CHROMOSOMAL EVOLUTION AND PHYLOGENY OF GOLDEN MOLES AND TENRECS (MAMMALIA: AFROSORICIDA)

CLÉMENT GILBERT

Dissertation presented for the Degree of Doctor of Philosophy (Zoology) at the University of Stellenbosch

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DECLARATION

I, the undersigned, hereby declare that this dissertation is my own original work that has not previously been submitted for any degree or examination at any other university.

Clément Gilbert Date: 14 February 2008

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ABSTRACT

Afrosoricida is a 65 million years old (my) eutherian order that together with the Tubulidentata (aardvark) and Macroscelidea (elephant shrews) form the Afroinsectiphillia, a subclade of Afrotheria. It includes two families – Chrysochloridae (nine genera of golden moles) and Tenrecidae (11 genera of tenrecs) – that collectively represent ~59% of the afrotherian generic diversity. This study presents the first comprehensive cytogenetic comparison between members of these two families (seven genera and 11 species/subspecies of golden moles, and two genera and 11 species of tenrecs) using G- and C-banding and chromosome painting. All detected rearrangements are interpreted in a strict cladistic framework. In the case of Chrysochloridae, this provides evidence for a sister relationship between *Chrysochloris* and *Cryptochloris*, the monophyly of the *Amblysomus* genus, and for the elevation of *A. hottentotus meesteri* to specific rank. The detection of telomeric-like repeats in the centromeres of all chromosomes of the *Amblysomus* species/subspecies but not in those of *A. h. meesteri* further strengthens its recognition as a distinct species. Parsimony analysis of chromosomal rearrangements within Tenrecidae, the second Afrotheria assemblage studied, showed that rearrangements which could be interpreted as Whole Arm Reciprocal Translocations (WARTs) were more likely to be the result of Robertsonian translocations. Four interspecific associations are recovered within *Microgale* that are consistent with morphological and molecular characters. It was also possible to infer ancestral karyotypes for the Chrysochloridae, Oryzorictinae and the two tenrecid genera, *Oryzorictes* and *Microgale*. Given the relatively high karyotypic diversity observed among some *Microgale* species and the prevailing debates on chromosomal evolution and regional palaeoenvironmental fluctuations, it is suggested that *Microgale* be added to the list of taxa where structural rearrangements are likely to have played a role in speciation. Using Genbank sequences and a relaxed Bayesian clock method, we estimate the age of the family Chrysochloridae at ~28.5 my and that of the genus *Microgale* at ~9.9 my. Based on these dates, it can be shown that most of the evolutionary branches are characterized by a slow rate of chromosomal change, but that markedly high rates are observed in some *Microgale* species and to a lesser extent in the lineage leading to *A. robustus*. The rates of chromosomal evolution and other cytogenetic features highlighted in this study are discussed in light of recent advances in understanding the molecular mechanims that underpin changes to genomic architecture.

OPSOMMING

Die Afrosoricida is ‘n eutheriaanse orde wat ongeveer 65 miljoen jaar oud is en wat saam met die Tubulidentata (aardvark) en Macroscelida (klaasneuse) geklassifiseer word as die Afroinsectiphillia, ‘n subklade binne die Afrotheria. Dit sluit twee families in – Chrysochloridae (nege genera van goue molle) en Tenrecidae (11 genera van tenreks) wat gesamentlik ~ 59% van die afrotheriaanse generiese diversiteit bevat. Hierdie studie is die eerste van sy soort wat ‘n sitogenetiese vergelyking tref tussen lede van die twee families (sewe genera en 11 species/subspecies van goue molle, en twee genera en 11 species van tenreks) met die gebruik van G- en C-bandbepaling asook chromosoom fluoressent hibridisasie. Alle chromosoom veranderinge word geinterpreteer in ’n streng kladistiese raamwerk. In die geval van Chrysochloridae is daar bewyse vir ‘n suster verwantskap tussen *Chrysochloris* en *Cryptochloris*, die monofilie van *Amblysomus*, en vir die opheffing van *A. hottentotus meesteri* tot spesiesvlak. Die waarneming van telomeriese-tipe herhalings in die sentromere van alle chromosome van die *Amblysomus* spesies/subspesies maar nie in die van *A. h. meesteri* nie, dien as addisionele bewys vir ‘n unieke species. Filogenetiese analise van chromosoom herrangskikkings binne die Tenrecidae, die tweede Afrotheria groep wat bestudeer is, het getoon dat die veranderinge wat geinterpreteer kon word as “Whole Arm Reciprocal Translocations (WARTs)” meer waarskynlik die resultaat van Robertsoniaanse translokasies is. Vier interspesifieke assosiasies was binne *Microgale* teenwoordig wat ooreenstem met morfologiese en molekulêre kenmerke. Dit was ook moontlik om die oerouer kariotipe vir die Chrysochloridae, Oryzorictinae en die twee tenrek genera, *Oryzorictes* en *Microgale* te bepaal. Gegee die hoë kariotipiese diversiteit waargeneem tussen sommige van die *Microgale* spesies en die debat oor chromosoom evolusie en streeks paleo-omgewings fluktuasies, word voorgestel dat *Microgale* gevoeg moet word tot die lys van taksa waar strukturele herrangskikkings waarskynlik ’n rol gespeel het in spesiasie. Met die gebruik van DNS basis bepaling vanaf “Genbank” en ‘n “Bayesian” klok metode is die ouderdom van die familie Chrysochloridae te bepaal. Dit word voorgestel dat die familie ongeveer ~28.5 my onstaan het en dat die genus *Microgale* ~ 9.9 my oud is. Gebaseer op hierdie data kan getoon word dat die evolusionêre takke gekenmerk word deur ‘n stadige tempo van chromosoom veranderinge, maar dat hoë tempos teenwoordig is binne sommige *Microgale* spesies en tot ‘n mindere mate binne die groep wat gelei het tot *A. robustus*. Die tempo van chromosoom evolusie en die ander sitogenetiese kenmerke teenwoordig

in die studie word bespreek in die lig van onlangse veranderinge ten opsigte van molekulêre meganismes wat genomiese veranderinge ondeskryf.

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During the two field trips I did in Madagascar to trap tenrecs (one week in January 2006 and one week in January 2007) I was under the supervision of Steve Goodman. I would like to thank him and his family for their hospitality and their kindness. But clearly the success of these field trips would not have been possible without the technical help and scientific expertise of Marie-Jeanne Raherilalao, Achille Raselimanana and Voahangy Soarimalala. I am therefore deeply indebted to them. Finally, during the time I spent in Madagascar I had the chance to interact in many ways with many students of the University of Tana and I would like to thank them all for their assistance in the field.

All the golden moles karyotyped in this study were trapped during several field trips conducted over the last five years by Gary Bronner, Nigel Bennett, and Sarita Maree. I wish to extend my appreciation to them for the lengths they went to in obtainming specimens for this study, often under difficult circumstances.

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CHAPTER I

GENERAL INTRODUCTION

Afrosoricida and the eutherian tree

During the last decade, the two families Chrysochloridae (golden moles) and Tenrecidae (tenrecs) have been among the pivotal taxa involved in changing our way of thinking about eutherian (or crown-group placental) phylogenetic relationships and evolution (Robinson and Seiffert 2004, Springer et al. 2004). For more than a century, morphologists have debated their relationships within Lipotyphla (formerly Insectivora) (Haeckel 1866, Simpson 1945, Butler 1988, MacPhee and Novacek 1993), a group that also includes hedgehogs (Erinaceidae), moles (Talpidae), shrews (Soricidae) and solenodons (Solenodontidae). However, there is now a considerable body of DNA sequences data and other molecular characters that group these two families within Afrotheria which, together with Laurasiatheria, Euarchontoglires and Xenarthra, form the four supraordinal mammalian clades that are currently recognized (Springer et al. 1997, Murphy et al. 2001a, b, Scally et al. 2001, Waddell et al. 2001, Amrine-Madsen et al. 2003) (Figure 1). Strongly supported evidence resulting from these studies challenges all morphological synapomorphies previously used to define the Lipotyphla. For example, hindgut simplification (with loss of the caecum), a pronounced reduction of the pubic symphysis, and a large maxillary contribution to the orbit and extrinsic snout musculature (Butler 1988, MacPhee and Novacek 1993, Whidden 2002) have either evolved independently or were ancestral characters that were retained in two of the most distantly related clades, Afrotheria and Laurasiatheria. Even more strikingly, these new relationships involve extreme ecological and behavioral convergence in the two clades with, among others, adaptation to a subterranean lifestyle shown both in golden moles (Chrysochloridae) and true moles (Talpidae), and the semi aquatic

carnivory developed both by otters (Carnivora) and otter shrews (Tenrecidae) (Madsen et al. 2001, Helgen 2003).

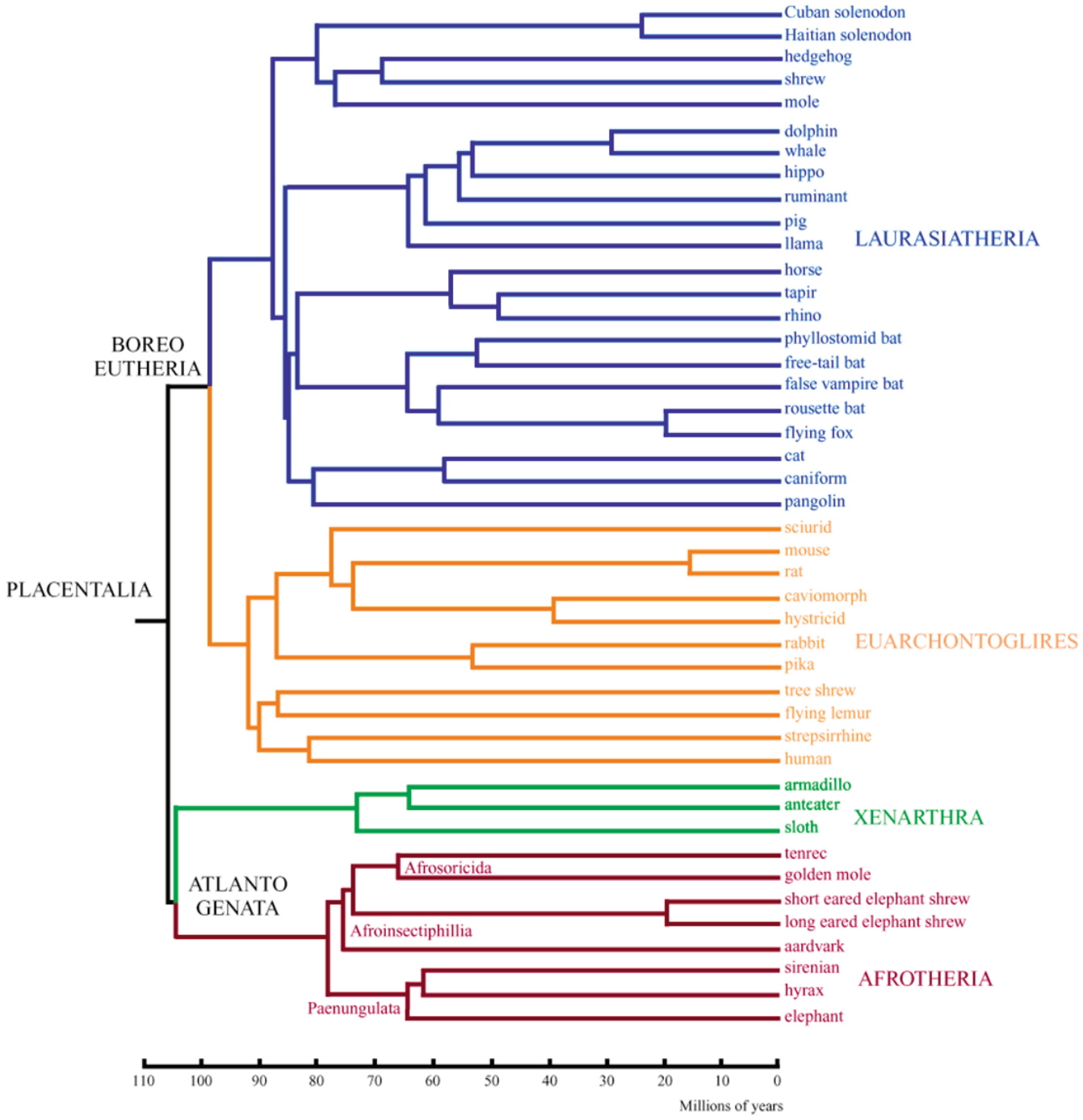


Figure 1. A dated phylogeny of eutherian mammals taken and modified from Murphy et al. (2007). Most of the nodes correspond to those obtained after the Bayesian analysis of a concatenation of 19 nuclear gene segments, the two complete mitochondrial ribosomal RNA subunit genes (12S rRNA and 16S rRNA) plus the intervening valine tRNA (Murphy et al. 2001a, Roca et al. 2004). In addition, the node supporting Atlantogenata derives from an analysis of coding indels and retroposon insertions (Murphy et al. 2007), while that grouping perissodactyls and bats (i.e. Pegasoferae) is from the analysis of retroposon insertions (Nishihara et al. 2006). Strong support was recently found for all clades indicated in capital letters based on an anlalysis of 1698 protein-encoding loci (Wildman et al. 2007).

In addition to Tenrecidae and Chrysochloridae, Afrotheria also includes elephant-shrews, aardvark and paenungulates (i.e., elephant, manatee and dugong, and hyrax) all of whom have an Afro-Arabian origin. Given the almost universal support provided by the sequence data, the grouping of Tenrecidae and Chrysochloridae as sister taxa within Afrotheria is largely favoured above alternative hypotheses (Robinson and Seiffert 2004, Springer et al. 2004, Helgen 2003 and references therein). The resulting clade has been named Afrosoricida after Stanhope et al. (1998) (but see Bronner and Jenkins 2005 for a discussion of this name). Together with Afrosoricida, Paenungulata is also a well-supported clade. Moreover, long concatenations of sequence data (Murphy et al. 2001b, Amrine-Madsen et al. 2003), a synapomorphic chromosomal association (Robinson et al. 2004), and a single SINE insertion (Nishihara et al. 2005) support the recognition of Afroinsectiphillia (after Waddell et al. 2001), a clade that groups Afrosoricida with the aardvark and elephant shrews. However, morphological characters are in conflict with the molecular signal since the analysis of 378 craniodental, postcranial and soft-tissue characters scored across 53 living and extinct afrotherians yielded support for a paenungulate + macroscelidean association (Seiffert 2003). The situation is more confused within Afroinsectiphillia. Whereas Amrine-Madsen et al. (2003) provided support for a clade termed "Afroinsectivora" (i.e., Afrosoricida + Macroscelidea) (Waddell et al. 2001) based on the analysis of ~18 kb of mitochondrial and nuclear DNA sequences, Robinson et al. (2004) found two chromosomal associations that unite aardvark and elephant shrews to the exclusion of golden moles. Nishihara et al. (2005) found two SINE insertions supporting the alternative hypothesis (Tubulidentata + Afrosoricida).

Molecular dating shows that the ancestor of Afrosoricida diverged from other Afrotheria approximately 75 millions years (my) ago (Springer et al. 2003, Murphy et al. 2007) (Figure 1). The subsequent split, which occurred at the Cretaceous/Tertiary

boundary (65 my), has given rise to two families, Tenrecidae and Chrysochloridae, that differ from each other in many evolutionary aspects and which together represent 59% of the afrotherian biodiversity (Figure 2).

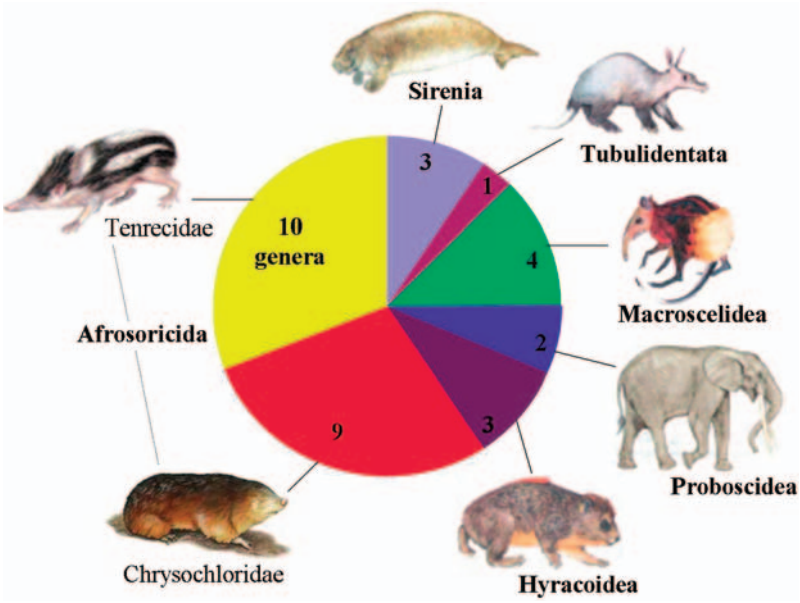


Figure 2. Number of genera in each of the six afrotherian orders showing that Afrosoricida contain the greatest diversity (~59%).

Phylogenomics, cytogenetics and cladistics

The recent "molecular revolution" in mammalian phylogenetics described above has benefited from the considerable progress made in genome-wide comparisons (Murphy et al. 2004, 2007). This relatively new field of investigation, known as phylogenomics, has been led by whole genome sequencing projects. The genomes of seven mammalian species (human, mouse, rat, dog, chimp, rhesus macaque, opossum) are now completely sequenced, although with different degrees of coverage (International Human Genome Sequencing Consortium 2001, Venter et al. 2001, Mouse Genome Sequencing Consortium 2002, Rat Genome Sequencing Consortium 2004, Lindblad-Toh et al. 2005, Mikkelsen et al. 2007, Rhesus Macaque Genome Sequencing

and Analysis Consortium 2007), and the genomes of several other species covering the whole diversity of the mammalian tree are currently well on their way (see Broad Institute website: http://www.broad.mit.edu/mammals/ and Ensembl website: http://www.ensembl.org).

Together with developments in large-scale sequencing, various analytical and experimental tools have been produced to make sense of the genomic architecture of these various species. Among the latter, comparative molecular cytogenetics involving the development and extensive use of Zoo-FISH ('zoo' Fluorescent In Situ Hybridization) or cross-species chromosome painting (see Speicher and Carter 2005 for the general principles underlying the method) has proved very useful in inferring the evolutionary history of genomes among and within the different eutherian orders (Ried et al. 1998, Wienberg 2004). This technique allows one to visualize homologies between chromosomes of distantly related species, and to identify conserved synteny blocks directly at the molecular level.

A large number of studies using Zoo-FISH have been published since its discovery nearly 20 years ago (Lichter et al. 1988, Wienberg et al. 1990). These investigations can generally be classified into two categories: (1) those aimed at constructing chromosomal maps between human and specific taxa and (2) those dealing with the karyotypic evolution of a particular clade of eutherian mammals. There is now at least one category 1 study published for all eutherian orders except Hyracoidea and Dermoptera (Table 1). Together these provide a good picture of the synteny associations in these taxa thus allowing for the reconstruction of a putative eutherian ancestral karyotype (Frönicke et al. 2003, Richard et al. 2003, Yang et al. 2003, Svartman et al. 2004, 2006, Ferguson-Smith and Trifonov 2007). These results, together with the details contained in category 2 studies that generally focus on lower taxonomic levels, allow for a precise description of the mode and tempo of chromosomal change characterizing

various branches of the eutherian tree (O'Brien et al. 2001, Murphy et al. 2004, Ferguson-Smith and Trifonov 2007).

Table 1. Human chromosomes have been mapped on the chromosomes of at least one representative of each of the eutherian orders with the exception of Dermoptera and Hyracoidea (the reference list is non-exhaustive).

Order	Reference
Afrosoricida	Robinson et al. (2004)
Macroscelidea	Robinson et al (2004), Svartman et al. (2004)
Tubulidentata	Yang et al. (2003)
Hyracoidea	no published map
Proboscidea	Frönicke et al. (2003), Yang et al. (2003)
Sirenia	Kellogg et al. (2007)
Xenarthra	Svartman et al. (2006)
Scandentia	Mueller et al. (1999)
Dermoptera	no published map
Primates	reviewed in Wienberg (2005)
Rodentia	Stanyon et al. (2003), Li et al. (2004)
Lagomorpha	Korstanje et al. (1999)
Eulipotyphla	Dixkens et al. (1998), Yang et al. (2006)
Chiroptera	Volleth et al. (2002)
Pholidota	Yang et al. (2006)
Carnivora	Frönicke et al. (1997), Nash et al. (1998), Yang et al. (2000), Graphodatsky et al. (2002), Perelman et al. (2005)
Perissodactyla	Richard et al. (2001)
Cetartiodactyla	Bielec et al. (1998), Frönicke and Wienberg (2001), Biltueva et al. (2004), Chaves et al. (2004)

The non-ambiguous assessment of homology between genomic segments of different species provided by Zoo-FISH also allows for genome rearrangements to be used as phylogenetic characters, expanding on investigations that rely on morphology, amino-acids and DNA sequences to infer phylogenetic relationships. As the tempo of karyotypic evolution (at the level of detection by FISH) is slower than that of nucleotide evolution, chromosomal rearrangements provide rare, but powerful signatures to common ancestry which serve as Rare Genomic Changes *sensu* Rokas and Holland (2000). These signatures (synapomorphic syntenic segmental associations) have been reported for many clades, and have proved useful in helping to decipher several unresolved nodes in the eutherian tree (see for example Frönicke et al. (2003), Robinson

et al. (2004), Svartman et al. (2004), Kellogg et al. (2007) and Pardini et al. (2007) for cases pertinent to Afrotheria). As the taxon sampling is often too limited, or the karyotypes too conserved, several studies simply map the chromosomal rearrangements to an existing, well resolved tree and/or discuss the chromosomal signatures in respect to previously formulated phylogenetic hypotheses (for example, Graphodatsky et al. 2001, 2002, Rambau et al. 2003, Bosma et al. 2004). However, some studies provide a comprehensive phylogenetic matrix by explicitly coding chromosomal rearrangements in different character states and base the analysis on parsimony (Ortells 1995, de Oliveira et al. 2002, Gerbeault-Serreau et al. 2004, Li et al. 2004, Veyrunes et al. 2006). The usefulness, value, and analysis of chromosomal rearrangements using different types of coding is reviewed in Dobigny et al. (2004). Their conclusions argue strongly for considering structural changes as characters, and their presence/absence as the character states.

Speciation and chromosomes

Speciation is a central issue in evolution and identifying the processes that lead to the origin of species has been a fundamental question since the origin of evolutionary biology. The intensity of the debates on this topic is well illustrated by the lack of consensus on a definition of the species, and the difficulties in testing hypotheses concerning proposed modes of speciation (for a general review on speciation, see Coyne and Orr 1998, Turelli et al. 2001).

The simple observation that reproduction between two different karyotypic forms can result in hybrids that exhibit a decrease in fertility (or viability) has led several authors to argue that chromosomal rearrangements are a primary cause of reproductive isolation, thus playing a key role in speciation (White 1978, King 1993). Various models of chromosomal speciation have been formulated (reviewed in Sites

and Moritz 1987, Rieseberg 2001). Most of them invoke the fixation of strongly underdominant rearrangements that causes a decrease in fitness of structural heterozygotes that result from the malsegregation of homologous chromosomes during the meiosis (White 1978, King 1993). Yet these models contain an unsolved paradox – the more meiotically disruptive a chromosomal mutation (for example by causing the complete sterility of heterozygotes), the less probable is its fixation in a population (see Robinson and Roux 1985). Consequently, several authors have argued that their applicability is contingent on drastic ecological, demographic and geographical prerequisites, and that karyotypic differences between species are more likely to be coincidental to speciation (Sites and Moritz 1987, Coyne and Orr 1998). Counter arguments to these criticisms are: (1) irrespective of whether rearrangements occur prior to or after speciation, they are nonetheless evident in extant karyotypes, implying that if drastic conditions are indeed necessary for fixation, these conditions must have been present at some stage during the evolutionary time span of the species (Dobigny et al. 2005); (2) some of the proposed models do not invoke strong underdominant mutations. This is perhaps best exemplified by Baker and Bickham (1986) who argue that if different neutral (or weakly underdominant) centric fusions are fixed in two isolated populations, the resulting monobrachial homologies induced in structural hybrids can impede normal segregation, and thus lead to speciation.

Most recently it has been proposed that underdominance of chromosomal rearrangements is not related to structural mispairing at meiosis but is rather associated with a recombination-suppression effect (Noor et al. 2001, Rieseberg 2001). The model described by Noor et al. (2001) considers two *Drosophila* species that display alleles which confer hybrid sterility on a heterospecific genetic background. The model predicts that long-term hybridization between two such species that do not differ by any chromosomal rearrangement will lead to the complete assimilation of the two species,

because recombination will eliminate deleterious alleles and retain only those alleles that are compatible with both genetic backgrounds. If these alleles are however situated in an inverted region where recombination is suppressed, it will be impossible to eliminate them and a barrier to gene flow will persist between the two species.

In conclusion, many models of chromosomal speciation remain largely untested and the relative importance of chromosomal versus genic and/or other factors in speciation still cannot be firmly assessed (Rieseberg 2001).

General aims of the study

Intraordinal comparative molecular cytogenetic studies within eutherians (category 2 studies described above) were, prior to the present investigation, largely limited to laurasiatherian and euarchontogliran taxa. The chromosomes of several afrotherian species had, however, already been mapped to the human genome (Yang et al. 2003, Robinson et al. 2004) as part of a large collaborative Wellcome Trust project between Professors T.J. Robinson and M.A. Ferguson-Smith (Center for Veterinary Science, University of Cambridge, Cambridge, UK). This led to the isolation of chromosome painting probes for each of the seven afrotherian families (see above) providing a valuable resource that could be used to investigate chromosomal relationships within each of these. Three projects were consequently initiated in our laboratory (Evolutionary Genomics Group, University of Stellenbosch) to address questions on chromosomal evolution within polytypic orders. The first on Paenungulata is complete (Pardini 2007, Pardini et al. 2007), and the second on Macroscelidae is nearing completion (Smit submitted). The final aspect entails the detailed analysis of the Afrosoricida which forms the substance of my dissertation.

