2

Recent advances and future prospects in xenarthran molecular phylogenetics

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Resumen

En los últimos 20 años, la reconstrucción de las relaciones filogenéticas de los xenartros ha sido revolucionada por datos moleculares. Las filogenias anteriores se basaban en caracteres morfológicos, citológicos, inmunológicos y de proteínas, pero ensayos más recientes se beneficiaron con la secuenciación de genes mitocondriales y nucleares. En este capítulo repasamos los avances recientes en sistemática molecular de los xenartros. Estos avances han conducido a la reconstrucción de una filogenia de Xenarthra a nivel genérico, con el reconocimiento de los clados Pilosa, Folivora, Vermilingua, Cingulata y Tolypeutinae + Euphractinae, pero dejando incierta la posición de Xenarthra entre los mamíferos placentarios. Este marco filogenético se utilizó posteriormente para definir una escala temporal molecular para la historia evolutiva de los xenartros vivientes, sugiriendo la potencial influencia de los cambios climáticos durante el Terciario sobre este orden endémico de América del Sur. El trabajo filogenético futuro sobre Xenarthra incluye la resolución de su relación con otros grupos placentarios usando datos genómicos y la reconstrucción de una filogenia comprehensiva a nivel específico. Junto con estudios filogeográficos a nivel poblacional, esto permitirá la caracterización adicional de la diversidad genética de este peculiar orden de placentarios y orientará planes para la conservación de sus especies en peligro.

Resumo

Nos últimos 20 anos a reconstrução dos relacionamentos filogenéticos dos xenarthros foi revolucionada por dados moleculares. As filogenias anteriores baseavam-se em carateres morfológicos, citológicos, imunológicos e de proteínas, mas as tentativas mais recentes beneficiaram-se da seqüenciação de genes mitocondriais e nucleares. Neste capítulo, nós revisamos os avanços recentes feitos na sistemática molecular dos xenarthros. Estes avanços conduziram à reconstrução de uma filogenia de Xenarthra em nível genérico, com o reconhecimento dos clados Pilosa, Folivora, Vermilingua, Cingulata, e Tolypeutinae + Euphractinae, mas deixando incerta a posição de Xenarthra dentro dos mamíferos placentários. Esta estrutura filogenética foi usada subseqüentemente para definir uma escala temporal molecular para a história evolutiva dos xenarthros existentes, sugerindo a influência potencial da mudança do clima no Terciário nesta ordem endêmica da América do Sul. O trabalho filogenético futuro em Xenarthra inclui resolver seu relacionamento com os outros grupos placentários usando dados genômicos e a reconstrução de uma filogenia detalhada em nível de espécie. Acoplado com estudos filogeográficos em nível de população, isto permitirá uma caracterização adicional da diversidade genética desta peculiar ordem placentária e fornecerá a orientação aos planos de conservação para suas espécies ameaçadas.

Introduction

Extant xenarthrans are currently represented by 31 living species of armadillos (Cingulata: Dasypodidae), anteaters (Vermilingua: Myrmecophagidae and Cyclopedidae) and sloths (Folivora [also known as Phyllophaga or Tardigrada, see Fariña and Vizcaíno 2003]: Bradypodidae and Megalonychidae), classified in 13 (possibly 14, see Gardner 2005) genera and distributed across the Americas, with most of the diversity centered

in South America (Wetzel 1985a; Vizcaíno 1995; Anderson and Handley 2001). This quite modest taxonomic diversity is in sharp contrast to that found in the fossil record (Patterson and Pascual 1972; McKenna and Bell 1997). Living species are relicts from an evolutionary radiation that occurred during the Tertiary isolation of South America (Patterson and Pascual 1972). In fact, the order was still quite diverse until the last mass extinction event just 10,000 years ago (Patterson and Pascual 1972; Lessa et al. 1997).

The use of molecular phylogenetics to reconstruct the evolutionary relationships among the three main xenarthran lineages dates back to the mid 1980s. The first attempts were based on evolutionary comparisons of protein sequences of ά-crystallin A (de Jong et al. 1985) and immunological distances derived from serum albumins (Sarich 1985). These early studies marked the dawn of the molecular era in xenarthran systematics which, until that time, had been restricted to the study of morphological and anatomical characters (Engelmann 1985). Since then, phylogenetic studies in xenarthrans (Delsuc et al. 2001, 2002, 2003) and, more generally, placental mammals (Madsen et al. 2001; Murphy, Eizirik, Johnson et al. 2001; Murphy, Eizirik, O'Brien et al. 2001; Amrine-Madsen et al. 2003), have benefited immensely from the sequencing of both mitochondrial and nuclear genes, giving access to a large number of phylogenetically useful characters. These studies allowed the reconstruction of a reliable phylogenetic framework for extant xenarthran genera (except *Chlamyphorus*) and provided the basis for the definition of a timescale for xenarthran evolution (Delsuc et al. 2004).

As we will review, modern studies, based on DNA sequence analyses using state-of-the- art probabilistic methods of phylogenetic reconstruction, have yielded numerous new insights into xenarthran systematics (Delsuc et al. 2001, 2002, 2003). They have also confirmed predictions of earlier molecular analyses, for example, by corroborating the independent evolution of xenarthrans and pangolins (de Jong et al. 1985, 1993; Sarich 1985). Additionally, we outline future prospects in the molecular phylogenetics of xenarthrans, among which are the resolution of their place in the placental tree using data from comparative genomics, and the construction of a comprehensive species-level phylogeny, which will be critical in elaborating conservation programs for the most threatened species.

The central place of Xenarthra within placental mammals

From edentates to xenarthran

Xenarthrans have long been of special interest to researchers involved in understanding the evolutionary origins and relationships of placental mammals. Despite their highly distinctive morphologies, armadillos, anteaters, and sloths have been recognized to form a monophyletic group on the basis of shared derived characters such as atypical articulations between the vertebrae (Engelmann 1985; Gaudin 1999b), from which the order name was derived (*xenos* = strange, and *arthros* = articulation in Greek). Xenarthrans were grouped with pangolins and aardvarks in early classifications into a group called edentates (Edentata, see Glass 1985). Aardvarks and pangolins were subsequently placed into their own orders, Tubulidentata and Pholidota, respectively. However, the name Edentata was retained for the superordinal grouping of Xenarthra with Pholidota (McKenna 1975; Novacek and Wyss 1986; Novacek 1992). Although abandoned by Simpson (1945), the hypothesis of a close relationship between Xenarthra and Pholidota was so popular among morphologists that it was resurrected by Novacek and Wyss (1986) despite contradictory anatomical evidence (Bugge 1979).

