Phylogeny, Recombination, and Mechanisms of Stepwise Mitochondrial Genome Reorganization in Mantellid Frogs from Madagascar

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In Malagasy frogs of the family Mantellidae, the genus *Mantella* is known to possess highly reorganized mitochondrial (mt) genomes with the following characteristics: 1) some rearranged gene positions, 2) 2 distinct genes and a pseudogene corresponding to the transfer RNA gene for methionine (*trnM*), and 3) 2 control regions (CRs) with almost identical nucleotide sequences. These unique genomic features were observed concentrated between the duplicated CRs surrounding cytochrome *b* (cob) and nicotinamide adenine dinucleotide dehydrogenase subunit 2 (cnad2) genes. To elucidate the mechanisms and evolutionary pathway that yielded the derived genome condition, we surveyed the reorganized genomic portion for all 12 mantellid genera. Our results show that the mt genomes of 7 genera retain the ancestral condition. In contrast, adding to *Mantella*, 4 genera of the subfamily Mantellinae, *Blommersia*, *Guibemantis*, *Wakea*, and *Spinomantis*, share several derived genomic characters. Furthermore, mt genomes of these mantellines showed additional structural divergences, resulting in different genome conditions between them. The high frequency of genomic reorganization does not correlate with nucleotide substitution rate. The encountered mt genomic conditions also suggest the occurrences of stepwise gene duplication and deletion events during the evolution of mantellines. Simultaneously, the majority of duplication events seems to be mediated by general (homologous) or illegitimate recombination, and general recombination also plays a role in concerted sequence evolution between multiple CRs. Considering our observations and recent conditional evidences, the following outlines can be expected for recombination processes in mt genome reorganization. 1) The CR is the "hot spot" of recombination; 2) highly frequent recombination between CRs may be mediated by a replication fork barrier lying in the CR; 3) general recombination has a potential to cause gene rearrangement in upstream regions of multiple CRs as the results of gene conversion and unequal crossing over processes. Our results also suggest that recombination activity is not a direct cause of convergent gene rearrangement; rather, homoplasious gene rearrangement seems to be mediated by persistence of a copied genomic condition through several lineage splits and subsequent parallel deletions.

Introduction

Animal mitochondrial (mt) DNA is a closed circular molecule, typically 16 kbp in size but ranging from 14 to 42 kbp (Wolstenholme 1992). The mt gene content is nearly identical across metazoans, with 13 protein-coding genes, 2 ribosomal RNA genes (*rrn*s), 22 transfer RNA genes (*trn*s), and 1 long noncoding region, the control region (CR; also referred to as D-loop region) that includes the signals necessary for regulation of mtDNA replication and transcription (Wolstenholme 1992; Boore 1999). Animal mt genomes generally lack introns and have very few noncoding regions and intergenic spacers (ISs), except for the CR (e.g., Wolstenholme 1992).

Genomic characters of animal mtDNA, that is, codon usage, gene content, secondary structures of *trn*s, and especially gene arrangement, have been considered to constitute effective phylogenetic markers for metazoans (see e.g., Brown 1985; Wolstenholme 1992; Boore and Brown 1998; Boore et al. 2005). This is partly supported by the apparent complete absence of recombination activity in the animal mt genomic system, which would suppress gene rearrangements and thereby lead to a low incidence of homoplasious rearrangement events (Brown 1985; Boore and Brown 1994). Based on the assumption of absence of recombination in mt genomes, rearrangements of mt genes have usually been interpreted as a result of tandem duplication caused by replication errors (the tandem duplication and random loss [TDRL] model; Moritz et al. 1987; Boore 2000). However, recent evidence for recombination in the animal mt genome (Thyagarajan et al. 1996; Lunt and Hyman 1997; Kajander et al. 2000, 2001; Kraytsberg et al. 2004; Rawson 2005; Sato et al. 2005; Tsaousis et al. 2005) urges to reconsider other duplication modes mediated by recombination (Dowton and Campbell 2001; Endo et al. 2005; Mueller and Boore 2005).

For metazoan mt genomes, it is often difficult to trace the evolutionary pathway of genome reorganization because of a generally low number of reorganization events, too drastic reorganizations in some animal lineages (e.g., nematodes, bivalves, snails, brachiopods, echinoderms, and ascidians; reviewed by Boore 1999 and see Noguchi et al. 2000; Yokobori et al. 2005), and, especially, very few species with intermediate genomic conditions (but see Inoue et al. 2003; Sano et al. 2005; San Mauro et al. 2006). One of the factors that is correlated with and may influence mt genome rearrangements are nucleotide substitution rate (Shao et al. 2003; Xu et al. 2006). Also, it is known that specific genomic regions (i.e., initiation and termination points of replication, and *trn* clusters) and potential secondary structures of *trn*s and noncoding sequences are involved in gene rearrangements (e.g., Macey et al. 1997; Kraytsberg et al. 2004; San Mauro et al. 2006). The understanding of mechanisms, patterns, and phylogenetic significance of mt genome rearrangements could be further fostered by analyzing an animal group with 1) a high

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frequency of genome rearrangements in one lineage and a low such frequency in other lineages and 2) intermediate conditions from a stepwise rearrangement process being conserved in some taxa.

