

The interphotoreceptor retinoid binding protein gene in therian mammals: Implications for higher level relationships and evidence for loss of function in the marsupial mole

(mammalian phylogenetics/*Notoryctes*/pseudogene)

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ABSTRACT The subclass Theria of Mammalia includes marsupials (infraclass Metatheria) and placentals (infraclass Eutheria). Within each group, interordinal relationships remain unclear. One limitation of many studies is incomplete ordinal representation. Here, we analyze DNA sequences for part of exon 1 of the interphotoreceptor retinoid binding protein gene, including 10 that are newly reported, for representatives of all therian orders. Among placentals, the most robust clades are Cetartiodactyla, Paenungulata, and an expanded African clade that includes paenungulates, tubulidentates, and macroscelideans. Anagalida, Archonta, Altungulata, Hyracoidea + Perissodactyla, Ungulata, and the “flying primate” hypothesis are rejected by statistical tests. Among marsupials, the most robust clade includes all orders except Didelphimorphia. The phylogenetic placement of the monito del monte and the marsupial mole remains unclear. However, the marsupial mole sequence contains three frameshift indels and numerous stop codons in all three reading frames. Given that the interphotoreceptor retinoid binding protein gene is a single-copy gene that functions in the visual cycle and that the marsupial mole is blind with degenerate eyes, this finding suggests that phenotypic degeneration of the eyes is accompanied by parallel changes at the molecular level as a result of relaxed selective constraints.

Marsupials (infraclass Metatheria) and placentals (infraclass Eutheria) together compose the subclass Theria of Mammalia. Among extant taxa, there are five to seven marsupial orders (1–3) and 18 placental orders (4) (Table 1). Within each subclass, it has proven difficult to resolve higher level relationships. Nevertheless, numerous phylogenies have been hypothesized and serve as a framework for examining questions in higher level mammal phylogeny.

Among living marsupials, there is uncertainty centering on the earliest cladogenic events. Most workers (1, 2, 5) advocate a fundamental division between American marsupials, excepting the South American microbiothere *Dromiciops gliroides* (monito del monte) and Australasian marsupials plus *Dromiciops*. The American taxa (Ameridelphia) are united by the presumed shared derived character of epididymal sperm-pairing (6). Australasian taxa and *Dromiciops* (Australidelphia), in turn, exhibit a derived morphology of the tarsus that facilitates improved grasping (3, 7). Other hypotheses reject this fundamental split between ameridelphians and australidelphians and postulate *Dromiciops* (8), caenolestids (3), and didelphids (9), respectively, as the sister taxon to other living marsupials.

Relationships among Australidelphia, assuming monophyly of this group, also are unclear. *Dromiciops* has been hypothesized as a sister taxon to (i) all other australidelphians (2, 7),

(ii) dasyuromorphs (3, 10), and (iii) diprotodontians (11, 12). The latter hypothesis derives from single-copy DNA hybridization experiments, which also place bandicoots outside of a clade that includes diprotodontians, *Dromiciops*, and dasyuromorphs (11, 12). The association of *Dromiciops* with diprotodontians is inconsistent with the conventional view that diprotodontians and bandicoots are sister taxa based on the occurrence of syndactyly in these taxa (13, 14). Syndactyly is a condition in which the second and third digits of the hind foot are variably reduced and always are joined together by a common integument (13, 14). One implication of the single-copy DNA results is that syndactyly may not be homologous in diprotodontians and bandicoots and that it evolved independently in these two groups or else was lost in other taxa (e.g., *Dromiciops* and dasyuromorphs) after evolving in a more basal common ancestor (12, 15).

Another enigmatic taxon is the marsupial mole, *Notoryctes typhlops*, which is the only living representative of the order Notoryctemorphia. *Notoryctes* possesses numerous autapomorphic features related to its fossorial lifestyle and has been hypothesized to be a relative of diprotodontians (2, 16, 17), dasyurids (18, 19), and bandicoots (3).

Relationships among placental orders are equally controversial. At the base of the placental radiation, several studies suggest that xenarthrans or xenarthrans plus pholidotans are the sister group to other placental mammals, collectively termed the “Epitheria” (20–26).

