Notes and Comments

Placental Invasiveness Mediates the Evolution of Hybrid Inviability in Mammals

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Submitted November 7, 2005; Accepted March 14, 2006; Electronically published May 19, 2006

abstract: A central question in evolutionary biology is why animal lineages differ strikingly in rates and patterns of the evolution of reproductive isolation. Here, we show that the maximum genetic distance at which interspecific mammalian pregnancies yield viable neonates is significantly greater in clades with invasive (hemochorial) placentation than in clades with noninvasive (epitheliochorial or endotheliochorial) placentation. Moreover, sister species with invasive placentation exhibit higher allopatry in their geographic ranges, suggesting that formerly separated populations in mammals with this placental type fuse more readily on recontact. These differences are apparently driven by the stronger downregulation of maternal immune responses under invasive placentation, where fetal antigens directly contact the maternal bloodstream. Our results suggest that placental invasiveness mediates a major component of reproductive isolation in mammals.

Keywords: reproductive isolation, mammals, placentation, hybrid inviability, immunology.

Speciation is a consequence of the evolution of barriers to gene flow between diverging populations. Studies in vertebrates indicate that barriers resulting from behavioral, ecological, life-history, and geographical divergence are common in disparate groups, including mammals, reptiles, birds, and amphibians. Despite the commonality of mechanisms generating speciation within the vertebrates, components of reproductive isolation such as hybrid inviability evolve at markedly different rates in each vertebrate group. For example, amphibians have been shown to evolve hybrid inviability 10 times more slowly than eutherian mammals (Wilson et al. 1974), and birds have been shown to do so around three times more slowly (Fitzpatrick 2004). Furthermore, different taxonomic groups of eutherian mammals exhibit considerable diversity in the rate at which hybrid inviability has evolved (Fitzpatrick 2004). Here we provide evidence that these marked differences in the rate of evolution of hybrid inviability in mammals can be accounted for in part by differences among clades in the degree of placental invasiveness, that is, how deeply the placenta invades the maternal uterine lining. In particular, clades with invasive placentation (contacting the maternal bloodstream; fig. 1) evolve hybrid inviability much more slowly, apparently as a result of increased downregulation of the maternal immune system during pregnancy.

Mammals are unique among the vertebrates in being almost universally viviparous. Unlike in the vast majority of other oviparous vertebrates, prenatal nutrition of eutherian mammals is accomplished by a chorioallantoic placenta, which is in close contact with both the fetal and maternal blood circulatory systems and which consequently provides an interface at which fetal antigens, including those of paternal origin, are presented to the maternal immune system (Bainbridge 2000). Pregnancy therefore represents an immunological paradox for the mammalian mother in that the conceptus can be considered a natural allograft that must be recognized as foreign but not rejected in order for pregnancy to proceed (Medawar 1953). While the initiation of pregnancy requires maternal recognition of the blastocyst as foreign tissue, the progress of pregnancy into later stages is accompanied by a transformation of the maternal immune environment that protects the developing fetal allograft from rejection.

Different taxonomic groups of eutherian mammals vary in the extent to which they have evolved means to suppress the immunological consequences of proximity between mother and fetus. Physiological research suggests that the maternal immune response is more strongly suppressed in taxa exhibiting "invasive" hemochorial placentation (in which maternal circulatory blood spaces are enclosed di-

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Am. Nat. 2006. Vol. 168, pp. 114–120. 0003-0147/2006/16801-41412$15.00.