Among vertebrate lineages the arrangement of mt genes tends to be conserved, with all 37 genes and the CR organized in a similar order. However, rearranged mt genomes have been reported from representatives of numerous vertebrate taxa (e.g., Macey et al. 1997; Boore 1999). This also applies to neobatrachian frogs, a phylogenetically nested clade comprising the vast majority of amphibian species (Hoegg et al. 2004; Frost et al. 2006). Basal lineages of frogs (a paraphyletic group sometimes referred to as archaeobatrachians) conserve the plesiomorphic vertebrate gene order (e.g., Roe et al. 1985; San Mauro et al. 2004; Gissi et al. 2006). In contrast, in most neobatrachians studied so far the genomic positions of 4 tRNA genes are rearranged and the reduced form of nicotinamide adenine dinucleotideNADH dehydrogenase subunit 5 gene (*nad5*) is commonly translocated from upstream *nad6* to downstream of CR in 2 neobatrachian families, Rhacophoridae and Mantellidae (see fig. 1). Furthermore, in the family Mantellidae, the members of the genus *Mantella* are known to possess a highly reorganized mt genome with the following characteristics: 1) some rearranged gene positions, 2) two distinct genes and a pseudogene corresponding to transfer RNA gene for methionine (*trnM*), and 3) duplicated CRs with almost identical nucleotide sequence (Kurabayashi et al. 2006). These unique genomic features were observed to concentrate between the duplicated CRs, surrounding the genes for cytochrome *b* (*cob*) and *nad2*. Because mt genomic organization is generally conserved within closely related taxa, the mantellid mt genomes with such a diversity in their organization seem to be a good model to elucidate molecular evolutionary and phylogenetic aspects of mt genomic rearrangements.

The family Mantellidae is an endemic frog radiation of Madagascar and the Comoro island of Mayotte (Bossuyt and Milinkovitch 2000; Vences, Andreone, et al. 2003; Vences, Vieites, et al. 2003). According to the most recent classification (Glaw and Vences 2006, 2007; Glaw et al. 2006), this family consists of 12 genera and is divided into three subfamilies, Boophinae (*Boophis*), Laliostominae (*Aglyptodactylus* and *Laliostoma*), and Mantellinae (*Blommersia*, *Boehmantis*, *Gephyromantis*, *Guibemantis*, *Mantella*, *Mantidactylus*, *Spinomantis*, *Tsingymantis*, and *Wakea*). Ecologically, mantellines usually breed in either ponds or streams. In both the Boophinae and in the Mantellinae, this reproductive preference seems to be phylogenetically conserved, and at least in boophines, pond breeders may be characterized by lower mt substitution rates (Vences et al. 2002). Despite intensive studies (see Vences et al. 2007), the relationships between various deep lineages in this family so far remain unresolved.

In this study, we newly examined the region of mt genomic reorganization known in *Mantella* for 12 mantellid species and compared the genome organizations among 17 species covering all known mantellid genera. Based on these results we 1) describe unique genomic features found in various mantellid species, 2) reconstruct the phylogenetic relationships among mantellid genera based on nucleotide sequence data, 3) infer possible mechanisms and pathways of mt genomic reorganizations, 4) search for correlates between rates of nucleotide substitution and genome rearrangement, and 5) discuss the phylogenetic utility of mt gene arrangement.

Materials and Methods

Frog Specimens

To survey, for all mantellid genera, the region of mt genomic reorganization (from *cob* to *nad2*) known from *Mantella*, we newly examined mt genomes of 12 species. Previous data of 5 mantellids, a rhacophorid (*Buergeria buergeri*; Sano et al. 2004), and a ranid (*Rana nigromaculata* [=*Pelophylax nigromaculatus*]; Sumida et al. 2001) were also included in the comparisons and analyses, and preliminary data of the length of the genomic reorganization fragment were obtained for further 48 mantellid species (supplementary table 2, Supplementary Material online). Voucher information and accession numbers of the determined DNA sequences are listed in table 1.

Polymerase Chain Reaction, Subcloning, and Sequencing

Total DNA was extracted from muscle tissues using DNeasy Tissue Kits (Qiagen, Hilden, Germany). The amplification and sequence strategy of mt fragments followed similar procedures as in a previous study (Kurabayashi et al. 2006). In brief, to prevent polymerase chain reaction (PCR) jumping (Pääbo et al. 1990) between homogenized CRs, a number of overlapping fragments representing the *cob–nad2* region were amplified with degenerated primer sets for each species (the sequences of primers used are available upon request to A.K.). PCR reactions were carried out using LA-Taq (Takara Bio Inc., Shiga), according to the manufacturer's instructions. The primer-walking method was employed for sequencing using an automated DNA sequencer (ABI 3100) with the BigDye Terminator Cycle Sequencing Kit (ABI Japan Ltd., Tokyo, Japan). PCR fragments containing long tandem repeats and/or mononucleotide tracts from which we could not sequence by primer walking were subcloned into *Escherichia coli* vector pCR-2.1 or pCR-XL using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Almost all fragments could be subcloned; however, we failed to get suitable clones for the CR of *Gephyromantis pseudoasper* (ca. 12 kbp) and *Mantidactylus grandidieri* (ca. 6 kbp). To determine the precise sequences of the long tandem repeats, a series of deleted subclones were made from the resultant subclones using the exonuclease III deletion method (Henikoff 1987). Both DNA strands were sequenced about half of the surveyed regions, but remaining regions, especially long tandem repeats within CRs, were determined for only one strand due to the lack of suitable deletion mutants. From the resulting sequences, genes were identified by comparisons with corresponding gene sequences from other vertebrates.