In broad terms, the investigation concentrated on the analysis of karyotypic diversity in Afrosoricida using conventional (banding) cytogenetic techniques. It also

comprises a comprehensive comparative molecular cytogenetic investigation that utilizes chromosome painting probes that were isolated from the Cape golden mole (*Chrysochloris asiatica*; Chrysochloridae) and the Taiva's shrew tenrec (*Microgale taiva*; Tenrecidae) by Cambridge (Center for Veterinary Science, University of Cambridge), and their subsequent characterization in Stellenbosch as part of my study.

In broad terms the aims were first, to describe the mode (i.e., the type of rearrangements) and tempo (the rate of accumulation) of chromosomal evolution in these two afrotherian families. Secondly, the data were examined for utility in deciphering the phylogenetic relationships of the constituent species, and the potential role of chromosomal rearrangements in their speciation.

Organization of the thesis

Most of the information contained in this thesis has been published. Citations to the papers encapsulated in the various chapters are:

Chapter II

Gilbert C, O'Brien PC, Bronner G, Yang F, Hassanin A, Ferguson-Smith MA, Robinson TJ (2006) Chromosome painting and molecular dating indicate a low rate of chromosomal evolution in golden moles (Mammalia, Chrysochloridae). *Chromosome Research* **14**: 793-803.

Gilbert C, Maree S, Robinson TJ (Submitted) Chromosomal evolution and distribution of telomeric repeats in golden moles (Chrysochloridae, Mammalia). *Cytogenetics and Genome Research*.

Chapter III

Gilbert C, Goodman SM, Soarimalala V, Olson LE, O'Brien PCM, Elder FFB, Yang F, Ferguson-Smith MA, Robinson TJ (In press) Chromosomal evolution in tenrecs

(*Microgale* and *Oryzorictes*, Tenrecidae) from the Central Highlands of Madagascar. *Chromosome Research*.

CHAPTER II

CHROMOSOMAL EVOLUTION IN GOLDEN MOLES

INTRODUCTION

General biology, taxonomy and geographic distribution

Golden moles are small subterranean mammals that somewhat resemble true moles in appearance. All species are morphologically very similar and display a mix of characters that are considered to be either plesiomorphic or highly derived within mammals. For example, they have retained a single urogenital opening (cloaca) (Butler 1988) but are the only mammals that show hyoid-dentary articulation (Bronner et al. 1990) and their hypertrophied malleus is the largest of all mammals relative to body size (Mason 2001). Their body length and weight varies from 76 mm/25 g in Grant's golden mole (*Eremitalpa granti*) to 235 mm/500 g in the giant golden mole (*Chrysospalax trevelyani*). They have no externally visible tail, their ears are small and concealed within the pelage, and their eyes are vestigial and covered by hairy skin; they are completely blind. Digging involves the short forelimbs (which bear a third digit armed with a powerful claw), and the muzzle which ends in a smooth, leathery pad (Nowak 1999, Bronner 1995a). Golden moles prefer deep sandy soils in a wide spectrum of biomes (desert to mountain forest), climates (arid to subtropical) and altitudes (sea level to >2 500m) (Bronner 1995b, 1997). They present relatively low and very labile body temperatures (Withers 1978, Fielden et al. 1990), and display K-selected reproductive strategies characterized by small litter size, slow post-natal development and extended periods of parental care (Bronner 1992, Bernard et al. 1994). According to the IUCN 2007 red list (http://www.iucn.org/themes/ssc/redlist2007

/index_redlist2007.htm), more than half of the species are considered threatened, the most likely reason being habitat fragmentation due to anthropogenic activities (Maree et al. 2003).

The family comprises 21 species grouped in two subfamilies (Bronner and Jenkins 2005). Chrysochlorinae that includes six genera (*Carpitalpa*, *Chlorotalpa*, *Chrysochloris*, *Chrysospalax*, *Cryptochloris* and *Eremitalpa*), and Amblysominae with three genera (*Amblysomus*, *Calcochloris* and *Neamblysomus*). The majority of the species (18 of 21) occur only in Southern Africa (Figure 3); with the three remaining species belonging to different genera that show a fragmented distribution in other parts of Africa. *Chrysochloris stuhlmani* is recorded locally in the Cameroon, Central African Republic, Congo, Burundi, Kenya, Rwanda, Tanzania and Uganda. *Calcochloris leucorhinus* also occurs in the Cameroon, Central African Republic and Congo, but its distribution extends southwards into northern Angola. In contrast, *Calcochloris tytonis* is known from only one specimen collected in Somalia. Several of the southern African species are relatively widely dispersed. For example, *Chrysochloris asiatica* is rather common in the southwestern Cape region, and *Amblysomus hottentotus* is found in the eastern parts of South Africa.

Phylogenetic relationships

After more than a century of research on golden moles, their taxonomy and phylogenetic relationships remain contentious. Here I follow Bronner and Jenkins (2005), the most recent nomenclatural work on the family, but include a brief historical perspective to facilitate interpretations of the evolutionary relationships suggested by the different hypotheses.

Broom (1907) was the first to provide a comprehensive and argued classification for Chrysochloridae. He recognized two main groups on the shape of the malleus. One

that includes *Chrysospalax*, *Cryptochloris* and *Chrysochloris* where the head of the malleus comprises a vesicular bulla, and the other that includes *Eremitalpa*, *Chlorotalpa*, *Calcochloris* and *Amblysomus* in which there is no vesicular bulla. Within this latter group he distinguished *Eremitalpa* and *Chlorotalpa*, both with an adult dentition of 40, and *Calcochloris* and *Amblysomus* with 36 teeth. However, Ellerman et al. (1953) argued that dental formulae were not valid generic characters within Chrysochloridae, and they consequently synonymized *Calcochloris*, *Chlorotalpa* and *Neamblysomus* with *Amblysomus*. This treatment was followed by Petter (1981) who included *Carpitalpa* (described by Lundholm in 1955) within *Amblysomus*.

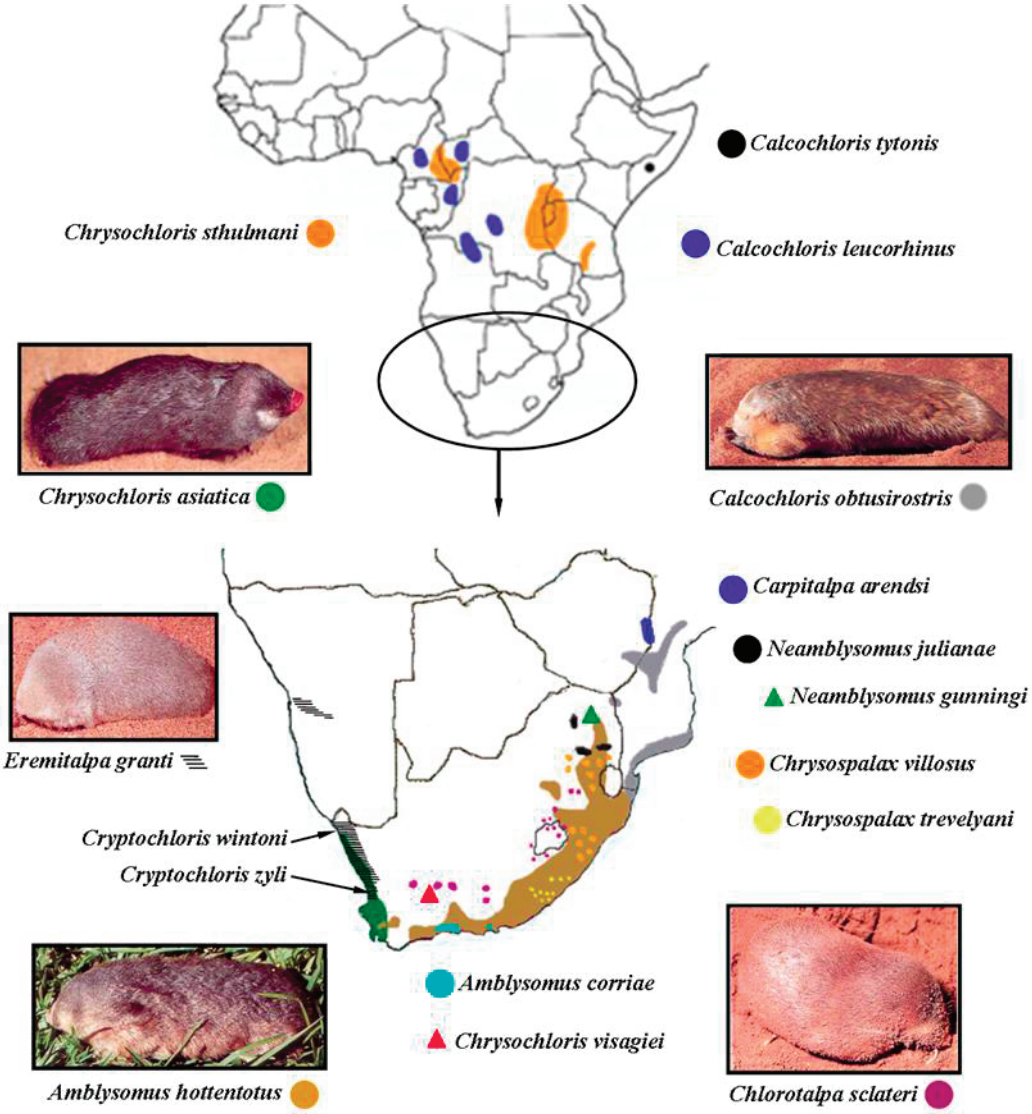


Figure 3. Geographic distribution of 17 species of golden moles in Southern Africa (redrawn from Bronner 1997, Kingdon, 1997).

Roberts (1924, 1951) showed that *Calcochloris*, *Chrysochloris*, *Cryptochloris* and *Eremitalpa* all share the lack of talonids on the lower molars and argued that they should be placed in a group distinct from the other genera. After analyzing several quantitative (body, mandibular and skull sizes) and discrete (malleus and epitympanic recess shape; presence/absence of talonid on lower molars) characters, Simonetta (1968) divided the family into the Chrysochlorinae (*Chrysochloris*, *Cryptochloris*, *Carpitalapa* and *Chlorotalapa*), the Amblysominae (*Amblysomus*, *Neamblysomus* and *Calcochloris*) and the Eremitalpinae (*Chrysospalax* and *Eremitalpa*). Meester (1974) and Meester et al. (1986) followed by Skinner and Smithers (1990) recognized *Chlorotalpa* and *Calcochloris* on the basis of cranial and dental characters, but retained *Neamblysomus* within *Amblysomus*, and *Carpitalpa* within *Chlorotalpa*.

The most recent treatment of Chrysochloridae entailed the cladistic analysis of eight binary and multistate characters from the hyoid bones of nine species of golden moles; regrettably this gives little resolution (Bronner 1991). Whereas the final consensus tree places *Calcochloris obtusirostris* sister to all other ingroup species, Bronner (1991) indicates that hyoid characters have little value for resolving intergeneric relationships. He bases his new classification (Bronner and Jenkins 2005) on the cladistic analysis of 10 quantitative craniometric ratios and five qualitative characters involving hyoid, dental and malleus morphology, and chromosomal data (Bronner 1995). Most importantly, however, there is still no published molecular phylogeny for Chrysochloridae. Preliminary results (Maree et al. 2003) based on complete mitochondrial cytochrome b and 12S rRNA sequences strongly confirmed the monophyly of all nine genera proposed by Bronner and Jenkins (2005), but failed to resolve the intergeneric relationships.

Paleontology and biogeography

The origins and biogeography of golden moles have not previously been addressed; in fact, none of the investigations that included eutherian divergence times has involved more than a single species of golden mole (Springer et al. 2003, Delsuc et al. 2004, Murphy et al. 2007). The most ancient chrysochlorid fossil (consisting of the anterior part of a skull) is found from the Lower Miocene in Kenya (Butler and Hopwood 1957). According to Butler and Hopwood (1957) and Butler (1984) differences in nine dental and two cranial characters justify its recognition as a new genus (*Prochrysochloris miocaenicus*), and its placement in a different subfamily (Prochrysochlorinae). Two fossils that date back to the Middle Pleistocene of South Africa (Broom 1941) are chronologically the next most ancient. According to Broom (1941) one resembles *Amblysomus* in general structure but its temporal region and tympanic bulla are sufficient to warrent placement in a new genus, *Proamblysomus antiquus*. He attributes the second fossil to a new species of *Chlorotalpa* (*C. spelea*) based on the general structure and measurements of the skull. Fossil evidence seems to favour an East African origin for the family and a subsequent dispersion and diversification in central and southern Africa. However, as morphological characters have been of little value in resolving intergeneric relationships within the family (and considering the relative paucity of characters available from the fossils), their position cannot be unambiguously assessed within Chrysochloridae. For example, Miocene fossils could represent independent lineages belonging to a stem group Chrysochloridae and so their distribution would not necessarily reflect that of the most recent ancestor of the extant species.

Cytogenetic data

Conventional karyotypes are available for 12 golden mole species representing six of nine genera (*Amblysomus*, *Neamblysomus*, *Calcochloris*, *Chlorotalpa*, *Chrysochloris*, and *Chrysospalax*) (Bronner 1995a, b). G-banded chromosomes have been reported only for *Chrysochloris asiatica* (2n=30 Robinson et al. 2004). Diploid numbers range from 2n=28 (*Calcochloris obtusirostris*) to 2n=36 in *Amblysomus robustus* (Bronner 1995a, b). It is noteworthy that Bronner (1995a) originally regarded *A. hottentotus* as comprising three allopatric cytotypes (2n=30, 34 and 36) but, based on morphometric evidence, the 2n=34 cytotype was subsequently described as a valid species, *A. septentrionalis* (Bronner 1996) with the 2n=36 form being assigned to *A. robustus* (Bronner 2000).

Context

This study represents the first comprehensive cytogenetic comparison among species within the Chrysochloridae. Standard G-banded karyotypes are reported for 10 species/subspecies representing 6 genera of golden moles and a comprehensive half-karyotype comparison between them and the chromosomes of *Chrysochloris asiatica* is established based on a combination of G-banded patterns and chromosome painting. The distribution of telomeric repeats among species is also described. Chromosomal rearrangements, the evolution of telomeric and other repeat sequences, and the potential support for several phylogenetic relationships are discussed in a cladistic framework. Finally, this study provides the first molecular time estimate for the origin of the Chrysochloridae allowing for the rigorous discussion of rates of chromosomal evolution in this unusual assemblage of mammals.

MATERIAL AND METHODS

Specimens, cell culture and chromosome preparation

A list of specimens included in this study and their associated voucher numbers is presented in Table 2. Cell lines were established from ribs and/or kidney fibroblasts using DMEM or Amniomax (Gibco) culture medium supplemented with 15 % foetal calf serum. Incubation was at 37°C with 5% CO_2. Chromosome harvests and slide preparation followed conventional procedures. G- and C-banding was by trypsin and barium hydroxide, respectively (Seabright 1971, Sumner 1972, Henegariu et al. 2001). Animals were collected under permits from the relevant conservation authorities issued to Prof N.C. Bennett, Dr S. Maree (both from the University of Pretoria) and Dr G. Bronner (University of Cape Town).

Table 2. Voucher numbers and origin of the golden mole specimens included in this study. All specimens were trapped in South-Africa and are kept in the Iziko museum (Cape Town).

	Museum voucher numbers	**Location**	**Co-ordinates**
Amblysomus hottentotus longiceps	SAM ZM 41631	Clarens	28º31'S - 28º25'E
A. h. pondoliae	SAM ZM 41632	Margate	30º51'S - 30º22'E
A. h. meesteri	SAM ZM 41634	Pilgrims Rest	24º25'S - 30º45'E
A. h. hottentotus	SAM ZM 41552	-	-
A. robustus	SAM ZM 41635	Dullstroom	25º25'S - 30º07'E
Calcochloris obtusirostris	SAM ZM 41636	Sodwana Bay	28º07'S - 32º46'E
Chrysospalax trevelyani	SAM ZM 41548	-	-
Cryptochloris zyli	SAM ZM 41550	-	-
Eremitalpa granti	SAM ZM 41551	-	-
Neamblysomus julianae	SAM ZM 41633	Pretoria	25º42'S - 28º13'E

Flow-sorting and generation of labeled chromosome-specific painting probes from *Chrysochloris asiatica*.

Chromosomes of *C. asiatica* were sorted on a dual laser cell sorter (FAC-Star Plus, Becton Dickinson) by fluorescence activated cell sorting. Flow-sorted chromosomes were amplified by degenerate oligonucleotide primed PCR (DOP-PCR,

Telenius et al. 1992). The primary PCR products were subsequently reamplified by DOP-PCR to make stock solutions; fluorescent labeling was with biotin- or digoxigenin-dUTP antigens (Roche) (Yang et al. 1997).

Chromosome painting

The fluorescence *in situ* hybridization (reviewed in Rens et al. 2006) was performed using painting probes from *C. asiatica* on metaphase chromosomes of 10 golden mole species. A total of 100-150 ng of probe was precipitated together with 50 ng of salmon sperm DNA in 1/10 volume of Na-Acetate and four volumes of 100% ethanol (-70°C for 2 hours). After 15 min centrifugation at 13000 rpm the pellet was washed in ice-cold 70% ethanol, dried for 30 min at 37°C and resuspended in 15µl hybridization buffer (50% deionised formamide, 10% dextran sulphate, 2x SSC, 0.5 M phosphate buffer, pH 7.3). There was an improvement in the quality of the hybridization signal when one volume of unlabeled probe (corresponding to one or two different chromosomes) was added to the precipitation mixture as a background suppressor. Probes were denaturated at 70°C for 10 min and preannealed at 37°C for 15-40 min depending on the painting probe used and the target species. Chromosome preparations were denaturated in a formamide 70%/0.6X SSC solution at 65°C for 10 - 45s and quenched in 70% ice cold ethanol for one min. Slides were dehydrated in an ethanol series (70%, 80%, 90% and 100% for 2 min in each) and dried at room temperature. The preannealed probe mixture was dropped onto the slide, cover-slipped and the edges sealed with rubber cement. Hybridization took place in a humid chamber for one or two nights at 37°C. After hybridization, slides were washed twice in formamide 50%/SSC 1X and SSC 1X or 2X for 5 min each and then in 4XT (SSC 4X, 0. 05% Tween 20) for 10 min. All five washes were at 40 - 45°C (variation dependent on the painting probe used). Detection involved 250 µl of a solution comprising 4XT/antibody (avidin-Cy3

for biotin, anti-DIG-FITC for DIG) at 37°C for 20 min. The slides were subsequently washed three times in 4XT at 37°C, counterstained with DAPI (6 µl DAPI 2 mg/ml in 50 ml SSC 2X) and mounted using an antifade solution (Vectashield).

FISH using telomeric probes

A telomeric probe containing the repeat motif $(TTAGGG)_n$ was constructed and biotin-labeled by PCR as decribed by Ijdo et al. (1991) with minor modification. We used the following primers: TR-A: 5' GGTTAGGGTTAGGGTAG 3' and TR-B: 5' AACCCTAACCCTAACCCT 3'. PCR was carried out at 95°C, 1 min; 30°C, 1 min; 72°C, 1 min (3 cycles); 94°C, 30 sec; 50°C, 1 min; 72°C, 1 min (17 cycles). Specifications for the amplification of the telomeric motif were: Buffer (10X): 2.5 µl, MgCl2 (25 mM): 2.5 µl, dNTP (20 mM): 2.5 µl, TR-A + TR-B (20 µM): 6 µl, Taq: 1.25 U, H2O: 11.25 µl. Those used for the labeling mix were: Buffer (10X): 2.5 µl, MgCl2 (25 mM): 2.5 µl, dACG (20 mM): 2.5 µl, dT (20 mM) 2 µl, biotin (1 mM): 2 µl, TR-A + TR-B (20 µM): 1.2 µl, Taq: 1.25 U, DNA (PCR product of the first amplification): 1-2 µl, H2O: 11.25 µl. Program: 94°C, 1 min; 50°C, 1 min; 72°C, 1 min (20 cycles).

Capture of images

Images were captured using the Genus software (Applied Imaging). Signals were assigned to specific chromosomes according to size, morphology and DAPI-banding. When the DAPI-bands were not sufficient to distinguish specific chromosomes, FISH was done on G-banded preparations. In these instances, and following capture of the G-banded images, slides were destained serially in methanol and 100% ethanol for 10 min in each. The times and temperatures used in the denaturation step (above) were decreased to 20s and 65°C, respectively.

Molecular dating

Molecular dating (the conversion of genetic distances into temporal framework) is widely used as a complement to the paleontological record to infer divergence times between taxa. This approach is based on the molecular clock principle, i.e., genetic distances between taxa are proportional to the time separating them (for a recent review on molecular dating, see Kumar 2005). In order to assess the rates of chromosomal change in golden moles we utilized nucleotide sequences available in Genbank (http://www.ncbi.nlm.nih.gov). Nucleotide sequences from five gene fragments (the subunit 2 of cytochrome oxidase (CO2), the subunit 2 of NADH dehydrogenase (NADH2), 12S and 16S rRNAs and tRNA-Valine (tRNA-Val)) were available for *Amblysomus* and *Chrysochloris*. Sequences from a further four gene fragments (12S rRNA, 16S rRNA, tRNA-Val and the 3' UTR of the nuclear gene CREM) were retrieved for *Amblysomus* and *Chrysospalax*. Our analyses could not accommodate all three species simultaneously since (1) the gene fragments (above) are not completely complimentary, and (2) the method used (see below) requires an input tree that is fully resolved which is presently not available for golden moles (see above). We thus conducted two separate analyses. In our first analysis (which included five gene fragments) *Amblysomus* and *Chrysochloris* were examined together with homologous sequences derived from the 39 other mammals species presented in Springer et al. (2003) but this excluded the two bat genera *Tadarida* and *Megaderma* for which CO2 sequences were not available. Our second analysis (four gene fragments) included *Amblysomus* and *Chrysospalax* together with 38 of the 39 mammal species referred to above. The rabbit, *Oryctolagus*, was excluded from the data set since the CREM sequence is unavailable. Accession numbers (and the associated references) of the sequences used in this study are provided in the Supplementary Data S1 of Gilbert et al.

(2006). Sequences were aligned using Bioedit v5.0.6 (Hall 2004). For the 12S rRNA, 16S rRNA, tRNA-Val and CREM sequences, we used the Springer et al. (2003) alignment as a reference (see the supporting data set 1 on the PNAS website) and simply added one new genus of golden mole (*Amblysomus* or *Chrysospalax*) and excluded the two bat genera (*Tadarida* and *Megaderma*) and the rabbit without changing the number and position of gaps. The alignment of the CO2 and NADH2 protein coding genes did not pose homology problems since it was based on the amino acid translation.

Molecular estimates were performed using a relaxed Bayesian molecular clock method for multigene datasets (Thorne et al. 1998, Thorne and Kishino 2002) which takes into account potential changes and differences in the rate of evolution of different genes. The parameters were set following the authors' instructions. We used the same input topology and calibration points as Springer et al. (2003) with the exception of the bat node (Pteropodidae + Megadermatidae) which was not included in our tree (see above). The Markov chains were sampled 10,000 times every 100 generations, and the "burn in" period was set at 100,000 generations.

RESULTS AND DISCUSSION

General description of the karyotypes and flow-sorted karyotype

G-banded karyotypes obtained for the ten new species or subspecies of golden moles described in this study (Table 2) are presented in Figure 4. The karyotype of *C. asiatica* was presented in Robinson et al. (2004). Diploid numbers of four species (*C. obtusirostris*, *C. trevelyani*, *N. julianae*, *A. robustus*) are consistent with the earlier report by Bronner (1995a) based on standard giemsa preparations. Out of the eleven species, only three have a diploid number that deviates from 2n = 30. These are *E. granti* (2n = 26), *C. obtusirostris* (2n = 28) and *A. robustus* (2n = 36). The G-banding

patterns were generally well conserved between taxa allowing the confident assessment of homology among chromosomes. However, in order to resolve any possible ambiguities and to strengthen phylogenetic inferences as well as to provide a more detailed understanding of the chromosomal rearrangements detected herein, we verified our G-band assessment by cross-species chromosome painting using *C. asiatica* (CAS) flow-sorted painting probes. This was done for all species except *N. julianae* (due to insufficient material).

The 30 chromosomes of a female *C. asiatica* specimen were resolved into 13 peaks (Figure 5). Nine peaks each contained a single chromosome (CAS 1, 2, 3, 7, 10, 11, 12, 13, 14), three peaks included two chromosomes each (CAS X+9, 4+5, 6+7), and one peak included three different chromosomes (CAS 8+9+X). It was possible to isolate CAS 8 in a subsequent attempt to separate single chromosomes from the impure flow sorts. Thus, the probes allow for the distinction of 10 of 15 CAS chromosomal pairs. Although a complete coverage of all 15 pairs of chromosomes, each by a specific painting probe was not possible (paints for CAS 4, 5, 6, 9, X were not obtained), we were able to resolve all ambiguities in the G-banded comparisons.

Description and polarization of intrachromosomal rearrangements

Figure 6 shows the half-karyotype comparisons among the 10 species/subspecies described in this study compared to that of *C. asiatica* (described in Robinson et al. 2004). Contrary to the other taxa included herein, chromosomes homologous to CAS 1-5, 10 and X in all species/subspecies of *Amblysomus* unambiguously show large, G-negative pericentric regions that correspond to C-positive heterochromatin (Figure 7) that are not hybridized by any of the CAS painting probes (e.g., Figure 8a, c, d, e). In the absence of a comprehensive phylogeny of golden moles, two equally parsimonious hypotheses must be considered *a priori* in order to explain this difference: (i) the large

pericentric regions correspond to a derived condition within Chrysochloridae and are the result of an increase in the amount of pericentric heterochromatin that occurred in the lineage leading to *Amblysomus*, and (ii) these large pericentric regions are plesiomorphic (= ancestral) within Chrysochloridae, and the amount of pericentric heterochromatin has been reduced in a common lineage that is ancestral to the other taxa.