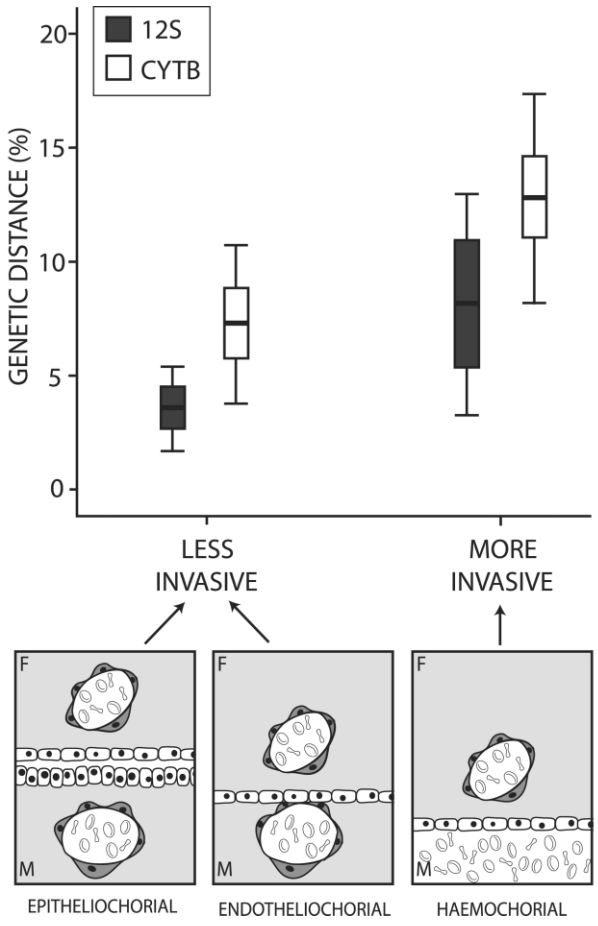


Figure 1: Placental invasiveness is quantified in terms of the number of maternal cell layers separating fetal tissues from the maternal circulatory system (see Mossman 1987; Wildman et al. 2006); with high invasiveness, there is a higher genetic distance between pairs of hybridizable mammal species. In species with epitheliochorial placentation—including all strepsirhine Primates, Cetartiodactyla, Perissodactyla, Pholidota, and some Insectivora—the fetal and maternal component of the interhemal membrane both typically consist of endothelium, connective tissue, and epithelium, making a total of six cell layers between the two circulatory systems. In species with endotheliochorial placentation—including all Carnivora, Serenia, and Proboscidea, some Rodentia, most Insectivora and Xenarthra, and the sole member of Tubulidentata—the maternal epithelium and connective tissue are stripped away during development of the placenta, resulting in an interhemal interface consisting of just four cell layers. Finally, in species with hemochorial placentation—including all haplorhine Primates, Lagomorpha, Dermoptera, and Hyracoidea, most members of Rodentia and Chiroptera, and many members of Afrotheria—all of the maternal cell layers are removed during development of the placenta, and maternal blood spaces are directly enclosed by the fetal chorion. The hemochorial placenta is thus unique in that it involves the presentation of fetal surface antigens directly to the blood circulatory system of the mother, unimpeded by cell layers of maternal origin. *Bottom*, schematic representation of blood flow relations between maternal (*M*) and fetal (*F*) sides of the three types of placenta. Open nucleated cells represent epithelial tissue; dark gray nucleated cells represent endothelial tissue; light gray areas represent connective tissue; open dumbbell-shaped cells represent hemocytes. *Top*, box plot showing cytochrome *b* and 12S genetic distance between hybridizable pairs of mammals with less invasive versus more invasive placentation. Horizontal bars show mean values; boxes show 95% confidence intervals; error bars show 1 SD either side of the mean. The mean value for mammals with less invasive placentation is approximately half that for mammals with more invasive placentation, indicating that the former species evolve reproductive isolation via hybrid inviability more rapidly than the latter.

rectly by fetal tissues) than in taxa exhibiting "noninvasive" endotheliochorial or epitheliochorial placentation (in which maternal blood is separated from fetal surface antigens by cell layers of maternal origin; Baker et al. 1999; Meeusen et al. 2001; fig. 1). Placental invasiveness is also associated with an increasingly strong downregulation of the adaptive antigen-specific immune response in favor of the innate inflammatory immune response (Hunt et al. 2000; Meeusen et al. 2001) and with reduced MHC antigen expression on the placental surface (Ait-Azzouzene et al. 1998; Baker et al. 1999).

Failure of pregnancy, both within and between species, is commonly due to inappropriate immunological interactions between mother and fetus (Croy et al. 1982; Ruffing et al. 1993; Clark 2003). On the basis of the apparent higher degree of downregulation of the maternal immune response in species with invasive placentation, we hypothesized that species with invasive placentation are less likely to abort interspecific conceptuses (or conceptuses from divergent populations) because of immunological incompatibility between mother and offspring. Consequently, our first prediction is that species with hemochorial (invasive) placentation should evolve hybrid inviability more slowly than species with epitheliochorial or endotheliochorial (noninvasive) placentation, because they experience reduced zygote loss during gestation.