Molecular Phylogenetic Analyses

A concatenated nucleotide alignment was made from the sequences of 4 protein-coding (cytochrome *b* [*cob*];

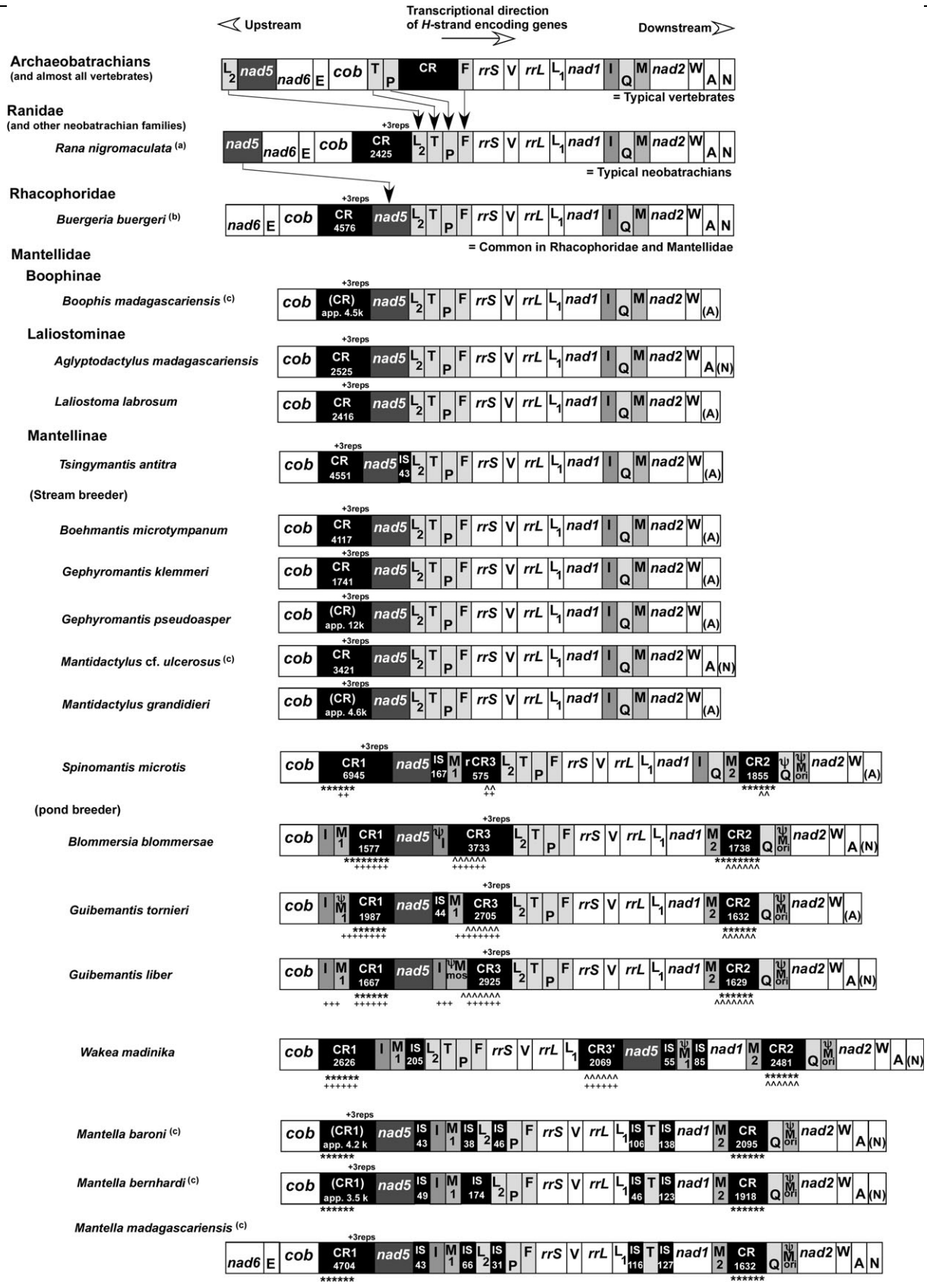

Upstream
Transcriptional direction of H-strand encoding genes
Downstream
Archaeobatrachians (and almost all vertebrates)
= Typical vertebrates
Ranidae (and other neobatrachian families)
Rana nigromaculata (a)
= Typical neobatrachians
Rhacophoridae
Buergeria buergeri (b)
= Common in Rhacophoridae and Mantellidae
Mantellidae
Boophinae
Boophis madagascariensis (c)
Laliostominae
Aglyptodactylus madagascariensis
Laliostoma labrosum
Mantellinae
Tsingymantis antitra
(Stream breeder)
Boehmantis microtympanum
Gephyromantis klemmeri
Gephyromantis pseudoasper
Mantidactylus cf. ulcerosus (c)
Mantidactylus grandidieri
Spinomantis microtis
(pond breeder)
Blommersia blommersae
Guibemantis tornieri
Guibemantis liber
Wakea madinika
Mantella baroni (c)
Mantella bernhardi (c)
Mantella madagascariensis (c)