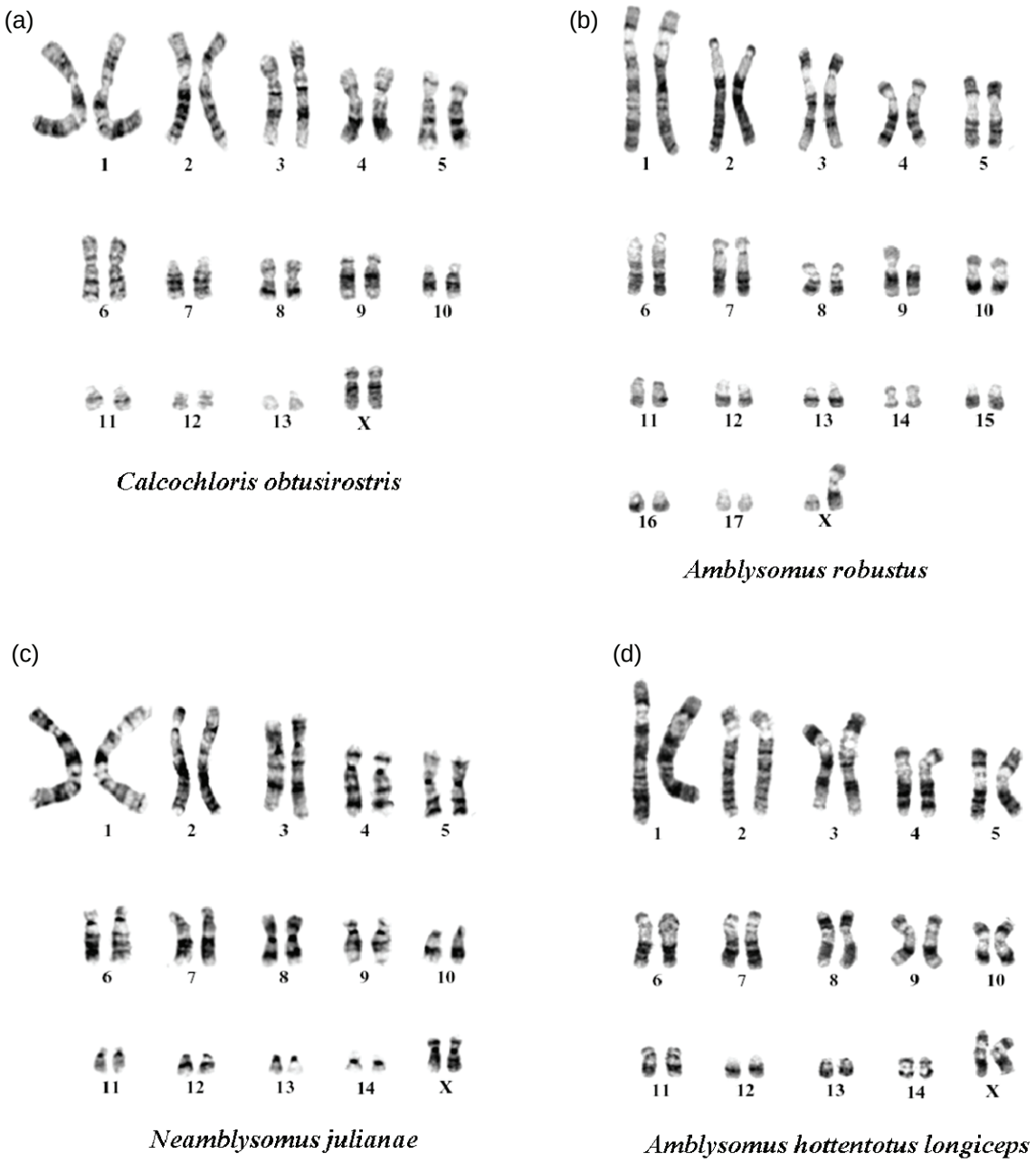
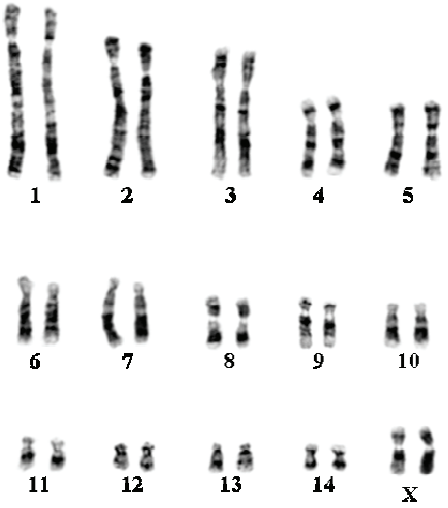


Figure 4. G-banded karyotypes of 10 species/subspecies of golden moles: **(a)** female *C. obtusirostris* (2n=28), **(b)** male *A. robustus* (2n=36), **(c)** female *N. julianae* (2n=30), **(d)** female *A. h. longiceps* (2n=30), **(e)** female *C. zyli* (2n=30), **(f)** female *A. h. hottentotus* (2n=30), **(g)** female *C. trevelyani* (2n=30), **(h)** female *E. granti granti* (2n=26), **(i)** female *A. h. meesteri* (2n=30), **(j)** female *A. h. pondoliae* (2n=30).

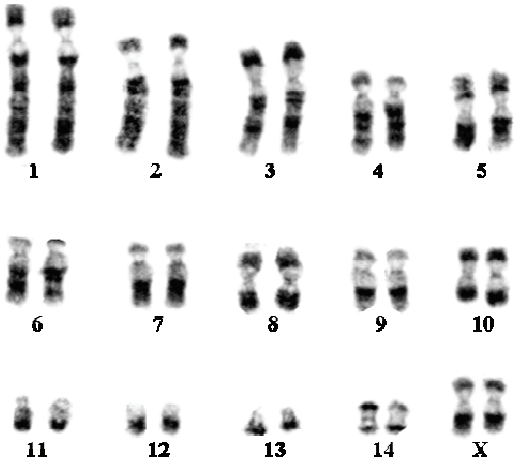
Figure 4 (continued).

(e)



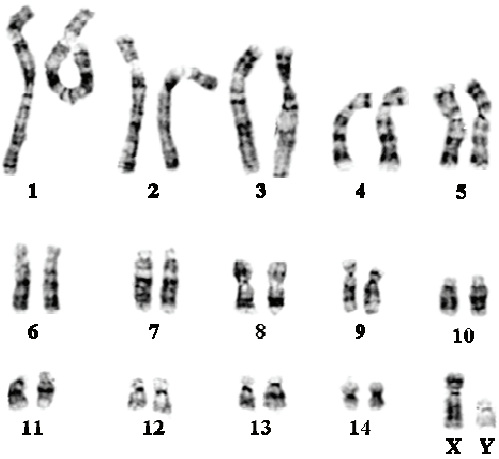
Cryptochloris zyli

(f)



Amblysomus hottentotus hottentotus

(g)

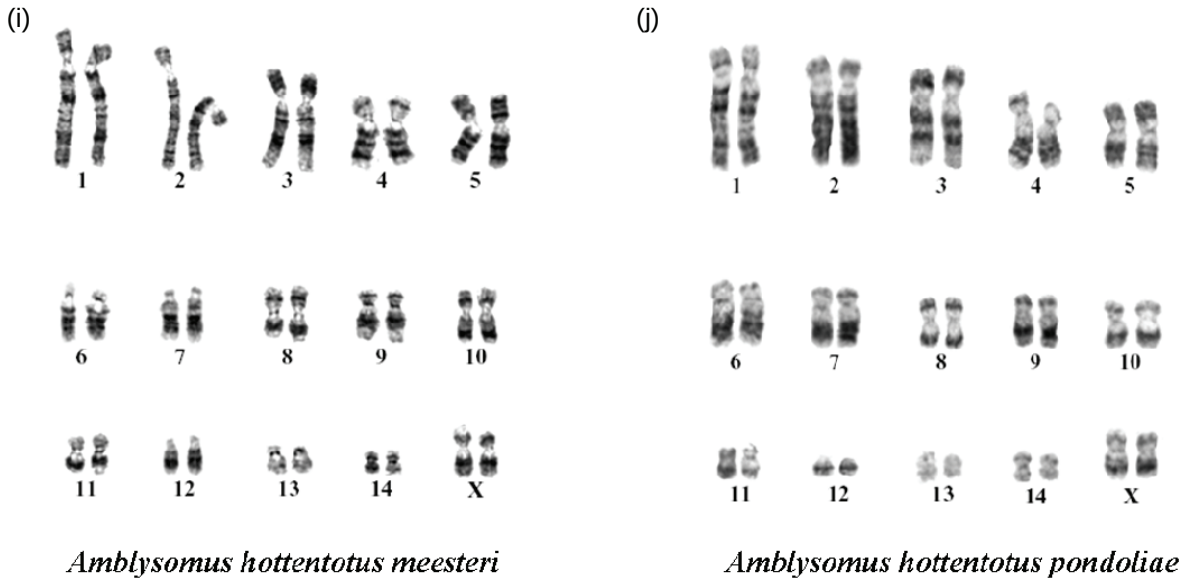


Chrysospalax trevelyani

(h)

Eremitalpa granti

Figure 4 (continued).



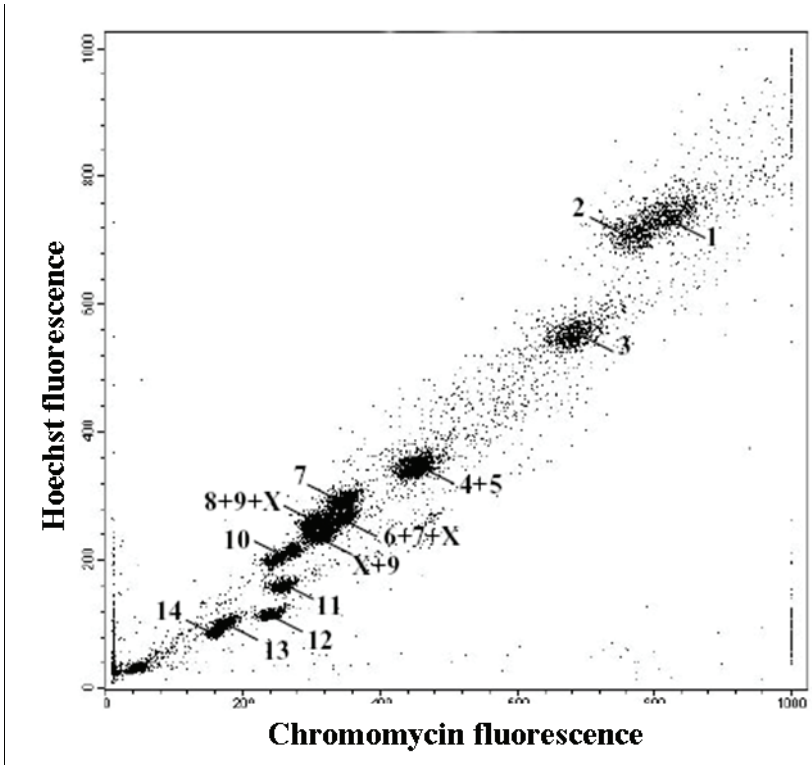


Figure 5. Flow-sorted karyotype of *C. asiatica* (CAS, 2n=30, XX) showing the correspondence between the peaks and CAS chromosomes. The probe set made from this flow-sort allows the clear distinction of 10 of the 14 autosomes in *C. asiatica* (see text for details).

Using the aardvark as an outgroup does not resolve which of these two hypotheses is more likely because homology between aardvark and golden mole centromeres cannot be assessed. Indeed, none of the aardvark centromeres is situated between the same synteny blocks as in golden moles (see Figure 3 in Robinson et al. 2004). That said, however, the two hypotheses are equally parsimonious only where the genus *Amblysomus* is sister to a clade that groups all other golden moles which, although feasible, would be in conflict with all previously published classifications based on morphology (Roberts 1951, Ellerman et al. 1953, Meester et al. 1986). Based on these considerations it is suggested that hypothesis (i) is more likely than hypothesis (ii), and that the large pericentric heterochromatin regions observed in chromosomes homologous to CAS 1-5, 10 and X of all *Amblysomus* spp. are cladistic characters that support the monophyly of the genus *Amblysomus* (Figure 9), a view that is consistent with an unpublished molecular phylogeny (S. Maree et al. unpublished).

Chromosomes homologous to CAS 10 in *A. h. hottentotus*, *A. h. meesteri*, *A. h. longiceps*, *A. h. pondoliae* and *A. robustus* differ significantly in G-banded pattern and morphology from the homologues in the other species (Figure 6). The homology of this chromosome to that of *C. asiatica* was unambiguously assessed by FISH (Figure 8b), showing that the difference in banding pattern is not due to an interchromosomal rearrangement, but is rather likely to result from intrachromosomal restructuring. Since the region homologous to CAS 10 in the aardvark has retained the same banding pattern as the six golden mole species *Calcochloris obtusirostris*, *N. julianae*, *E. granti*, *Cryptochloris zyli*, *Chrysospalax trevelyani* and *Chrysochloris asiatica*, we can infer that this rearrangement is indicative of *Amblysomus* common ancestry, and whatever the nature of this change, it constitutes an additional synapomorphy confirming the monophyly of this genus (Figure 9).

Similar reasoning applies to the chromosome homologous to CAS 7 where two distinct G-banded patterns can be observed. This time, however, although four species/subspecies of the genus *Amblysomus* (namely *A. h. hottentotus*, *A. h. longiceps*, *A. h. pondoliae* and *A. robustus*) show a pattern that differs from the other genera, *A. h. meesteri* shows the same pattern as the other genera (Figure 6). Again, the homology of this chromosome to that of *C. asiatica* was unambiguously assessed by FISH (Figure 8a), showing that the difference in banding pattern is not due to an interchromosomal rearrangement but is rather likely to be the result of an intrachromosomal rearrangement. Moreover, since the region homologous to CAS 7 in the aardvark has retained the same G-banded pattern as in the seven golden mole species *Calcochloris obtusirostris*, *N. julianae*, *E. granti*, *Cryptochloris zyli*, *Chrysospalax trevelyani*, *Chrysochloris asiatica*, and *A. h. meesteri*, we can infer that this rearrangement occurred in the common ancestor of *A. h. hottentotus*, *A. h. longiceps*, *A. robustus* and *A. h. pondoliae* and it, thus, constitutes a further synapomorphy supporting the grouping of these species (Figure 9). This conclusion is supported by Maree et al. (unpublished), and by the distribution of telomeric repeats (see below).

The two painting probes CAS 11 and 12 produced particularly interesting signals on metaphase chromosomes of *C. zyli*. These two probes not only hybridized to their homologues CZY 11 and 12, but they also produced strong cross-signals in the *C. zyli* karyotype. Specifically CAS 11 hybridized to the heterochromatic CZY 12p (Figure 8g), and CAS 12 to the heterochromatic CZY 11p (Figure 8h). In addition to these cross-signals, CAS 11 and 12 also hybridized to the centromeric regions of CZY 3 and 4 (Figure 8g, h). These cross-signals were also observed on CAS 3 and 4 when hybridizing CAS 11 and 12 onto *C. asiatica* metaphases. This indicates that the satellite sequences that constitute these heterochromatic regions are shared between the p arms

of chromosomes 11 and 12 and the centromeres of chromosomes 3 and 4 of *C. zyli* and *C. asiatica*.

As shown in Figure 8i, j, CAS 11 and 12 did not hybridize to the p arms of their homologues in any other species included here, nor did they produce signals on the centromeres of the other chromosomes in these species. These data indicate that the satellite sequences are shared by *C. asiatica* and *C. zyli*, but not by the other species (Figure 9). This character provides the first unambiguous evidence of a generic level phylogenetic association within Chrysochloridae. Interestingly, at first glance there is morphological support for a sister taxon relationship between *Chrysochloris* and *Cryptochloris*. These two genera are the only small-bodied golden moles which present temporal bullae that house hypertrophied, club-shaped malleus bones (Roberts 1951, Ellerman et al. 1953, Meester et al. 1986, Mason 2003). However, a strict cladistic assessment of the morphological characters used in the available identification schemes (Roberts 1951, Ellerman et al. 1953, Meester et al. 1986) is not possible since they all rely on plesiomorphic and apomorphic characters in their construction, and therefore remain equivocal on this issue.

As illustrated on Figures 6 and 10, the G-banding patterns of chromosomes homologous to CAS 1 and 2 in *Calcochloris obtusirostris* are clearly distinct from the other species. Although the centromere of both chromosomes is situated in region 2 in *C. obtusirostris*, it is situated between region 1 and 2 in the other species (Figure 10). The homology of these two chromosomes to those of *Chrysochloris asiatica* was unambiguously assessed by FISH (Figure 8k, l), showing that these differences are likely to be due to intrachromosomal rather than to interchromosomal rearrangements.

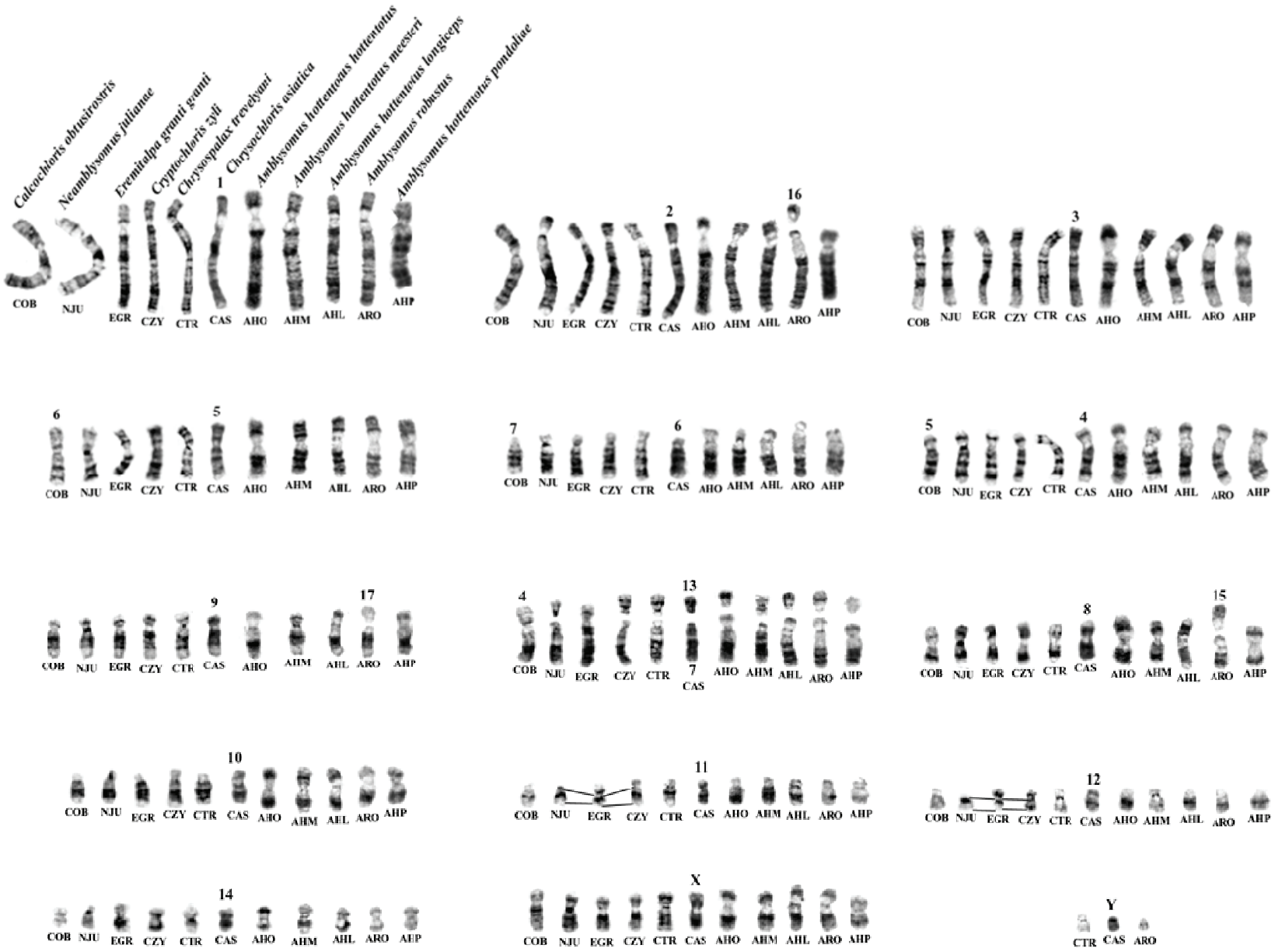


Figure 6. G-banded half-karyotype comparison among the species/subspecies analysed herein showing the genome wide correspondence defined by painting and banding homologies.

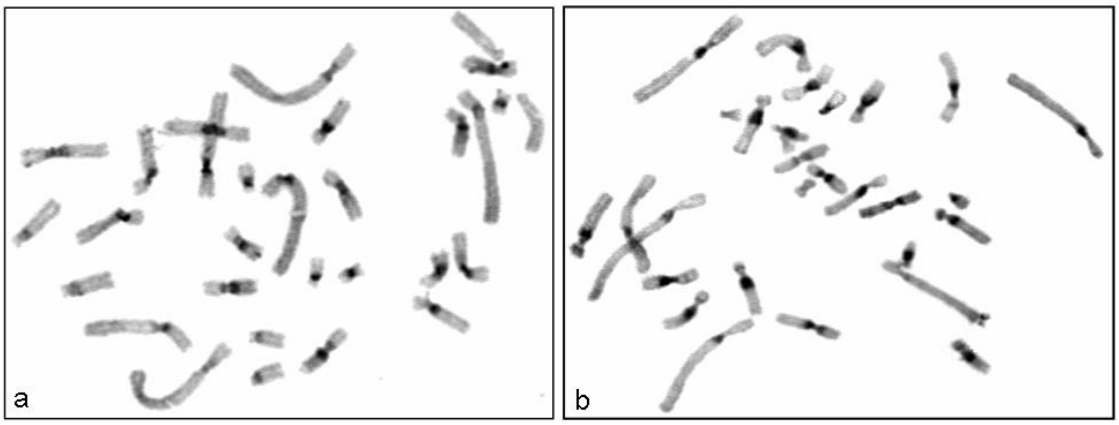


Figure 7. C-banding pattern of *A. h. meesteri* **(a)** and *A. h. hottentotus* **(b)**. No significant difference in the amount of pericentric heterochromatin is observed between the two species.

Two types of rearrangements could explain these differences: (1) a pericentric inversion, or (2) a centromeric shift. The definitive polarization of this character using the aardvark as outgroup is not possible because the position of these two centromeres is not conserved between the aardvark and golden moles (see Figure 3 in Robinson et al. 2004). In other words, at present it is equally parsimonious to infer a shift in centromere position either (1) on the lineage leading to *C. obtusirostris* or (2), in the ancestor of a clade grouping the remaining genera. As was the case for the differences in pericentric heterochromatin content between *Amblysomus* spp. and the other species (see above), hypothesis (2) would imply a topology that is in conflict with all previously published morphological classifications of golden moles. We thus believe that hypothesis (1) is more likely (Figure 9). It will be interesting to see whether these rearrangements are shared by the two other recognized species of *Calcochloris* (*C. leucorhinus* and *C. tytoni*).

