of *C. vellerosus*).

The molecular phylogenetic reconstructions led to apparent topological conflicts between non-coding and protein-coding nuclear markers. The non-coding nuclear partition suggests the paraphyly of the genus *Chaetophractus* by grouping *C. vellerosus* + *C. nationi* with *Z. pichiy* with strong statistical support, whereas *C. villosus* clusters with *E. sexcinctus* with more moderate support. The protein-coding partition yielded an alternative topology where the genus *Chaetophractus* appeared monophyletic and grouped with *E. sexcinctus*, *Z.*

pichiy being the first euphractine lineage to emerge. However, this topology received only moderate bootstrap support from maximum likelihood whereas the Bayesian approach resulted in an unresolved multifurcation within Euphractinae. The concatenation of all 8 nuclear markers supported the exact same topology as the non-coding nuclear markers for euphractines, with strong support for the paraphyly of the genus *Chaetophractus* by grouping *C. vellerosus* with *Z. pichiy*. Such a relationship would be compatible with karyotypic data because *C. vellerosus* is similar to *Z. pichiy* ($2n = 62$), but different from *C. villosus* ($2n = 60$ —Jorge et al. 1977), and *E. sexcinctus* ($2n = 58$ —Jorge et al. 1977). The sister-group relationship between *E. sexcinctus* and *C. villosus* was more moderately supported and could still be considered uncertain, all the more so because such a relationship appeared incompatible by the 19-nucleotide deletion shared by *C. villosus*, *Z. pichiy*, and *C. vellerosus* in the An5 non-coding nuclear marker.

The apparently conflicting results obtained with the 2 types of nuclear genes could easily be explained by the contrasting phylogenetic informativeness of the 2 partitions. Indeed, the lack of statistical support observed for the protein-coding partition may be a consequence of the number of informative sites available for discriminating between alternative branching schemes. The combination of the 2 nuclear exons comprises 4,014 nucleotide sites, of which 55 were variable (1.4%) in euphractines, and only 5 were parsimony informative (0.1%). The non-coding partition was half the length of the protein-coding partition with a total of 2,131 nucleotide sites for the concatenation of the 6 markers, but it appeared more informative with 55 variable (2.5%) and 13 parsimony informative (0.6%) sites. From these figures it can thus be extrapolated that for every 1,000 base pairs sequenced from the genomes of Euphractinae, about 6 informative sites in the non-coding fraction compared to only 1 informative site in the coding fraction will be found for deciphering their phylogenetic relationships. The paucity of informative sites for resolving

the relationships among euphractine species highlights the difficulties of establishing an adequate phylogenetic framework for these morphologically and ecologically similar armadillos (Delsuc et al. 2003; Delsuc and Douzery 2008). It also suggests the occurrence of a rapid diversification event, possibly related to paleoenvironmental changes (Delsuc et al. 2004), with opportunity for incomplete lineage sorting events to occur and create gene tree-species tree discordances. Further resolution of the relationships within Euphractinae will thus involve gathering large-scale comparative genomic data, coupled with the use of gene tree-species tree reconstruction methods based on multispecies coalescent models (Liu et al. 2009; Heled and Drummond 2010). In this perspective, the targeted-sequence capture and sequencing of genomic regions flanking ultra-conserved elements (McCormack et al. 2012; McCormack et al. 2013) might be a promising strategy.

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SUPPORTING INFORMATION

Supporting Information S1.—Specimens examined for cephalic shield data.

Supporting Information S2.—Specimens examined for skull morphological data.

Supporting Information S3.—Cranial landmarks names, definitions, and position.

Supporting Information S4.—New PCR primers used to amplify the six non-coding markers in hairy armadillos.

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Figure legends

Fig. 1.—Landmark and semilandmarks 1–55 on the right side and midline, shown on outline of skull of *C. vellerosus*. Names and definitions of landmarks are given in Supporting Information S3. Skull is shown in 3 views: lateral (above), dorsal (middle), and ventral (below). Semilandmarks are indicated in brackets. Landmark and semilandmarks 56 to 100 on the left side are not shown.