Differences among mammal clades and placental forms in the rate of evolution of hybrid inviability should translate into effects on reproductive isolation and speciation. Allopatric speciation is widely regarded as the norm in vertebrates because their ability to travel relatively large distances promotes gene flow between divergent populations or incipient species (Coyne and Orr 2004). Sympatry combined with the ability to hybridize is known to result in the loss of species via fusion, demonstrated, for example, by concern over the future of endangered animals such as the dingo *Canis lupus dingo* and the wildcat *Felis sylvestris* in the face of widespread hybridization with domesticated sister taxa (Daniels and Corbett 2003). We expect a sustained period of allopatry to be found more frequently in sister species that have the ability to maintain interspecific pregnancy by virtue of their placental morphology, because sympatry of divergent populations within such species

should more readily result in the fusion of populations by hybridization and the loss of incipient species. Consequently, our second prediction is that hemochorial sister species will, on average, exhibit a lower degree of sympatry than epitheliochorial and endotheliochorial sister taxa. We test our two predictions using data on placental type, ability of pairs of species to hybridize, genetic distances between pairs of species that can hybridize, and species ranges.

Methods

Database of Hybridizable Mammals

We used the compendium of mammalian species known to hybridize successfully by Gray (1972). An interspecific pregnancy was judged to be successful if it resulted in the birth of live offspring; we make no assumption about the likelihood of gene flow resulting from such hybridizations, though many such hybridizations are known to result in fertile or semifertile adults.

Measurement of Genetic Distances

We obtained genetic distance data via GenBank for mammals known to hybridize successfully. We report genetic distances as the percentage of sites that have undergone substitutions, using two putatively neutral loci (cytochrome *b* and 12S ribosomal RNA). In order to obtain a phylogenetically independent sample of hybridizable species pairs within clades where all species can hybridize, only the most divergent nonoverlapping hybridizable species within each clade were included in the analysis; this resulted in a data set unaffected by the bias caused by some clades being more heavily sampled than others. Genetic distances were calculated according to Kimura's two-parameter method, as implemented in the "distmat" program of EMBOSS (http://emboss.sourceforge.net/). Distances calculated using this method are based on the number of transitions/transversions apparent from comparison of a pair of nucleotide sequences, corrected for the probability of multiple substitutions at a single site.

Measurement of Range Sympatry

We obtained species range maps from a number of sources including NatureServe (http://www.natureserve.org) and the African Mammal Database (http://www.gisbau.uniroma1.it/amd/). Maps not available in digital format were digitized before analysis. Species range area was calculated by counting pixels in Adobe Photoshop. Range maps were overlaid to calculate the area of overlap between species ranges. Sympatry was measured by dividing the area of overlap between the range of two species by the area of the smaller species range. This measure results in a value between 0 (no overlap) and 1 (the smaller range is entirely enclosed by the larger range). Before analysis, measures of sympatry were normalized by arcsine transformation.

Analysis of Hybridizable Species Pairs

We used ANOVA to test for the difference in the mean genetic distance found between pairs of hybridizable species grouped by placental type. In order to account for the phylogenetic nonindependence of data, we also repeated the analysis using only the most divergent nonoverlapping pairs of hybridizable species within each clade that contained multiple pairs of hybridizable species, as described in "Measurement of Genetic Distances." We used data from studies of the mammalian molecular clock to test the alternative hypothesis that the observed patterns of genetic divergence between hybridizing sister species can be explained by differences in the age of each clade rather than differences in placentation. Finally, we also analyzed the genetic distance data using overall mean values for higher taxa (families or subfamilies) that were homogeneous for placental type; this highly conservative analysis assumes that the effects of placental type on hybridizability are independent only at the highest taxonomic levels at which placental variation is found.