Among the epitherian superorders, Archonta, Glires, Paenungulata, and Cetartiodactyla have received considerable attention. Archonta (27) includes the orders Chiroptera, Primates, Scandentia, and Dermoptera. Only a few anatomical features (e.g., pendulous penis, tarsal specializations) support the monophyly of this superorder (21, 28). Within this group, Novacek (29) and Simmons (30) suggest an association of chiropterans and dermopterans (=Volitantia) based on morphological data, and Pettigrew (31) has proposed a “flying primate” hypothesis in which Old World fruitbats (suborder Megachiroptera) are more closely related to dermopterans and primates than to microchiropterans. Molecular data provide some support for the association of Primates, Scandentia, and Dermoptera together but argue against the inclusion of chiropterans with this group (32–35).

The superorder Glires, which encompasses rodents and lagomorphs, is supported by craniodental and fetal membrane characters (22, 36). Morphological data also suggest an association of macroscelideans (elephant shrews) with Glires in the superorder Anagalida (37). Glires has been challenged based on an analysis of 91 orthologous protein sequences (38). Furthermore, Anagalida is contradicted by both nuclear and mitochondrial gene studies (35, 39–40). Even the seemingly

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Table 1. Classification of therian mammals

Subclass Theria
Infraclass Metatheria (marsupials)
Order Dasyuromorphia (<i>Phascogale</i> = phascogale)
Order Didelphimorphia (<i>Didelphis</i> = opossum)
Order Diprotodontia (<i>Pseudochirops</i> = ringtail possum; <i>Vombatus</i> = wombat)
Order Microbiotheria (<i>Dromiciops</i> = monito del monte)
Order Notoryctemorphia (<i>Notoryctes</i> = marsupial mole)
Order Paucituberculata (<i>Caenolestes</i> = shrew opossum)
Order Peramelina (<i>Echymipera</i> = bandicoot)
Infraclass Eutheria (placentals)
Order Artiodactyla (<i>Bos</i> = cow; <i>Sus</i> = pig)
Order Carnivora (<i>Felis</i> = cat)
Order Cetacea (<i>Balaenoptera</i> = whale; <i>Steno</i> = rough-toothed dolphin)
Order Chiroptera (<i>Cynopterus</i> = dog-faced bat; <i>Megaderma</i> = false vampire bat; <i>Pteropus</i> = flying fox; <i>Tonatia</i> = round-eared bat)
Order Dermoptera (<i>Cynocephalus</i> = flying lemur)
Order Hyracoidea (<i>Procavia</i> = hyrax)
Order Insectivora (<i>Erinaceus</i> = hedgehogs; <i>Sorex</i> = shrew)
Order Lagomorpha (<i>Oryctolagus</i> = rabbit)
Order Macroscelidea (<i>Elephantulus</i> = elephant shrew)
Order Perissodactyla (<i>Equus</i> = horse)
Order Pholidota (<i>Manis</i> = pangolin)
Order Primates (<i>Galago</i> = galago; <i>Homo</i> = human; <i>Tarsius</i> = tarsier)
Order Proboscidea (<i>Loxodonta</i> = elephant)
Order Rodentia (<i>Mus</i> = mouse; <i>Spalax</i> = blind mole rat)
Order Scandentia (<i>Tupaia</i> = tree-shrew)
Order Sirenia (<i>Dugong</i> = dugong; <i>Trichechus</i> = manatee)
Order Tubulidentata (<i>Orycteropus</i> = aardvark)
Order Xenarthra (<i>Bradypus</i> = sloth)

Genera included in this study are given in parentheses. The classification for marsupials follows Marshall *et al.* (2). The classification for placentals follows Novacek (68).

secure monophyly of Rodentia is inconsistent by some molecular studies (42, 43) wherein myomorphs and caviomorphs do not cluster together.

Paenungulata (44) includes Proboscidea, Sirenia, and Hyracoidea. Although most workers agree that proboscideans and sirenians are closely related, the association of hyracooids with these orders is controversial, and a competing hypothesis unites hyraxes with perissodactyls. Morphological evidence for these competing hypotheses is divided (20, 23, 37, 45–49) whereas molecular evidence supports Paenungulata (35, 39, 41, 50, 51).

One of the strongest supported associations of placental orders is Cetartiodactyla (=Cetacea + Artiodactyla). Molecular evidence supports this association (35, 52) and further suggests that artiodactyls are paraphyletic with hippopotamids as a possible sister taxon to cetaceans (53–54). Ferungulata [*sensu* Krettek *et al.* (55)] is a more inclusive group that contains Cetartiodactyla, Perissodactyla, and Carnivora. Ungulata, as advocated by McKenna (20), includes cetartiodactyls, perissodactyls, and paenungulates. Archibald (56) suggested a more expansive Ungulata that also embraces tubulidentates. Presumed evidence for Ungulata includes the rectangular shape of the upper molars (56).