The first molecular studies, using immunological and protein-based characters, clearly separated xenarthrans from pholidotes (de Jong et al. 1985; Sarich 1985; Shoshani 1986). These results forced morphologists to reconsider the evidence for Edentata, and it was concluded that support for such a relationship was actually very weak (Rose and Emry 1993). Further results from the analysis of ά-crystallin A protein sequences led de Jong et al. (1993) to first propose a possible sister-group relationship between Pholidota and Carnivora, a hypothesis that has been adopted by some morphologists (Shoshani and McKenna 1998). The lack of evolutionary affinities and the independent evolution of Xenarthra and Pholidota has since been confirmed in numerous phylogenetic studies (van Dijk et al. 1999; Delsuc et al. 2001, 2002; Madsen et al. 2001; Murphy, Eizirik, Johnson et al. 2001; Murphy, Eizirik, O'Brien et al. 2001; Lin et al. 2002; Amrine-Madsen et al. 2003; Hudelot et al. 2003; Reyes et al. 2004); some of these also found strong support for grouping Pholidota with Carnivora (Murphy, Eizirik, O'Brien et al. 2001; Delsuc et al. 2002; Amrine-Madsen et al. 2003). As a consequence, the

morphological similarities between xenarthrans and pangolins, including the trend toward dental reduction (Ferigolo 1985), are now generally considered adaptive convergences associated with their fossorial and myrmecophagous habits (Rose et al. 2005).

Xenarthra and Epitheria

The phylogenetic relationships of armadillos, anteaters, and sloths to other placental mammals have been of special interest for quite some time because of their seemingly "archaic" morphology. Indeed, retention of anatomical and physiological characters thought to be plesiomorphic for placental mammals, such as a low and poorly regulated body temperature, a lack of clear differentiation between uterus and vagina, and the intra-abdominal position of the testes, has led to their being considered an early offshoot of the placental mammal radiation (Gregory 1910). McKenna (1975) was the first to propose that Xenarthra represents the sister group to all other eutherians, which were collectively named Epitheria. This view has been widespread among morphologists (Novacek and Wyss 1986; Novacek 1992) and was adopted in the most recent morphologically based classification of mammals (McKenna and Bell 1997). However, morphological synapomorphies defining epitherians are actually weak and their phylogenetic distribution among placentals is equivocal (Gaudin et al. 1996). Shoshani and McKenna (1998) summarized the morphological view of placental radiation with the recognition of 18 orders whose interrelationships remain largely hypothetical, except the grouping of rodents and lagomorphs into Glires and a monophyletic origin of elephants, hyraxes, and sirenians (Paenungulata). In this synthetic tree, xenarthrans constitute the earliest diverging branch, in agreement with the Epitheria hypothesis, even though they are separated from Pholidota based on available molecular evidence (Shoshani and McKenna 1998).

The molecular revolution

The morphological view of placental phylogeny was shattered in 2001 with the simultaneous publication of two independent studies based on phylogenetic analyses of multiple genes (Madsen et al. 2001; Murphy, Eizirik, Johnson et al. 2001). These studies identified four major placental clades: Afrotheria (aardvarks, elephant-shrews, golden-moles, tenrecs, hyraxes, elephants, and sirenians), Xenarthra (armadillos, anteaters, and sloths), Euarchontoglires (tree-shrews, flying-lemurs, primates, rodents, and lagomorphs), and Laurasiatheria (eulipotyphlans, bats, pangolins, carnivores, perissodactyls, and cetartiodactyls), of which only Xenarthra had been previously recognized by morphological studies. The subsequent combination of these two datasets (Murphy, Eizirik, O'Brien et al. 2001) also supported the grouping of Euarchontoglires and Laurasiatheria. This superclade was named Boreoeutheria to reflect its Northern Hemisphere origin, as opposed to Afrotheria and Xenarthra, which both originated in the Southern Hemisphere (Springer and de Jong 2001). The recognition of such biogeographical clades suggests that plate tectonics may have played an important role in shaping the early stages of placental diversification (Murphy, Eizirik, O'Brien et al. 2001). Additional phylogenetic analyses, mainly of nuclear genes (Delsuc et al. 2002; Amrine-Madsen et al. 2003; Waddell and Shelley 2003), mitochondrial RNAs (Hudelot et al. 2003), and recent studies of complete mitochondrial genomes (Lin et al. 2002; Reyes et al. 2004), all support recognition of the four major placental clades. These studies have also revealed the extent of morphological convergence, a problem that has blurred any phylogenetic signal based on morphology (see Springer et al. 2004 for a recent review).

The question of the placental root

That xenarthrans represent one of the four major placental lineages underlines the evolutionary significance of this often neglected group. However, none of the multigene studies (Madsen et al. 2001; Murphy, Eizirik, Johnson et al. 2001; Murphy, Eizirik, O'Brien et al. 2001; Delsuc et al. 2002; Amrine-Madsen et al. 2003) found statistically significant support for locating Xenarthra within placentals with confidence. Indeed, using maximum likelihood (ML) and Bayesian analyses, all the studies favored a basal position of Afrotheria, with Xenarthra as a sister group to Boreoeutheria. However, these statistical tests have been influenced by both character and taxon sampling (Delsuc et al. 2002; Holland et al. 2005). Thus, it has proved difficult to distinguish between three competing topologies for the position of the placental root: (1) basal Afrotheria, (2) basal Xenarthra (= Epitheria), or (3) Afrotheria + Xenarthra.

The difficulty of the question is illustrated by the results reported in table 2.1. This information was obtained after new analyses of the multigene dataset of

Amrine-Madsen et al. (2003), which consists of 17,736 unambiguously aligned nucleotide sites for 42 placental taxa and two marsupial outgroups. The results of likelihood-based SH tests (Shimodaira and Hasegawa 1999), performed for different partitions of the complete dataset, show that the Epitheria and the Afrotheria + Xenarthra hypotheses cannot be rejected, although each partition gives the highest likelihood score for the basal Afrotheria hypothesis (table 2.1). Moreover, as shown previously (Delsuc et al. 2002), character sampling influences the results, with the three alternatives becoming almost indistinguishable in terms of likelihood scores when only the first two codon positions of nuclear genes are used. In this case, the Afrotheria + Xenarthra hypothesis, which makes more biogeographical sense because it divides placentals into Northern and Southern Hemisphere clades (Waddell et al. 1999), differs from the highest likelihood topology only at the decimal level, whereas the Epitheria hypothesis is slightly less likely (table 2.1). While the nuclear first and second codon position partition can be considered to contain the more reliable sites in terms of mutational saturation (Delsuc et al. 2002), the question of the position of the root and thereby the place of xenarthrans within placentals is left unresolved by the data currently available. The future application of refined methods dedicated to the identification of the most reliable sites for phylogenetic inference (Brinkmann and Philippe 1999; Burleigh and Mathews 2004; Pisani 2004) might help to further evaluate the three competing alternatives.