Analysis of Sympatry Data

We based our analysis of sympatry on the comparison of sister species. Where possible, sister species were determined from published phylogenetic sources. For 14 of our sister pairs, no published phylogeny could be found, so phylogenies for undisputed monophyletic groups were reconstructed using genetic sequences available in GenBank. For a number of our species pairs, no published phylogeny was available, nor were genetic sequences available within GenBank. Twenty-five such pairs were included in our analysis on the basis of taxonomy, given that they occur in genera containing only two species. We used ANOVA to test for a difference between the mean sympatry of sister species grouped by placental type. To assess the extent to which our results are robust across different clades, we repeated the analysis separately for each order of mammals exhibiting both invasive and noninvasive placental forms. We used genetic distance data (as a surrogate for time since divergence) to test the alternative hypothesis that the observed patterns in range sympatry can be explained by differences in the age of each sister pair.

Results

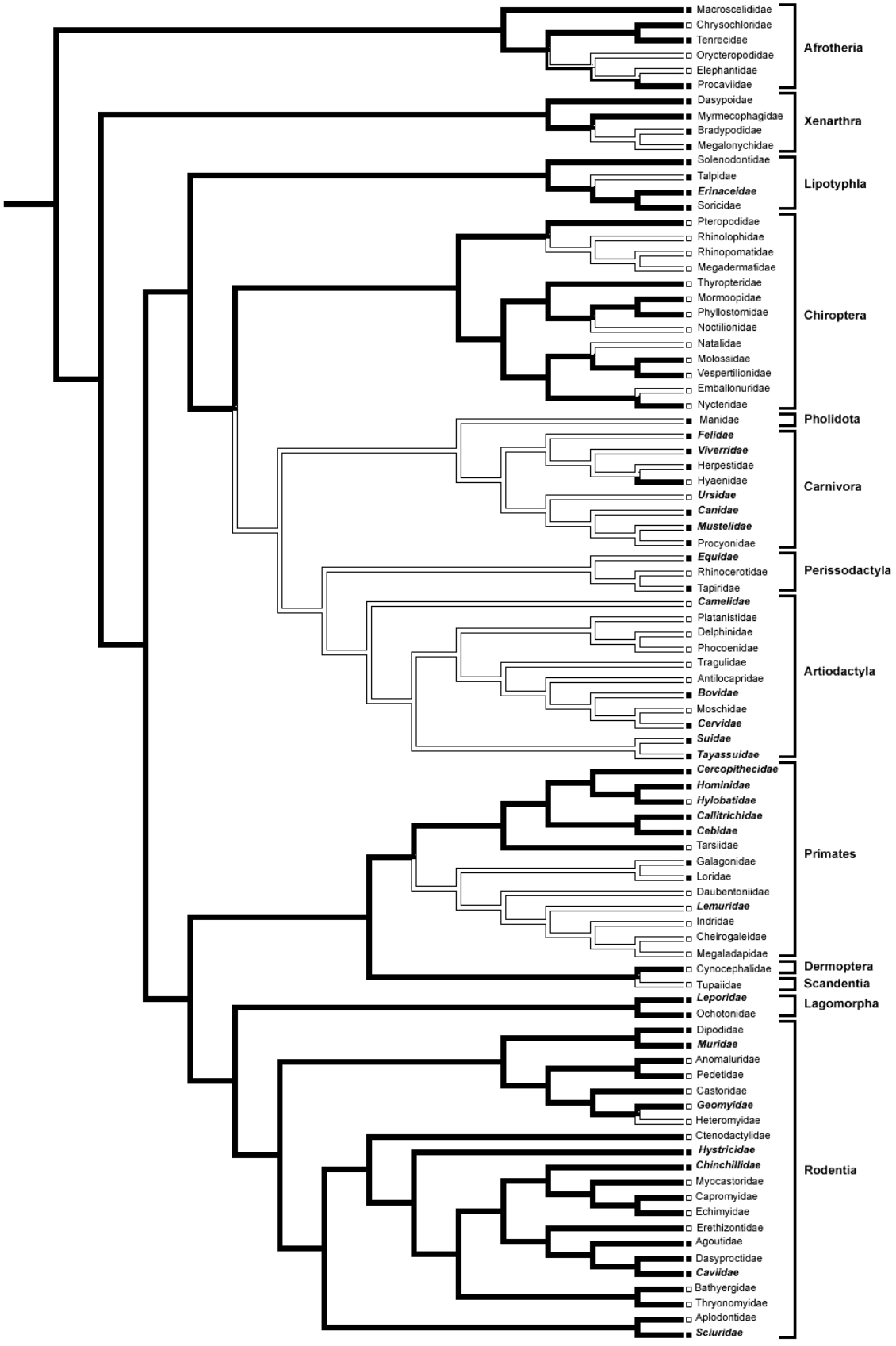
To test the hypothesis that species with invasive placentation evolve hybrid inviability more slowly than species with noninvasive placentation, we examined 208 pairs of mammalian species (representing seven orders and 23 families) known to be hybridizable (capable of bringing interspecific pregnancies to term and birthing live offspring; Gray 1972). We predicted that mean genetic distances would be lower between pairs of species exhibiting noninvasive placentation than they are between hybridizable pairs of species exhibiting invasive placentation, because the former group must, in general, be more closely related if they are to be immunologically compatible in utero.