Among the gene sequences that have figured in testing and developing interordinal phylogenetic hypotheses is exon 1 of the interphotoreceptor retinoid binding protein (IRBP) gene (34, 35). IRBP is a single-copy gene that functions in the regeneration of rhodopsin in the visual cycle (57). Stanhope *et al.* (35) reported on a data set that included representatives of 17 of the 18 placental orders. By using xenarthrans as an outgroup, Stanhope *et al.* (35) found strong support for Cetartiodactyla, Paenungulata, and an expanded clade con-

taining paenungulates, macroscelideans, and tubulidentates. However, it is questionable whether or not xenarthrans are an outgroup to other placental mammals (26, 58, 59). Here, we report 10 IRBP sequences and expand the data set of Stanhope *et al.* (35). The sequences include a hedgehog, a manatee, a pangolin, and seven marsupials. The present collection of sequences is a data set for a nuclear gene that includes representatives of every placental and marsupial order. This allows us to examine higher level questions in mammalian evolution in the context of a broad phylogenetic framework in which numerous marsupials and placentals serve as reciprocal outgroups to each other. The inclusion of multiple representatives from each mammalian infraclass also allows for subdivision of long branches to minimize the effects of homoplasy. In addition to the phylogenetic implications of the IRBP data, we report on the evolution of the IRBP gene in the marsupial mole, which is blind and has eyes that lack an iris and a lens. Specifically, indels and stop codons suggest that the *Notoryctes* IRBP gene is no longer evolving under purifying selection.

METHODS

DNA was extracted from seven marsupials (*Caenolestes fuliginosus*, *Dromiciops gliroides*, *Echymipera kalubu*, *Phascogale tapoatafa*, *Notoryctes typhlops*, *Vombatus ursinus*, and *Pseudochirops cupreus*) and three placentals (*Manis* sp., *Erinaceus europaeus*, and *Trichechus manatus*) as described elsewhere (34, 60). Part of exon 1 of the IRBP gene was amplified by using the primers +IRBP217 and –IRBP1531 (34) except for *Caenolestes* and *Phascogale* for which the primers IR-H (5'-aacctaattgggatgcaagaag-3') and IR-I (5'-tccgagcccaaatgctg-gcca-3'), respectively, were substituted for IR-A. Marsupial PCR products were cloned into PCR II (Invitrogen) and sequenced (at least three clones) or sequenced directly by using the PCR Product Sequencing Kit (United States Biochemical). Placental PCR products were sequenced as described in Stanhope *et al.* (35). In all cases, both strands were sequenced. Accession numbers for the sequences are AF025381–AF025390. Additional sequences derive from previous analyses (34, 35). Table 1 lists the ordinal representation of taxa included in our study. Sequences were aligned by using CLUSTAL W (61) with minor adjustments by eye.

All phylogenetic analyses and tests were performed with PAUP 4.0d54, test version written by David L. Swofford. We retained third positions of codons in our analyses because they retain phylogenetic signal (62) at the interordinal level, i.e., by using a single representative from each of the 25 orders, $g_1 = -0.818$ (significant at $P = 0.01$). Full heuristic searches were used with maximum parsimony and maximum likelihood (ML) under the Hasegawa *et al.* (63) model of sequence evolution. Previous hypotheses (Table 2) were evaluated by using Templeton (64), Kishino–Hasegawa (65), and winning sites (66) tests. In cases in which hypothesized clades were supported on the best tree(s), we made comparisons with the best trees that constrained against the monophyly of these clades. When hypothesized clades did not occur on the best tree(s), we made comparisons with the best trees that constrained the monophyly of these clades.