Table 1
Mantellid Specimens Used in This Study

Subfamily Species	Voucher Specimen	Accession Numbers (sequenced portion: length)
Boophinae		
Boophis madagascariensis[a]	IABHU 6798	AB239570 (*Cob*–CR5′: 3 287 bp), AB239572 (CR3′–*trnA*: 7 180 bp)
Laliostominae		
Aglyptodactylus madagascariensis	IABHU 6942	AB325874 (*Cob*–*trnN*: 10 674 bp)
Laliostoma labrosum	Not preserved	AB325875 (*Cob*–*trnA*: 10 497 bp)
Mantellinae		
Blommersia blommersae	UADBA (ZCMV 31)	AB325876 (*Cob*–*trnN*: 15 396 bp)
Boehmantis microtympanum	UADBA (FGZC 140)	AB325877 (*Cob*–*trnA*: 12 173 bp)
Gephyromantis klemmeri	ZSM (ZCMV 2063)	AB325878 (*Cob*–*trnA*: 9 769 bp)
Gephyromantis pseudoasper	ZMA (FGMV 2002.732)	AB325879 (*Cob*–CR-5′: 2 472 bp), AB325880 (CR-3′–*trnN*: 8 451 bp)
Guibemantis liber	IABHU (AK12)	AB325881 (*Cob*–*trnN*:14 652 bp)
Guibemantis tornieri	IABHU 6817	AB325882 (*Cob*–*trnA*: 14 603 bp)
Mantella baroni[a]	UADBA (AK041208-09)	AB239567 (*Cob*–CR1-5′: 3 902 bp), AB239568 (CR1-3′–*trnN*: 10 814 bp)
Mantella bernhardi[a]	UADBA (AK041213-01)	AB239569 (*Cob*–CR1-5′: 3 075 bp), AB239570 (CR1-3′–*trnN*: 10 218 bp)
Mantella madagascariensis[a]	IABHU 6960	AB212225 (Complete genome: 22 874 bp)
Mantidactylus grandidieri	UADBA (AK041209-01)	AB325883 (*Cob*–CR5′: 2 776 bp), AB325884 (*CR3′*–*trnA*: 7 715 bp)
Mantidactylus cf. *ulcerosus*[a]	IABHU 6814	AB239573(*Cob*–*trnN*: 11 500 bp)
Spinomantis microtis	ZSM (FGZC 2437)	AB325885 (*Cob*–*trnA*: 17 792 bp)
Tsingymantis antitra	UADBA 24766 (FGZC 531)	AB325886 (*Cob*–*trnA*: 12 659 bp)
Wakea madinika	MVDNA 2001F44	AB325887 (*Cob*–*trnN*: 15 832 bp)

NOTE.—UADBA, Département de Biologie Animale, Université d'Antananarivo; FGZC, Frank Glaw Zoological Collection; FGMV, Field number of M.V. and F.G.; ZCMV, Zoological Collection M.V., IABHU, Institute for Amphibian Biology, Hiroshima University; and MVDNA, tissue collection of M.V. For some specimens, the corresponding specimens have not yet been catalogued and we here give instead their corresponding field numbers (AK, FGMV, FGZC, ZCMV) in parentheses.

[a] Data from Kurabayashi et al. (2006).

reduced form of nicotinamide adenine dinucleotide dehydrogenase subunits 1, 2, and 5 [*nad1*, *2*, and *5*]), 12S and 16S ribosomal RNA (*rrnS* and *rrnL*), and 9 transfer RNA (*trnA*, *F*, *Lcun*, *Luur*, *P*, *Q*, *T*, *V*, and *W*) genes. For protein-coding genes, the elucidated amino acid sequence of each gene was initially aligned using ClustalX 1.83 (Thompson et al. 1997). Gaps and ambiguous areas were excluded using Gblocks 0.91b (Castresana 2000) with default parameters; then the corresponding codon sequences (4 503 sites) were used for further analysis. The rRNA and tRNA alignments (2 178 and 584 sites, respectively) were also constructed using the same programs. The final alignment encompassed 19 taxa and 7 265 nt sites, of which 3 148 sites were parsimony informative.