Fig. 2.—Drawings of the squamosal-jugal suture in skulls of putative *C. nationi* from Oruro (Bolivia, type locality) and *C. vellerosus* from Magdalena (Buenos Aires, Argentina). In both groups there are specimens with rounded or angular sutures.

Fig. 3.—Size variation (with box and whisker plot showing median, quartile, and range) in: a) proportions of cephalic shield (width/length) organized by localities of *C. vellerosus* and putative *C. nationi*, altitude (m above sea level) on secondary x-axis; b) body size, as indicated by centroid size, of the 5 species of euphractine armadillos compared to putative *C. nationi*; c) body size, as indicated by centroid size, of *C. vellerosus* from southern, low altitude to northern and high altitude populations (left to right) compared to putative *C. nationi*. Results of post-hoc Tukey Honest Significant Difference tests among pairs are indicated (*: significant difference; ns: non-significant difference).

Fig. 4.—A) Morphospace defined by the first 2 principal components of tangent shape space: putative *C. nationi* (black circles); *C. vellerosus* (gray circles); *C. villosus* (open circles); *Z. pichiy* (open squares); *E. sexcinctus* (black squares). B) Principal component axis 1 and C) principal component axis 2 shape surface meshes (lateral, dorsal, and ventral norm) after application of a thin-plate spline interpolation to extreme negative and positive values of each component from consensus (enlarged by a factor of 2).

Fig. 5.—A) Regression of shape coordinates on log-transformed (base 10) centroid size as proxy for body size. Putative *C. nationi* (black circles); *C. vellerosus* (grey circles); *C. villosus* (open circles); *Z. pichiy* (open squares); *E. sexcinctus* (black squares). B) Shape surface meshes (lateral, dorsal, and ventral norm) after application of a thin-plate spline interpolation to extreme negative and positive values of regression shape scores from consensus (enlarged by a factor of 2).

Fig. 6.—Relationships between *C. nationi* (green), *C. vellerosus* (red), *C. villosus* (magenta), and *Z. pichiy* (blue) as represented by a neighbor-net network based on Kimura-2-parameter distances inferred from the mitochondrial D-loop data set including 127 sequences and 483 nucleotide sites.

Fig. 7.—Phylogenetic relationships of armadillo subfamilies (Dasypodinae, Tolypeutinae, Chlamyphorinae, and Euphractinae) represented as maximum likelihood phylograms inferred from the concatenation of a) the 6 nuclear non-coding markers; b) the 2 nuclear protein-coding genes BRCA1 and VWF; and c) all 8 non-coding and protein-coding nuclear markers. Values at nodes represent bootstrap percentages/Bayesian posterior probabilities. A dash (-) indicates that the node was not recovered in the corresponding analysis. The scale bar represents the mean number of substitutions per site.

Table 1.—Procrustes distances for the mean shape of each Euphractinae species. Elements above the diagonal are from shape space, and below are from the residuals of the regression of shape versus log₁₀ transformed centroid size. All comparison pairs were significant ($P < 0.0001$) after 10,000 rounds of permutation test.