RESULTS

Alignment. Fig. 1 shows portions of the 1302-nt multiple alignment for four marsupials (*Echymipera*, *Dromiciops*, *Vombatus*, and *Notoryctes*). With the exception of *Notoryctes*, all sequences in the alignment were from ORFs without stop codons. *Notoryctes* shows four indels, three of which are not in multiples of three nucleotides. The first is an 11-nt deletion extending from position 38 to position 48, second is a 1-nt insertion at position 477, and, finally, a 1-nt deletion at position 1120. These indels do not complement each other to restore a correct reading frame; translation of the *Notoryctes* sequence

Table 2. Results of Kishino–Hasegawa, Templeton, and winning-sites tests

Clade	Shortest tree with clade	Shortest tree without clade	KH-P	Templeton	Winning sites	-Ln likelihood with clade	-Ln likelihood without clade	KH-ML
Marsupial clades								
All except <i>Didelphis</i>	3724 (2)	3732	0.5737	0.6158	0.7078	18921.9577	18943.7639	0.3597
All except <i>Dromiciops</i>	3742	3724 (2)	0.0605	0.1000	0.0405*	18943.5070	18921.9577	0.1998
Ameridelphia	3732	3724 (2)	0.5737	0.6158	0.7078	18937.6790	18921.9577	0.3862
Australidelphia	3724 (2)	3727	0.6311	0.6714	0.8676	18921.9577	18926.6137	0.7369
<i>Notoryctes</i> +								
Diprotodontia	3730 (2)	3724 (2)	0.0488*	0.0769	0.0886	18923.3736	18921.9577	0.8575
Diprotodontia +								
Peramelina	3731 (3)	3724 (2)	0.2543	0.3161	0.3179	18932.0427	18921.9577	0.5595
Peramelina +								
Dasyuromorphia	3724 (2)	3728 (2)	0.4725	0.5305	0.5903	18921.9577	18931.3186	0.5900
<i>Notoryctes</i> +								
Dasyuromorphia	3729 (4)	3724 (2)	0.3654	0.4292	0.4690	18931.3186	18921.9577	0.5900
Placental clades								
Africana	3724 (2)	3753 (6)	0.0204*	0.0429*	0.0248*	18921.9577	19003.3833	0.0004*
Altungulata	3778 (2)	3724 (2)	<0.0001*	<0.0001*	<0.0001*	19086.8585	18921.9577	<0.0001*
Anagalida	3775 (2)	3724 (2)	<0.0001*	<0.0001*	<0.0001*	19045.6117	18921.9577	<0.0001*
Archonta	3754 (2)	3724 (2)	0.0317*	0.0614	0.0290*	18996.6624	18921.9577	0.0084*
Archonta (-bats)	3728 (10)	3724 (2)	0.4656	0.5200	0.5993	18922.8399	18921.9577	0.9063
Cetartiodactyla	3724 (2)	3744 (10)	0.0709	0.0995	0.0969	18921.9577	18962.4634	0.0791
Edentata	3740 (4)	3724 (2)	0.2232	0.2579	0.3283	18939.9958	18921.9577	0.3295
Epitheria I	3731 (12)	3724 (2)	0.5135	0.5622	0.6662	18937.9144	18921.9577	0.1871
Epitheria II	3753 (4)	3724 (2)	0.0116*	0.0239*	0.0235*	18944.7378	18921.9577	0.1507
Ferungulata	3728 (2)	3724 (2)	0.5147	0.5676	0.6752	18921.9577	18926.9409	0.7198
“Flying primate”	3763 (1)	3724 (2)	0.0075*	0.0137*	0.0236*	19045.1750	18921.9577	0.0003*
Glires	3724 (2)	3727 (6)	0.7633	0.7992	0.6617	18921.9577	18932.9675	0.5426
Hyracoidea +								
Perissodactyla	3800 (2)	3724 (2)	<0.0001*	<0.0001*	<0.0001*	19155.6222	18921.9577	<0.0001*
Paenungulata	3724 (2)	3735 (6)	0.1689	0.2275	0.2229	18921.9577	18943.0474	0.0602
Tethytheria	3730 (2)	3724 (2)	0.1393	0.1883	0.2085	18930.8171	18921.9577	0.5398
Ungulata I	3785 (2)	3724 (2)	<0.0001*	<0.0001*	<0.0001*	19072.9924	18921.9577	<0.0001*
Ungulata II	3787 (2)	3724 (2)	<0.0001*	<0.0001*	<0.0001*	19118.4440	18921.9577	<0.0001*
Volitantia	3785 (50)	3724 (2)	0.0538	0.0879	0.1790	19002.5185	18921.9577	0.0208*