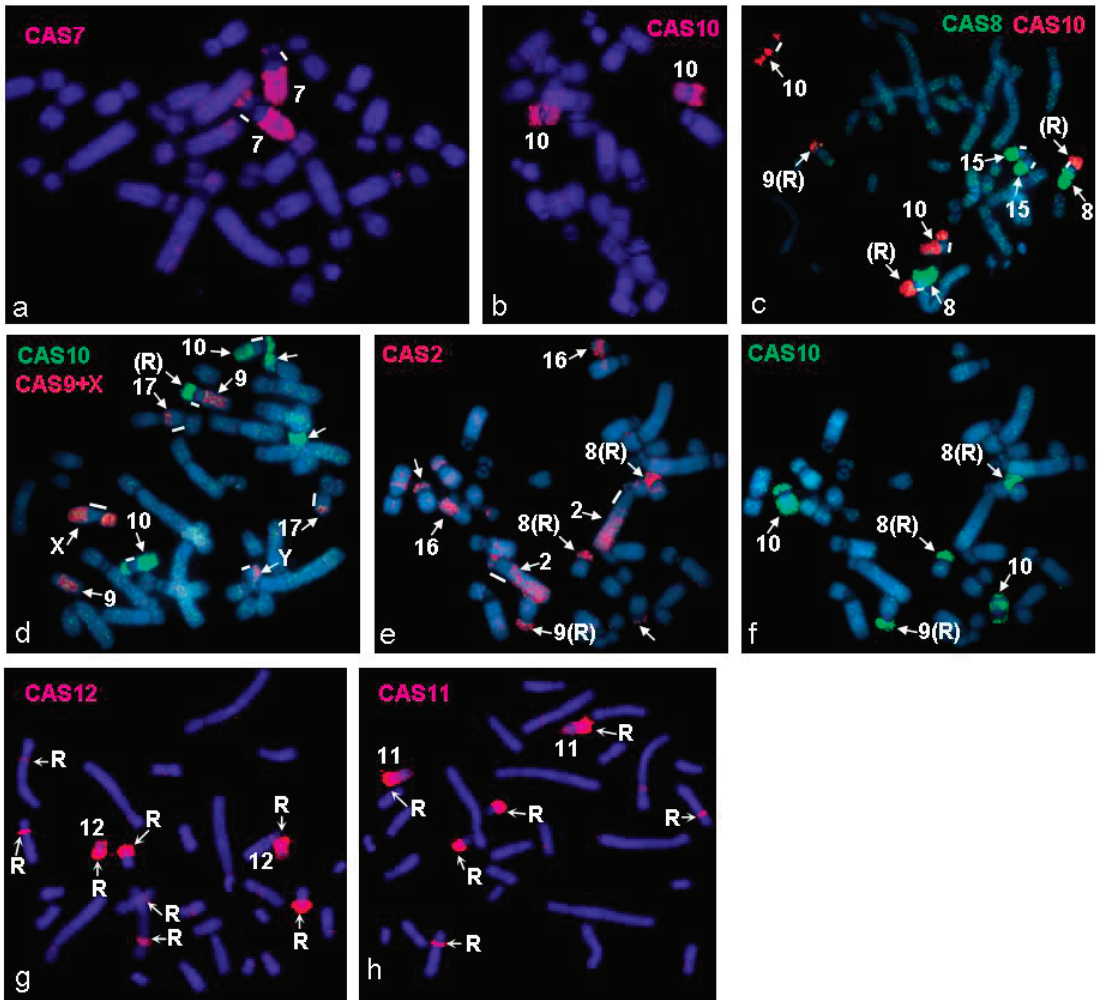


Figure 8. Examples of FISH using *C. asiatica* (CAS) chromosome specific painting probes on other species of golden moles. White arrows indicate regions of interest. Chromosome numbers of the target species are indicated in white while CAS probes are indicated in green (DIG-labelled) or red (biotin-labelled). White bars indicate absence of hybridization in the large G-negative pericentric regions of *A. h. hottentotus* (AHO) and *A. robustus* (ARO). Panels **(a)** and **(b)** present FISH of CAS 7 and CAS 10 on metaphase chromosomes of *A. h. hottentotus* showing that hybridization extends along the full length of the euchromatic portion of AHO 7 and AHO 10 respectively. **(c)** FISH of CAS 10 and 8 on metaphase chromosomes of *A. robustus* showing the split of CAS 8, the conservation of CAS 10, and the hybridization of repeat sequences (R) from CAS 10 on the p arm of the submetacentric ARO 9 and on those of ARO 8. **(d)** hybridization of CAS 9 and X and CAS 10 on metaphase chromosomes of *A. robustus* showing the conservation of CAS X and 10, the split of CAS 9 and the hybridization of repeat sequences (R) from CAS 10 on the p arm of the submetacentric ARO 9 and on those of ARO 8. **(e)** and **(f)** same metaphase of *A. robustus* hybridized with (e) CAS 2 and (f) CAS 10 showing the conservation of CAS 10, the split of CAS 2 and the overlapping hybridization of repeat sequences (R) from CAS 2 and CAS 10 on the p arm of the submetacentric ARO 9 and on those of ARO 8 and 10. **(g)** and **(h)** hybridization of CAS 12 and 11 on metaphase chromosomes of *C. zyli* indicating that these two chromosomes and CZY 11 and CZY 12 share the same family of repeat sequences (R) on their small heterochromatic arms.

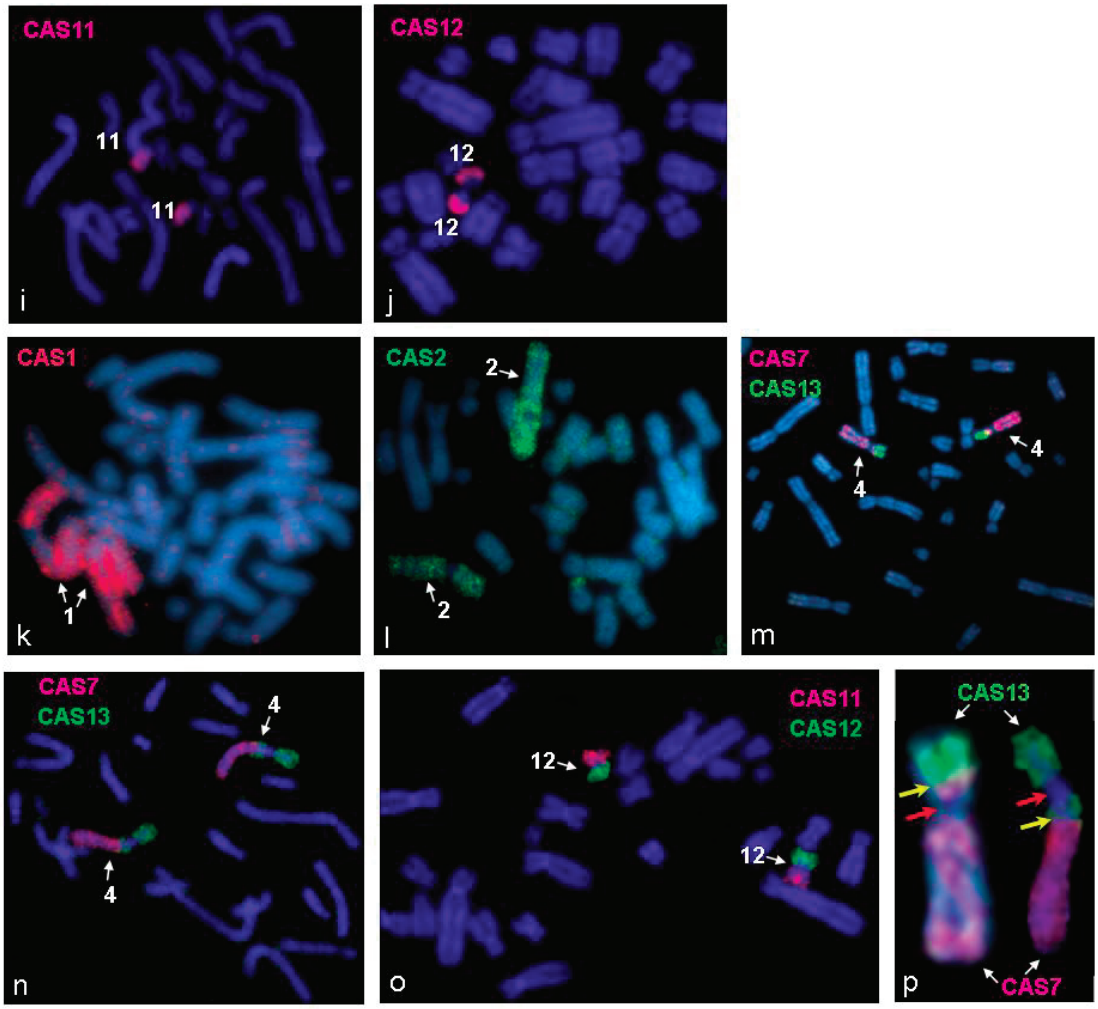


Figure 8 (continued). (i) and **(j)** hybridization of CAS 12 and CAS 11 on metaphase chromosomes of *C. trevelyani* (CTR) (i) and *A. h. hottentotus* (AHO) (j). The heterochromatic arms of CTR11 and AHO12 are not hybridized. **(k)** and **(l)** hybridization of CAS 1 and CAS 2 on *C. obtusirotris* metaphase chromosomes showing complete conservation of these chromosomes between the two species. **(m)** and **(n)** hybridization of CAS 13 and 7 on metaphase chromosomes of *C. obtusirostris* **(m)** and *E. granti* **(n)** showing that these two chromosomes are fused in both species. **(o)** hybridization of CAS 11 and 12 on *E. granti* metaphase chromosomes showing the fusion of these two chromosomes. **(p)** Enlargement of EGR 4 and COB 4 showing painting results using CAS 13 and 7 painting probes. The fusion of two chromosomes corresponding to CAS 13 and 7 giving rise to chromosome 4 of *E. granti* (EGR) and *C. obtusirostris* (COB) (taken from panel m and n) is evident, as are the differences in the location of the breakpoint region (red arrow) and that of the centromere (yellow arrow) between the two species.

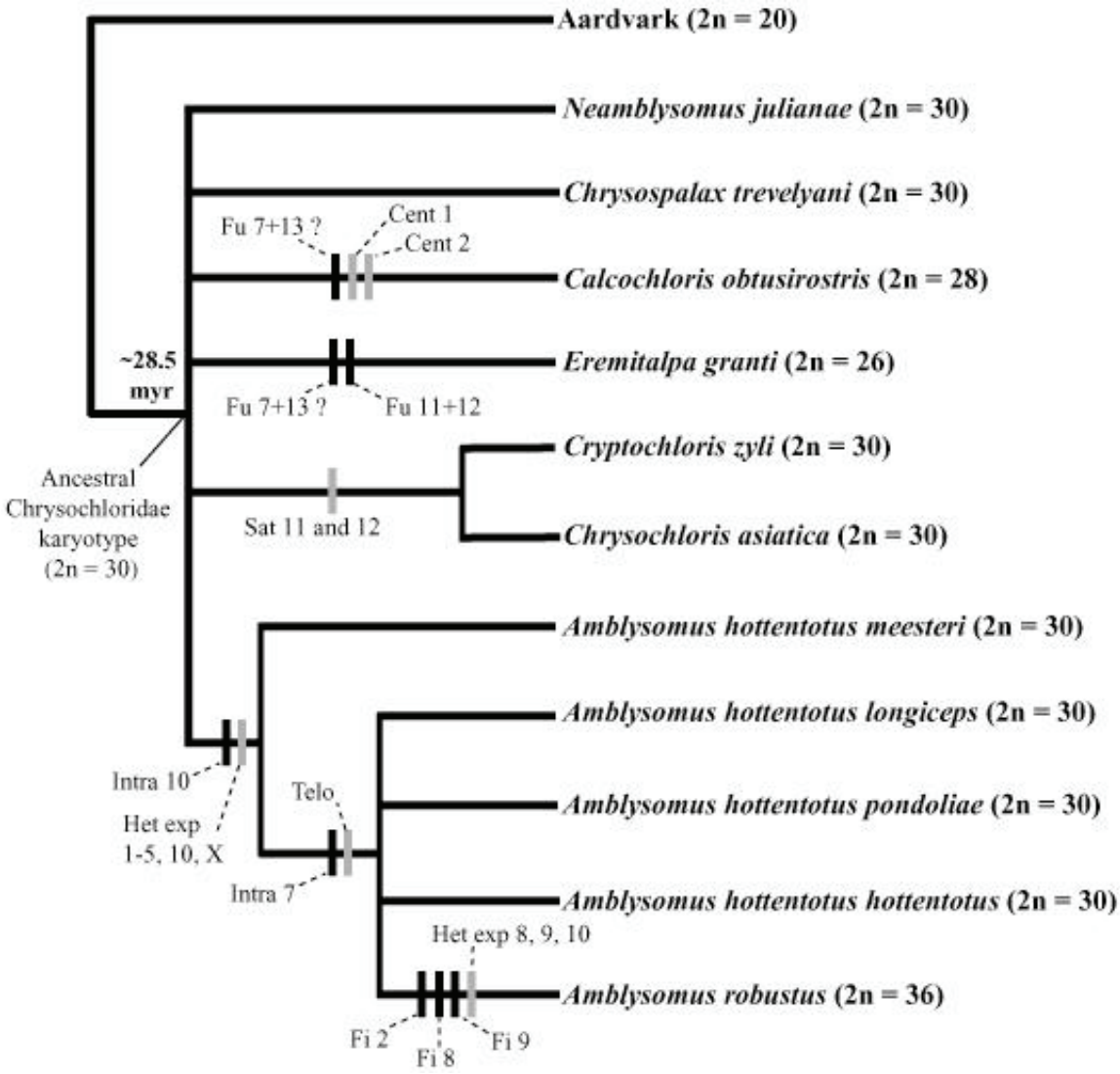


Figure 9. Cladogram depicting the phylogenetic relationships between the 11 species/subspecies of golden moles included in this study based on the cladistic interpretation of each of the rearrangements detected. The numbering corresponds to the chromosomes of *C. asiatica*. Cent = centromeric shift or pericentric inversion (see Figures 8k, l and 10); Fu = fusion; Fi = fission; Intra = indeterminant intrachromosomal rearrangement; Het exp = heterochromatic expansion (see Figures 4, 5 and 8); Sat = sharing of the same satellite DNA family (see Figure 8g, h); Telo = presence of telomeric-like sequences in the pericentromeric region of most chromosomes (see Figure 11). ▮ = rearrangements of the euchromatin; ▯ = rearrangements of the heterochromatin. Question marks indicate the ambiguity regarding the fusion 13+7 (see text for details).

Description and polarization of interchromosomal rearrangements

No interchromosomal rearrangements could be detected between the eight species/subspecies that have 2n = 30 chromosomes (Figure 6). Interchromosomal variation is thus restricted to differences between a group comprising these eight species and *A. robustus*, *E. granti* and *C. obtusirostris*.

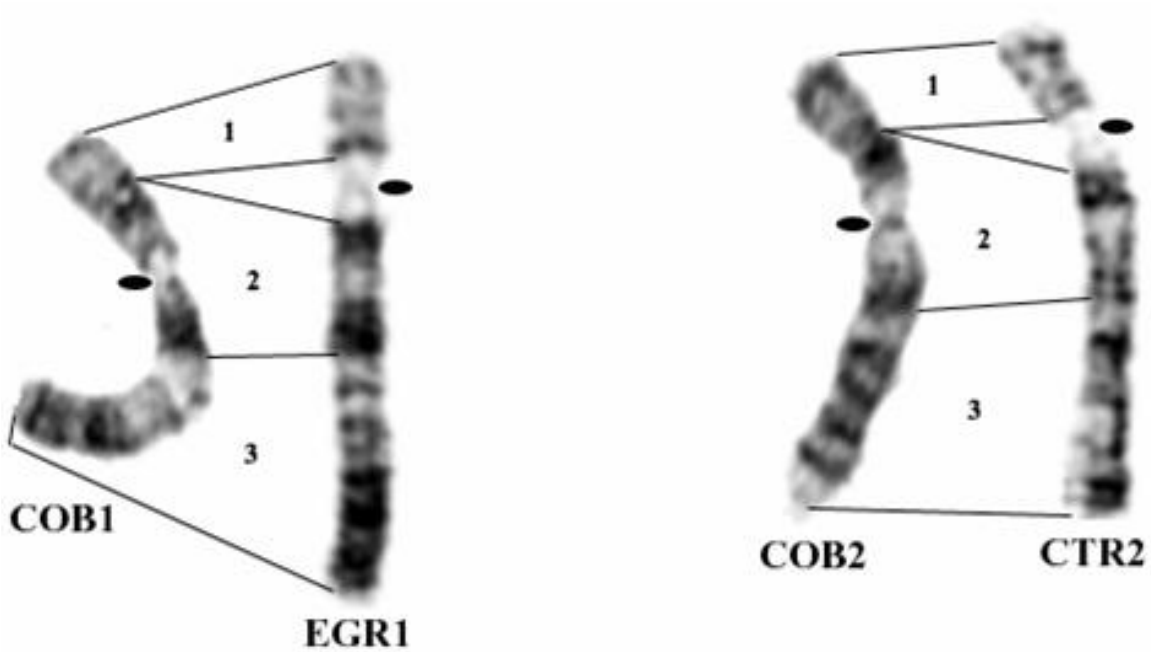


Figure 10. Detailed comparison of the G-banding patterns of chromosome 1 and 2 of *C. obtusirostris* (COB) with those of *E. granti* (EGR1) and *C. trevelyani* (CTR2) showing a shift in the position of the centromeres. EGR1 and CTR2 show the same pattern as all other species (see Figure 6). Three chromosomal regions are delimited in order to facilitate the comparison (see text). The ovals indicate the position of the centromeres.

Differences in *A. robustus* concern chromosomes homologous to CAS 2, 8 and 9. As illustrated by the G-banding alignment (Figure 6) and the painting results (Figure 8c, d, e, f), each of these chromosomes corresponds to two autosomal elements in the *A. robustus* karyotype. Given that the genus *Amblysomus* is monophyletic (this study), and that all other *Amblysomus* species/subspecies show the same state for these three chromosomes as *Calcochloris*, *Cryptochloris*, *Chrysochloris*, *Chrysospalax*, *Eremitalpa* and *Neamblysomus*, the differences between *A. robustus* and other taxa can be

interpreted to result from three autapomorphic fissions that must have occurred in the *A. robustus* lineage (Figure 9).

The fission of the chromosome homologous to CAS 2 gave rise to ARO 2 and 16. ARO 2 has a very large G-negative pericentric region (Figure 4) that failed to hybridize when using the probe corresponding to CAS 2 as painting probe (Figure 8e). However, the fact that this probe produced a signal on the small G-positive p arm of ARO 2 indicates that the breakpoint of the fission was situated in the p arm of the chromosome homologous to CAS 2.

The fission of the chromosome homologous to CAS 8 gave rise to ARO 8 and 15, and that of the chromosome homologous to CAS 9 to ARO 9 and 16. The ARO 9 pair is heteromorphic comprising one submetacentric and one acrocentric chromosome (Figure 4). Chromosomal regions corresponding to the p arm of the submetacentric ARO 9 and to those of ARO 8 do not have homologues in the other species (Figure 6) suggesting that they are comprised of repetitive sequences that were accumulated in the lineage leading to *A. robustus*. The painting results support this hypothesis. The two painting probes corresponding to CAS 10 and CAS 2 produced overlapping signals on the p arm of the submetacentric ARO 9 and on the p arm of ARO 8 and 10 (Figure 8c, d, e, f). Moreover, the probe corresponding to CAS 9 did not hybridize the p arm of the submetacentric ARO 9 and that corresponding to CAS 8 did not hybridize ARO 8p (Figure 8c, d). Overlapping signals were not observed using these probes in any of the other species of *Amblysomus* (not shown). Together these observations indicate that the p arm of the submetacentric ARO 9, and those of ARO 8 and 10, are made of repeat sequences of which at least some components are shared between ARO 8, 9 and 10 and CAS 2 and 10, and that within *Amblysomus*, the amplification of these sequences is restricted to *A. robustus*. Moreover, as illustrated in Figure 8d the probe corresponding to CAS 9 + X not only hybridizes homologous regions in *A. robustus*, but it also

produced a signal on the Y chromosome of this species. Since the flow-sorted *C. asiatica* specimen was female, the most likely explanation for this pattern is that the *A. robustus* Y contains repetitive sequences of which at least some components are shared with CAS 9 and/or X. In summary, the three fissions detected in *A. robustus* are not phylogenetically informative. It will, however, be interesting to see whether *A. septentrionalis* with 2n = 34 (Bronner 1995b, 1996) which was not available to us, shares some of the fissions observed in this species, or whether the increase in diploid number is the result of independent rearrangements.

G-banding comparisons suggest that COB 4 and EGR 4 correspond to two separate autosomal elements in the other taxa (Figure 6). Painting results show that these differences involve the same chromosomes in *C. granti* and *C. obtusirostris*, i.e., chromosomes homologous to CAS 13 and 7 (Figure 8m, n). The FISH also confirms that these two chromosomes are retained as single chromosomes in all other species/subspecies of golden moles (not shown). As a syntenic association involving these two chromosomes is not observed in the aardvark and human, we can infer that the association between CAS 13 and 7 identified in *C. obtusirostris* and *E. granti* is the result of a fusion. At first glance it is tempting to suggest that *Eremitalpa* and *Calcochloris* are closely related, and that the fusion of CAS 13 and 7 occurred only once in their common ancestor. However, as illustrated in Figure 8p, EGR 4 and COB 4 differ in the position of their centromeres. Whereas the centromere of EGR 4 lies in the region that is homologous to CAS 13, that of COB 4 is situated in the region homologous to CAS 7. Although it is likely that both EGR 4 and COB 4 are the result of the same type of fusion, i.e., an end-to-end fusion (telomere:telomere fusion), two equally parsimonious hypotheses must be considered to explain the difference in centromeric position between EGR 4 and COB 4: (1) the end-to-end fusion effectively occurred only once in the ancestor of a clade grouping *C. obtusirotris* and *E. granti*

followed by an intrachromosomal rearrangement (most probably a centromeric shift) that occurred in one of the two the species, or (2) that *C. obtusirostris* and *E. granti* are not closely related, and each of these independently underwent an end-to-end fusion between CAS 13 and CAS 7. According to this hypothesis the difference in centromeric position would best be explained by the fact that the inactivated centromere is not the same in both species, i.e., it corresponds to that of CAS 7 in *E. granti*, and to that of CAS 13 in *C. obtusirostris*.

These two species occur in areas that are very distant from each other in Southern Africa. *Eremitalpa granti* occurs in the coastal dunes from Western and Northern Cape Provinces of South Africa as well as in the Namib Desert in Namibia, whereas *C. obtusirostris* occurs in the KwaZulu-Natal and Northern Province in South Africa as well as in the south of Zimbabwe and south of Mozambique (Bronner and Jenkins 2005). In addition, morphological characters are equivocal on a possible sister relationship between *Calcochloris* and *Eremitalpa*. Similarities between the two genera include the lack of talonids on the lower molars (Roberts 1924, 1951) and the absence of vesicular bulla on the head of the malleus (Broom 1907). It is, however, not clear whether these characters are plesio- or apomorphic within Chrysochloridae. A sister relationship between *Calcochloris* and *Eremitalpa* has not been proposed and the two genera have often been separated into different taxonomic categories (Ellerman et al. 1953, Simonetta 1968, Petter 1981) as is evident from the recent Bronner and Jenkins (2005) classification in which *Calcochloris* has been assigned to the Amblysominae and *Eremitalpa* to Chrysochlorinae. Given the lack of evidence suggesting a close relationship between the two genera, a definitive statement on which of the two phylogenetic interpretations of the CAS 7 and 13 fusion discussed above accurately reflects the natural situation must await the generation of a well resolved molecular phylogeny for chrysochlorids.

The second and last difference observed in *E. granti* concerns EGR 12. Each arm of this chromosome has been unambiguously mapped to CAS 11q and CAS 12q using FISH (Figure 8o). CAS 11 and 12 are retained as two separate autosomal elements in all other species (e.g., Figure 8g, h, i, j) and syntenic associations involving these two chromosomes are not observed in the aardvark or human genome. We can thus infer that the association observed in *E. granti* is the result of an autapomorphic fusion. As mentioned previously, the painting probes CAS 11 and 12 hybridize to only one arm of the chromosome resulting from their fusion in *E. granti* (EGR 12) (Figure 8o). The p arms of these two chromosomes are fully heterochromatic in *C. asiatica* (Robinson et al. 2004) as well as in most of the other species (not shown). It is therefore likely that these heterochromatic regions have been lost during the fusion leading to EGR 12.

Distribution of telomeric sequences

As is the case in all other vertebrates, telomeric signals were detected at the ends of all chromosomes in golden moles (Figure 11). This is consistent with suggestions that telomeres are essential for maintaining the integrity and stability of the genome (recently reviewed in Bolzan and Bianchi 2006). In addition to the expected pattern, several studies have shown that interstitial telomeric sequences (ITS) can sometimes be detected at breakpoint sites of ancient fusions (Lee et al. 1993, Lear 2001, Metcalfe et al. 2002, Dobigny et al. 2003 among others), but this observation is by no means universal (Meyne et al. 1990, Garagna et al. 1995, Viera et al. 2004 among others). No telomeric signals were noted at the breakpoint sites of the three fusions involved in the genesis of EGR 12, EGR 4 and COB 4 (Figure 11a, b). This was anticipated in EGR 12 since the heterochromatic arms of each of the chromosomes was lost during the fusion process (see above). However, the absence of telomeric signals at the EGR4 and COB4

breakpoint regions may reflect (1) that these sequences have been completely lost during the fusion events, implying that the breaks occurred in a region proximal to the telomeres, (2) that the telomeric sequences have decayed since the fusion event, or (3) that the amount of telomeric repeat sequence remaining at the fusion site is too small to be detected by FISH.

Although ITSs were not detected at the breakpoints of the three fusions, strong signals were observed in the large G-negative pericentromeric regions of most chromosomes in all *Amblysomus* species/subspecies (Figure 11f, g, h, j) – the only exception being *A. h. meesteri* (Figure 11e). More specifically, pericentric telomeric signals were observed in all *A. h. longiceps* chromosomes as well as in all *A. h. hottentotus* chromosomes except the X. The patterns observed in *A. h. pondoliae* were similar except that the telomeres of the X, 3, 5, and Y showed no hybridization, whereas in *A. robustus* the Y and the acrocentric 9 failed to fluoresce.

As is evident from Figure 7, no significant difference in C-banding patterns was detected between *A. h. meesteri* and the other *Amblysomus* spp. suggesting that the presence/absence of pericentric telomere signal reflects differences in the sequence composition of the pericentromeric heterochromatin, rather than differences in the amounts of this material. As pericentric regions are not involved in fusions in *Amblysomus* species, the presence of telomeric sequences in the pericentric region of *A. h. hottentotus*, *A. h. pondoliae*, *A. h. longiceps* and *A. robustus* does not correlate with the location of ancient breakpoints. Similar patterns have been reported in other taxa (see for example Garagna et al. 1997, Faravelli et al. 1998, Metcalfe et al. 2004) and in these cases, the telomeric motif is thought to be a component of a satellite family constituting these heterochromatic regions, an observation that has been substantiated at the sequence level in unrelated species (Southern 1970, Arnasson and Widegren 1989). Interestingly, since pericentric telomeres were not observed in *A. h. meesteri* or in any

of the other golden mole genera, it is possible to infer that the pericentric telomere motifs were amplified only once in an ancestor common to *A. h. hottentotus*, *A. robustus*, *A. h. longiceps* and *A. h. pondoliae*.