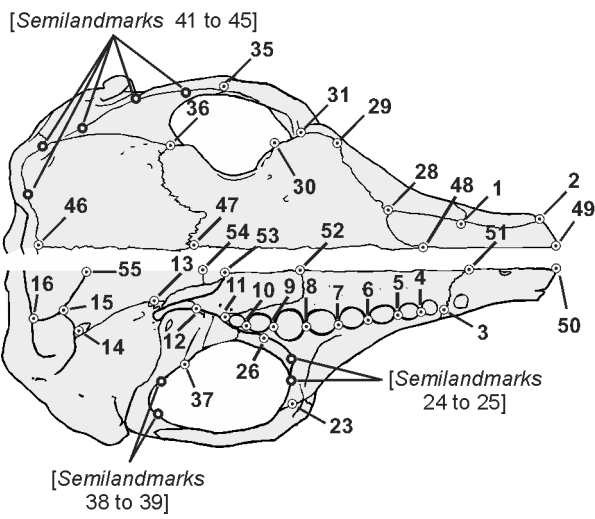
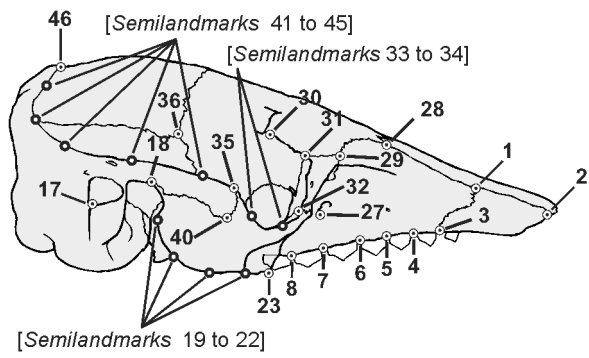
Species	<i>n</i>	<i>C. nationi</i>	<i>C. vellerosus</i>	<i>C. villosus</i>	<i>E. sexcinctus</i>	<i>Z. pichiy</i>
<i>C. nationi</i>	9		0.0334	0.0635	0.0954	0.0768
<i>C. vellerosus</i>	73	0.0346		0.0553	0.0915	0.0725
<i>C. villosus</i>	26	0.0507	0.0406		0.0693	0.0750
<i>E. sexcinctus</i>	19	0.0471	0.0357	0.0534		0.1074
<i>Z. pichiy</i>	31	0.0760	0.0717	0.0560	0.0486	

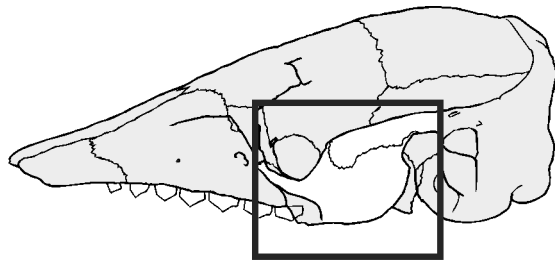
Table 2.—Mean percentages of individuals of Euphractinae classified according to species by hold-out cross-validation. Bold fonts indicate correct classifications.

Species	<i>n</i>	<i>C. nationi</i>	<i>C. vellerosus</i>	<i>C. villosus</i>	<i>E. sexcinctus</i>	<i>Z. pichiy</i>
Based on half cranium using shape coordinates						
<i>C. nationi</i>	9	93.45 %	4.76 %	1.78 %	0 %	0 %
<i>C. vellerosus</i>	33	6.04 %	92.88 %	1.02 %	0 %	0.06 %
<i>C. villosus</i>	26	1.47 %	1.44 %	95.99 %	0.52 %	0.6 %
<i>E. sexcinctus</i>	19	0.06 %	0.17 %	1.13 %	98.58 %	0.06 %
<i>Z. pichiy</i>	31	0.5 %	1.26 %	1.99 %	0 %	96.24 %
Using first 25 principal component scores						
<i>C. nationi</i>	9	81.55 %	14.29 %	2.38 %	0 %	1.79 %
<i>C. vellerosus</i>	33	12.22 %	81.32 %	3.37 %	0.14 %	2.95 %
<i>C. villosus</i>	26	4.76 %	5.21 %	85.12 %	3.85 %	1.06 %
<i>E. sexcinctus</i>	19	1.53 %	1.36 %	7.54 %	88.83 %	0.74 %
<i>Z. pichiy</i>	31	4.56 %	8.55 %	2.06 %	0.44 %	84.39 %

Table 3.—Mean percentages of individuals of *C. vellerosus* and *C. nationi* (from Oruro, type locality) classified according to localities by hold-out cross-validation. Bold fonts indicate correct classifications.