The molecular phylogeny of living xenarthrans

Phylogenetic relationships among xenarthrans have been investigated using both mitochondrial and nuclear genes. The first study considered a combination of mitochondrial 12S and 16S ribosomal RNA (rRNA) genes and nuclear exon 28 of the von Willebrand Factor (VWF) for eight of the 13 living xenarthran genera (Delsuc et al. 2001). Subsequently, taxon sampling was increased to 12 genera, with only *Chlamyphorus* missing, in analyses of three genetically independent protein-coding nuclear genes: the intronless ά-2B Adrenergic receptor gene (ADRA2B), exon 11 of the Breast Cancer Susceptibility gene (BRCA1), and, once again, the VWF gene (Delsuc et al. 2002). Gene sampling in this latter study was later expanded by the addition of two mitochondrial genes: 12S rRNA and NADH dehydrogenase 1 (ND1), representing a total of 6,968 nucleotide sites (Delsuc et al. 2003). These three studies allowed the reconstruction of a comprehensive phylogeny of extant xenarthrans at the genus level with only two remaining uncertainties, both within armadillos.

The monophyly of the order

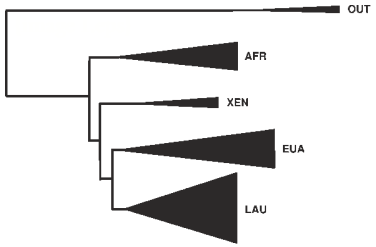
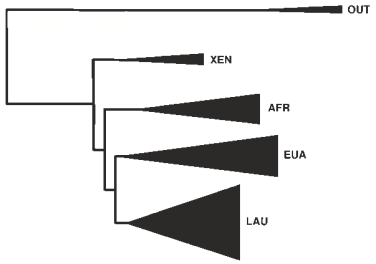
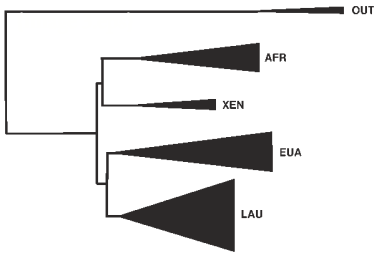
The monophyly of Xenarthra is well defined morphologically by characters generally thought to reflect adaptation toward fossoriality and myrmecophagy (Gaudin 1999b; see also Gaudin and McDonald this volume). The common ancestry of extant xenarthrans was suggested by early molecular studies (de Jong et al. 1985; Sarich 1985) and has been retrieved with strong statistical support in all subsequent sequence-based phylogenetic studies (Delsuc et al. 2001, 2002; Madsen et al. 2001; Murphy, Eizirik, Johnson et al. 2001). Furthermore, these results are also supported by the occurrence of a rare three amino acid deletion in the ά-crystallin A chain, offering a diagnostic molecular signature for Xenarthra (van Dijk et al. 1999).

Xenarthra intraordinal relationships

The respective monophyly of armadillos (Cingulata), anteaters (Vermilingua), and sloths (Folivora) is also well defined morphologically (Engelmann 1985; Gaudin and McDonald this volume). In addition, unambiguous support for a monophyletic origin of each xenarthran lineage has been found in our molecular studies (Delsuc et al. 2002, 2003).

The interrelationships of the three xenarthran lineages were a matter of conflict among morphologists (compare Guth 1961; Bugge 1979 with Engelmann 1985; Patterson et al. 1992). The most recent classification (McKenna and Bell 1997) groups anteaters and sloths into a clade called Pilosa, which refers to their coat. Early molecular studies did not contribute much to this debate (de Jong et al. 1985; Sarich 1985). Our molecular data (Delsuc et al. 2001, 2002, 2003), which included samples from all anteater and sloth genera, provided strong support for Pilosa, extending the results of Madsen et al. (2001) and Murphy, Eizirik, Johnson et al. (2001), which included fewer taxa. These results contradicted studies of the ear region (Guth 1961) and cephalic arterial patterns (Bugge 1979) that favored the early emergence of anteaters within Xenarthra. It seems likely that these studies (Guth 1961; Bugge 1979) were misled by the extreme specialization of the skull toward myrmecophagy in anteaters. Indeed, subsequent cladistic studies of characters from the ear region (Patterson et al. 1992) and other morphological and anatomical features (Engelmann 1985) have provided synapomor-

Table 2.1. Where Do Xenarthrans Fit among Placentals?

Competing topologies	All genes (17,736 sites) -lnL	pSH	Nuclear genes (16,089 sites) -lnL	pSH	Nuclear coding genes (14,327 sites) -lnL	pSH	Positions 1+2 of nuclear coding genes (9,551 sites) -lnL	pSH
	[23,0533.50]	Best	[21,1791.04]	Best	[19,2901.33]	Best	[9,4238.35]	Best
	23,0540.43 DlnL = 6.93	0.20	21,1797.86 DlnL = 6.82	0.16	19,2906.53 DlnL = 5.20	0.21	9,4238.56 DlnL = 0.21	0.62
	23,0541.92 DlnL = 8.42	0.13	21,1797.82 DlnL = 6.78	0.18	19,2906.97 DlnL = 5.64	0.18	9,4239.90 DlnL = 1.55	0.44

Notes: Results are from SH statistical tests (Shimodaira and Hasegawa 1999) for the position of the placental root using different partitions of the multiple gene dataset of Amrine-Madsen et al. (2003). These tests were computed with PAUP* 4.0b10 (Swofford 2002) under a concatenated GTR+G8+I model with parameters estimated for each alternative topology. The highest log-likelihood value is shown in brackets for each dataset and the log-likelihood difference (DlnL) relative to the best topology is given. The last hypothesis corresponds to Epitheria. pSH = probability of the SH test. Triangles in trees are drawn proportional to taxon diversity and branch length of the corresponding clade. Abbreviations: OUT = Marsupial outgroup, AFR = Afrotheria, XEN = Xenarthra, EUA = Euarchontoglires, LAU = Laurasiatheria.

phies for Pilosa, such as the interruption of the zygomatic arch and the intra-pelvian location of the testes.

Phylogeny of anteaters (Vermilingua)

The classical arrangement of Vermilingua groups the giant anteater (*Myrmecophaga*) with lesser anteaters (*Tamandua*) to the exclusion of the pygmy anteater (*Cyclopes*), which is considered morphologically divergent from the others (Engelmann 1985; Reiss 1997; Gaudin and Branham 1998). Molecular results confirmed this view by favoring the early emergence of the pygmy anteater (Delsuc et al. 2001, 2002, 2003; Barros et al. 2003), a phylogenetic hypothesis also supported by cranial muscle (Reiss 1997) and morphological (Gaudin and Branham 1998) characters.