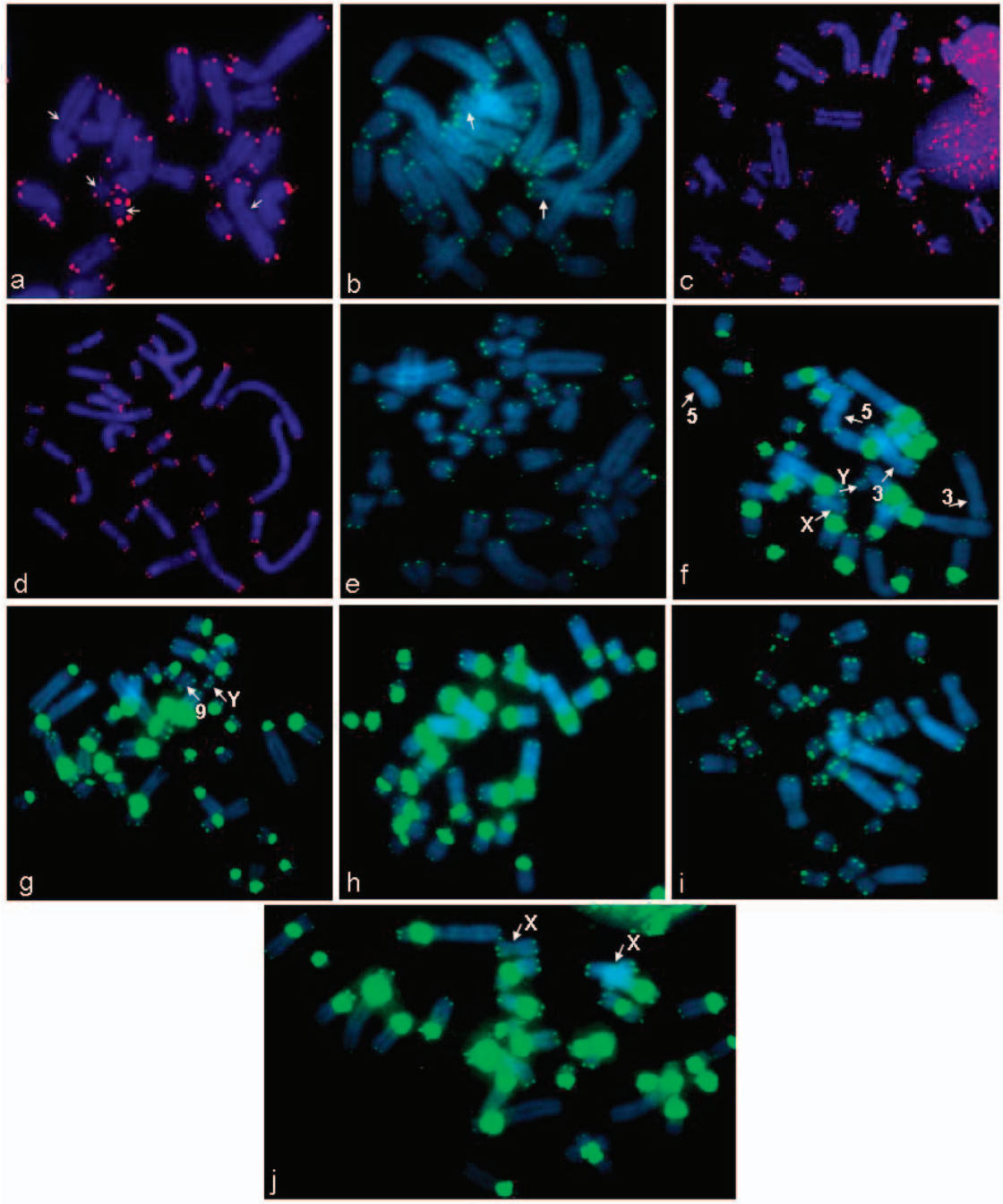


Figure 11. Distribution of telomeric repeats $(TTAGGG)_n$ on metaphase chromosomes of **(a)** *E. granti* **(b)** *C. obtusirostris*, **(c)** *C. trevelyani*, **(d)** *C. zyli*, **(e)** *A. h. meesteri*, **(f)** *A. h. pondoliae* **(g)** *A. robustus*, **(h)** *A. h. longiceps*, **(i)** *C. asiatica*, and **(j)** *A. h. hottentotus.* Note the absence of interstitial telomeric sequences at the breakpoint of the fusion between CAS 13 and 7 in *E. granti* and *C. obtusirostris* and between CAS 11 and 12 in *E. granti* (panels (a) and (b), white arrow) and the absence of telomeric sequences in the pericentromeric region of all chromosomes of *A. h. meesteri* and in some chromosomes of the other *Amblysomus* species. Numbers and letters on panels (f), (g), (j) refer to the chromosome numbers in Figures 4 and 6.

Consequently, in addition to the sharing of an intrachromosomal rearrangements (see above), the presence of telomeric repeat motifs around the centromeres represents yet another character supporting the grouping of these four species/subspecies to the exclusion of *A. h. meesteri* (Figure 9). This indicates that *A. hottentotus* is currently paraphyletic and in order to resolve this, *A. h. meesteri* should be recognized as a full species.

Because centromeric specific satellites are thought to undergo recurrent fixation of new variants and expansions and/or contractions that can increase the probability of a centromere being pulled towards the meiotic pole in oogenesis, they have been viewed as selfish structures which can make use of female meiotic drive to increase their frequency in natural populations (Henikoff et al. 2001, Henikoff and Malik 2002, Malik and Henikoff 2002, see also Pardo Manuel de Villena and Sapienza 2001). In this context it has been shown that centromeric-specific histones have adaptatively evolved to counterbalance the potentially deleterious effects associated with the resulting distortion in centromere segregation (Malik and Henikoff 2001, Talbert et al. 2002, 2004). The two centromeric components (satellite sequences and histones) are consequently believed to have coevolved in a host/parasite-like fashion. An extension of this model is that the independent coevolution of centromeric specific proteins and satellite sequences in two isolated populations of the same species could lead to speciation (Henikoff et al. 2001). Indeed, if the two populations remain isolated for a sufficient period of time, the two centromeric components may become incompatible when present together in hybrids leading to reproductive isolation. Irrespective of whether this process is a cause or consequence of speciation within *Amblysomus*, it can realistically be invoked to explain the differences in the composition of pericentric satellites (i.e., whether telomeric motifs are present or absent) among the different species/subspecies of this genus. There are, regrettably, no data available on *A. h.*

meesteri hybridization and its effects; however, since the composition of pericentric sequences in all or most chromosomes differ among the *Amblysomus* species/subspecies taxa, it is not unlikely that hybrids, should they occur, would be expected to show centromeric histone/centromeric sequence incompatibility. The differences in pericentric sequences between *A. h. meesteri* and other *Amblysomus* species/subspecies therefore reinforces suggestions that *A. h. meesteri* is indeed reproductively isolated from its conspecifics (interestingly, it can unambiguously be distinguished from other *Amblysomus* by the presence of a mid-dorsal reddish black stripe; Bronner 1996, 2000) and that it therefore warrants consideration as a full species.

Should this hold, the effects of centromeric incompatibility discussed above would be anticipated to be less pronounced in hybrids among any two of the four remaining species/subspecies (i.e., *A. h. hottentotus*, *pondoliae*, *longiceps* and *A. robustus*) since the composition of the pericentric repeats was found to differ only in a subset of their chromosomes (at least at the level of detection permissable in our study). However, as pointed out by Henikoff et al. (2001), the effects of centromeric incompatibility on reproductive isolation are expected to be stronger in the heterogametic sex since the centromeres of the sex chromosomes would always be the most dissimilar. Interestingly, in most instances the differences in the pericentric repeat composition among the *Amblysomus* species/subspecies involved the sex chromosomes, and if these differences are sufficiently pronounced, it is possible that other *Amblysomus* subspecies too may warrant specific rank.

Age, ancestral karyotype, and rate of chromosomal evolution of the Chrysochloridae

The molecular dating analysis based on gene fragments NADH2, CO2, 12S rRNA, 16S rRNA, tRNA-Val for *Amblysomus*, *Chrysochloris* and 39 other mammals

(see Material and Methods) resulted in an estimated divergence of 28.5 my (Cred. Int. = 21.5 - 36.5 my) for golden moles (i.e. Chrysochloridae). The second analysis, which was based on gene fragments 12S rRNA, 16S rRNA, tRNA-Val and the 3'UTR of CREM for *Amblysomus*, *Chrysospalax* and 38 other mammals suggested a divergence of 26.9 my (Cred. Int. = 14.7 - 41.8 my). The average differences between these two estimates and that of Springer et al. (2003), which was calculated on all the nodes outside Chrysochloridae, was 4.8 my on the first analysis and 4.4 my on the second analysis. Moreover, most of the divergence times that we obtained within Afrotheria were comparable to those of Springer et al. (2003). For example, we estimate the origin of the Afrosoricida at 67.9 my (Cred. Int = 58.5 - 77.7) *cf.* 66.36 my (Cred. Int. = 59.5 - 72.4 my) in Springer et al. (2003). Thus, although we included far less gene fragments than Springer et al. (2003), our results are reasonably consistent with this study suggesting that the reduction in gene sampling did not lead to significant biases in estimating divergence times. Although both results place the origin of the extant Chrysochloridae in the Oligocene, they differ substantially in their credibility intervals (21.5 - 36.5 my *vs.* 14.7 - 41.8 my). This is to be expected since the second analysis is based on a smaller dataset (2049 bp *vs.* 3366 bp); therefore preference is given to the former when placing the rates of chromosomal change in a temporal context.

The oldest Chrysochloridae fossils date back to the lower Miocene (16-24 my) of Kenya (Butler and Hopwood 1957, Butler 1984) which marginally overlaps with our older molecular estimate (i.e. 28.5 my; Cred. Int. = 21.5 - 36.5 my). However, these fossils strikingly resemble the extant species in overall morphology of the skull suggesting that all the distinctive cranial features of the extant species were already present at the beginning of the Miocene (Butler and Hopwood 1957, Butler 1984). This indicates that the origin of extant species is probably older than that intimated by the fossil dating, which is in keeping with suggestions that although the fossil record

provides a definitive date by which individual clades must have been present, this does not define when they arose (see Smith and Peterson 2002 for an excellent review of the shortcomings of both molecular and paleontological dating).

We have shown that four genera of golden moles (*Chrysochloris asiatica*, *Chrysospalax trevelyani*, *Cryptochloris zyli*, *N. julianae*) included in our investigation have identical karyotypes in terms of diploid number, morphology and banding patterns. Moreover, all rearrangements identified among the other species are autapomorphies. Commonality does not necessarily imply the ancestral condition, but it is noteworthy that at least one species in each of the two genera of Chrysochloridae (*Carpitalpa* and *Chlorotalpa*) not included in our study also has a diploid number of 2n = 30 (Bronner 1995a). It is therefore not unlikely that the ancestral karyotype of the family had 2n = 30 and, given the highly conserved karyotypes within chrysochlorids, that this was very similar to that retained in the extant *Chrysochloris asiatica*, *Chrysospalax trevelyani*, *Cryptochloris zyli*, and *N. julianae*. Taking only the rearrangements of the euchromatic parts of the genome into consideration, this suggests an average rate of 0.7 rearrangements per 10 my (Cred. Int. = 0.54 - 0.93) for the branches leading to *E. granti* (two fusions in 28.5 my) and to *A. h. hottentotus*, *A. h. longiceps* and *A. h. pondoliae* (two intrachromosomal rearrangements). An average rate of 0.35 rearrangements per 10 my (Cred. Int. = 0.27 – 0.46) can also be calculated for the branches leading to *C. obtusirostris* (one fusion in 28.5 my) and *A. h. meesteri* (one intrachromosomal rearrangement in 28.5 my). These rates are clearly lower than the "default rate" of mammalian chromosomal evolution which has been estimated at one change per 10 million years (O'Brien and Stanyon 1999, O'Brien et al. 1999, Murphy et al. 2001b, Weinberg 2004). Moreover, they are at least twice as low as the average eutherian rate of ~ 1.9 chromosomal rearrangements /10 my (Frönicke 2005). In addition to these low rates, the chromosomal stasis observed in *Chrysochloris*, *Cryptochloris*, *Chrysospalax*

and *Neamblysomus* during the 28.5 my that separate them from their common ancestor clearly place the Chrysochloridae among the most karyotypically conserved families of mammals.

Given this extreme karyotypic conservatism, the three fissions observed in *A. robustus* (see above) are noteworthy for two reasons. First, the three fissions occurred along the *A. robustus* branch after its divergence from the ancestor of a clade that unites *A. h. hottentotus*, *longiceps* and *pondoliae*. Although the age of this clade cannot be calculated on present data, it is certainly less than the ~28.5 my estimated for the origin of Chrysochloridae, implying that the rate of chromosomal change significantly increased in the *A. robustus* lineage. Secondly, only one type of interchromosomal rearrangement (a fusion) was detected among all other golden mole species studied indicating that other rearrangements such as fissions are very unlikely, or have a very low probability of fixation in Chrysochloridae. One of the possible factors that might have facilitated the accelerated fixation of fissions in *A. robustus* is the presence of telomeric motifs in the pericentric area of most of its chromosomes. Following a fission in this region, the location of these motifs at the ends of the resultant two independent chromosomes may have facilitated the genesis and activation of neotelomeres, thereby increasing the probability for the two chromosomes to be viable (see Zhdanova et al. 2005). This hypothesis might apply to ARO 8, 9, 15 and 17 (Figure 4). In this context, the heterochromatic short arms of ARO 8 and that of the submetacentric ARO 9 would have been amplified subsequent to their fissioning in *A. robustus*. The recruitment of pericentric telomeric motifs to generate neotelomeres after fission is, however, less evident in ARO 2 and 16 since the fission breakpoint is not situated in the pericentric region but rather in the p arm of CAS 2.

In conclusion, the pattern of chromosomal evolution evidenced by comparative molecular and conventional cytogenetic studies of seven of the nine golden mole genera

is one of constrained change. While it seems unlikely that the inclusion of the outstanding C*hlorotalpa* and *Carpitalpa* will significantly alter this (given that two of the three species included in these genera have unbanded karyotypes whose morphology is very similar to that of *C. asiatica*, see Bronner 1995a), we are nonetheless of the opinion that intraspecific cytogenetic investigations should continue to be encouraged. The detection of cytogenetic evidence supporting the elevation of *A. h. meesteri* to specific status in the present study underscores the utility of this approach in the search for cryptic species among these afrotherians, many of which are highly endangered.

CHAPTER III

CHROMOSOMAL EVOLUTION IN TENRECS

INTRODUCTION

Biology, taxonomy, and geographical distribution

The family Tenrecidae comprises 34 species that are divided into four subfamilies and 10 genera (Bronner and Jenkins 2005, Goodman et al. 2006). One subfamily, Potamogalinae, includes the only two genera occurring in Africa: *Potamogale* (one species: *P. velox*) that is found throughout a large part of central Africa, and *Micropotamogale* (two species), which occurs in west (*M. lamottei*) and central (*M. ruwenzori*) Africa (Kingdon 1997) (Figure 12). These two genera are otter-like in appearance and have developed a semi- or completely aquatic carnivorous way of life, foraging mainly by night in a rather large variety of stream-types (Nowak 1999). All their relatives are endemic to Madagascar (Figure 12). Their colonization of the ecological niches usually occupied by the eulipotyphlans (true shrews, hedgehogs, moles and solenodon) elsewhere, has been accompanied by an impressive range of morphological and ecological adaptations (Olson and Goodman 2003). Among them the subfamily Geogalinae is monotypic and includes only *Geogale aurita* which occurs in the western and southern dry forests of the island. It is mouse-like in appearance and is unique in that females have a post-partum oestrous (Stephenson 2003a). The Tenrecinae comprise four genera of spiny tenrecs, of which one, *Tenrec ecaudatus*, is the largest of the family with males weighing up to 2 kg and measuring up to 40 cm in length (Nicoll 2003). The second spiny-tenrec, *Hemicentetes,* includes two species, *H. semispinosus* which lives in the lowlands of the eastern rainforest and *H. nigriceps* which is restricted to a smaller, more elevated area of the central plateau (Stephenson 2003b). These two "streaked tenrecs" frequently live in family groups of approximately 20 individuals

(covering three related generations) that communicate by mean of non-vocal sounds produced by specialized quills situated on the middle of the back behind the neck, the so-called stridulating organ. In addition, these animals can produce a tongue click that is probably a form of echolocation that is used in prey localization (Eisenberg and Gould 1970).

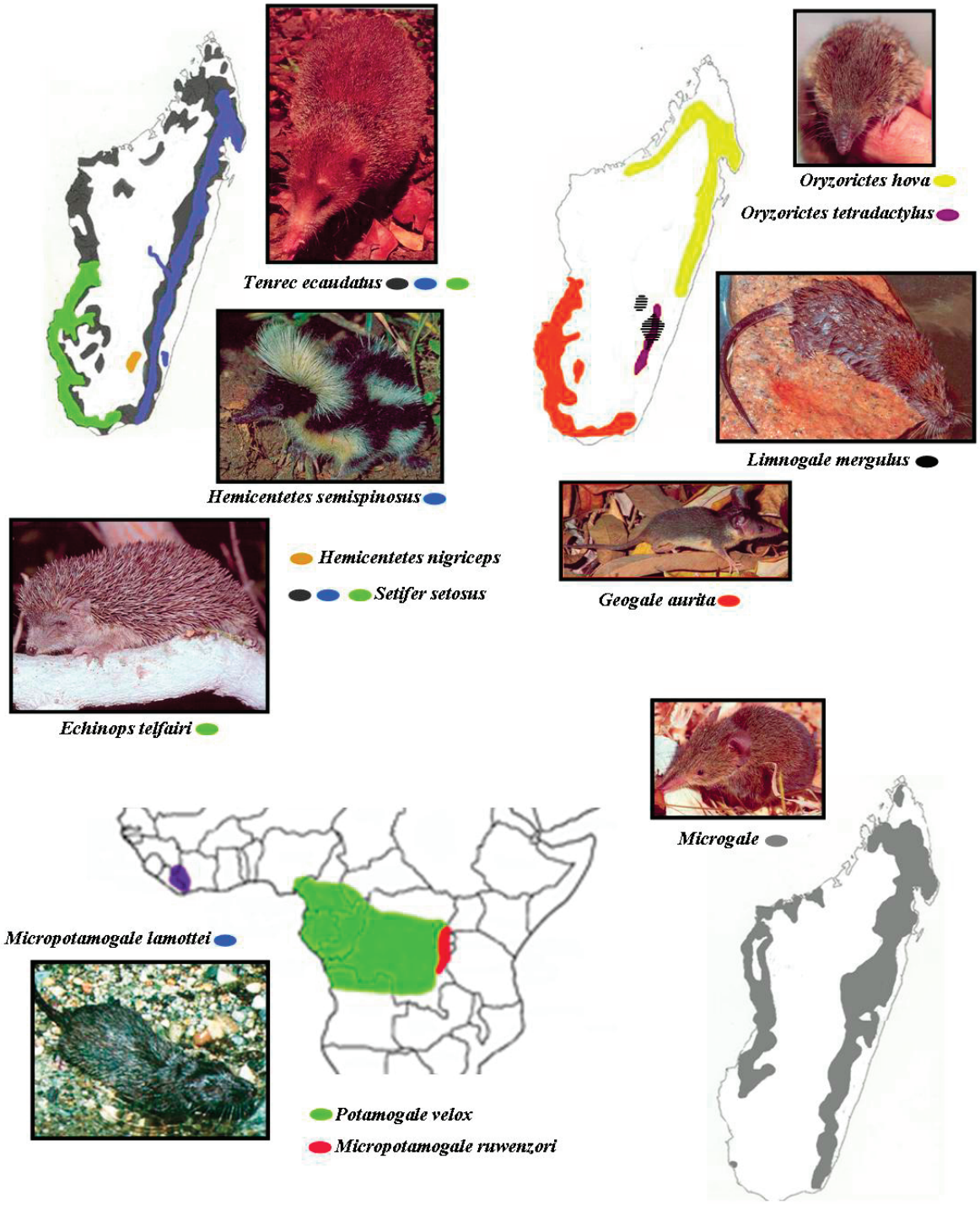


Figure 12. Geographic distribution of Tenrecidae on Madagascar and continental Africa (redrawn from Garbutt 1999, Kingdon 1997).

The two remaining genera of the subfamily greatly resemble the eulipotyphlans hedgehogs with their rounded, short-legged bodies and spiny fur. *Setifer setosus* is found throughout the island and *Echinops telfairi* is found only in the western and southwestern dry forest (Garbutt 1999). The subfamily Oryzorictinae includes *Limnogale mergulus*, sole mammalian occupant of an aquatic niche on Madagascar. *Limnogale* occurs in the vicinity of the faster flowing streams of the central highlands (Benstead and Olson 2003). Within this subfamily, two species of *Oryzorictes*, *O. hova* and *O. tetradactylus* are adapted to a fossorial lifestyle, and strikingly resemble true moles of the family Talpidae. *Oryzorictes hova* is broadly distributed in the humid forest zone of the island and in marshes of the bottom land, while *O. tetradactylus* is thought to be restricted to montane areas of the central eastern portion of the central highlands (Goodman 2003). Finally, the subfamily Oryzorictinae includes *Microgale*, the most speciose mammalian genus on Madagascar with no less than 21 currently recognized species (Goodman et al. 2006). Most of these small, shrew-like tenrecs (between 3 and 40 g) are found in the eastern humid forests of the island where they generally have a broad distribution, with many species occurring sympatrically (Jenkins 2003).

The taxonomy of *Microgale* has undergone extensive revision since its original description by Thomas (1882). For example, MacPhee (1987) retained only 10 of the 22 species described during the preceding century. During the past 20 years, however, extensive field surveys coupled with comprehensive morphometric and/or molecular investigations resulted in a considerable refinement of their taxonomy and patterns of distribution, with 11 new or resurrected species recognised in newer treatments (e.g., Jenkins 1993, Jenkins et al. 1997, Jenkins and Goodman 1999, Goodman and Soarimalala 2004, Olson et al. 2004, Goodman et al. 2006).

Phylogenetic relathionships

Recent phylogenies based on molecular and morphological characters are in good agreement with the classification of Bronner and Jenkins (2005) (Figure 13). They strongly support the monophyly of the Malagasy tenrecs and their further division in two main clades, the Tenrecinae and the Oryzorictinae (Poux et al. 2005, Asher and Hofreiter). The placement of *Geogale* is however uncertain, being either sister to all Malagasy tenrecs (Olson and Goodman 2003), consistent with the recognition of the subfamily Geogalinae, or occupying an unresolved position within Oryzorictinae (Asher and Hofreiter 2006) which implies paraphyly of the Oryzorictinae. Within Oryzorictinae, Olson and Goodman (2003) found *Limnogale* nested within *Microgale*, a position supported by several non-ambiguous molecular characters. Moreover, although subsequent studies included only one species of *Microgale* and could therefore not test the monophyly of this genus, strong support was found for a *Limnogale* + *Microgale* clade (Asher and Hofreiter 2006, Poux et al. 2005). Although intergeneric relationships of the Tenrecidae are rather well resolved, there is no well-supported phylogenetic hypothesis detailing interspecific relationships within *Microgale*. Olson and Goodman's (2003) cladistic analysis of the Tenrecidae is the most comprehensive in terms of taxonomic sampling, including as it does a large number of species of shrew tenrecs. Importantly, however, relationships within *Microgale* were not discussed in this study. Finally, although two recent studies have utilised both molecular and morphometric characters to define species limits in selected shrew tenrec taxa (Olson et al. 2004, Goodman et al. 2006), neither was intended to produce a comprehensive phylogeny of the genus.

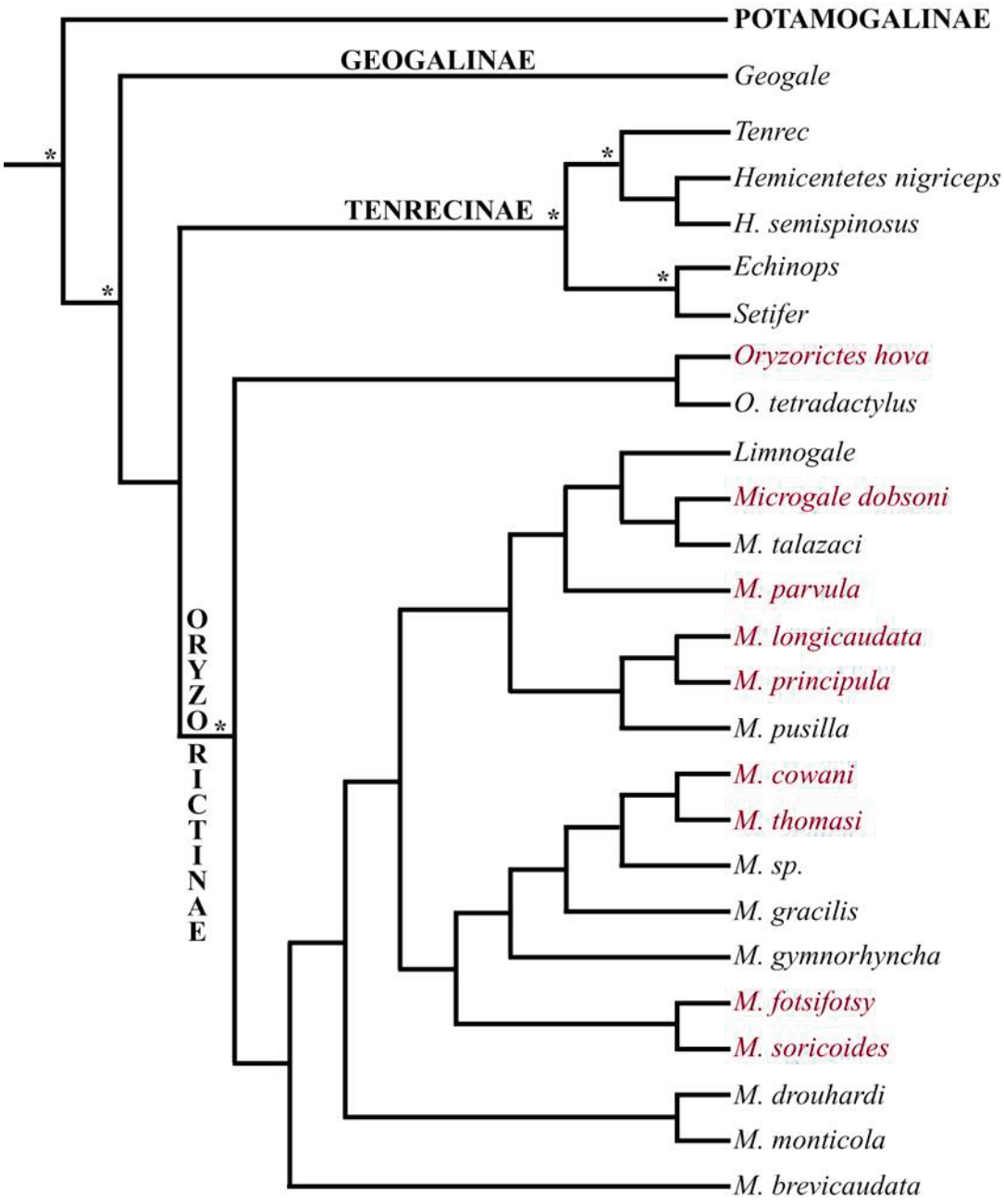


Figure 13. Single most parsimonious tree of the family Tenrecidae recovered from an analysis of the mitochondrial genes 12S rRNA, tRNA-Valine, and ND2, and exon 28 of the nuclear von Willebrand Factor gene (taken from Olson and Goodman 2003). Asterisks indicate nodes that are consistent with the study of Poux et al. (2005) and Asher and Hofreiter (2006) which included only a single representative of each genus. Taxa included in this study are shown in red. In addition, the present investigation also includes *M. majori* and *M. taiva*.