Phylogenetic analyses based on the concatenated alignment data were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. In all analyses, *R. nigromaculata* was used as out-group. MP analysis was performed using PAUP*4.0b10 (Swofford 2003). A heuristic search option with 10 random addition replicates and tree bisection-reconnection (TBR) branch swapping was used, and all sites were of equal weighting. Clade support under MP was evaluated using 1 000 replicates of nonparametric bootstrapping. For ML and BI analyses, appropriate substitution models (general time-reversible model with gamma distribution substitution rates and proportion of invariable sites [GTR + G + I]) were chosen using the Akaike information criterion implemented in Modeltest 3.7 (Posada and Crandall 1998). The ML analysis based on the concatenated data was performed using PAUP* with heuristic search and TBR swapping. Nonparametric BPs under ML were calculated using PHYML 2.4.4 (Guindon and Gascuel 2003) with 300 replicates. BI analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). To determine a suitable partitioning strategy for BI analysis, we calculated the Bayes factor of 6 different partitioning strategies (see supplementary table 1, Supplementary Material online) and compared the resulting values by the same procedure proposed by Brandley et al. (2005); and the 5-partition

←

FIG. 1.—Gene organizationof mantellid mtDNAs (color version of this figure is available as Supplementary Material online). The transcriptional direction of *H*-strand–encoding gene is shown by open arrows, and open arrowheads indicate upstream and downstream notations used in this paper. The *H*-strand–encoded and *L*-strand–encoded genes are denoted above and below each gene box, respectively. The sizes of the gene boxes do not reflect actual gene length. Closed arrows show the rearranged genes from the typical vertebrate arrangement and direction of rearrangement. The hypothesized gene rearrangement pathway within mantellids is not indicated in this figure (supplementary fig. 4, Supplementary Material online), but gray boxes represent the rearranged genes. Transfer-RNA genes (*trn*s) are designated by single-letter amino acid codes. L1 and L2 indicate *trn*s for Leu(UUR) and Leu(CUN), respectively. M1 and M2 denote 2 distinct *trnM*s. ψ indicates a pseudogene of its corresponding gene; ψ*M*ori and ψ*M*os (ps*M*ori and ps*M*os in the text) show the pseudogene of original single copied *trnM* and the mosaic pseudogene having both *trnM1* and *M2* characteristics (see supplementary fig. 1, Supplementary Material online), respectively. Other genes are abbreviated as follows: *rrS* and *rrL*, 12S and 16S ribosomal RNA genes; Cob, cytochrome *b*; nad1, 2, and 5, NADH dehydrogenase subunits 1, 2, and 5. Multiple CRs within a species are named CR1–3 (and 3′), and rCR3 indicates a CR3 remnant (see text). CRs and long (>30 bp) ISs are shown by black boxes with their lengths. The homogenized regions between multiple CRs are shown by "*** (CR1 vs. CR2)," "+++ (CR1 vs. CR3)", and "^^^(CR2 vs. CR3)" (see text and table 2). The CRs containing 3′ side repeats are denoted by "+3rep." The CRs, *trnA*, and *trnN* within parentheses indicate that these regions were partially sequenced. [a–c] Data from previous studies; [a]Sumida et al. (2001), [b]Sano et al. (2004), and [c]Kurabayashi et al. (2006).

strategy (firstst/second/third codons of protein-coding genes + *rrn*s/*trn*s) was selected. The following settings were also used for the BI analysis: number of Markov chain Monte Carlo generations = 15×10^5, sampling frequency = 10. The burn-in size was determined by checking convergences of –log likelihood ($-\ln L$) values, and first 1×10^5 generations were discarded. The statistical support of the BI tree was evaluated by Bayesian posterior probabilities (BPP). Relative rate tests were conducted using Phyltest (Kumar 1996).

Sixteen alternative phylogenetic hypotheses for mantellid frogs were compared using approximately unbiased (AU) and Kishino–Hasegawa (KH) tests implemented in CONSEL 0.1h (Shimodaira and Hasegawa 2001). Sitewise ln *L* values were calculated using PAUP* and used as input for the program. Resampling of estimated Log-likelihood of sites (Hasegawa and Kishino 1994) was conducted in 1×10^4 resamplings.

Divergence times of mantellid taxa were estimated from the concatenated sequence data by using the Bayesian molecular clock method implemented in the MultiDivtime program package (Thorne and Kishino 2002). We used the root of mantellids (=the last split between Mantellidae and other ranoids) as a single calibration point and 2 alternative constraints, 61 MYA) and 55–72 MYA, at this point were applied. These ages were estimated by Van der Meijden et al. (2007) (61 ± 11); and at least an independent study suggested a similar age (62–67 MYA, Roelants et al. 2007). Several studies indicated different time ranges for the root of mantellids: 53.6–92.6 MYA (Bossuyt and Milinkovitch 2001), 56–86 MYA (Van Bocxlaer et al. 2006), 55–91 MYA (Igawa et al. 2008), and 58–117 MYA (San Mauro et al. 2005). Thus, we also tried an alternative constraint, 53.6–92.6 MYA, at the root of mantellids. These reference point settings did not strongly affect the estimated branching ages of mantellid taxa (though the latter setting resulted in slightly older ages; data not shown); and thus, we only describe the result from the former setting. In this analysis, we excluded *Tsingymantis* because of its ambiguous phylogenetic position. Although our ML and BI analyses recovered the *Spinomantis* + stream-breeder clade, we constrained the *Spinomantis* + pond-breeder clade in branching time estimation. The *Spinomantis* + pond-breeder clade was recovered in the MP tree in this study and is strongly supported by the mt genomic characters elucidated here (see below) and by ML and BI analyses based on combined data of nuclear and mt genes (Vences et al. 2007).