Phylogeny of sloths (Folivora)

The two living genera of three-toed (*Bradypus*) and two-toed (*Choloepus*) sloths are unknown as fossils and have been classified into distinct families (respectively Bradypodidae and Megalonychidae) on the basis of their numerous morphological differences and a presumably diphyletic origin from two different fossil lineages (Patterson and Pascual 1972; Webb 1985a). This taxonomic distinction was supported by early immunological data demonstrating considerable evolutionary distance between the albumins of the two genera (Sarich 1985). The diphyly hypothesis also found some support from ancient DNA studies that sequenced mitochondrial 12S and 16S rRNA fragments from fossil sloths (Höss et al. 1996; Greenwood et al. 2001). These studies indicated that modern two-toed sloths (Megalonychidae) are closely related to the giant ground sloth *Mylodon darwini* (Mylodontidae), whereas three-toed sloths (Bradypodidae) appear closer to the Shasta ground sloth *Nothrotheriops shastensis* (Megatheriidae). However, these results have been contradicted by the inclusion of additional modern sloth species in phylogenetic analyses of partial 16S sequences (Barros et al. 2003). Here, moderate support was obtained for grouping *Mylodon darwini* with three-toed sloths instead of two-toed sloths. The sequencing of complete mitochondrial genomes from fossil sloths might help to resolve the controversy (H. Poinar personal communication).

The 16S rRNA study (Barros et al. 2003) was the first to include three of the four living species from the genus *Bradypus*. These authors found evidence of a sister-group relationship between the pale-throated (*B. tridactylus*) and brown-throated (*B. variegatus*) sloths to the exclusion of the endangered maned sloth (*B. torquatus*). The one species for which no molecular data are currently available is the newly described dwarf three-toed sloth (*B. pygmaeus*), endemic to the small island of Bocas del Toro in Panama (Anderson and Handley 2001). Acquisition of molecular data from this species will be important in deciding whether it deserves species status or represents a morphologically divergent population of *B. variegatus*.

There are only two living species of two-toed sloths currently recognized: the southern two-toed sloth (*Choloepus didactylus*) and Hoffmman's two-toed sloth (*Choloepus hoffmanni*). However, given the large differences in chromosome number reported between specimens within the genus (Jorge et al. 1985a; Jorge and Pereira this volume), future molecular data may be important in revealing cryptic species.

Phylogeny of armadillos (Cingulata)

With 21 living species classified into 8 genera (Wetzel 1985a; Vizcaíno 1995), armadillos (Cingulata, Dasypodidae) are the most speciose xenarthran lineage. Taxonomically, the family Dasypodidae is generally divided into five tribes: Dasypodini, Euphractini, Tolypeutini, Priodontini, and Chlamyphorini. These appear well defined morphologically (Wetzel 1985a; McKenna and Bell 1997) and are consistent with the cytological structure of their spermatozoa (Cetica et al. 1998; Cetica and Merani this volume). The tribe Dasypodini is composed of seven species of long-nosed armadillos classified in the single genus *Dasypus* (Wetzel and Mondolfi 1979; Vizcaíno 1995). The three genera of hairy armadillos—*Chaetophractus*, *Euphractus*, and *Zaedyus* (Euphractini)—are very similar morphologically and ecologically, and their interrelationships have been difficult to decipher on solely morphological grounds (Engelmann 1985; Gaudin and Wible 2006). The two species of three-banded armadillos (*Tolypeutes*) are the only members of the tribe Tolypeutini and are famous for being able to roll entirely into a ball thanks to the articulation of their carapace (Wetzel 1985a). The giant armadillo (*Priodontes maximus*) and naked-tailed armadillos (genus *Cabassous*) are traditionally united within the tribe Priodontini (Engelman 1985; Wetzel 1985a). One feature uniting them is their unusual spoon-shaped spermatozoa, which are among the largest found in mammals (Cetica et al. 1998; Cetica and Merani this volume). Finally, the tribe Chlamyphorini contains two species of fairy armadillos (genus *Chlamyphorus*; Gardner [2005] has argued that one of these