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Paleontology and biogeography

The question of which biogeographical scenario best explains the actual partitioning of tenrecs on the African continent and Madagascar is part of a wider issue dealing with the origin and diversification of the Malagasy biota. Given that the island has a very ancient origin, and was once connected to all the other continents (de Witt 2003), a fundamental question concerns which of the two biogeographical models – vicariance or dispersal – best explains the composition of the modern fauna. Biophysical and geological evidence suggest that Madagascar broke-off from its African connections ~160 my ago and subsequently its direct link with Antarctica 20 my later. It finally severed its ties with India ~50 my ago (de Witt 2003). In a recent review, Yoder and Nowak (2006) showed that most of the modern Malagasy biota originated during the Cenozoic and have an African sister group. They therefore concluded that dispersion from Africa, rather than vicariance or dispersion from another continent, was the predominant mode of colonization of the island. Malagasy tenrecs follow this rule. Their sister group is African (i.e. the Potamogalinae and other Afrotheria), and their origin is estimated to have taken place 25.3 myr ago (Cred. Int. = 31.8 - 19.7) (Poux et al. 2005). Given this time estimate, as well as the time at which the ancestor of Malagasy tenrecs split from their closest sister group, the African tenrecs, Poux et al. (2005) calculated that tenrecs dispersed from Africa to Madagascar between 42 and 25 my ago. However, just how the ancestors of the Malagasy tenrecs crossed the Mozambique Channel is a matter of controversy. McCall (1997) suggested that a land bridge along the Davie Fracture Zone of the Mozambique Channel could have connected Africa to Madagascar between the mid-Eocene and the early Miocene. If the four endemic Malagasy clades of terrestrial mammals (tenrecs, rodents, carnivorans, primates) were to disperse through this, one would expect that they would all have colonized the island during the same period, i.e., when the land bridge was present

(mid-Eocene – early Miocene). In contrast, under the hypothesis of sweepstake dispersal a more random pattern of colonization is expected as these could have occurred at any time subsequent to the divergence of the Malagasy clade from its African sister group. Poux et al. (2005) tested this hypothesis and found that the colonization times of the four Malagasy clades of terrestrial mammals overlap only marginally, thereby suggesting that the ancestors of these taxa most probably dispersed from Africa to Madagascar by rafting on flotsam. The results of this study, coupled to more recent geological evidence (Rogers et al. 2000), challenge the hypothesis of the presence of a land bridge that provided a dispersal route from Africa as suggested by McCall (1997). However, the results of Poux et al. (2005) are also contradictory with Stankiewicz et al. (2006) who recently showed that the directions of the surface currents and prevailing winds were probably not favourable for rafting from Africa to Madagascar during the Cenozoic, and that other dispersal models including crossing by McCall's (1997) hypothesized land bridge should be sought.

Tenrec fossil material has been described from the Miocene of East Africa (*Protenrec*, *Erythrozootes*, *Parageogale*) (Butler and Hopwood 1957, Butler 1969, 1984) and of Namibia (Mein and Pickford 2003). Parsimony analyses of morphological characters, including the three east African fossils, support a close relationship between these taxa and *Geogale* rendering Malagasy tenrecs paraphyletic (Asher and Hofreiter 2006). Although this topology similarly implies only one dispersal event from Africa to Madagascar, it also requires one reversal from Madagascar back to Africa. It is worth noting that the suggestion of a single Africa to Madagascar event could not be statistically rejected in this study. Interestingly, however, according to Stankiewicz et al. (2006) surface currents and winds were much more favourable for movement from Madagascar to Africa. Clearly, the discovery of well-preserved fossils on both the

African continent and Madagascar would greatly help in clarifying this fascinating issue.

Cytogenetic data

Diploid numbers are known for all genera with the exception of *Potamogale* and *Limnogale*. They vary from 2n = 14 (*G. aurita*, Olson et al. unpublished) to 2n = 54 (*M. cowani*, Borgaonkar and Gould 1969). Unbanded karyotypes are available for *T. ecaudatus* (2n = 38), *M. dobsoni*, *M. talazaci* (2n = 30) (Borgaonkar and Gould 1968), (2n = 54), *E. telfairi* (2n = 42) and *H. nigriceps* (2n = 38) (Hsu and Bernischke 1974).

Context

The present investigation represents the first comprehensive cytogenetic comparison of the family Tenrecidae. New standard G-banded karyotypes are reported for 10 species of the genus *Microgale* and for *O. hova*. A comprehensive half-karyotype comparison between the 10 *Microgale* species and *O. hova* was established based on a combination of G-banding patterns and chromosome painting. The observed rearrangements are placed in a cladistic framework and these are examined with respect to two hypotheses of chromosomal evolution - one involving Whole Arm Reciprocal Translocations (WARTs), and the other involving only Robertsonian translocations. We place our findings in a temporal framework by expanding the Poux et al. (2005) molecular clock analysis and show that extreme rate differences exist in the chromosomal evolution of the *Microgale* species. Using these data we critically examine a role for chromosomal rearrangements in tenrec speciation.

MATERIAL AND METHODS

Tissues samples and cytogenetics

Tissue samples were collected during three inventory surveys of study sites situated in the rainforests of the central highlands of Madagascar (Table 3). Establishment of fibroblast cell lines, chromosome harvest and preparation, as well as G- and C-banding, followed the same protocols as for the golden moles (see Chapter II) except that the concentration of the trypsin was decreased to 0.0025% for G-banding. Chromosomes were ordered in decreasing size and centromere position, or according to the *M. taiva* format when the chromosome complement was conserved. FISH followed the protocol described in Chapter II with the exception that chromosome preparations were denatured for 10 s in 70 % formamid/0.6 % SSC solution at 65°C rather than 30-45 s at the 70 °C.

Table 3. List of species included in this study and associated voucher numbers of specimens. Site 1: surveyed in November 2003, Province de Fianarantsoa, Parc National de Midongy-Sud, NE slope of Mt. Papango, 3.5 km SW Befotaka, 23º50.3'S, 46º 57.5'E, alt. 1250. Site 2: surveyed in January 2006, Province d'Antananarivo, Fivondronana d'Anjozorobe, Forêt d'Iaban'Ikoto, 5.5 km E Alakamisy, 18º31.3'S, 47º58.4'E, alt. 1280 m. Site 3: surveyed in January 2007, Province d'Antananarivo, Réserve Spéciale d'Ambohitantely, Jardin Botanique, 18º10.3'S, 47º16.9'E, alt. 1450 m. The locations of Anjozorobe and Ambohitantely are illustrated in Olson et al. (2004). All specimens are housed in the Field Museum of Natural History (FMNH).

Species	Site #	Voucher number
Microgale cowani	3	FMNH 194138
M. dobsoni	3	FMNH 194140
M. fotsifotsy	2	FMNH 188723
M. longicaudata	3	FMNH 194143
M. majori	2	FMNH 188726
M. parvula	2	FMNH 188729
M. principula	3	FMNH 194146
M. soricoides	2	FMNH 188732
M. thomasi	2	FMNH 188744
Oryzorictes hova	3	FMNH 194150
M. taiva	1	FMNH 178756

Parsimony analysis

Parsimony analyses of the chromosomal rearrangements characterising the interspecific relationships within *Microgale* were conducted by scoring chromosomal changes as characters and their presence/absence as the character state (Dobigny et al. 2004). Ancestral karyotypes for *Microgale*, *Oryzorictes* and the Oryzorictinae could be inferred *a priori* (see Results and Discussion). These ancestral karyotypes were used to polarize the characters. Consistent differences in G-banding patterns were observed in two instances that probably reflect complex intrachromosomal rearrangements. We could not assess the precise nature of these rearrangements but included them in the analyses since they result in distinct, easily identifiable G-banding patterns, and could therefore potentially be of interest to future studies that include other species of *Microgale*. These rearrangements were coded as "presence/absence of an undetermined intra-chromosomal change" (Table 4). Additionally, patterns corresponding to what would be anticipated following a Whole Arm Reciprocal Translocation (WART) were observed in some instances (Winking 1986, Searle et al. 1990). This type of rearrangement necessitates an exchange between chromosomal arms of two metacentrics, between one metacentric and one acrocentric, or between two metacentrics and one acrocentric chromosome (respectively type a, b and c WART in Hauffe and Pialek 1997). The effect of these rearrangements on fitness is thought to vary depending on the type of WART involved in the rearrangement, with type c and b likely to be more detrimental than type a (Searle 1993, Hauffe and Pialek 1997). WARTs are considered rare in mammals. They are thought to result in complex meiotic pairing configurations (such as chromosomal rings or chains) when in the heterozygous condition, but detailed information on the expected underdominance associated with these types of rearrangements is scarce.

When there is no additional information other than simply the presence of monobrachial homology (as it is the case in our study), it is impossible to distinguish between a WART on one hand, and a series of simple Robertsonian (Rb) translocations on the other. The same pattern is expected after a WART between two metacentrics, and after two fissions of these metacentrics followed by two fusions of the resultant four acrocentrics. The fission/fusion hypothesis generally implies a greater number of steps and thus seems less parsimonious (Dobigny et al. 2004). However, since it has been shown that Rb translocations have a minimal impact on the fitness (for example in the house mouse, Nachman and Searle 1995), this class of rearrangement could, in spite of the greater number of steps, be considered more likely than WARTs.

We have therefore erred on the side of caution and for this reason two character matrices were constructed. In the first, all interchromosomal rearrangements were coded as fissions or fusions; in this case, WARTs, if present, were viewed to have resulted from two fissions followed by two fusions. In the second matrix, WARTs were coded as such (i.e., one step) wherever possible. The results obtained under these two hypotheses of chromosomal evolution are critically discussed and compared to other studies in order to determine whether one hypothesis received greater support than the other in our analyses. The two matrices are provided in Table 4. The most parsimonious tree inferred from each matrix was retrieved using an exhaustive search in PAUP* 4.0b10 (Swofford 2002). Bootstrap analyses were performed using 1000 replicates of the original matrices.

RESULTS AND DISCUSSION

G-banded karyotypes of 11 species of the Oryzorictinae are presented in Figure 14. They represent the first banded karyotypes published for the Tenrecidae. Diploid numbers vary from 30 to 56 with five species characterised by 2n = 32 (*M. fotsifotsy*, *M. parvula*, *M. soricoides*, *M. taiva* and *O. hova*), four with 2n = 30 (*M. dobsoni*, *M.*

longicaudata, *M. majori* and *M. principula*), one with 2n = 38 (*M. cowani*), and one having 2n = 56 chromosomes (*M. thomasi*). Although an early report by Borgaonkar and Gould (1968) confirms the 2n=30 recorded by us for *M. dobsoni*, in a subsequent paper these authors document a 2n=54 for *M. cowani* (Borgaonkar and Gould 1969), a diploid number that differs markedly from the 2n=38 observed in the present study. The ambiguity is compounded by the fact that no voucher specimens were collected or reported by these authors. In many ways, *M. cowani* exemplifies the complicated taxonomic history of shrew tenrecs. MacPhee (1987) synonymized five nominal species and one subspecies with *M. cowani*, two of which (*M. taiva* and *M. drouhardi*) have since been resurrected (see Jenkins 2003). Given the absence of a preserved voucher, the identification of the specimen karyotyped by Borgaonkar and Gould (1969) cannot be confirmed, and the notable difference in 2n between their specimen and FMNH 194138 (present study) will remain a mystery.

Although diploid numbers most commonly varied between 2n=30 and 2n=32 in the species examined, it was nonetheless often difficult to establish chromosomal homologies among them and the more rearranged (2n=38 and 2n=56) karyotypes using only the G-banding patterns. Consequently chromosome painting using the flow-sorted chromosomes of *M. taiva* as painting probes was implemented to clarify homologies, and to identify complex rearrangements among species. Figure 15 shows the flow-karyotype of a male *M. taiva* specimen (MTA). The 30 chromosomes were resolved into 12 peaks. Eight peaks each contained a single chromosome pair (MTA 1-3, 4, 7, 13-15) and four peaks each contained a mix of two different chromosomes (MTA 9+8, 5+6, 11+12, X+10). The complete resolution of all 15-chromosome pairs was thus not possible, but this suite of painting probes in conjunction with the G-bands was sufficient to confidently resolve all chromosomal homologies among the tenrecids examined herein.

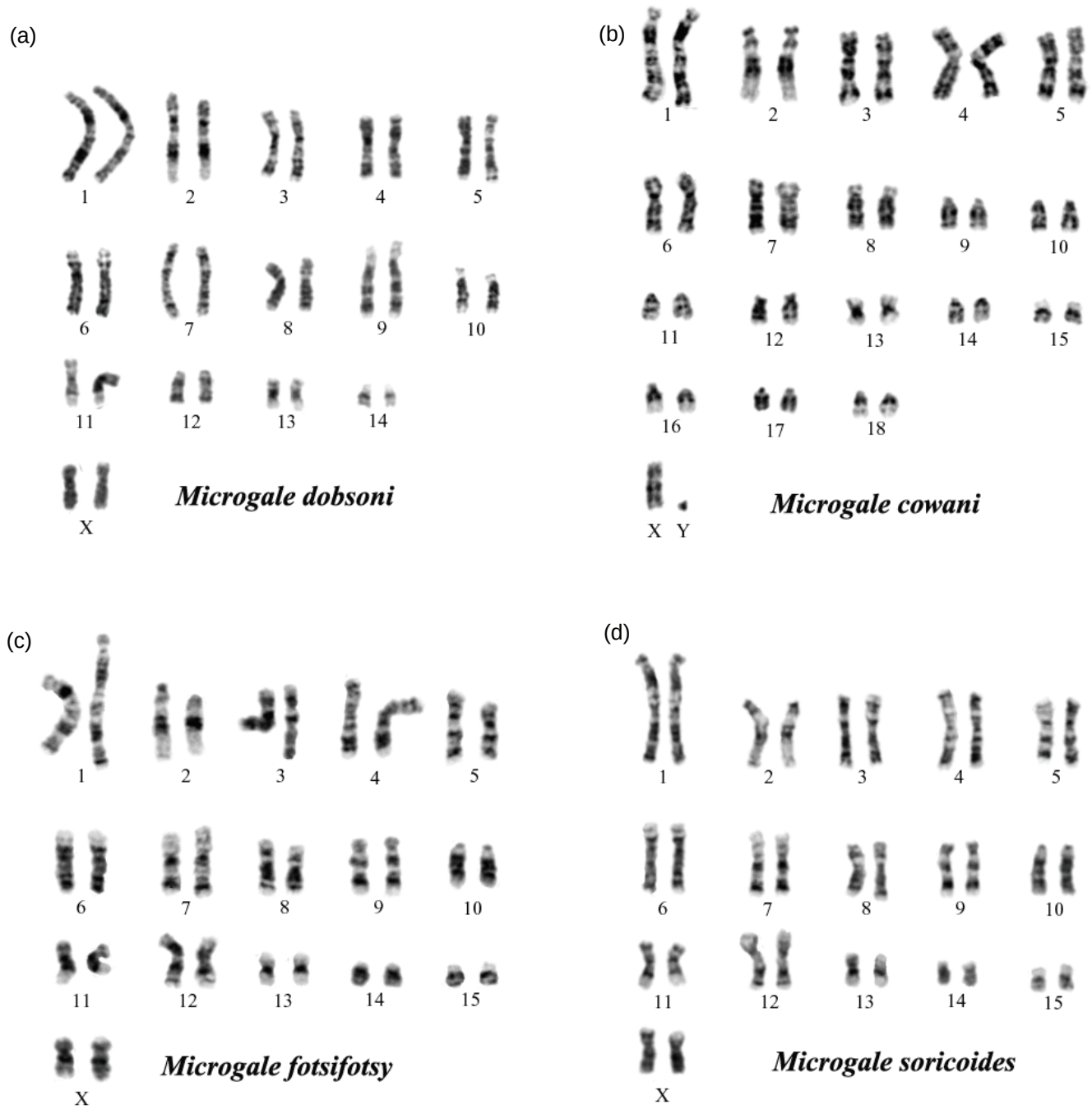
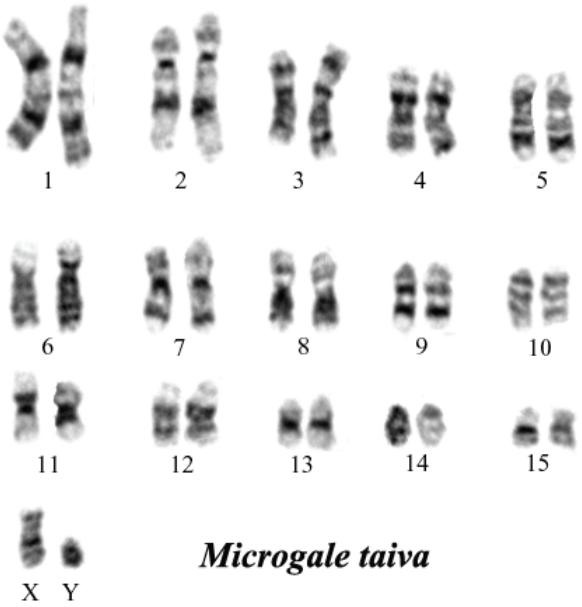


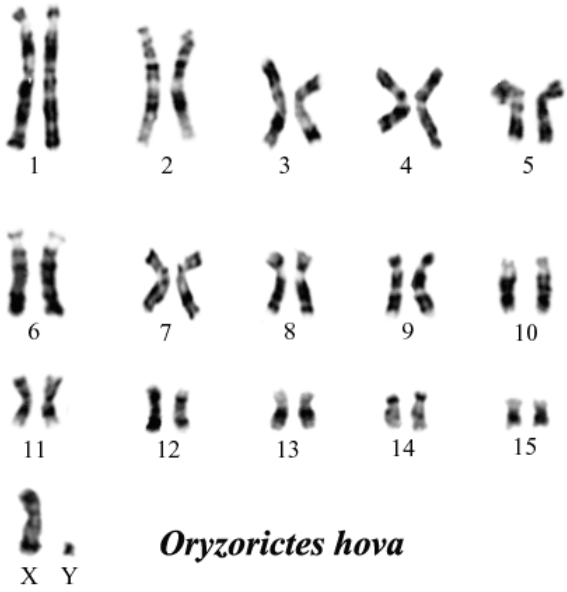
Figure 14. G-banded karyotypes of the 11 species of the Oryzorictinae included in this study: **(a)** male *M. dobsoni* (2n = 30; FMNH 194140), **(b)** male *M. cowani* (2n = 38; FMNH 194138), **(c)** female *M. fotsifotsy* (2n = 32; FMNH 188723), **(d)** female *M. soricoides* (2n = 32; FMNH 188732), **(e)** male *M. taiva* (FMNH 178756), **(f)** male *O. hova* (FMNH 194150), **(g)** female *M. thomasi* (FMNH 188744), **(h)** male *M. parvula* (FMNH 188729), **(i)** male *M. longicaudata* (FMNH 194143), **(j)** female *M. principula* (FMNH 194146), **(k)** female *M. majori* (FMNH 188726).

Figure 14 (continued).

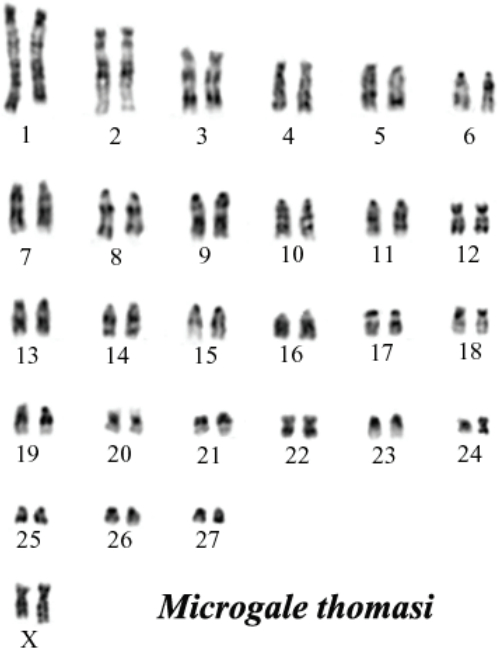
(e)



(f)



(g)



(h)

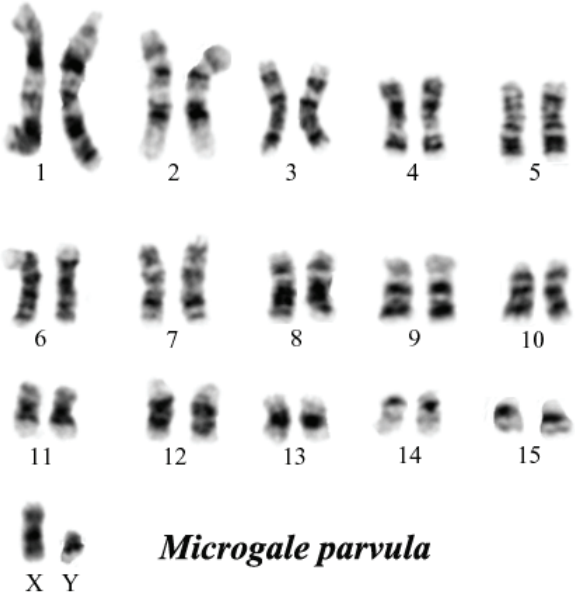
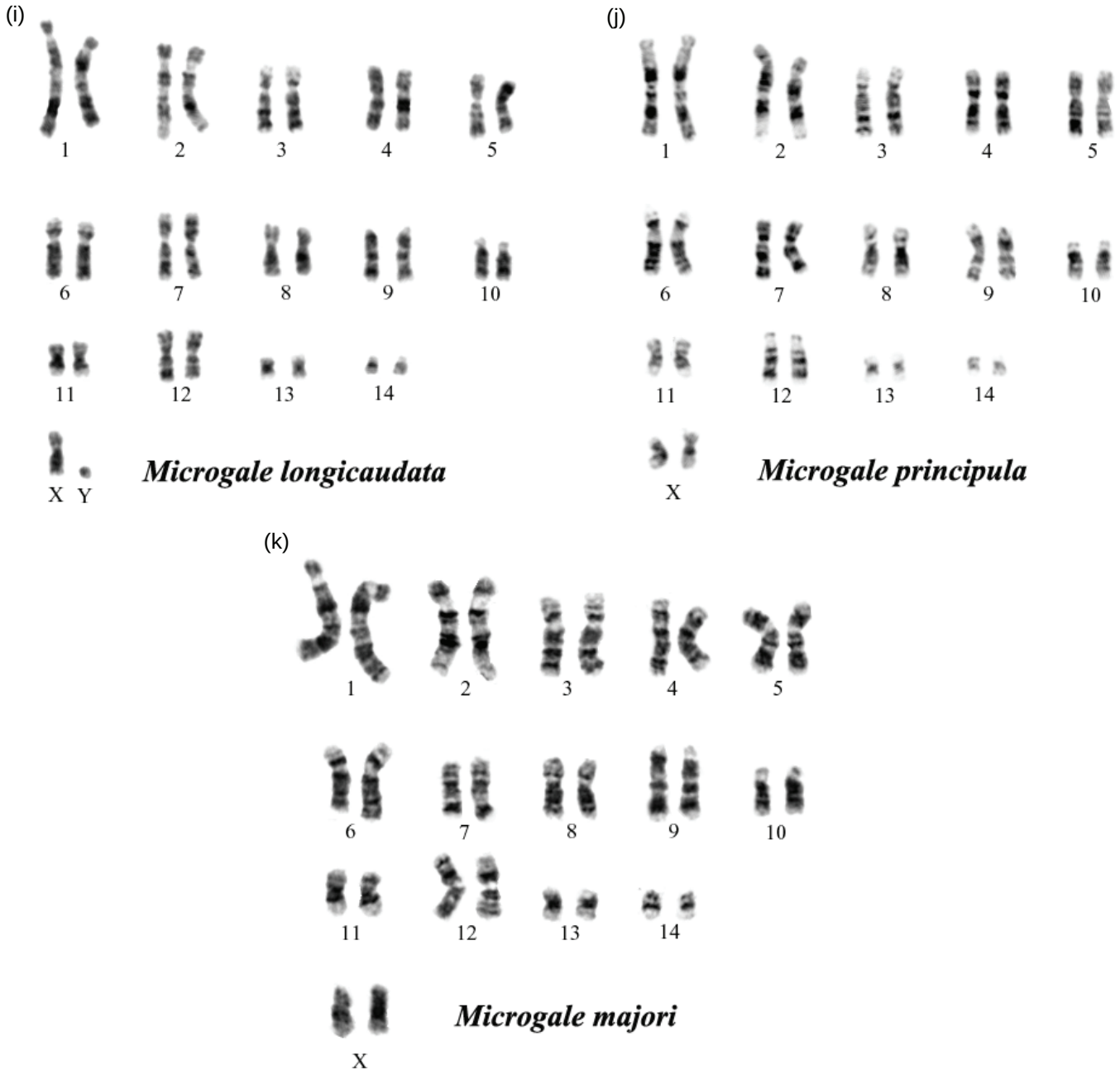


Figure 14 (continued).