As predicted, hybridizable pairs of species with noninvasive placentation are much less genetically distant than hybridizable pairs of species with invasive placentation (mean = 10.28 ± 1.84 and 6.69 ± 0.92 substitutions per hundred base pairs of cytochrome *b* for species with invasive and noninvasive placentation, respectively; $P < .001$, $N = 159$, $F = 46.5$; mean = 6.00 ± 1.27 and 2.84 ± 0.51 substitutions per hundred base pairs of 12S for species with invasive and noninvasive placentation, respectively; $P < .001$, $N = 143$, $F = 50.0$). This analysis may overrepresent certain laboratory, farm, and zoo animals that have been subject to many attempted hybridizations and are thus present in multiple pairs of species in our data set. In order to circumvent this possible bias, we repeated the analysis using only the most divergent pairs of taxa within each monophyletic group (fig. 1). The results with this subset of most divergent species pairs were consistent with the previous analysis (mean = 12.64 ± 1.79 and 7.37 ± 1.2 substitutions per hundred base pairs of cytochrome *b* for species with invasive and noninvasive placentation, respectively; $P < .001$, $N = 61$, $F = 26.7$; mean = 7.97 ± 2.7 and 3.71 ± 0.83 substitutions per hundred base pairs of 12S for species with invasive and noninvasive placentation, respectively; $P < .001$, $N = 35$, $F = 14.74$). Finally, these results are not biased by the relative ages of clades with invasive versus noninvasive placentation; analysis of molecular clock data for orders and suborders shows that these clades do not differ in estimated divergence dates ($P > .30$ by ANOVA, using topologies and branch length data from recent mammalian trees in Murphy et al. 2001, Hasegawa et al. 2003, or Springer et al. 2003, comparing the placentally invasive Haplorhine primates—Insectivora, Rodentia, and Lagomorpha—with the noninvasive Strepsirhine primates—Carnivora, Perissodactyla, and Artiodactyla).

Data within each clade on the evolution of hybrid inviability and on placental type are not statistically independent under usual models of character evolution, such as Brownian motion (Felsenstein 1985; Martins 2000). Given that only four major clades in our analysis exhibit invasive placentation (Haplorhine primates, Insectivora, Rodentia, and Lagomorpha) and four major clades show noninvasive placentation (Strepsirhine primates, Carnivora, Perissodactyla, and Artiodactyla), the tests conducted above may be subject to unsuitably high risk of Type I error. However, analyses using mean values for each of these eight major groups yielded essentially the same results, with hemochorial (invasive) taxa exhibiting roughly twofold higher genetic distances for hybridizable species pairs (cytochrome *b*, mean genetic distance 6.41 ± 1.46 vs. 3.01 ± 0.95, Mann-Whitney *U*-test, $P = .021$, $N = 8$; 12S, mean genetic distance 11.52 ± 1.92 vs. 6.29 ± 1.12, Mann-Whitney *U*-test, $P = .034$, $N = 7$; both results also remain significant if the related groups Perissodactyla and Artiodactyla are combined). These results demonstrate the strength and among-taxon consistency, across major eutherian orders, of the differences in hybridization effects between placental type.