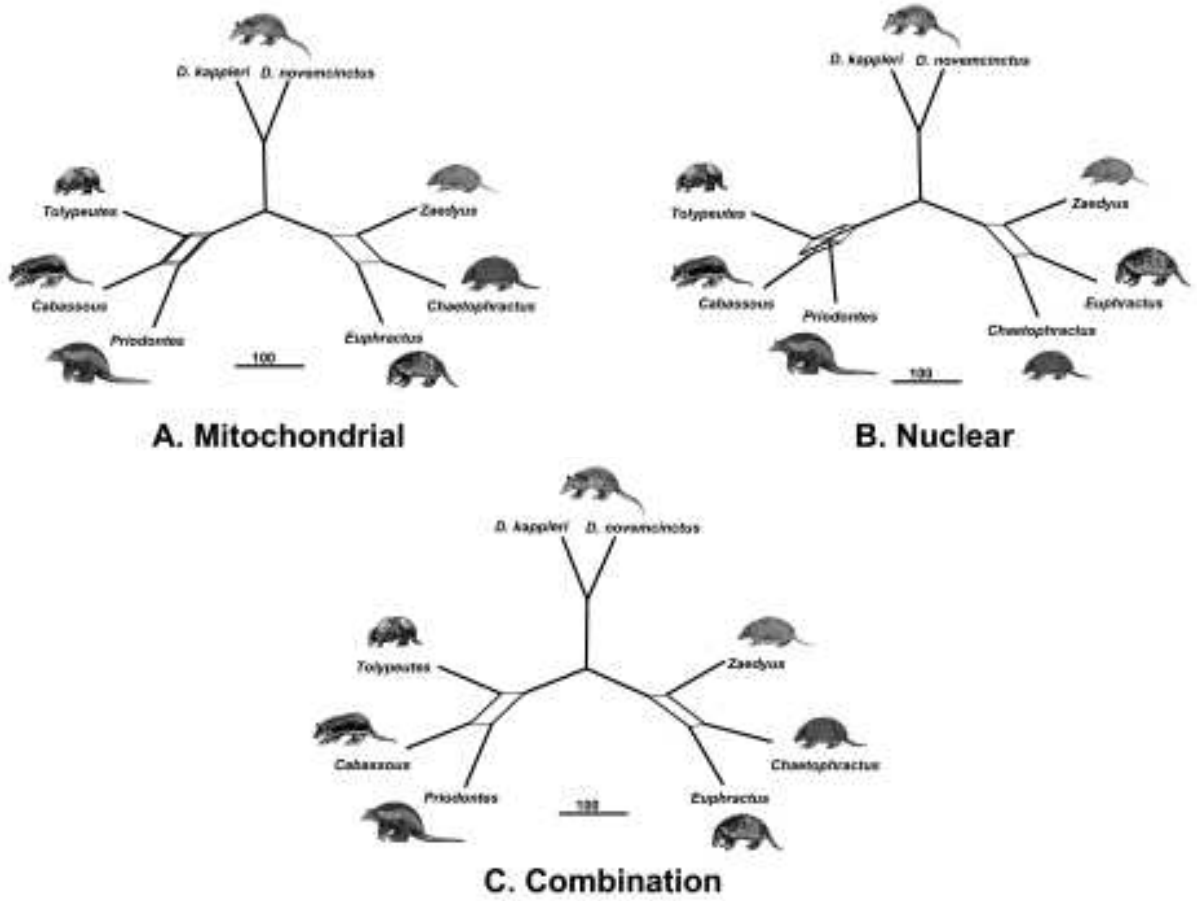


Figure 2.1. Illustration of the two remaining uncertainties in armadillo molecular phylogeny. Eight armadillo species (Delsuc et al. 2003) were analyzed using mitochondrial (A), nuclear (B), and their combination (C) data. A consensus network was computed (Holland et al. 2005) of the 100 maximum likelihood bootstrap trees obtained under the GTR+G_8 model using PAUP* 4.0b10 (Swofford 2002), with a threshold of 10% in SplitsTree 4.1 (Huson and Bryant 2006). The consensus networks therefore represent all splits that appear in more than 10 of the 100 bootstrap trees, with edge lengths corresponding to bootstrap percentages.

species, *Chl. retusus*, should be placed in its own genus, *Calyptophractus*, which would increase the total number of armadillo genera to 9). The extensively subterranean lifestyle of these cryptic animals renders the study of their biology particularly difficult (Meritt 1985c).

The phylogeny of living armadillos was poorly studied for many years, with only one cladistic analysis of morphological characters in fossil and extant taxa (Engelmann 1985). However, a recent craniodental analysis has allowed testing Engelmann's hypotheses in a cladistic context and revealed numerous incongruities (Gaudin and Wible 2006). Our molecular studies (Delsuc et al. 2001, 2002, 2003) clearly identified three main lineages of armadillos corresponding to the subfamilies defined in the classification of McKenna and Bell (1997): Dasypodinae (*Dasypus*), Euphractinae (*Chaetophractus*, *Euphractus*, and *Zaedyus*) and Tolypeutinae (*Priodontes*, *Cabassous*, and *Tolypeutes*). The early emergence of Dasypodinae was also strongly supported, with Tolypeutinae and Euphractinae unequivocally clustering together. Such a relationship is congruent with the study of spermatozoa (Cetica et al. 1998) but contradicts the morphological studies of both Engelmann (1985) and Gaudin and Wible (2006).

Molecular data failed to resolve relationships within the subfamilies Tolypeutinae and Euphractinae, with contradictory results obtained from mitochondrial versus nuclear genes, which in turn led to poorly supported relationships in ML analyses when the data were combined (Delsuc et al. 2003). The presence of contradictory signals in molecular data is illustrated in figure 2.1, which depicts phylogenies generated by the use of consensus networks (Holland and Moulton 2003). This method allows graphical representation of the uncer-

tainty in phylogenetic relationships by displaying alternative hypotheses in the form of a network, where edge lengths are proportional to bootstrap support (Holland et al. 2005). The consensus network obtained from ML bootstrap analysis of two combined mitochondrial genes (12S rRNA and ND1) displays a three-dimensional box for the relationships within Tolypeutinae, and a two-dimensional cycle for the relationships within Euphractinae (figure 2.1A). The three-dimensional box in Tolypeutinae indicates that the three possible alternatives have at least some support, with *Cabassous* + *Priodontes* as the most likely hypothesis, followed by *Cabassous* + *Tolypeutes*, with *Tolypeutes* + *Priodontes* being only marginally supported. Within Euphractinae, the two-dimensional cycle shows that there are only two competing alternatives, with *Chaetophractus* + *Euphractus* being slightly favored over *Chaetophractus* + *Zaedyus*. The same network structure is observed for the combination of three nuclear genes (ADRA2B, BRCA1, and VWF) except that the hierarchy of alternative arrangements is different (figure 2.1B). Within Tolypeutinae, *Cabassous* + *Tolypeutes* becomes the favorite hypothesis, then *Cabassous* + *Priodontes*, but *Tolypeutes* + *Priodontes* still ranks as the least well-supported. For Euphractinae, nuclear data favor *Chaetophractus* + *Euphractus* over *Chaetophractus* + *Zaedyus*. Combining all five genes results in a consensus network with two rectangular cycles describing the relationships within the two subfamilies (figure 2.1C). Two competing hypotheses remain for each subfamily, with *Cabassous* + *Priodontes* being favored over *Cabassous* + *Tolypeutes* within Tolypeutinae, and *Chaetophractus* + *Euphractus* being preferred over *Chaetophractus* + *Zaedyus* within Euphractinae (figure 2.1C).

Within tolypeutines, a close relationship between *Cabassous* and *Priodontes* would be consistent with their classification in the tribe Priodontini, if their very similar morphologies, spermatozoa (Cetica et al. 1998; Cetica and Merani this volume), and characters related to their fossorial habits, such as their enlarged manus claws (Engelmann 1985; Wetzel 1985a; McKenna and Bell 1997), are interpreted as synapomorphies rather than symplesiomorphies. Concerning euphractines, the grouping of *Euphractus* + *Chaetophractus* to the exclusion of *Zaedyus* is congruent with study of craniodental characters (Gaudin and Wible 2006). Given the independent support coming from morphological studies, it is tempting to consider these two schemes as the best current phylogenetic hypotheses for the relationships within the subfamilies Tolypeutinae and Euphractinae. It is hoped that sampling of additional genes with different selective constraints, such as nuclear introns, will confirm our predictions.

From a more general viewpoint, even if the global molecular picture of armadillo phylogenetic relationships (Delsuc et al. 2003) remains somewhat incongruent with the most recent cladistic analysis (Gaudin and Wible 2006), it is interesting to note that the two phylogenetic hypotheses are actually quite close in terms of parsimony scores (Gaudin and Wible 2006). In fact, the molecular tree appears far more compatible with the craniodental evidence than all other morphologically-based hypotheses (see Gaudin and Wible 2006). Molecular and morphological phylogenies of Cingulata are thus closer to agreement than ever.