Ancestral karyotypes of *Microgale, Oryzorictes* and the Oryzorictinae

Chromosomal homologies between the 10 *Microgale* species and *O. hova* (a representative of the closely-related genus *Oryzorictes*) are illustrated in Figure 16. All the homologies are supported by chromosome painting data, several examples of which are presented in Figure 17. A striking result to emerge from these comparisons is that not a single interchromosomal rearrangement was detected between *M. taiva, M. parvula* and *O. hova* (see Figure 16 and 17a-e) underscoring their karyotypic conservatism since common ancestry. Moreover, the G-banding patterns are rather well conserved suggesting little internal rearrangement within chromosomes. These data suggest therefore, that the common ancestor of *Oryzorictes* and *Microgale* had a karyotype that was virtually identical to that observed in these three extant species, both with respect to diploid number (2n = 32) and G-banding pattern.

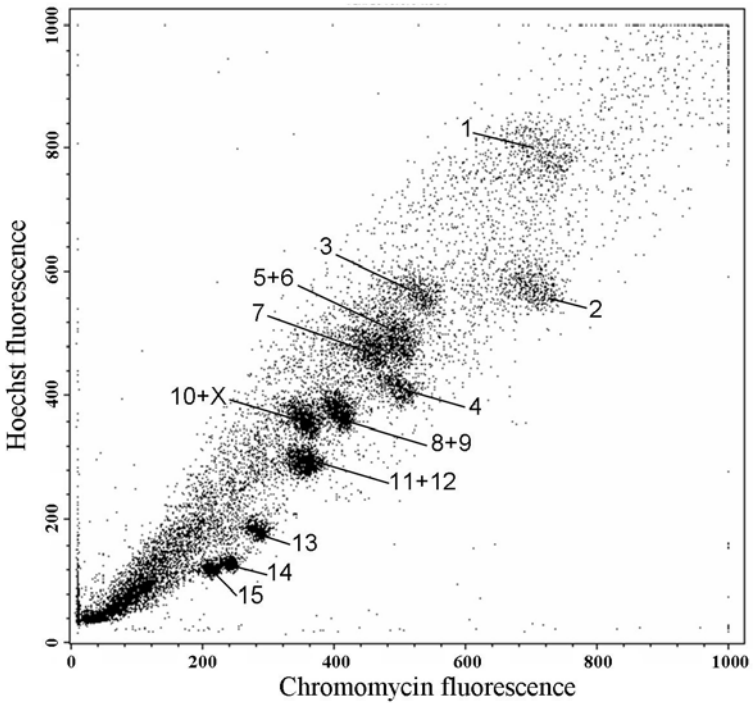


Figure 15. Flow-sorted karyotype of *M. taiva* FMNH 178756 (MTA, 2n = 32, XY) showing the correspondence between the peaks and MTA chromosomes (see text for details).

Interestingly, the ancestral karyotypes of the two genera *Oryzorictes* and *Microgale* are also likely to reflect the ancestral karyotype of the subfamily Oryzorictinae because the only other genus of this subfamily, the monotypic *Limnogale mergulus*, is nested within *Microgale* (Olson and Goodman 2003). It follows therefore, that all rearrangements detected within *Microgale* can consequently be polarized using these inferred ancestral karyotypes as the outgroup. It is not possible to undertake a detailed comparison of the karyotypes described here with those of other tenrecids since only limited data are available for these taxa (i.e., unbanded karyotypes or only the diploid numbers). Interestingly, however, it is possible to infer that the oryzorictine ancestral karyotype is not found outside Oryzorictinae since all non-oryzorictines tenrecids have diploid numbers that differ from the 2n=32 of the ancestral oryzorictine (Borgaonkar and Gould 1965, Borgaonkar 1967, Borgaonkar and Gould 1968, Bernischke 1969, Borgaonkar and Gould 1969).

WARTs vs. fissions/fusions

Chromosomal changes detected in 12 of the 15 *M. taiva* (MTA, 2n = 30) chromosomes (Figure 16) were polarized as detailed above and coded as either present or absent in order to infer interspecific relationships within the genus *Microgale* (Table 4). Irrespective of whether WARTs were included to explain differences in chromosomal states between the species, or excluded in favour of the alternative hypothesis of Robertsonian (Rb) translocations, the same single most parsimonious tree was obtained in both instances (Figure 18). The length of the "WART tree" was three steps shorter than that of the "Rb tree" (22 *cf.* 25 steps). Both matrices were homoplasy free as inferred by the high consistency indexes (CI = 1 for all characters and both matrices), but bootstrap values increased when only Rb translocations were considered.

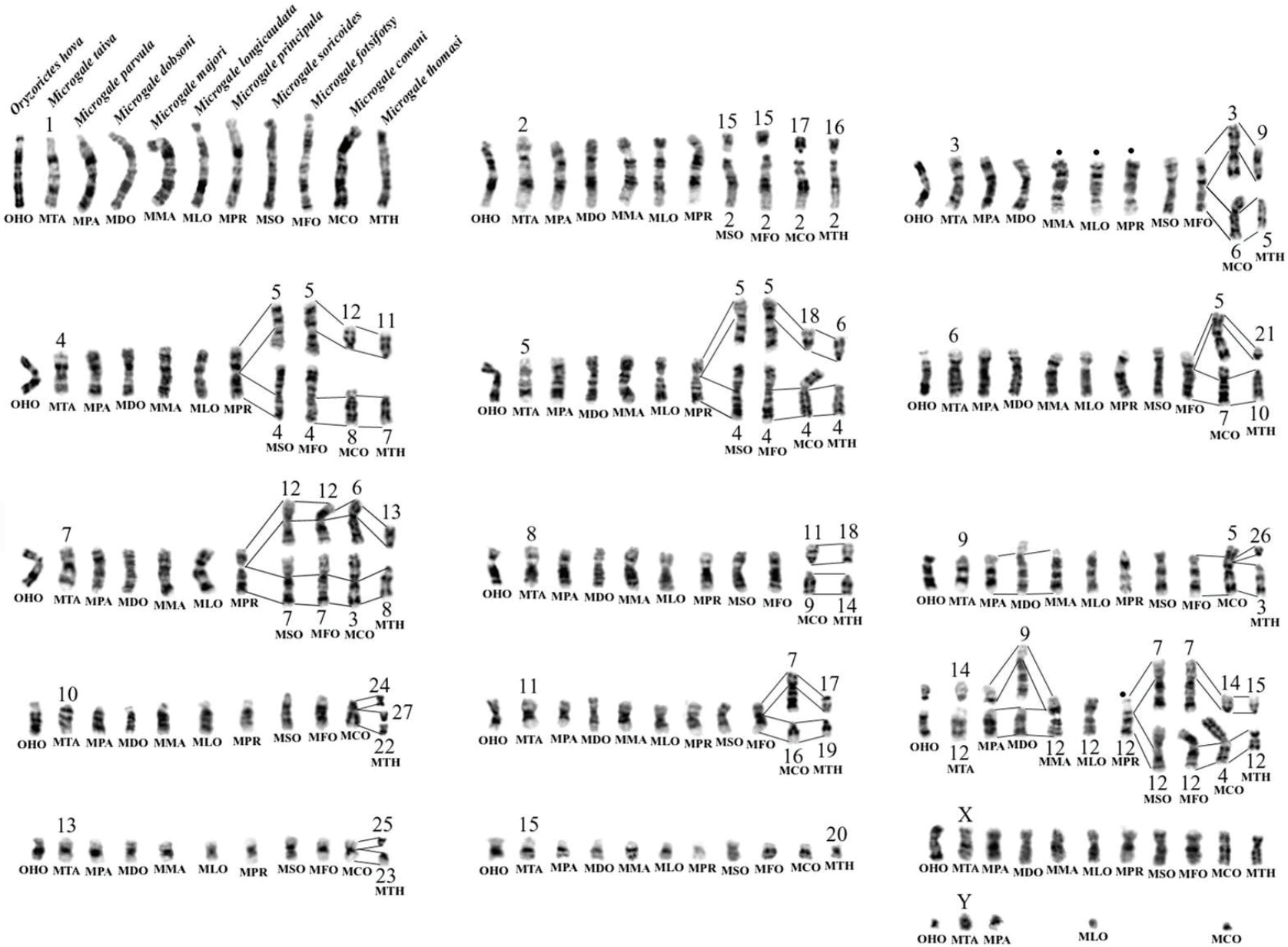


Figure 16. G-banded half-karyotype comparison between 11 species of the Oryzorictinae showing the genome-wide chromosomal correspondence defined by painting and banding homologies. Closed circles indicate chromosomes that have undergone intrachromosomal rearrangements. Chromosome numbers are indicated for *M. taiva* and for the rearranged chromosomes of the other species in order to facilitate the correspondence with the diploid karyotypes (Figure 14).

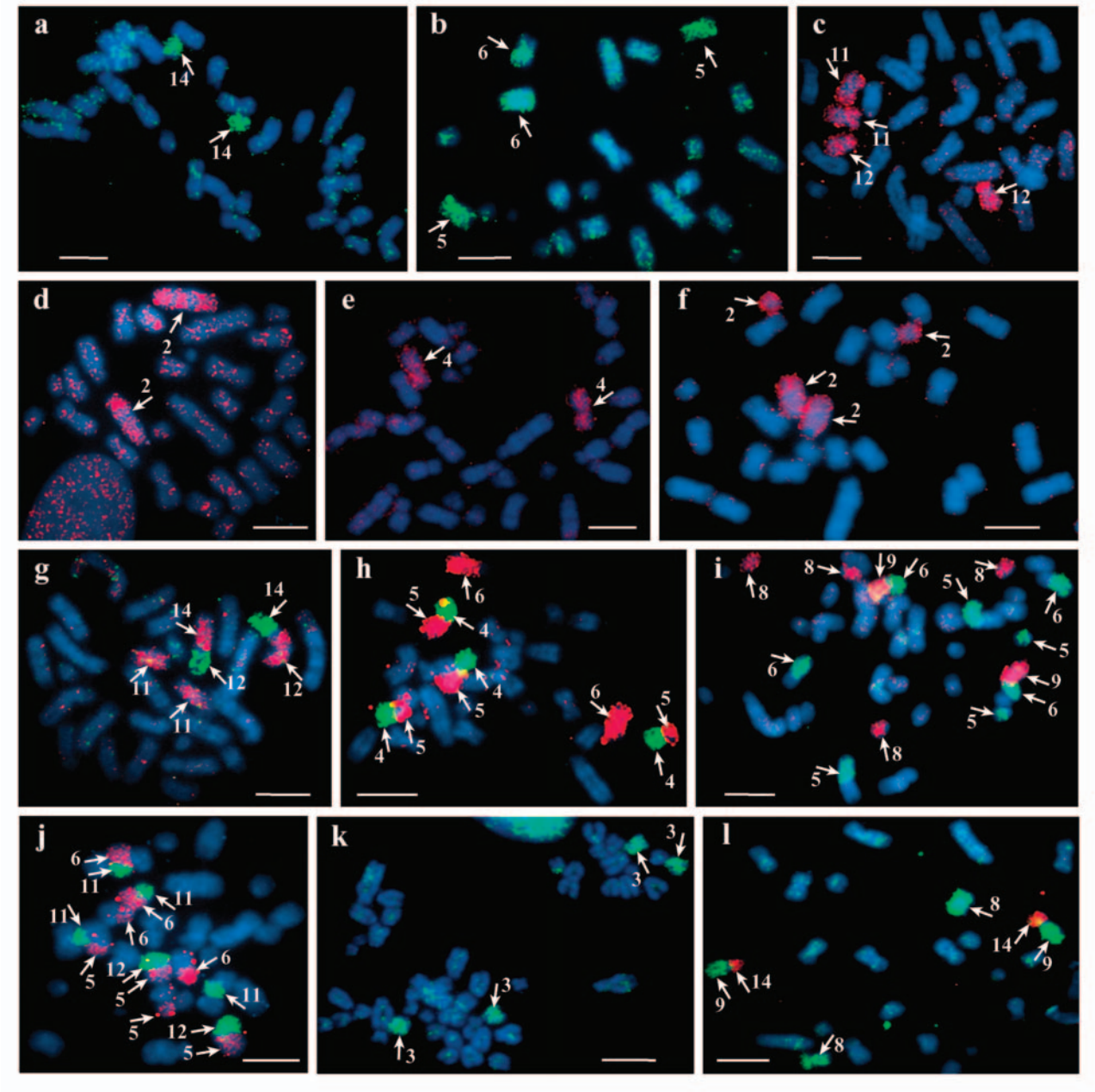


Figure 17. Examples of FISH using *M. taiva* (MTA) chromosome-specific painting probes. White arrows highlight the chromosome of interest on all panels. Numbers refer to MTA chromosomes. Panels **(a)**, **(b)**, **(c)**, **(d)**, **(e)** present FISH of MTA 14, 5/6, 11/12, 2 and 4 respectively on metaphase of *Oryzorictes hova* showing that no interchromosomal break occurred in these chromosomes between *M. taiva* and *O. hova*. As illustrated by the following panels, these chromosomes are however, all rearranged in other *Microgale* species. Panel **(f)** shows that MTA 2 has undergone a fission in *M. soricoides*. The same pattern was observed in *M. fotsifotsy*, *M. cowani* and *M. thomasi*. Panel **(g)** shows that MTA 14 (green) is fused with MTA 12 (red) in *M. longicaudata*. The same pattern was observed in *M. principula* and *M. majori*. Panel **(h)** illustrates the monobrachial homologies of MTA 4 (green) and MTA 5 (red) observed in *M. fotsifotsy*. MTA 6 (red) is not rearranged in this species. The same pattern was observed in *M. soricoides*. Panel **(i)** illustrates monobrachial homologies of MTA 9 (red) and 6 (green) observed in *M. cowani* and the fission of MTA 8 (red). The fission of MTA 8 was also observed in *M. thomasi*. Panel **(j)** illustrates monobrachial homologies of MTA 5 (red) and 12 (green) and of MTA 6 (red) and 11 (green) observed in *M. cowani*. Panel **(k)** shows that MTA 3 has undergone a fission in *M. thomasi* and panel **(l)** shows that MTA 9 has been fused to MTA 14 in *M. dobsoni*.

These findings are strikingly different to those obtained for the house mouse races from the Raethian Alps of northern Italy and southern Switzerland (Hauffe and Pialek 1997), as well as those from the Island of Madeira (Britton-Davidian et al. 2005). The inclusion of WARTs in these studies resulted in tree topologies that were not only different to those based on Rb translocations, but were also much more parsimonious being characterised by a maximum of nine (in Alpine mice) and five (in Madeiran mice) mutational steps less than what were retrieved using the Rb translocations data. Additionally, the inclusion of WARTs reduced the level of homoplasy from a maximum of eight convergent events to only one in Madeiran mice, and increased support for all nodes (as measured by bootstrap and Bremer decay indexes) in this population (Britton-Davidian et al. 2005), both trends that contrast strongly with our analysis of the tenrec data. Thus, while these observations strongly suggest that WARTs, in addition to Rb translocations, occurred in the house mouse (Hauffe and Pialek 1997, Britton-Davidian et al. 2005, Pialek et al. 2005), this clearly begs a more detailed analysis in *Microgale*.

The fact that the analysis of the Rb translocations data matrix resulted in an increase in the bootstrap values for the *Microgale* species' nodes does not appear sufficient in itself to favour this hypothesis. This increase is simply the result of a more homogeneous distribution of rearrangements along the branches of the tree (Figure 18), and there is no *a priori* justification for choosing this above the heterogeneous distribution evident when testing the WART hypothesis. Interestingly, however, in the case of the house mouse, the WART hypothesis is upheld by the fact that the fissions of Rb metacentrics are thought unlikely since telomeric and large amounts of centromeric satellite sequences are lost during Rb fusions in this species, and thus the subsequent fission of these Rb metacentrics would lead to acrocentrics deficient in these sequences (Garagna et al. 1995, Nanda et al. 1995). In contrast, fissions seem to be likely in *Microgale* as they are the most common rearrangements detected in our study.

Table 4. Matrices of taxa/characters, (a) including whole arm reciprocal translocations (WARTs), or (b) considering only fusion and fissions. Characters in bold are present in both matrices. Chromosomal changes are considered to be characters and their presence (1) / absence (0) the character states. Fi = fission; Fu = fusion; W(a) = WART between two metacentric chromosomes (type a WART in Hauffe and Pialek 1997); W(b) = WART between one metacentric and one acrocentric chromosome (type b WART in Hauffe and Pialek 1997); W(c) = WART between two metacentric chromosomes and one acrocentric chromosome (type c WART in Hauffe and Pialek 1997); undet. intra-chr. change = undetermined chromosomal change (see Material and Methods for more details). Numbers associated with rearrangements refer to *Microgale taiva* chromosomes.

a.

Characters		**OHO**	**MTA**	**MPA**	**MDO**	**MMA**	**MLO**	**MPR**	**MSO**	**MFO**	**MCO**	**MTH**
1	**Fi 2**	0	0	0	0	0	0	0	1	1	1	1
2	**Fi 3**	0	0	0	0	0	0	0	0	0	0	1
3	**Fi 4**	0	0	0	0	0	0	0	0	0	1	1
4	**Fi 5**	0	0	0	0	0	0	0	0	0	0	1
5	**Fi 6**	0	0	0	0	0	0	0	0	0	0	1
6	**Fi 7**	0	0	0	0	0	0	0	0	0	0	1
7	**Fi 8**	0	0	0	0	0	0	0	0	0	1	1
8	**Fi 9**	0	0	0	0	0	0	0	0	0	0	1
9	**Fi 10**	0	0	0	0	0	0	0	0	0	0	1
10	**Fi 10dist**	0	0	0	0	0	0	0	0	0	0	1
11	**Fi 11**	0	0	0	0	0	0	0	0	0	0	1
12	**Fi 13**	0	0	0	0	0	0	0	0	0	0	1
13	**Fu 12+14**	0	0	0	0	1	1	1	0	0	0	0
14	**Fu 9+14**	0	0	0	1	0	0	0	0	0	0	0
15	**Fu 7q+14**	0	0	0	0	0	0	0	1	1	0	0
16	**undet. intra-chr. change 1**	0	0	0	0	0	0	1	0	0	0	0
17	**undet. intra-chr. change 2**	0	0	0	0	1	1	1	0	0	0	0
a	W(a) 4/5	0	0	0	0	0	0	0	1	1	0	0
b	W(b) 5/12	0	0	0	0	0	0	0	0	0	1	0
c	W(b) 7/12	0	0	0	0	0	0	0	1	1	0	0
d	W(a) 3/7	0	0	0	0	0	0	0	0	0	1	0
e	W(c) 6/11/9	0	0	0	0	0	0	0	0	0	1	0

All chromosomes potentially involved in WARTs are also fissioned in at least one other species (Figure 16). This finding, together with the fact that WARTs (especially those of type b and c) are considered to be highly detrimental when in the heterozygous condition (Hauffe and Pialek 1997), would tend to support the observation that what holds true for the house mouse, does not apparently do so for *Microgale*. Put succinctly, WARTs are much less likely to have occurred in *Microgale* than is the case with the house mouse.

Table 4 (continued).

b.

Characters		OHO	MTA	MPA	MDO	MMA	MLO	MPR	MSO	MFO	MCO	MTH
1	**Fi 2**	0	0	0	0	0	0	0	1	1	1	1
2	**Fi 3**	0	0	0	0	0	0	0	0	0	1	1
3	**Fi 4**	0	0	0	0	0	0	0	1	1	1	1
4	**Fi 5**	0	0	0	0	0	0	0	1	1	1	1
5	**Fi 6**	0	0	0	0	0	0	0	0	0	1	1
6	**Fi 7**	0	0	0	0	0	0	0	1	1	1	1
7	**Fi 8**	0	0	0	0	0	0	0	0	0	1	1
8	**Fi 9**	0	0	0	0	0	0	0	0	0	0	1
9	**Fi 10**	0	0	0	0	0	0	0	0	0	0	1
10	**Fi 10dist**	0	0	0	0	0	0	0	0	0	0	1
11	**Fi 11**	0	0	0	0	0	0	0	0	0	1	1
12	**Fi 13**	0	0	0	0	0	0	0	0	0	0	1
13	**Fu 12+14**	0	0	0	0	1	1	1	0	0	0	0
14	**Fu 9+14**	0	0	0	1	0	0	0	0	0	0	0
15	**Fu 7q+14**	0	0	0	0	0	0	0	1	1	0	0
16	**undet. intra-chr. change 1**	0	0	0	0	0	0	1	0	0	0	0
17	**undet. intra-chr. change 2**	0	0	0	0	1	1	1	0	0	0	0
f	Fu 7p+12	0	0	0	0	0	0	0	1	1	0	0
g	Fu 5q+4q	0	0	0	0	0	0	0	1	1	0	0
h	Fu 5p+4p	0	0	0	0	0	0	0	1	1	0	0
i	Fu 5q+12	0	0	0	0	0	0	0	0	0	1	0
j	Fu 3p+7q	0	0	0	0	0	0	0	0	0	1	0
k	Fu 3q+7p	0	0	0	0	0	0	0	0	0	1	0
l	Fu 6p+9	0	0	0	0	0	0	0	0	0	1	0
m	Fu 6q+11p	0	0	0	0	0	0	0	0	0	1	0

Interspecific relationships within *Microgale*

Both matrices were homoplasy free (see above) resulting in four species clades with generally high bootstrap values ($BP_W = BP_{WARTS;}$ $BP_{fi/fu} = BP_{fusion/fission}$) despite the inclusion of a relatively low number of characters (Table 4, Figure 18). The first node groups *M. longicaudata*, *M. majori* and *M. principula* ($BP_W = 87$; $BP_{fi/fu} = 87$) on the basis that they share one fusion and one intrachromosomal rearrangement. The second node recovers *M. fotsifotsy* and *M. soricoides* as sister taxa ($BP_W = 96$; $BP_{fi/fu} = 99$) supported by either four fusions, or one fusion and two WARTs (depending on which matrix is considered in the analysis), and the third groups *M. cowani* and *M. thomasi* ($BP_W = 88$; $BP_{fi/fu} = 99$) on the basis of either four or two shared fissions. Finally, the last node clusters the *fotsifotsy* + *soricoides* lineage as sister to *cowani* + *thomasi* (BP_W

= 63; $BP_{fi/fu}$ = 99), an association supported by either one or four fissions (depending on the matrix used).

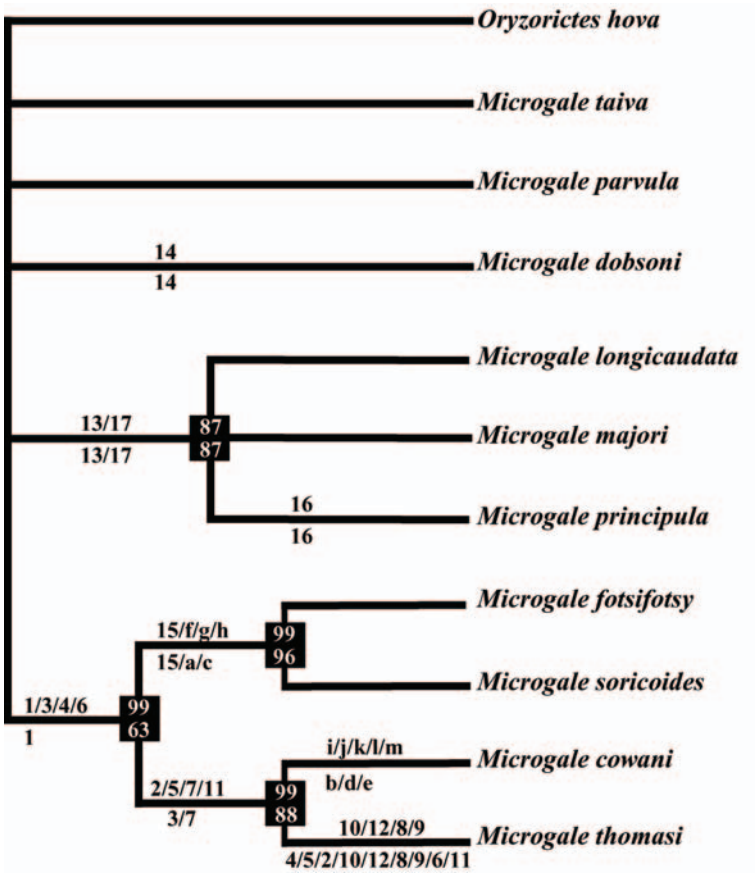


Figure 18. Single most parsimonious cladogram obtained after analysis of the two matrices presented in Table 4. Numbers and letters on branches refer to characters described in Table 4a (WART; bottom of the branches) and 4b (fusions/fissions; top of the branches). Bootstrap values based on the analysis of the two matrices are given at each node (Table 4a, bottom; Table 4b, top). Both matrices are homoplasy free (Consistency Indexes = 1).

These groupings are in perfect agreement with the topology obtained by Olson and Goodman (2003) (see also Figure 13) derived from parsimony analysis of mitochondrial and nuclear gene sequences. In addition, the recognition of *M. longicaudata* and *M. principula* as sister species is supported by the parsimony analysis of morphological characters (Olson and Goodman 2003) and, interestingly, both (*M. soricoides* + *M. fotsifotsy*) and (*M. longicaudata* + *M. principula*) groupings correspond

to distinct phenetic clusters based on overall similarities in their craniodental morphology, and the proportions thereof (MacPhee 1987, Jenkins 1993, Jenkins et al. 1997). It is important to note that *M. majori* was not considered in these earlier studies given that it has only recently been resurrected from synonymy with *M. longicaudata* (based on molecular and morphometric analyses of a large number of specimens, see Olson et al. 2004). Unfortunately, the chromosomes are not informative in this regard since *M. majori* and *M. longicaudata* are karyotypically identical at the level of resolution permitted by their G-band patterns. However, the G-banded pattern of the chromosome resulting from the fusion of MTA 12 and 14 clearly differentiates *M. principula* from *M. majori* and *M. longicaudata* (Figure 16) representing as it does an autapomorphy for *M. principula*.