An explicitly phylogeny-based test of our hypothesis requires inferring a species-level phylogeny for the bulk of eutherian mammals, inferring the transitions between invasive and noninvasive placentation, and relating these transitions to changes in the genetic distances at which species can hybridize. Nine to 11 evolutionary transitions in placental type can be inferred on such a phylogeny, but data on hybridization distances are currently available for taxa spanning only two of these transitions, and in both cases, the clades with invasive versus noninvasive placentation are much more distant than sister lineages (fig. 2; see also Wildman et al. [2006], who inferred 11 such transitions). Both inferred changes in hybridization distance are in the predicted direction, but robust phylogeny-based tests of our hypothesis require data on hybridization from additional taxonomic groups that closely bracket the transitions in placental type. Until then, our analysis of this prediction can be considered as a species-level test that describes current patterns of trait association (Ricklefs and Stark 1996), subject to the presumptions that the immunological effects of invasive placentation on hybrid inviability exhibit macroevolutionary lability and that unobserved, phylogenetically distributed third variables do not drive the observed patterns (Ridley 1989). Given the >100 million years of mammalian radiation represented here, the wide range of taxa already included in our analyses, and the striking magnitude (around twofold) of the differences between placental types in hybridization distance, we believe that the strength of these species-level results should compel more comprehensive tests.

An additional means of testing our hypothesis that placental invasiveness mediates aspects of speciation is to use independent lines of evidence. The hypothesis also predicts that a sustained period of allopatry should be found more


Macroscelididae
Chrysochloridae
Tenrecidae
Orycteropodidae
Elephantidae
Procaviidae
Afrotheria
Dasypoidae
Myrmecophagidae
Bradypodidae
Megalonychidae
Xenarthra
Solenodontidae
Talpidae
Erinaceidae
Soricidae
Lipotyphla
Pteropodidae
Rhinolophidae
Rhinopomatidae
Megadermatidae
Thyropteridae
Mormoopidae
Phyllostomidae
Noctilionidae
Natalidae
Molossidae
Vespertilionidae
Emballonuridae
Nycteridae
Chiroptera
Manidae
Pholidota
Felidae
Viverridae
Herpestidae
Hyaenidae
Ursidae
Canidae
Mustelidae
Procyonidae
Carnivora
Equidae
Rhinocerotidae
Tapiridae
Perissodactyla
Camelidae
Platanistidae
Delphinidae
Phocoenidae
Tragulidae
Antilocapridae
Bovidae
Moschidae
Cervidae
Suidae
Tayassuidae
Artiodactyla
Cercopithecidae
Hominidae
Hylobatidae
Callitrichidae
Cebidae
Tarsiidae
Galagonidae
Loridae
Daubentoniidae
Lemuridae
Indridae
Cheirogaleidae
Megaladapidae
Primates
Cynocephalidae
Dermoptera
Tupaiidae
Scandentia
Leporidae
Ochotonidae
Lagomorpha
Dipodidae
Muridae
Anomaluridae
Pedetidae
Castoridae
Geomyidae
Heteromyidae
Ctenodactylidae
Hystricidae
Chinchillidae
Myocastoridae
Capromyidae
Echimyidae
Erethizontidae
Agoutidae
Dasyproctidae
Caviidae
Bathyergidae
Thryonomyidae
Aplodontidae
Sciuridae
Rodentia

frequently in sister species that have the ability to maintain interspecific pregnancy by virtue of their placental morphology, because sympatry of divergent populations within such species should more readily result in the fusion of populations by hybridization and the loss of incipient species. We tested this prediction by gathering data on species ranges for 166 pairs of sister taxa of known placental type (representing 11 orders and 46 families of nonaquatic, nonvolant mammals) and testing for a mean difference in allopatry between groups with invasive versus noninvasive placentation. We followed Barraclough and Vogler (2000) in measuring the degree of allopatry between two species as the area of range overlap divided by the area of the smaller species range, normalized by arcsine transformation. By considering only recent biological sister species, as determined from published phylogenies or analyses of published DNA sequences, we avoided the phylogenetic inference of ancestral species ranges from present-day species ranges, which has come under criticism (Losos and Glor 2003), and we thus minimized the potentially confounding effect of range shifts since speciation.