A molecular timescale for xenarthran evolution

The newly established phylogenetic framework for extant xenarthrans was subsequently used to derive a molecular timescale for their evolutionary history. Because pervasive among-lineages substitution rate variations were recorded for the genes compared, a Bayesian method that relaxed strict molecular clock assumptions (Thorne et al. 1998) and allowed for among-gene rate heterogeneity (Thorne and Kishino 2002) was employed (Delsuc et al. 2004). This method also had the advantage of considering time intervals defined by the paleontological record instead of fixed calibration points (Kishino et al. 2001). Based on the study of three nuclear genes (ADRA2B, BRCA1, and VWF) the age of the xenarthran crown group was estimated at 65 ± 5 million years (myr), close to the Cretaceous/Tertiary boundary (figure 2.2). This is fully compatible with the age obtained by Springer et al. (2003) with a similar set of nuclear exon characters and using the same calibration points, but is more recent than previous estimates suggesting a date around 80 myr (Sarich 1985; Höss et al. 1996). This younger estimate makes Xenarthra the major placental clade with the most recently diverged extant lineages, but also with the deepest stem lineage. The gap observed between the first occurrence of fossil xenarthrans in the late Paleocene of Brazil (ca. 58 myr ago [MYA]), in the form of the earliest armadillo scutes (Scillato-Yané 1976b; Oliveira and Bergqvist 1998; Bergqvist et al. 2004), and the molecular estimate of their purported origin around 105 MYA (Springer et al. 2003; Delsuc et al. 2004), suggests the existence of

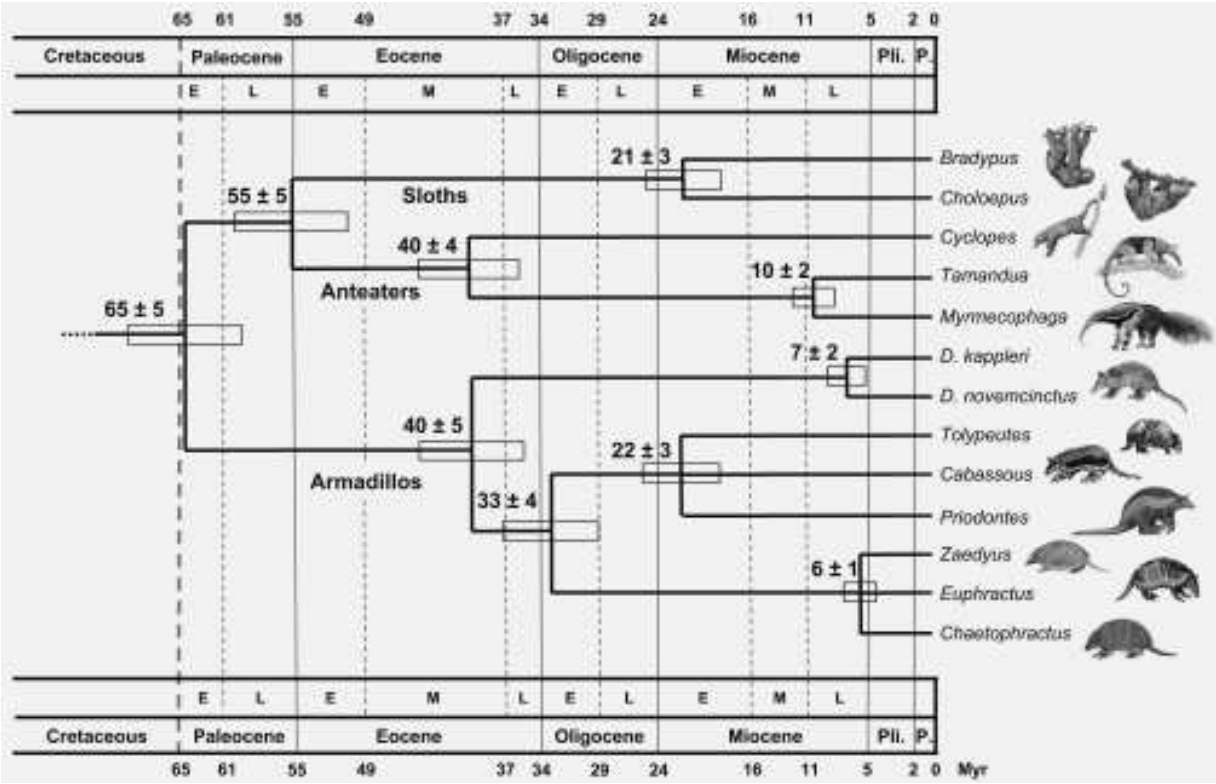


Figure 2.2. Phylogenetic relationships and molecular timescale for extant xenarthran genera based on analyses of three nuclear genes and a Bayesian relaxed molecular clock (modified from Delsuc et al. 2004). The time scale is given in million years. The mean age estimate ± SD is given for all nodes. Horizontal rectangles depict the uncertainty of age estimates based on 95% confidence intervals. Note that the relationships within both Tolypeutinae (*Tolypeutes*, *Cabassous*, and *Priodontes*) and Euphractinae (*Zaedyus*, *Euphractus*, and *Chaetophractus*) are left as unresolved to reflect the current phylogenetic uncertainty about these nodes. Vertical lines demarcate geological periods. Abbreviations: E = Early, M = Middle, L = Late, Pli. = Pliocene, P. = Pleistocene, D. = *Dasypus*.

an ancestral "ghost" lineage for almost 50 myr. This inference concurs with the view that the origin of xenarthrans constitutes a paleontological and biogeographic enigma (McKenna 1975; Engelmann 1985) that only the discovery of new fossils might help to resolve.

Results about the timing of xenarthran diversification estimated the early split between anteaters and sloths at the transition between the Paleocene and Eocene some 55 ± 5 MYA (figure 2.2). Within anteaters, molecular dating emphasized the antiquity of the pygmy anteater (*Cyclopes*) lineage, estimated as emerging in the middle Eocene around 40 ± 4 MYA, relative to the *Tamandua* and *Myrmecophaga* lineages, which diverged only 10 ± 2 MYA. Also, the considerable divergence between two-toed (*Choloepus*) and three-toed (*Bradypus*) sloths was confirmed, with an estimate of their separation at around 21 ± 3 MYA. This result gives further credit to their taxonomic placement in two distinct families, as described above. Among armadillos, the early emergence of *Dasypus* was estimated to have occurred during the middle Eocene around 40 ± 5 MYA, followed by the split between Tolypeutinae and Euphractinae at about 33 ± 4 MYA (figure 2.2). Diversification in the latter two subfamilies happened relatively quickly, but in markedly different epochs: the diversification of tolypeutines appeared quite ancient (ca. 22 ± 3 MYA), whereas euphractines diversified much more recently (ca. 6 ± 1 MYA). These results reveal that the family Dasypodidae contains lineages of fairly ancient origin, as might be expected given their distinctive morphologies (Wetzel 1985a) and marked structural differences in spermatozoa (Cetica et al. 1998; Cetica and Merani this volume).