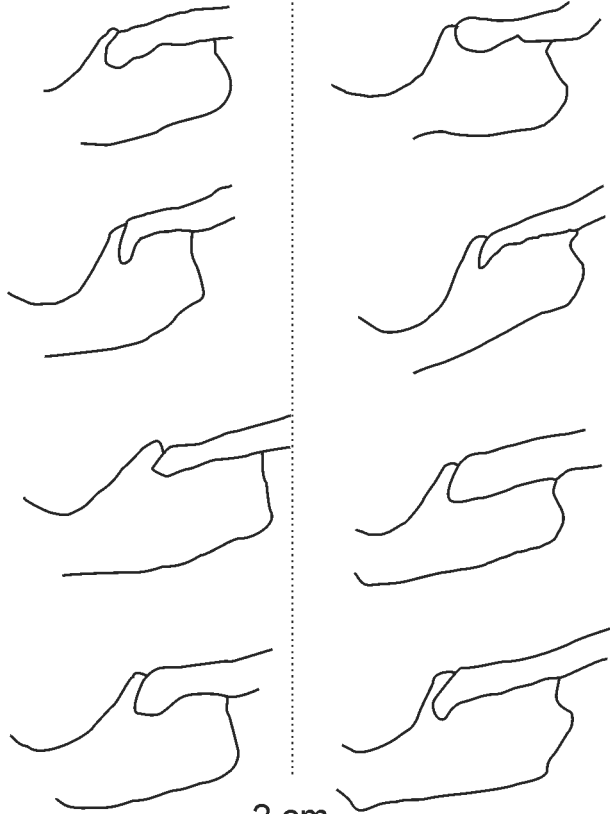
Localities	<i>n</i>	Buenos Aires	Córdoba	Catamarca	Oruro
Buenos Aires	33	45.28 %	25.25 %	27.23 %	2.23 %
Córdoba	8	33.93 %	38.69 %	19.64 %	7.73 %
Catamarca	9	31.70%	29.46 %	27.68 %	11.16 %
Oruro	9	4.02%	5.35 %	4.46 %	86.16 %





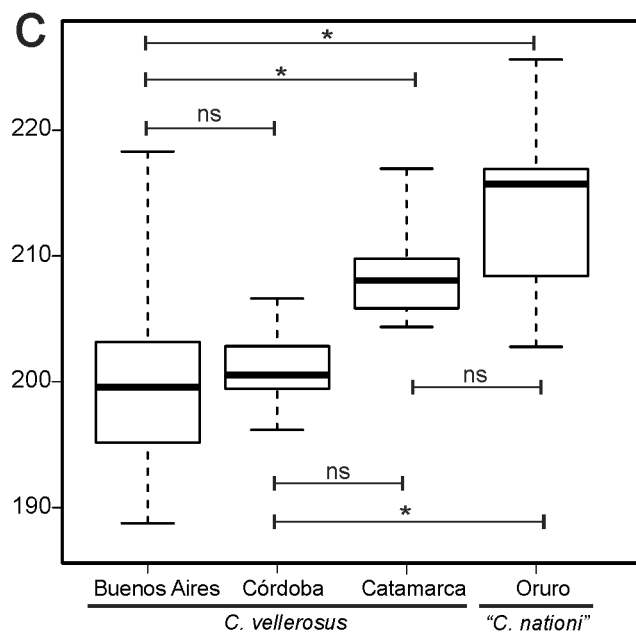
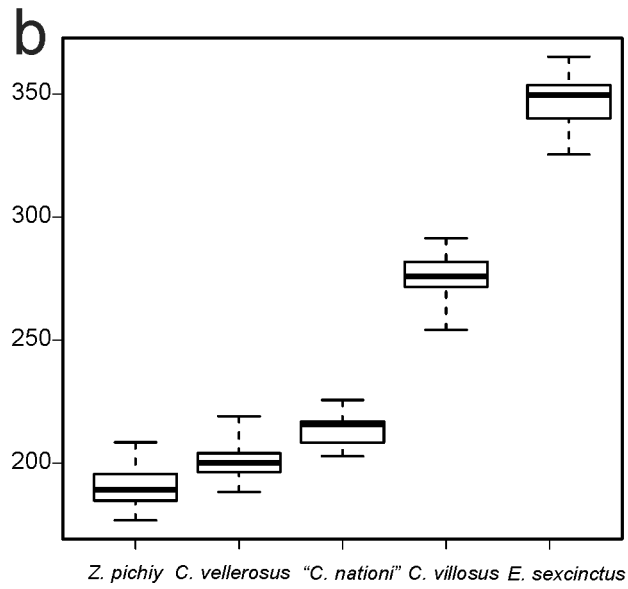
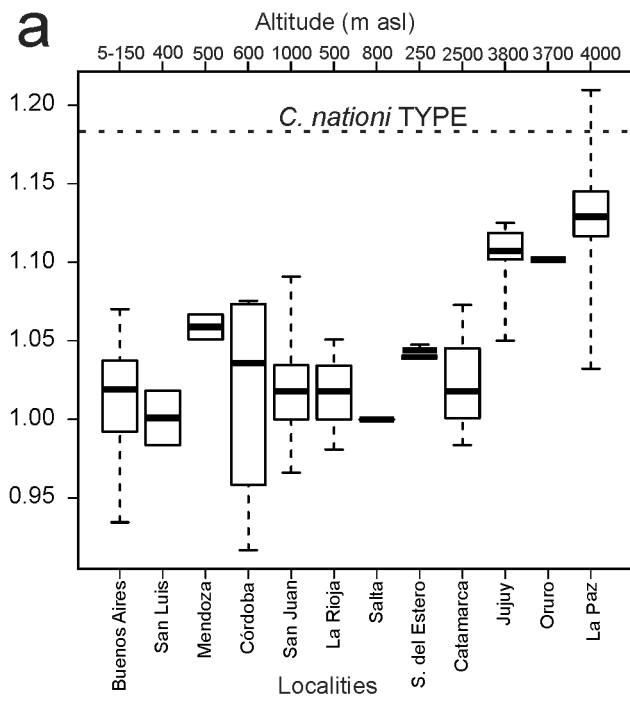
ORURO, BOLIVIA