Composition of superordinal clades is as follows: Ameridelphia = Didelphimorphia + Paucituberculata; Australidelphia = Dasyuromorphia + Diprotodontia + Microbiotheria + Notoryctemorphia + Peramelina; Africana = Macroscelidea + Paenungulata + Tubulidentata; Altungulata = Paenungulata + Perissodactyla; Anagalida = Glires + Macroscelidea; Archonta = Chiroptera + Dermoptera + Primates + Scandentia; Cetartiodactyla = Artiodactyla + Cetacea; Epitheria I = all placentals except Xenarthra; Epitheria II = Pholidota + Xenarthra as sister-group to other placentals; Ferungulata = Carnivora + Cetartiodactyls + Perissodactyla; “Flying Primate” = Dermoptera + Megachiroptera + Primates; Glires = Lagomorpha + Rodentia; Paenungulata = Hyracoidea + Proboscidea + Sirenia; Tethytheria = Proboscidea + Sirenia; Ungulata I = Cetartiodactyla + Paenungulata + Perissodactyla + Tubulidentata; Ungulata II = Cetartiodactyla + Paenungulata + Perissodactyla; Volitantia = Chiroptera + Dermoptera. Additional placental clades that could not be accepted or rejected based on statistical tests include Insectivora, Artiodactyla, and Chiroptera. Among marsupials, *Dromiciops* + Diprotodontia and *Notoryctes* + Peramelina were evaluated but could not be accepted or rejected. Asterisks indicate significant at $P = 0.05$. KH-P, Kishino–Hasegawa test for parsimony; KH-ML, Kishino–Hasegawa test for maximum likelihood.

in all three reading frames indicates the occurrence of nine, 15, and 10 stop codons, respectively.

Phylogenetic Analyses. The best ML tree is illustrated in Fig. 2. Two minimum length parsimony trees (not shown) were discovered at 3724 steps (consistency index = 0.436). A majority rule bootstrap consensus tree based on parsimony is depicted in Fig. 3. Among marsupials, *Didelphis* is excluded from a clade that includes representatives of all other marsupial orders on both the ML and maximum parsimony trees. Bootstrap support uniting all marsupials to the exclusion of *Didelphis* was 80%. None of the other interordinal associations among marsupials was this well supported. Several placental clades were supported above the 95% bootstrap level including Paenungulata (99%), the African clade (i.e., Paenungulata + Macroscelidea + Tubulidentata; 100%), and Cetacea + Artiodactyla (100%).

Templeton, Kishino–Hasegawa, and winning sites tests indicated that the African clade was significantly better than the best trees that constrained against the monophyly of these groups. Other clades, including Paenungulata and Cetartiodactyl, were not significantly better than the best trees that constrained against these groups. Clades that were rejected at

the $P = 0.05$ level included (i) Anagalida, (ii) Archonta, (iii) Dermoptera + Megachiroptera + Primates, (iv) Hyracoidea + Perissodactyla, and (v) Ungulata, with or without the inclusion of tubulidentates. Epitheria was rejected by all of the parsimony tests if xenarthrans and pholidotans were the sister group to other placentals but not if Xenarthra alone was the sister group to other placentals. Other hypotheses, including Archonta (excepting chiropterans), Edentata, Ferungulata, Glires, and Tethytheria, could not be accepted or rejected based on statistical tests.

Most of the marsupial hypotheses were either not supported or could not be rejected at the $P = 0.05$ level. Exceptions included rejection of the Hershkovitz hypothesis (i.e., *Dromiciops* as a sister group to all other marsupials) based on the winning sites test and rejection of the diprotodontian–*Notoryctes* alliance based on the Kishino–Hasegawa test (parsimony).

DISCUSSION

Mammalian Phylogeny. Stanhope *et al.* (35) found strong support for Paenungulata, a more inclusive African clade (Paenungulata + Macroscelidea + Tubulidentata), and Cetartiodactyla in an IRBP study that included representatives of 17

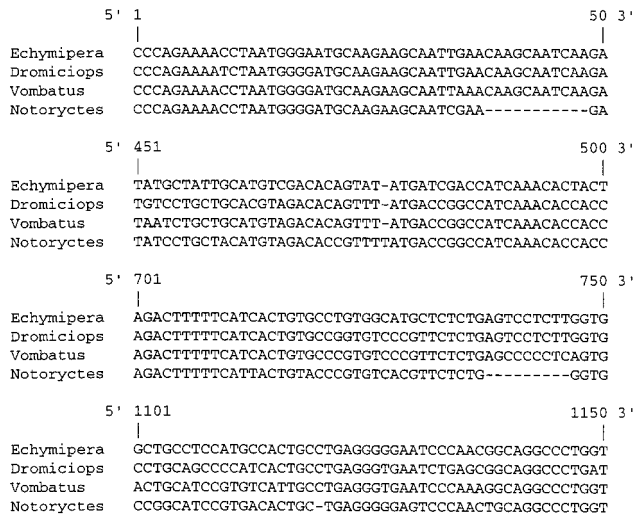


FIG. 1. Selected regions of the IRBP alignment for four marsupials to illustrate the location of three deletions and one insertion in the *Notoryctes* sequence relative to other marsupials.