Finally, the molecular estimates of xenarthran divergence dates were correlated with the relatively well-documented paleoenvironmental changes that occurred

during the Tertiary of South America (Patterson and Pascual 1972; Pascual and Ortiz Jaureguizar 1990; Marshall and Sempere 1993). This allowed us to unravel the potentially important role played by paleoenvironmental changes in the diversification of living xenarthrans (Delsuc et al. 2004). Indeed, molecular dating revealed a striking synchronicity in some diversification events among independent xenarthran lineages. For instance, the separation of *Cyclopes* from other anteaters was correlated with the separation of the *Dasypus* lineage from other armadillos in the middle Eocene around 40 MYA (figure 2.2). Similarly, the diversification of Tolypeutinae paralleled the separation between the two modern sloth genera around 21–22 MYA in the early Miocene. Finally, the recent diversification of Euphractinae matched well with the separation between *Dasypus novemcinctus* and *Dasypus kappleri* some 6–7 MYA in the late Miocene (figure 2.2). Although we cannot rule out the possibility that such a correlated history between independent lineages occurred by chance, it is more likely these biological events reflect the impact of environmental changes. Actually, all three synchronous diversification events appear to follow periods of important environmental changes, possibly triggered by major phases of Andean uplift (Marshall and Sempere 1993). The evolutionary history of extant xenarthrans therefore seems to have been influenced by the environmental changes that occurred during the Tertiary of South America.

Future prospects in xenarthran molecular phylogenetics

The promises of comparative genomics

As described above, molecular phylogenetic studies based on multiple genes currently leave the position of Xenarthra within placental mammals unresolved, with three alternative positions for the root being almost equally likely (see table 2.1). The solution to this difficult problem, as with many other uncertainties in the tree of life, might come from future phylogenomic studies taking advantage of the wealth of genomic data generated by ongoing large-scale sequencing projects (Delsuc et al. 2005). Indeed, genomes of 27 mammalian species, including representatives from all the major placental clades, are currently being sequenced at varying levels of coverage, an effort offering great promise for mammalian phylogenomics (Murphy et al. 2004). Among these species, of particular importance are the platypus (*Ornithorhynchus anatinus*) and two marsupials (the Tammar wallaby, *Macropus eugenii*, and the gray short-tailed opossum, *Monodelphis domestica*) that can be used as outgroups in both comparative genomic and phylogenetic studies. Within Xenarthra, two species—the nine-banded armadillo (*Dasypus novemcinctus*) and Hoffman's two-toed sloth (*Choloepus hoffmanni*)—have been or will be sequenced (see Chang and Adams this volume).

Genomic data have the potential to provide answers to many remaining questions in placental mammal phylogeny, most importantly the position of the root, and thereby the relationships among Afrotheria, Xenarthra, and Boreoeutheria. Indeed, the wealth of data accumulated by the phylogenomic approach offers the luxury of selecting only the most reliable sites for classical sequence-based phylogenetic analyses (Delsuc et al. 2005). In the context of mammalian phylogenomics, consideration of the platypus as an additional outgroup might prove particularly useful for rooting the placental tree, because it might break the problematic long branch of marsupials, as was previously demonstrated with complete mitochondrial genomes (Cao et al. 1997; Philippe 1997). Moreover, access to complete genomes affords the opportunity to mine them for signatures of common ancestry (Delsuc et al. 2005). Useful signatures include rare genomic changes such as diagnostic insertion/deletion events, intron positions, transposable element (SINEs and LINEs) integrations, gene fission/fusion events, and evidence of shared gene families or chemical pathways. These whole-genome features can be used as an independent source for corroboration of sequence-based phylogenies (Delsuc et al. 2005). Of special interest for resolving the relationships of Afrotheria and Xenarthra is the study of SINEs integration: specific families have been identified in both groups (Nikaido et al. 2003; Churakov et al. 2005) and successfully used at the intraordinal level in placental phylogenetics (Nikaido et al. 1999; Schmitz and Zischler 2003; Nishihara et al. 2005; Schmitz et al. 2005). Such an approach was recently applied by Kriegs et al. (2006), who identified two retroposon markers found to be absent in Xenarthra but present in all other placental Afrotheria and Boreoeutheria, providing some support for the Epitheria hypothesis. However, conclusions are tentative because evidence that the markers are also missing in marsupials is still uncertain. The sequencing of marsupial and monotreme complete

genomes will soon provide the opportunity to check whether these elements are really absent in placental outgroups.

Toward a phylogeny of living xenarthran species

Ultimately, continued efforts on the molecular front should result in a well-resolved phylogeny of extant xenarthrans at the species level. This will provide answers to evolutionary questions, some of which we highlight below.

The evolutionary affinities of fairy armadillos

At present, the only lineage of armadillos for which there are no molecular data is the tribe Chlamyphorini (fairy armadillos or pichiciegos), which includes the single genus *Chlamyphorus* Harlan 1825 (but see Gardner 2005). Two species, *Chl. truncatus* (pink fairy armadillo) and *Chl. retusus* (greater fairy armadillo), are traditionally recognized. They are rather similar morphologically in being highly adapted to burrowing, with enlarged digging claws and reduced eyes. However, marked differences between the two species exist, most notably in the structure of the carapace and the shape of the cephalic shield. To reflect the degree of morphological differentiation between these two species, the larger species, *Chl. retusus*, was assigned to its own genus, *Burmeisteria* Gray, 1865 or *Calyptophractus* Fitzinger, 1871. Moeller (1968) proposed retaining *Burmeisteria* (however, this name is preoccupied, so the appropriate choice should have been *Calyptophractus*, see Gardner 2005). In contrast, cladistic analyses of morphological characters indicate the two species of fairy armadillos are monophyletic and phylogenetically related to members of the family Euphractinae (Engelmann 1985; Gaudin and Wible 2006; Gaudin and McDonald this volume). However, as it has never been molecularly tested, the possibility of a diphyletic origin of the two species cannot be rejected *a priori*. Indeed, based on characters like the shape and color pattern of the carapace, as well as the shape of the anterior claws, *Chl. retusus* resembles naked-tailed armadillos from the tribe Priodontini, whereas *Chl. truncatus* seems to be closer to Euphractini. These observations leave open the possibility that the morphological similarities between fairy armadillos might be the result of convergence due to extreme selective pressures induced by their subterranean lifestyle. Given their rarity and the difficulties in obtaining biological material from these species, molecular analyses of fairy armadillos may have to exploit museum specimens, using ancient DNA techniques. This might limit the potential molecular markers to mitochondrial genes, which are much easier to amplify in this context. The ND1 and 12S rRNA genes, for which data from all other armadillo genera are already available (Delsuc et al. 2003), seem therefore to represent the best candidates. Perhaps with these data we will finally be able to resolve the phylogenetic affinities of fairy armadillos and thus better understand the evolution of morphological characters in these enigmatic animals.