Results and Discussion

Gene Organization of the *cob–trnA* Region of Mantellid mtDNAs

In this study, the partial mt genomic portion from *cob* to *trnA* (or *trnN*), covering the region of genomic reorganization known for mantellid frogs, has been newly analyzed for 12 mantellid species (table 1 and fig. 1). Adding to our previous results (Kurabayashi et al. 2006), DNA sequences of 17 species of all 12 mantellid genera are now available to compare the mt genome organization. The length of the surveyed portion varies among species from 9 676 bp (*Gephyromantis klemmeri*) to 17 792 bp (*Spinomantis microtis*), mainly due to the length difference of CR and/or copy number of CRs. In all analyzed mantellid species we identified, in this genomic portion, one CR and 17 single-copy genes typical for vertebrate mtDNAs (4 protein-coding genes: *cob*, *nad1*, *nad2*, and *nad5*; 2 ribosomal RNA genes: *rrnS* and *rrnL*; 11 transfer RNA genes for A, F, I, Lcun, Luur, M, P, Q, T, V, and W) (fig. 1). In the studied representatives of the genera *Boophis*, *Aglyptodactylus*, *Laliostoma*, *Tsingymantis*, *Boehmantis*, *Gephyromantis*, and *Mantidactylus*, these genes occur in 1 copy each, with a gene order identical among all these taxa and to the rhacophorid frog *B. buergeri* (Sano et al. 2004).

On the contrary, the eight studied species of the genera *Spinomantis*, *Blommersia*, *Guibemantis*, *Wakea*, and *Mantella* carry additional copied CRs (CR1–CR3 and/or CR3′; fig. 1). Excluding extra repeat units in particular CRs, the copied CRs have nearly identical sequences within each species (97.8–99.8%; table 2 and see fig. 2), and the homogenized sequence spans from near the 5′ end (often including the 5′ flanking gene) to downstream of the conserved sequence block (CSB) 3, a characteristic sequence element of vertebrate CR (see section below). A remnant of a third CR (rCR3), 575 bp in length, was also found in *S. microtis*. Although this rCR3 lacks CSB motifs, a part of it (ca. 280 bp) shows 95% similarity with the other 2 CRs of this species. The eight species with duplicated CRs uniformly contain two distinct *trnM*s (*trnM1* and *M2*; fig. 1, supplementary fig. 1, Supplementary Material online), although only single *trnM* is encoded in almost all metazoan mtDNAs. All of these species also contain a pseudo *trnM*: this pseudogene located just upstream of *nad2* corresponds to the original position of this gene (and is thus named ps*M*ori) and has been considered to have a function in *nad2* mRNA processing (Mabuchi et al. 2004; Kurabayashi et al. 2006). The *trnM1* and *M2* can be defined by both nucleotide similarity (average identity between two *trnM*s = 79.5%) and by a characteristic nucleotide of the 5′ adjoining anticodon; that is, "T" in *trnM1* and "C" in *trnM2*, respectively (supplementary fig. 1, Supplementary Material online). The single-copy *trnM* in other mantellids also has a C nucleotide at this position and shows a high nucleotide similarity with *trnM2* (90.1%) rather than with *trnM1* (83.5%), indicating that *trnM1* and *trnM2* correspond to the paralogue and homologue of the single copied *trnM*, respectively. *Guibemantis tornieri* and *Wakea madinika* harbor an obvious pseudo *trnM1* at the 3′ side of *nad5*, and *Guibemantis liber* also has an additional pseudo *trnM* at a similar location (between *nad5* and CR2). Notably, the pseudo *trnM* in *G. liber* shows sequence similarity with both *trnM1* (95% in 5′—22 bp) and *trnM2* (100% in 3′—35 bp), and therefore seems to be a "mosaic pseudogene" of these genes (ps*M*mos) (see fig. 2 and supplementary fig. 1, Supplementary Material online). *Guibemantis liber* furthermore encodes two copied *trnI*s (only 3 mutations between them; see fig. 2) whereas one pseudo *trnI* is found in *Blommersia blommersae*. *Spinomantis microtis* contains a possible pseudo *trnQ* (having 50% nucleotide similarity with *trnQ* in 56 bp) located just upstream of ps*M*ori.

Summarizing, eight species among the surveyed mantellids, namely the members of pond-breeding mantellines

Table 2
Similarities and Boundaries of Homogenized Sequence between Multiple CRs within Species