of 18 placental orders and that used a xenarthran (sloth) as an outgroup to other placentals. With the addition of the remaining placental order (pangolins) as well as a subdivided marsupial outgroup, these associations remain robust and are all supported at or above the 99% bootstrap level. These clades also are supported by other molecular data sets and are emerging as the most secure associations of placental orders (39–41). In the case of Paenungulata, several independent data sets now provide strong support in favor of this hypothesis

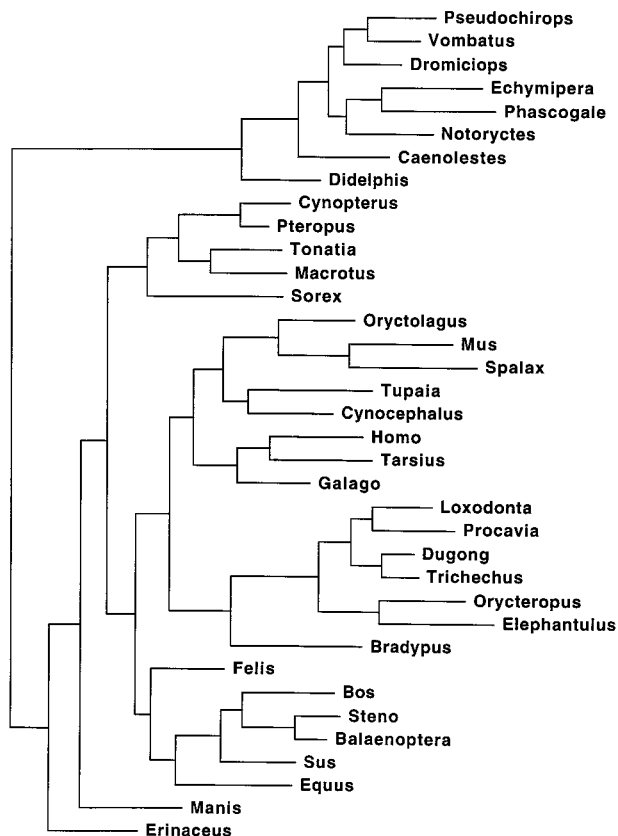


FIG. 2. ML tree (-ln likelihood = 18921.95773) under the HKY85 model of sequence evolution. For scale, the terminal branch leading to *Erinaceus* represents 11.6% nucleotide substitution.

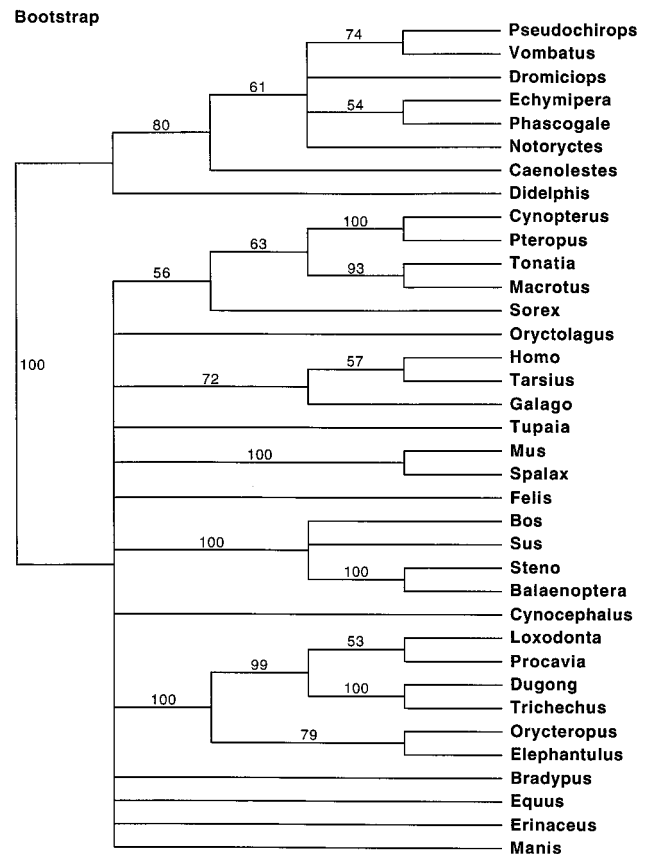


FIG. 3. Majority rule bootstrap parsimony tree showing clades supported at or above the 50% level.