Tracking the origin of polyembryony in long-nosed armadillos

One of the most fascinating features observed in xenarthrans is the occurrence of monozygotic polyembryony—the splitting of one sexually produced embryo into many—in long-nosed armadillos (genus *Dasypus*, see the chapters by Enders, Prodöhl et al., and McDonough and Loughry this volume). In these species, the origin and evolution of polyembryony is generally interpreted as a response to the phylogenetic constraint represented by an unusual uterine shape with only one implantation site (Galbreath 1985; but see Enders this volume). Galbreath proposed an evolutionary scenario in which the specialized uterus evolved first, thus preadapting for monozygotic polyembryony any species that underwent selection for increasing litter size. Testing this evolutionary hypothesis requires, first, molecular confirmation of the occurrence of polyembryony in each of the seven *Dasypus* species (cf. Prodöhl et al. this volume) and, second, reconstruction of their phylogenetic relationships. Such analyses are a prerequisite for understanding the evolution of twin number (i.e., litter size) and the structure and organization of the reproductive tract (Enders this volume). The reconstruction of a species-level phylogeny for the genus *Dasypus* would therefore provide new insights into the origin and evolution of polyembryony.

Modern cytogenetics and molecular phylogenetics

Cytological and karyological studies in xenarthrans have long been restricted to classical studies in which karyotypes were described using techniques such as G-banding (Jorge et al. 1985a; Jorge and Pereira this volume). However, molecular phylogenetics is not the only discipline where advances in technology have led to renewed interest. Indeed, cytogenetics is currently experiencing a rebirth thanks to the development of

new techniques such as chromosome painting (FISH: fluorescence *in situ* hybridization) and radiation hybrid mapping, both of which allow the fine-tuned study of chromosomal evolution (Murphy, Stanyon, and O'Brien 2001). These techniques were recently applied successfully to characterize the dynamics of chromosome evolution in placental mammals as a first step toward reconstructing their ancestral karyotype (Murphy et al. 2005).

Unfortunately, data from xenarthrans, which are pivotal for inferring the ancestral placental karyotype, were missing until recently. A first attempt at applying these new techniques to xenarthran cytogenetics was recently made using chromosome painting comparisons among the three major lineages. Data came from a two-toed (*Choloepus didactylus*, 2n = 64) and three-toed sloth (*Bradypus tridactylus*, 2n = 52), the lesser anteater (*Tamandua tetradactyla*, 2n = 54), and the six-banded armadillo (*Euphractus sexcinctus*, 2n = 58, Dobigny et al. 2005). By using the phylogenetic framework and timescale derived from previous molecular studies (Delsuc et al. 2003, 2004) to map inferred chromosomal changes, these authors revealed a low rate of genomic repatterning in Xenarthra relative to other placentals (Dobigny et al. 2005). Moreover, by identifying homologous chromosomal segments that have been conserved among members of the three lineages, this study provides clues to the likely architecture of the ancestral karyotype for extant xenarthrans (Dobigny et al. 2005). Additional insight into this ancestral karyotype was provided recently by Svartman et al. (2006), who hybridized human chromosome probes to metaphases of *Dasypus novemcinctus*, *Tamandua tetradactyla*, and *Choloepus hoffmanii*. They showed that the two-toed sloth, *C. hoffmanii*, (2n = 50), exhibited a chromosome complement strikingly similar to the proposed 2n = 48 ancestral Eutherian karyotype. Future consideration of additional species with less conserved genome architecture (see Jorge and Pereira this volume), such as three-banded armadillos (*Tolypeutes*) and the silky anteater (*Cyclopes didactylus*), will critically test the conclusion of low genomic repatterning in Xenarthra. Also, the inclusion of armadillo species belonging to the two currently unsampled groups (Tolypeutinae and Euphractinae) will allow a more precise delineation of the ancestral xenarthran karyotype.

Phylogeny and conservation genetics

As reviewed by Prodöhl et al. (this volume), population genetic studies of living xenarthrans are just beginning, with most efforts concentrated on the most common and widespread xenarthran species: the nine-banded armadillo (*Dasypus novemcinctus*). However, the nine-banded armadillo constitutes a case study illustrating well the insights that can be gained from molecular approaches, that is, combining fine-scale population genetics with phylogeographic studies at a larger scale. Such molecular studies have the potential to enhance our understanding of proximal and historical factors that influenced the evolutionary history of xenarthran species and might help define conservation strategies.

Conservation genetic studies within Xenarthra so far have been limited to flagship species such as the endangered maned sloth (*Bradypus torquatus*) of the remnant Brazilian Atlantic forest (Moraes-Barros et al. 2002), and the giant anteater (*Myrmecophaga tridactyla*), for which microsatellite loci are only beginning to be developed (Garcia et al. 2005). Unfortunately, Xenarthra contains a number of vulnerable and endangered species (Aguiar and Fonseca this volume) for which virtually no data exist on their genetic diversity. Consequently, species delineation still relies on morphological, geographical, and ecological observations. Given the high rate at which biological diversity is currently being eroded, phylogeographic and population genetic studies are urgently needed to characterize the extent of genetic diversity in xenarthran populations and thus identify biologically important conservation entities. The definition of a comprehensive phylogenetic and taxonomic framework is a prerequisite for designing efficient conservation plans for living xenarthran species (Purvis et al. 2005).

Conclusions

The use of molecular data to assess xenarthran phylogenetic relationships has provided several new insights into the evolutionary history of this often neglected major lineage of placental mammals. However, much still needs to be done on the molecular side to answer such fundamental questions as the position of Xenarthra within placentals. The new genomic era appears full of promise to adequately locate the root of the placental tree, and modern cytogenetics has the potential to reveal how the genomic architecture of xenarthrans has evolved. The ultimate goal of obtaining a fully resolved molecular phylogeny for extant xenarthrans will be achieved only through the collaborative and con-

certed efforts of the xenarthran research community. Such a phylogenetic framework is urgently needed to help design conservation strategies for the numerous endangered xenarthran species (see Aguiar and Fonseca this volume).

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