Species	CR1 versus CR2 Nucleotide Similarity: Alignable Sites[b] Position of 5′ and 3′ Boundaries	CR1 versus CR3[a] Nucleotide Similarity: Alignable Sites[b] Position of 5′ and 3′ Boundaries	CR2 versus CR3[a] Nucleotide Similarity: Alignable Sites[b] Position of 5′ and 3′ boundaries[c]
Blommersia blommersae	99.7%:1520 bp	99.4%:1493 bp	99.7%:1711 bp
5′ boundary	−9 (61st nt of *trnM1*) −9 (62nd nt of *trnM2*)	+7 +16	+7 +16
3′ boundary	+44 +44	+30 +30	+30 +30
Guibemantis tornieri	99.7%:1477 bp	99.8%:1565 bp	99.5%:1610 bp
5′ boundary	+13 +16	−35 (18th nt of ps*trnM1*) −35 (35th nt of *trnM1*)	+16 +13
3′ boundary	+44 +44	+44 +44	+45 +45
		First homologous region / Second homologous region	
Guibemantis liber	99.6%:1589 bp	99.3%:1630 bp / 97.1%:103 bp	99.6%:1598 bp
5′ boundary	+14 +6	−150 (third nt of *trnI*) −150 (third nt of *trnI*) / +13 +5	−36 (34th of *trnM2*) −36 (34th of ps*M*mos)
3′ boundary	+43 +43	−48 (22nd nt of *trnM1*) −48 (22nd nt of ps*M*mos) / +43 +43	+44 +44
Spinomantis microtis	98.9%:1598 bp	95.8%:284 bp (CR1 vs. rCR3)	95.4%:285 bp (CR2 vs. rCR3)
5′ boundary	+12 +57	+1070 +198	+1313 +198
3′ boundary	+71 +71	−176 lacking CSB3	−176 lacking CSB3
Wakea madinika	98.5%:2218 bp	97.9%: 1942 bp (CR1 vs. CR3′)	98.5%:1936 bp (CR2 vs. CR3′)
5′ boundary	+19 +49	+37 +10	+67 +10
3′ boundary	+46 +46	+45 + 45	+45 +45
Mantella baroni[d]	99.8%:1977 bp		
5′ boundary	+96 +1	None	None
3′ boundary	+66 +66		
Mantella bernhardi[d]	99.5%:1856 bp		
5′ boundary	+80 +2	None	None
3′ boundary	+71 +71		
Mantella madagascariensis[d]	99.4%:2047 bp		
5′ boundary	+174 +150	None	None
3′ boundary	+71 +71		

[a] CR3 includes rCR3 in *Spinomantis* and CR3′ in *Wakea*. For *Spinomantis* rCR3, the 3′ boundary is not indicated due to the lack of CSB3 in this region.

[b] The length of alignable homogenized sequence between CRs. Additional tandem repeats in one of the pair of CRs are not counted but minor insertion sites are included. Several alignable site numbers are longer than the original sequences of compared CRs by the insertion sites (e.g., original alignable sequence of *Spinomantis* rCR3 is 279 bp).

[c] 5′ boundary of homogenized sequence is indicated by the nucleotide number from the 1st nucleotide of each CR (e.g., "+7" shows that the homologous sequence starts from seventh nucleotide of the CR; see fig. 2*B*). Negative numbers indicate the upstream location of the boundary from the 1st nucleotide of the CR, and the corresponding gene position is shown in parentheses. Similarly, 3′ boundary is indicated by the nucleotide number from the last nucleotide of the CSB3 motif (see fig. 2*C*). For the 1st region of two separated homologous regions between CR1 and CR3 of *Guibemantis liber*, both 5′ and 3′ boundaries are numbered from the 1st nucleotide of each CR.

[d] Data from Kurabayashi et al. (2006).

and of *Spinomantis*, possess divergent mt genomes. Although the mt genomic structures of this group of taxa differ in many respects, there are a number of derived gene arrangements shared by some or all of them (figs. 1 and 4): 1) A derived arrangement, *trnM2*–CR2–*trnQ*–ps*M*ori located upstream side of *nad2*, is shared by all these species, excluding pseudo *trnQ* between CR2 and ps*M*ori in *S. microtis*. 2) A novel *trnI–trnM1* arrangement is commonly found. Including their pseudogenes in the count, 7 species possess this arrangement. Although *S. microtis* lacks this *trnI–trnM1* arrangement, an IS (167 bp) is found upstream of its *trnM1*. The *trnI* and/or *trnM1* is basically positioned downstream side of *nad5*; all eight species possess either the *trnI–trnM1* arrangement, or at least one of these genes, or a pseudogene of *trnI* or *trnM1* in this region. 3) An additional *trnI–trnM1* gene block in a unique position between *cob* and CR1 is found in species of *Blommersia* and *Guibemantis*. 4) Four mantellids belonging to *Spinomantis*, *Blommersia*, and *Guibemantis* carry a third CR (CR3) or its remnant (rCR3) on the upstream side of the LTPF *trn* cluster. 5) *Wakea* and *Mantella* species lack this third CR, but these species have an IS of considerable length (43–205 bp) at the same position. *Wakea madinika* has, in addition, a third CR positioned upstream side of *nad5*, and the CR–*nad5*–pseudo *trnM1* block is translocated from its original position (upstream side of the LTPF *trn* cluster) to the upstream side of *nad1* (thus, the *Wakea* CR3′ seems to be a paralogue of its first CR and not homologous to the CR3 of the other species). 6) *trnT* is translocated from its original position in the LTPF *trn* cluster to the upstream side of *nad1* in species of *Mantella*.

The distribution of these derived gene arrangements among taxa supports the respective synapomorphic origin of these rearrangements at different levels of mantellid phylogeny because alternative scenarios would require assumptions of convergent occurrence of complex molecular evolutionary steps (see section below).