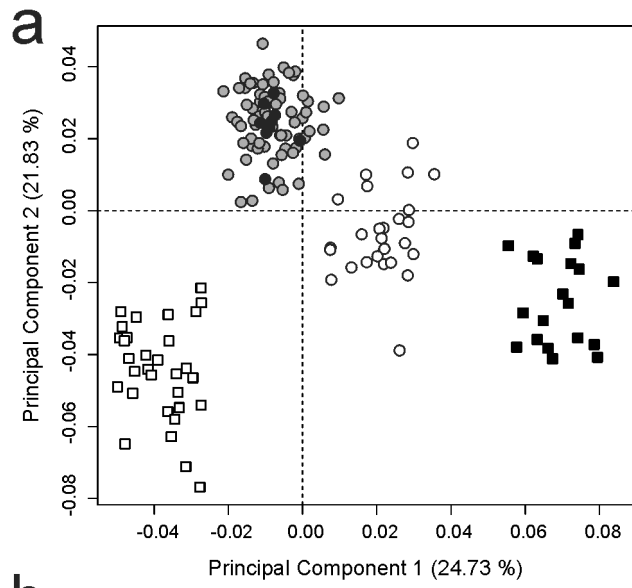
MAGDALENA, ARGENTINA



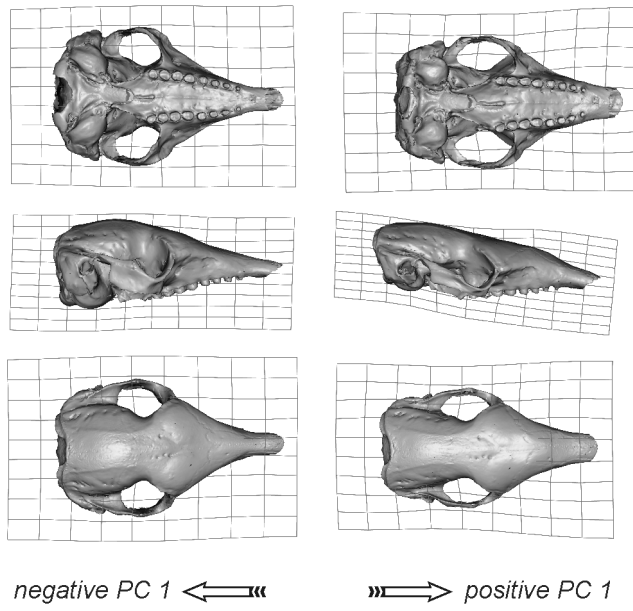
2 cm