and against the competing hypotheses that associates perissodactyls with hyracoids (39, 41, 51, 67). An analysis of morphological characters that includes fossil data (68) also favors Paenungulata. Likewise, molecular support for Cetartiodactyla corroborates recent fossil discoveries that document that cetaceans are derived from an ancestral stock with a paraxonic arrangement of the digits, as in artiodactyls (69). In contrast to whole mitochondrial genome studies (43, 55), IRBP does not provide strong support for Ferungulata. It remains to be seen whether this clade is robust in the mitochondrial genome studies when additional placental orders are represented.

At the base of the placental radiation, IRBP data do not make a convincing case for the basal split. Epitheria is not supported on the best trees but cannot be rejected based on statistical tests unless xenarthrans and pholidotans are the sister group to other placental mammals. The maximum parsimony and ML trees both associate the representative xenarthran with the African clade; this arrangement is not robust but raises the intriguing possibility of an expanded Gondwanan clade that may include the endemic African groups plus the South American xenarthrans. Arnason *et al.* (59) suggested a ferungulate association for xenarthrans based on mitochondrial protein coding gene sequences; however, that study did not include representatives of the African clade and does not allow for direct comparisons with the IRBP results.

Archonta is rejected by statistical tests, but we cannot reject an association that includes primates, dermopterans, and scandentians. Bats are monophyletic on both the ML and maximum parsimony trees, but support for bat monophyly is weak in the context of taxa included in our study. Stanhope *et al.* (34) found strong support for bat monophyly based on IRBP sequences but with less diverse ordinal representation. Even with weak support for bat monophyly, there remains strong support against Pettigrew's (31) flying primate hypothesis.

All statistical tests reject Hyracoidea + Perissodactyla, Altungulata, Ungulata, and Anagalida at $P < 0.0001$. None of the other superordinal hypotheses is rejected so convincingly. This is not surprising because all of these superorders conflict with the African clade that includes paenungulates, aardvarks, and elephant shrews. Exon 28 of the von Willebrand factor and mitochondrial rRNA sequences also support the African clade and contradict the alternative hypotheses (39, 41). Here, morphological and molecular data are in striking contrast. Archibald's (56) cladistic analyses support Ungulata and Anagalida. Furthermore, Archibald (56) proposed Ungulatomorpha as a superorder for ungulates and the extinct zhelestids (70) and suggested that zhelestids are a paraphyletic group that is near the base of the ungulate radiation. In this scheme, rectangular upper molars and other characters are synapomorphies for these superorders. Anagalida, which includes the Late Cretaceous Asian genera *Zalambdalestes* and *Barunlestes*, is not closely related to Ungulata in Archibald's (56) scheme. However, the inclusion of elephant shrews in the African clade [also golden moles (41)] dissociates paenungulates from ferungulates. This either implies the independent evolution of ungulate characters in these two ungulate groups or alternatively the loss of such features in elephant shrews and golden moles (at least) after evolving in a more basal common ancestor.

The most robust marsupial clade is an association of all australidelphian orders plus caenolestids to the exclusion of Didelphimorphia. Separation of didelphids and caenolestids suggests independent evolution of sperm-pairing in these taxa. Recent evidence (71) corroborates this view because the style of pairing is different in these two groups. The phylogenetic position of bandicoots, which is controversial and has implications for the evolution of syndactyly, is not resolved by IRBP data. The association of a bandicoot (Echymipera) and a dasyurid (Phascogale) based on IRBP sequences is consistent with serological results (15), but we cannot reject the competing Eometatheria hypothesis of Kirsch *et al.* (12), according to which dasyurids are more closely related to diprotodontians, *Dromiciops*, and *Notoryctes* than to peramelinans. The phylogenetic position of bandicoots, which has implications for the evolution of syndactyly, remains one of the most problematic questions in marsupial systematics. Likewise, the position of the highly derived marsupial mole remains enigmatic based on IRBP data although at least one test argues against the *Notoryctes*-Diprotodontia hypothesis.