Consistent with our prediction, hemochorial sister species were on average less sympatric (mean 0.22 ± 0.06) than grouped epitheliochorial and endotheliochorial (noninvasive) species (mean 0.55 ± 0.1; $P \leq .01$, $N = 166$, $F = 33.46$; ANOVA). This difference does not result from the possibility that sister species with invasive placentation tend to be younger than those with noninvasive placentation and have had less time for their species ranges to become sympatric: cytochrome *b* and 12S genetic distance, surrogates for time since divergence (Gissi et al. 2000), did not differ between invasive and noninvasive groups (cytochrome *b*: $P = .51$, $N = 58$, $F = 0.4$; 12S: $P = .26$, $N = 50$, $F = 1.3$; ANOVA), irrespective of their ability to hybridize. Hemochorial species were also less sympatric, on average, in the two orders (Primates and Insectivora) where both invasive and noninvasive placental forms are represented in our data set (primates: $P = .001$, $F = 12.41$; insectivores: $P = .007$, $F = 14.42$; ANOVA), which suggests that the overall mean difference was robust to taxon-specific effects other than placentation. Such reduced range overlap between sister species pairs and invasive placentation is an expected biogeographic consequence of their slower evolution of hybrid inviability.

Discussion

Our analyses suggest that invasive placentation engenders an approximate halving of the rate at which eutherian mammal species evolve hybrid incompatibility, compared with species with noninvasive placentation. The results provide evidence for the importance of physiological adaptations to placental viviparity in determining the rate at which different clades of mammals evolve reproductive isolation. In particular, the balance between placental antigenicity and maternal immune response may differ between taxa with invasive versus noninvasive placentation, such that immune system downregulation in taxa with invasive placentation allows for viable hybrid production at much greater genetic distances. The lower degree of sympatry between sister species in hemochorial clades provides independent support for this hypothesis and suggests that placental invasiveness has important implications for modes of speciation. The evolution of placental form and function, under conditions of ongoing conflict over resource allocation between mother and offspring (Haig 1993; Zeh and Zeh 2000; Crespi and Semeniuk 2004; Wildman et al. 2006), may thus generate strong effects on rates and patterns in the evolution of reproductive isolation. Further studies of eutherian phylogeny, comparative reproductive immunology, the causes of hybrid inviability in mammals, and placental evolution should illuminate the mechanisms driving these results.

Acknowledgments

We would like to thank J. Dale, J. Joy, A. Mooers, P. Nosil, two anonymous reviewers, and members of the Simon Fraser University FAB-lab for useful comments and the

Figure 2: Distribution of our placental and hybridization data on a phylogenetic tree of eutherian mammals. This tree of mammalian families was inferred from five genes—RAG-1 (recombination activating gene 1), RAG-2 (recombination activating gene 2), cytochrome *b*, VWF (von Willebrand's factor), and IRBP (interphotoreceptor binding protein)—and 405 species using maximum likelihood (M. G. Elliott and B. J. Crespi, unpublished data), and it is fully compatible with other recent mammalian trees (see Wildman et al. 2006). Placental invasiveness data was taken from Luckett (1974), Gopalakrishna and Karim (1979), Benirschke and Miller (1982), Mossman (1987), King (1993), Rasweiler (1993), Mess (2003), and Carter et al. (2004; see also Wildman et al. 2006). Placental invasiveness was mapped onto the branches of the tree by maximum likelihood reconstruction using Mesquite (Maddison and Maddison 2005), and the reconstructions correspond very closely with those of Wildman et al. (2006). Solid branches represent more invasive placentation (hemochorial), and open branches represent less invasive placentation (endotheliochorial or epitheliochorial). Families of mammals whose names are written in bold are those for which hybridizability data and genetic distance data are available. Families whose names are written in regular type are those for which data were not available and are not included in this study. Each family has a small square, either solid or open, to the left of its name. Solid squares denote the availability of species range data (resulting in a measure of sympatry) for sister species within each family; open squares denote the current unavailability of such data.

Natural Sciences and Engineering Research Council for financial support.

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