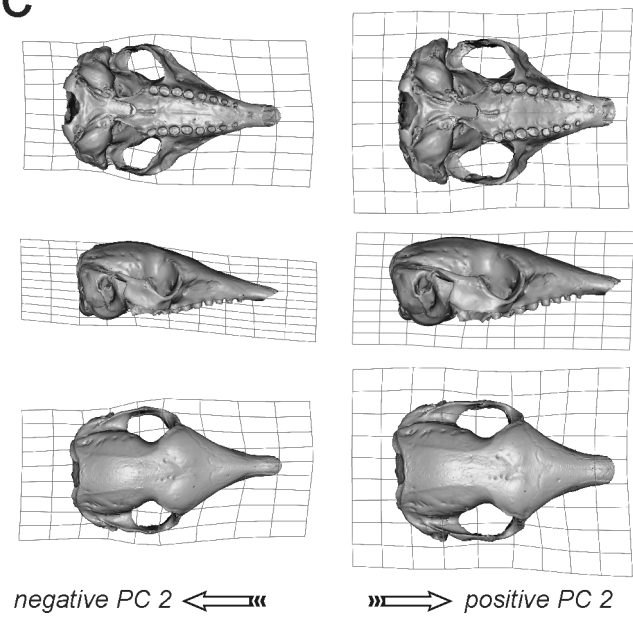


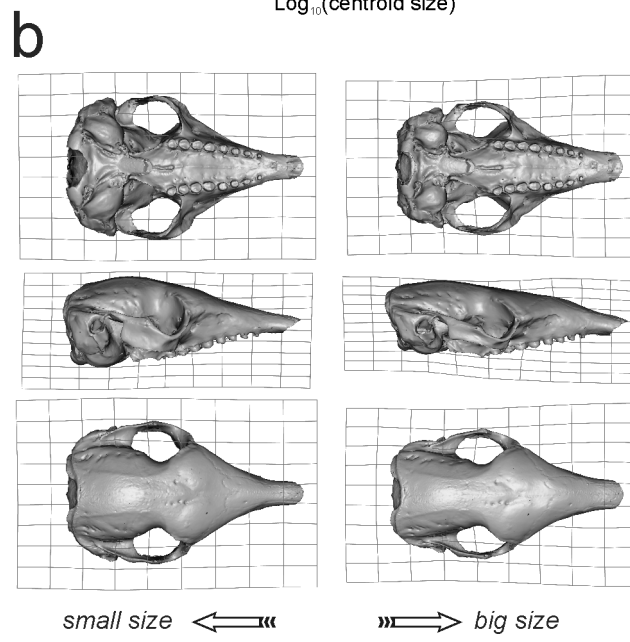
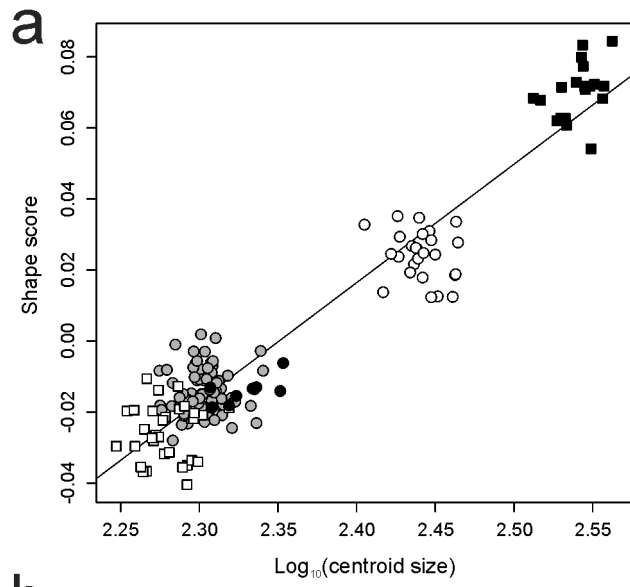


b



c





0.01