Finally, the last of the chromosomally distinct lineages, that of *M. dobsoni*, is characterised by a fusion between MTA 9 and 14 (Figures 17 and 18), which represents an autapomorphy for this species. Although not informative in our tree, this character may prove to be phylogenetically important in future studies involving other *Microgale* spp. In particular it will be interesting to see whether this rearrangement is present in *M. talazaci*, a species which is phenotypically (MacPhee 1987) and genetically (Olson and Goodman 2003) thought most closely associated to *M. dobsoni*.

Rates of chromosomal evolution within the Oryzorictinae

The subfamily Oryzorictinae includes the Malagasy tenrecs *Limnogale*, *Microgale* and *Oryzorictes*; *Limnogale* has, however, recently been considered to be nested within *Microgale* (see Olson and Goodman 2003). Poux et al. (2005) provide a molecular date for the *Oryzorictes* and *Limnogale* divergence (and thus an estimate for the Oryzorictinae). They calculated the divergence at 18.9 my (Cred. Int. = 14.1 - 24.7) using sequences from the nuclear exonic ADRA2B, AR and vWF gene fragments.

Although *M. brevicaudata* was sequenced in the Poux et al. (2005) study, the species was not included in their dating analysis because of missing vWF sequence. Since *Limnogale* is nested within *Microgale,* the analysis of sequences from *M. brevicaudata* as well as *L. mergulus* provides a means for dating the origin of *Microgale*, and thus a more refined timeframe for the discussion of the rates of chromosomal evolution within Oryzorictinae. We therefore repeated Poux et al.'s (2005) analysis using their sequence matrix (available in Treebase; accession number: M2279) and the same criterion for discarding ambiguous regions in the alignment, the same calibration points and identical Bayesian methods (which can handle missing data, see Thorne et al. 2002), but including *M. brevicaudata* (i.e., the ADRA2B and AR sequences that were not analysed in the original study). We estimate the *L. mergulus* /*M. brevicaudata* split at ~9.9 my (Cred. Int. = 6.3 - 14.8) which can be interpreted as a minimum age for *Microgale*.

To place our discussion in context, it is important to emphasize (i) that no interchromosomal change occurred during the ~18.9 my that separates *O. hova* from the oryzorictine ancestor (Poux et al. 2005), and (ii) that chromosomal stasis similarly characterises both the 9 my (i.e., 18.9 - 9.9 my) separating the oryzorictine ancestor from the *Microgale* ancestor, and the ~9.9 my distinguishing the *Microgale* ancestor from *M. taiva* and *M. parvula*. Moreover, during the same period (i.e., ~9.9 my), only one interchromosomal change was detected in the lineage leading to *M. dobsoni* and two were detected in the lineage leading to *M. longicaudata*, *M. majori* and *M. principula* (Figure 18). These observations are in keeping with the low rates that have been reported in the Chrysochloridae (See Chapter II), sister family to the Tenrecidae, and are consistent with a more generalised slow rate for the Afroinsectiphillia (Afrosoricida + Macroscelidae + Tubulidentata). In contrast 12 chromosomal changes are detected in *M. thomasi*, and between 6 and 13 (depending on which matrix is considered) in *M. cowani* punctuate the ~9.9 my that separate these two species from

the *Microgale* ancestor (Figure 18), mimicking the karyotypic megaevolution of certain bat species (Baker and Bickham 1980). Although these rates are lower than those observed in several mammals (e.g., Britton-Davidian et al. 2000, Wang and Lan 2000, Dobigny et al. 2005), they are clearly accelerated with respect to most Afrotheria, the only exception being the Sirenia where at least four chromosomal changes separate *Trichechus inunguis* and *T. manatus* (Pardini et al. 2007), taxa that are thought to have diverged 1-4 my ago (Catanhede et al. 2005; Vianna et al. 2005).

Chromosomal speciation in *Microgale*

The tenrec species included in our study all occur in sympatry in the humid forests of the central highlands of Madagascar (Goodman and Rakotondravony 2000, Jenkins 2003 and references therein). Contemporary distributions do not, however, necessarily reflect the ancestral condition, requiring that temporal as well as climatic aspects must be considered in any discussion of the potential causes of speciation in a specific group of taxa. Wilmé et al. (2006) have recently provided a compelling biogeographic model to explain the high number of speciation events that the extant vertebrate fauna of Madagascar has undergone. These authors suggest that during the Quaternary glacial maxima, when climatic conditions were cooler and drier and animals sought refuge in more mesic riverine forest, watersheds with their sources at lower elevations would have been dispersal dead ends resulting in areas in which extensive allopatric speciation could have occurred. In addition, a recent study by Olivieri et al. (2007) involving a comprehensive taxonomic sample of mouse lemur species (*Microcebus*) argued that factors such as ancestral distribution, species-specific habitat preference, as well as the role of rivers and mountains as barriers to gene flow (initially proposed by Martin et al. 1972, 1995), are fundamental to understanding the diversification and present distribution of mouse lemurs.

Interestingly, our estimated minimum age for the origin of *Microgale* (9.9 my, Cred. Int. = 6.3 - 14.8) is close to that calculated for the lemur genera *Microcebus* (8.9 my, Cred. Int. = 5.5 - 13.2) and *Eulemur* (9.7 my, Cred. Int. = 6.5 - 13.7) (Yoder and Yang 2004). Although we may have underestimated the age of *Microgale*, and this may be refined through greater taxonomic representation, it nonetheless shows that the evolutionary histories of *Microcebus*, *Eulemur* and *Microgale* are largely concordant, and thus that the mechanisms that have shaped the diversification of lemurs might have also influenced the evolutionary history of shrew tenrecs. Should this hold, the observed sympatric patterns exhibited by most species of shrew tenrecs are the result of secondary contact that occurred subsequently to allopatric speciation.

Although chromosomal speciation is not ubiquitous in *Microgale* (two sets of well defined species, *M. taiva*/*M. parvula*, and *M. fotsifotsy*/*M. soricoides* have an identical karyotype), a causal role for chromosomal rearrangements in speciation is plausible where marked differences in karyotypes are found. When inferring a causal mechanisms of speciation it is clearly necessary to ascertain that it is really pairs of sister species where we find such distinct chromosomal differences and, at this point, there are no data on geographic karyotypic variation and no independent, reliable information on the identity of definitive sister species in this genus. In spite of these limitations, however, it is not unreasonable to suggest that the extensive chromosomal rearrangements detected in our study may have driven speciation in *Microgale* through the negative effects of underdominance (heterozygote meiotic breakdown, White 1978, Baker and Bickham 1986, King 1993, see Rieseberg 2001 for critical discussion), forming as it does, an hypothesis that can be tested empirically in subsequent studies.

In most models, it is generally assumed that chromosomal rearrangements must be somewhat deleterious in the heterozygous condition requiring extreme conditions for their fixation. These include small population size and inbreeding among others (the

monobrachial homology model of Baker and Bickham [1986] being an exception), factors that could quite plausibly have existed under the Wilmé *et al*. (2006) model, at least during the climatic shifts of the Pliocene/Pleistocene.

During this period populations of a previously widespread species of *Microgale* may have been isolated in several low elevation watersheds in a glacial maximum. In the subsequent glacial minimum, hybrids carrying heterozygous rearrangements that resulted from crosses between specimens from previously isolated populations could have exhibited reduced fertility or, in extreme instances, complete reproductive breakdown. The complex meiotic configurations anticipated to result from the multiple rearrangements that define many of the species examined herein (chains and/or rings of chromosomes) could reasonably be expected to result in malsegregation and/or germ cell death.

Table 5. Number and type of abnormal meiotic configurations expected in all possible hybrids resulting from theoretical crossings of any pair of chromosomally different species of *Microgale* included herein (based on Figure 16). Only interchromosomal rearrangements are considered.

	MSO,MFO	**MPR,MMA,MLO**	**MTH**	**MCO**	**MDO**
MTA,MPA	1 chain of 3 1 chain of 5 1 ring of 4				
MSO,MFO	1 chain of 3	1 chain of 3 2 rings of 4			
MPR,MMA,MLO	10 chains of 3 1 chain of 4	11 chains of 3 1 chain of 4	11 chains of 3 1 chain of 4		
MTH	3 chains of 3 1 ring of 4 1 chain of 4 1 chain of 6	1 chain of 9 3 chains of 3 1 chain of 6 1 chain of 4	3 chains of 3 1chain of 5 1 chain of 6 1 ring of 4	5 chains of 3 2 chains of 4	
MCO	1 chain of 3	1 chain of 3 1 chain of 6 1 ring of 4	1 chain of 4	9 chains of 3 2 chains of 4	3 chains of 3 1 chain of 4 1 chain of 7 1 ring of 4

In the case of the house mouse races, hybrid fertility varies considerably with respect to the number of heterozygous rearrangements present in carriers (Nachman and

Searle 1995). In some instances a single heterozygous rearrangement may be sufficient to adversely affect fertility (Hauffe and Searle 1998) while in others, those carrying few heterozygous rearrangements do not generally show a decrease in fertility (Winking et al. 1988, Viroux and Bauchau 1992, Wallace et al. 1992). On the contrary, however, hybrids heterozygous for more than three rearrangements generally show elevated levels of aneuploidy and/or germ cell death (Redi and Capanna 1978, Garagna et al. 1990, Saïd et al. 1993). It is also anticipated that meiotic chains are more detrimental than rings of chromosomes since they present unpaired axes and the more meiotic abnormal configurations present, the less fertile the hybrid (Hauffe and Pialek 1997). We determined the number of complex meiotic configurations theoretically expected in hybrids that would result from crosses among the chromosomally distinct shrew tenrec species identified by our investigation (Table 5). Nine of the possible 15 interspecific crosses would result in hybrid meiosis characterised by a high number of chains and/or rings (between 6 and 11 abnormal pairing configurations per specimen), an observation that warrants further detailed empirical analysis, among others, through captive breeding experiments.

We recognize that it could be argued that the final arbiter of correct segregation is the meiotic spindle (Eichenlaub-Ritter and Winking 1990, King 1993) and that not all instances of shrew tenrec hybridization depicted in Table 5 may have been possible (due to geographic or the development of other premating barriers before the possibility of secondary contact). Nonetheless, given Madagascar's paleoclimatic oscillations and the spectacular shrew tenrec species diversity, which is often underpinned by marked differences among karyotypes, a case can be made for including *Microgale* in the suite of taxa (*Spalax*: Nevo et al. 1994, *Muntiacus*: Wang and Lan 2000, *Mus musculus domesticus*: reviewed in Capanna and Castiglia 2004, *Taterillus*: Dobigny et al. 2005

among others) for which the fixation of underdominant chromosomal rearrangements may have played a role in cladogenesis.

CHAPTER IV

CONCLUDING COMMENTS

Information on the chromosomes of the 55 afrosoricidan species was previously restricted to one G-banded karyotype, that of *C. asiatica* (Robinson et al. 2004), and few unbanded karyotypes or reports of diploid numbers (Borgaonkar and Gould 1965, 1968, 1969, Borgaonkar 1967, Hsu and Bernishke 1974, Bronner 1995a, b). The description of G-banded karyotypes of 18 new species and three subspecies of Afrosoricida presented here thus provides solid basis for understanding chromosomal evolution within this mammalian order. Although this study provides a comprehensive picture of the chromosomal evolution within the Chrysochloridae (seven of nine genera included), a large portion of generic diversity within Tenrecidae nonetheless remains to be described (only two of 10 genera were included).

The study of chromosomal evolution at the cytogenetic level does not generally allow hypotheses concerning the detailed mechanisms involved in chromosomal rearrangements since the level of resolution provided by these techniques is low. As a consequence, and as emphasized by White (1973) ‘‘Eventually the story of the chromosomal mechanisms and its evolution will have to be entirely rewritten in molecular terms’’. Since there has been an exponential growth in the knowledge of the molecular mechanisms underlying the structural modifications of mammalian genomes since White (1973) wrote this sentence, I would like to conclude by placing the outcomes of this study in a broader context, one that concerns and discusses several hypotheses pertaining to the molecular mechanisms and forces that drive chromosomal evolution.

Fissions and telomeres

The comprehensive assessment of chromosomal homologies among golden moles and between the relatively large number of tenrec species included in this study reveals that fusions and fissions are the predominant structural rearrangements that have shaped the evolution of the karyotypes in these taxa. This observation is, in itself, not surprising given that these two types of rearrangements are the most frequent in mammals (Slijepcevic 1998, Kolnicki 2000). As discussed in Chapter II, the fixation of the fissions in *Amblysomus robustus* may have been facilitated by the presence of telomeric-like repeats in the centromeres of the chromosomes. In contrast to the situation in golden moles, the centromeres of *Microgale taiva*, *M. thomasi* and *M. cowani* are devoid of telomeric-like sequences (not shown) and thus the fixation of fissions in these species was probably not enhanced by the recruitment of pre-existing telomeric motifs at the neo-chromosomal ends. Alternatively, one could argue that the ends of chromosomes resulting from fissions in these taxa might have been "capped" by the *de novo* formation of telomeres, or by the capture of telomeric repeats situated on other chromosomes through non-reciprocal translocation (reviewed in Murnane 2006). *De novo* formation of telomeres is frequent in yeast where it involves the action of the telomerase coupled with several co-factors (reviewed in Pennaneach et al. 2006). Although the underlying mechanisms are not well described in mammals, *de novo* addition of telomeres on broken chromosomes has been observed in human tumour cells (Fouladi et al. 2000) and mouse embryonic stem cells (Sprung et al. 1999). This process has also been invoked to explain the presence of telomeres at the proximal end of the neo-acrocentric chromosome 12 that resulted from a WART in one specimen of the house mouse (Catalan et al. 2000).

Chromosomes, speciation and centromere drive

As mentioned in the introduction to this study (Chapter I), the role of chromosomal rearrangements in speciation through heterozygous underdominance has been extensively debated. The data presented here clearly do not provide conclusive evidence of this but nonetheless suggest that given Madagascar's paleoclimatic fluctuations, chromosomal speciation is plausible in *Microgale*. In large part this is underpinned by the observation that the karyotypes of several species-pairs show levels of difference that are at least as high as those observed between parents of hybrid mice showing reduced fertility and/or germ cell death. It will be interesting to further test this hypothesis by exhaustively determining the phylogenetic relationships and divergence times among all *Microgale* species and, secondly, by experimentally assessing the potential role of factors such as behaviour and genetic divergences on breeding and reproductive success, and hence their potential impact on speciation.

Similarly, comprehensive information on the divergence times within Chrysochloridae may also prove useful in future debate on golden mole speciation. In particular, knowledge on the *A. h. meesteri* and the other *Amblysomus* species/subspecies divergences would provide some indication, albeit untested, as to whether these taxa are likely to produce viable hybrids. If hybridization could be manipulated under captive conditions, it would allow for observation on the coexistence of two different sets of centromeres *in vivo* and, in conjunction with hybrid fertility, could provide insights on whether one set of centromeres is more frequently transmitted than the other. In essence, therefore, it could serve as a model to test the hypothesis of centromere drive (Henikof and Malik 2001) and its possible consequences on the speciation of golden moles.

Genome-wide homogenization of centromeric tandem repeats

In addition, estimates of the degree of separation between *A. h. meesteri* and the other *Amblysomus* species/subspecies will allow inferences on the spread of a telomeric-like motif containing satellite family that is present in the centromeres of all, or most chromosomes in *A. h. hottentotus*, *A. h. longiceps*, *A. h. pondoliae* and *A. robustus*. The principal mechanism thought to explain the formation/homogenization of a tandem repeat array is unequal crossover between sister chromatids during meiosis and germ cell mitosis (Smith 1976, and see below). This mechanism, together with conversion, slippage, transposition and retrotransposition is responsible for the non-Mendelian increase in the frequency of a genetic variant in a population through a process called molecular drive (Dover et al. 1982, Dover 2002). This results in an observed pattern of concerted evolution, i.e. all copies of a satellite family are more identical within a species than between species (Dover 1982 et al., Dover 2002). Although sister chromatid recombination can (at least partially) explain the evolution of a satellite family within a chromosome, there seem to be no clear consensus in the literature on which mechanism is responsible for the genome-wide homogenization of centromeric satellites. This phenomenon is indirectly observed in this study (Chapter II) since knowing that telomeric-like motifs are present in the centromere of every chromosomes of a species does not necessarily imply that the satellite family constituting these chromosomes have a common ancestor. In other words, it could be argued that telomeric-like motifs have been independently inserted in different satellite families that are found at the centromeres of each chromosome.

However, genome wide homogenization of satellite families is a well known phenomenon and this is well illustrated by the presence of α-satellites at all centromeres of primate (including human) chromosomes (Willard 1991, Alexandrov et al. 2001). Intuitively, genome-wide homogenization patterns could be explained by the formation

of non-homologous (between different chromosomes) meiotic crossovers. However, as meiotic crossovers are suppressed at centromeres (Choo 1998, Gerton et al. 2000), this mechanism is believed to have little if any impact on the evolution of centromeric satellites (Ma and Bennetzen 2006). The most likely mechanism involved in genome wide homogenization of centromeric repeats thus appears to be equal and unequal conversion events, a type of recombination that does not involve the formation of crossovers (Baudat and de Massy 2007, Chen et al. 2007). Clear evidence of this mechanism has been found in the centromere of rice chromosome 8 for example (Ma and Bennetzen 2006). This mechanism could operate not only between chromosomes, but also between chromosomes and extrachromosomal circular DNA (eccDNA).

Extrachromosomal circular DNA is found in a wide variety of eukaryotes (Gaubatz 1990) and is thought to derive from rolling circle amplification of chromosomal DNA (Cohen et al. 2005). In *Drosophila*, eccDNA varies in length (<1kb and >20kb) and can constitute up to 10% of the total repetitive DNA content; many show high similarity to centromeric satellites (Cohen et al. 2003). Indeed, Walsh (1987) proposed that rolling circle amplification of eccDNA was the principal mechanism of the formation of a tandem array, rather than unequal crossover between sister chromatids (Smith 1976). Although the relative importance of the two mechanisms has not been critically assessed in any organism, empirical evidences for both have been observed (e.g., Rossi et al. 1990 for rolling circle amplification of eccDNA; Kapitonov et al. 1998 for unequal crossover). Intuitively therefore, it seems likely that equal and unequal conversion events between chromosomes and eccDNA - rather than between chromosomes - could be a highly efficient mechanism leading to the genome wide expansion and homogeneisation of satellite families such as the centromeric α-satellite of primates, or the telomere-containing satellites of golden moles. A simple test of this

hypothesis would be to characterize the population of eccDNA in these species as has been done in *Drosophila* (Cohen et al. 2003).

The rate of chromosome evolution

Overall, the rates of chromosomal rearrangement calculated in this study are slow and in line with the generally constrained trend observed across the mammalian tree. A global or "default" rate of one rearrangement per 10 my was suggested by Murphy et al. (2001b) based on the fact that the karyotypes of several distantly related species of boreoeutherians such as cat, mink, ferret, dolphin and human were highly conserved. Indeed, the high karyotypic conservation recently observed in the two-toed sloth (Svartman et al. 2006) and in the aardvark (Yang et al. 2003), two Atlantogenata representatives, have largely confirmed Murphy et al. (2001b) hypothesis on an ancestral slow rate for eutherian mammals. However, as has been repeatedly suggested, high rates of change are observed on many branches of the eutherian tree (reviewed in Murphy et al. 2001b, Ferguson-Smith and Trifonov 2007). This phenomenon is particularly well documented in *Microgale* species with a ten-fold increase in *M. thomasi* compared to *M. dobsoni*, for example, and to a lesser extent in golden moles where the tempo increases in the lineage leading to *Amblysomus robustus*. When both slow and high rates documented for eutherians are taken into account, a value of 1.9 rearrangements per 10 my has been estimated (Frönicke 2005) which is almost the double of the ancestral rate. Together, these observations lead to what is perhaps the most fascinating question related to chromosomal evolution: what are the factors that can explain the contrasting rates of rearrangements on different branches of the eutherian tree?

Knowledge of the molecular mechanisms that underlie both large and small-scale chromosomal rearrangements has grown considerably during the last 20 years

following the exponential availability of genomic sequences. Comparisons between several mammalian species whose genomes are fully or partially sequenced have clearly shown that evolutionary breakpoints are not randomly distributed in the genome but tend to cluster in hotspots that are enriched in tandem repeats that are significantly associated with fragile sites (Murphy et al. 2005, Robinson et al. 2006, Ruiz-Herrera et al. 2006, Gordon et al. 2007, Ruiz-Herrera and Robinson, 2007). It is also now well accepted that one of the major mechanism leading to rearrangement of chromosomal segments is ectopic recombination at meiosis between repetitive sequences such as transposable elements and low copy repeats (or segmental duplications) (Lupski and Stankiewicz 2005, Han et al. 2007, Kehrer-Sawatski and Cooper 2007). A recent study also shows that the erroneous repair of staggered double strand breaks (DSB) seems to be another major source of rearrangements, at least with respect to inversions in *Drosophila* (Casals and Navarro 2007, Ranz et al. 2007). More generally, any chromosomal rearrangement can simply be viewed as staggered DSB that are not properly repaired (Morgan et al. 1998, Agarwal et al. 2006). Evolutionary rearrangements only represent a small fraction of those that occur since they need to occur in germ cell mitotic divisions and/or during meiosis to have an evolutionary effect.

The two main pathways of DSB repair acting in mammalian cells are respectively non-homologous end joining, which includes single-strand annealing, and homologous recombination (Burma et al. 2006). The latter mechanism also occurs during meiotic crossovers (Helleday et al. 2007). Recent evidence suggests that the mechanism through which retrotransposons insert in a new genomic locus also plays a role in DSB repair, although the relative importance of this pathway compared to the two highlighted above is unknown (Eickbush 2002, Sen et al. 2007). Interestingly, given that meiotic recombination is suppressed at centromeres in mammals, and given

that evolutionary breakpoints are concentrated in centromeres, erroneous repair of DSB that would not involve illegitimate meiotic recombination, such as those illustrated in Ranz et al. (2007) in *Drosophila*, could also have had a major impact on the dynamics of mammalian chromosomes.

Despite these enormous advances in the knowledge of the mechanistic aspects of chromosomal rearrangements, to my knowledge no convincing explanation exists to explain the different rates of chromosomal evolution observed in mammals. The rate of chromosomal evolution is a fascinating issue since it embraces a myriad of complex biological processes. In the context of the above discussion, rates of chromosomal evolution can be expressed as the rate of improperly repaired DSBs x the rate of fixation. The rate of improperly repaired DSB can be further expressed as the product of the rate of DSB x the rate of improper repair. In turn, the rate of fixation depends on several forces such as selection (as measured by the impact of a rearrangement on the fitness), genetic drift (which is directly dependent on population size) and meiotic drive.

There is good, though indirect, evidence to show that variation in genetic drift may have been the major force leading to accelerated rates of change in some mammals, perhaps the most striking example of which is the house mice on Madeira Island (Britton-Davidian et al. 2000). Likewise, selection (e.g., Nevo et al. 1994) and meiotic drive (e.g., Pardo-Manuel de Villena and Sapienza, 2001) are believed to have had a significant impact on chromosome evolution, however their precise influence on the rates at which rearrangements occur has never been comprehensively ascertained. One of the most exciting unanswered questions is whether genomic traits (i.e., rate of DSB and rate of improper repair) have had a significant influence when compared to factors influencing the rate of fixation. In other words, does an increase in the number of DSBs or a decrease in the accuracy of repair mechanisms lead to an increase in the number of rearrangements, or are these factors negligible compared to genetic drift, selection and

meiotic drive? A recent elegant experimental study shows that female mice with a defective DSB repair mechanism produce a significantly higher proportion of chromosomal aberrations after mating with males irradiated with ionising radiation (Marchetti et al. 2007). Although this suggests that variation in the accuracy of DSB repair can indeed lead to variation in rates of chromosomal rearrangements, the extent to which accuracy in DSB repair can vary through time is unknown.

The mediation of rearrangements by transposable elements (TEs) through numerous mechanisms is well demonstrated (see above and also Hedges and Deininger 2007). Interestingly, the activity level of these elements generally varies greatly through time (e.g., Khan et al. 2006, Pace and Feschotte 2007) in a fashion that could provide an explaination of the variation in the rate of chromosome breaks and, by extension, rates of rearrangements. Indeed, it seems straightforward that an increase in transposition of one or more different families of TEs would lead to an increase in DSBs in the host genome, simply because insertion of both classes of TEs (DNA transposons and retrotransposons) starts with the creation of at least one DSB (or two staggered single strand breaks, Craig et al. 2002). Moreover, since the efficiency of meiotic recombination depends somewhat on similarity between the two recombining sequences (see Carrington and Cullen 2004), a burst of transposition leading to the spread of many highly similar or even identical TE sequences throughout the genome in a short period should, theoretically, lead to a considerable increase in the probability of ectopic meiotic recombination, and thus to more rearrangements during this short period.

Although attractive, hypotheses linking variation in TE activity to the rate of chromosomal evolution are not yet supported by any experimental evidence. The absence of evidence does not necessarily imply the evidence of the absence, and despite the considerable advances in molecular biology the data necessary to test these hypotheses are not yet available. More precisely, the number and taxonomic diversity of

complete mammalian genome sequence assemblies is still too limited to accurately estimate the relative importance of the different factors that could have triggered the observed variatioin in rates of rearrangements over the eutherian tree. However, given that the costs of producing a whole genome sequences will decrease dramatically in the near future (Pennisi 2006), it should be possible to provide insights to these and other fascinating questions that are pertinent to mammalian genome evolution.

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