Several interordinal associations of mammals are emerging as robust including Cetartiodactyla, Paenungulata, and an even larger endemic African clade. Other hypotheses, including Archonta, Ungulata, and Anagalida, are rejected. Additional advances in our understanding of higher level relationships will require concatenation of multiple data sets. It is also important to increase taxonomic representation, especially where taxonomic diversity is great and there are questions about monophyly, e.g., Rodentia, Artiodactyla, and Insectivora.

IRBP Evolution in the Marsupial Mole. The finding that exon 1 of the IRBP gene in the *Notoryctes* contains frameshift indels and stop codons in all three reading frames indicates that this sequence is from a pseudogene. Given this finding, we accounted for indel regions to restore the *Notoryctes* sequence into the correct reading frame and used MEGA 1.01 (72) to calculate uncorrected synonymous/nonsynonymous ratios. The average synonymous/nonsynonymous ratio in pairwise comparisons involving *Notoryctes* (4.02) is lower than in comparisons for other pairs of marsupials (5.18), but in all cases these ratios provide evidence of purifying selection. This is expected because, even in comparisons involving the *Notoryctes* pseudogene, the second sequence is from a functional IRBP gene. Also, *Notoryctes* diverged from other marsupials 66–81 million years ago (12), and most of the evolutionary history along the *Notoryctes* branch (see below) may have occurred under purifying selection.

Conceivably, the *Notoryctes* IRBP sequence is from a processed pseudogene that is not orthologous to the other IRBP sequences. We cannot reject this hypothesis. However, the marsupial mole is blind and has vestigial eyes that are hidden under the skin. Furthermore, the lens and pupil are absent, and the optic nerve is reduced (73). Given this degeneration and loss of function of the eyes, it might be expected that genes for proteins that function solely in vision should evolve as pseudogenes if functional copies no longer confer a fitness advantage. Evidence indicates that IRBP is a single-copy gene in mammals, and among taxa that have been investigated, there is no evidence for processed pseudogenes (35, 57). This finding suggests that the *Notoryctes* IRBP PCR product derives from the same locus that codes for a functional gene in other mammals but that in *Notoryctes* indels has accumulated because of the relaxation of purifying selection. Indeed, DNA sequencing of PCR products in seven other marsupials has yielded only intact sequences within each species; variation within a species, when it occurs, is limited to one to two nucleotide substitutions per species and can be attributed to PCR error and/or allelic variation. In the case of *Notoryctes*, both direct sequencing and sequencing of cloned PCR products result in the same sequence, including the indels. If a functional copy and a processed pseudogene are both present, we might expect to amplify and sequence both the functional and nonfunctional copies rather than just the single PCR product that we detected.

In a second blind mammal (*Spalax*), we do not find evidence for loss of function in the IRBP gene such as indels or stop codons. However, the branch leading to *Spalax* is longer than the branch leading to its sister lineage (*Mus*) (Fig. 1), and the ratio of changes at first + second positions to third positions is higher in *Spalax* (42:59) than *Mus* (29:55).

Saitou and Ueda (74) estimated the rate at which indels occur in primate pseudogenes at 0.17/kb/million years. Based on this rate, we estimate that *Notoryctes* IRBP has been nonfunctional since the late Oligocene-early Miocene, i.e., 18.5–24.7 million years ago based on the occurrence of three to four indels. Aplin and Archer (1) argued that *Notoryctes* is one of the most arid-adapted Australian mammals. Our estimate for the time frame over which *Notoryctes* IRBP became nonfunctional corresponds to a period of aridification in Australia (75). Possibly this is the time period when *Notoryctes* adapted to its fossorial lifestyle with relaxed selection pressures on vision.

Darwin (76) noted that the eyes of moles and some burrowing rodents are rudimentary in size and in some cases are covered by skin and fur. He attributed this to disuse, perhaps aided by natural selection. In the case of *Notoryctes*, the lack of functional eyes ostensibly eliminates any selective advantages associated with a functional IRBP gene product because there is no need to regenerate rhodopsin. The *Notoryctes* sequence that we report is consistent with this view and suggests that phenotypic degeneration of the eyes may be accompanied by parallel changes at the molecular level. In contrast, Crandall and Hillis (77) investigated the evolution of rhodopsin genes in blind, cave-dwelling crayfish relative to surface-dwelling forms and did not find evidence for loss of functional constraint. In this case, they suggest an additional, previously unrecognized function that is unrelated to light absorption—perhaps a role in circadian rhythms (77).

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