Mantellid Phylogeny and Synapomorphic mt Gene Arrangements

The ML tree ($-\ln L = 71\,527.04$; fig. 3) obtained from the concatenated mt gene sequence (7 265 sites) fully agrees in topology with the BI analysis. Various alternative phylogenetic hypotheses have been suggested in previous studies for the Mantellidae (see the references denoted in table 3). Our ML tree supports the most recent taxonomic and phylogenetic proposals of Glaw and Vences (2006) and Glaw et al. (2006), in particular regarding monophyly of the following clades: 1) the family Mantellidae, 2) each of the three mantellid subfamilies (if not considering *Tsingymantis*), 3) the Boophinae + Laliostominae clade, 4) two major clades corresponding to pond breeders and stream breeders within the subfamily Mantellinae, 5) relationship of *Boehmantis* + (*Gephyromantis* + *Mantidactylus*) within the stream-breeder clade, and 6) two subclades corresponding to *Blommersia* + *Guibemantis* and *Wakea* + *Mantella* within pond breeders. These clades are supported by very high BPP and/or ML BP values, but the support for the position of *Tsingymantis* (in a clade with Laliostominae + Boophinae) is low (ML and MP bootstrap values <50 and 69, respectively, and BPP = 90). This relationship differs from the hypothesis of Glaw et al. (2006), where *Tsingymantis* occupied the most basal position of Mantellinae and was included this subfamily. Also poorly supported is the basal position of *Spinomantis* within the clade of stream breeders in our ML and BI trees. Although BPP of this clade is very high (=100), BPs of ML and MP are moderate (=77 and 63, respectively). Furthermore, the MP tree did not recover this position of *Spinomantis* but instead placed *Spinomantis* with pond breeders (hypothesis 2 in table 3).

We compared 16 alternative phylogenetic hypotheses regarding the positions of *Spinomantis* and *Tsingymantis* (table 3) using AU and KH tests. For the position of *Spinomantis*, six alternative hypotheses (hypotheses 3–8) are rejected by both tests at $P < 0.05$, but the hypothesis placing *Spinomantis* sister to the pond-breeding mantellines (hypothesis 2) is not rejected. For the position of *Tsingymantis*, two alternative hypotheses, a basal position of *Tsingymantis* in the Mantellinae (hypothesis 12) and a basal position in the Mantellidae (hypothesis 13) are not rejected. Alternative hypotheses for other taxa are largely rejected by these tests. For example, the nodal support of *Boehmantis* + *Gephyromantis* and *Mantidactylus* is low in BP of MP (=57), but alternative *Boehmantis* positions are rejected by the tests (see hypotheses 4, 5, and 8 in table 3).

Interestingly, some aspects of mantellid phylogeny are also supported by mt genomic data. Whereas boophines (*Boophis*), laliostomines (*Aglyptodactylus* and *Laliostoma*), *Tsingymantis*, and most stream-breeding and direct-developing mantellines (*Boehmantis*, *Gephyromantis*, *Mantidactylus*) share the plesiomorphic gene order, the mainly pond-breeding genera (*Blommersia*, *Guibemantis*, *Mantella*, *Wakea*) and *Spinomantis* are characterized by the derived condition, which supports the placement of *Spinomantis* sister to the pond breeders (hypothesis 2 in table 3 and fig. 4). Indeed, recent analysis including sequence data from various mt and two nuclear genes (Rhodopsin and Rag-2) recovered the clade consisting of *Spinomantis* plus pond breeders with relatively high statistical supports (BI > 99 and BP of ML = 90; Vences et al. 2007). Furthermore, some genomic characters also support the monophyly of pond breeders and their subgroups (i.e., *Blommersia* + *Guibemantis*, *Wakea* + *Mantella*) that are also unanimously supported by our ML/BI/MP analyses (fig. 4).

Frequency of Gene Rearrangements among Mantellid Lineages

Our data indicate a multiple occurrence of mt gene rearrangements in pond-breeding mantellines plus *Spinomantis*. This led to (at least minor) autapomorphic features in the mt genomes of basically every representative of the lineage for which we determined the gene order (fig. 1 and see supplementary fig. 4, Supplementary Material online). In contrast, our data provide evidence that representatives of all other mantellid genera have maintained the ancestral gene order, thus suggesting that rearrangement rates were much lower in these lineages. To test this assumption beyond the analysis of the 17 mantellid species analyzed here, we amplified in 48 additional mantellid

species a fragment spanning from the *rrns* to *nad2* and thus encompassing the area where in the pond-breeding species a large part of the duplicated and translocated genes and CRs are situated. We subsequently electrophoretically determined the approximate length of the amplified fragment to identify deviations from the ancestral gene arrangement (which corresponds to a fragment length of ca. 3.2–3.3 kbp). The complete list of taxa examined is found in the supplementary table 2 (Supplementary Material online).

All 21 additionally examined species in the pond-breeding lineage (6 *Blommersia*, 6 *Mantella*, 6 *Guibemantis*, and 3 *Spinomantis*) showed apomorphic fragment lengths, and fragment length variability among taxa was high (4.9–13 kbp; supplementary table 2, Supplementary Material online). In contrast, 22 other mantellids (1 *Aglyptodactylus*, 6 *Boophis*, 3 *Gephyromantis*, and 12 *Mantidactylus*) showed typical fragment length, indicating that in this region of these genomes, no major gene translocations or duplications had occurred. However, 2 *Boophis*, 1 *Gephyromantis*, and 2 *Mantidactylus* species showed variable lengths of 5.0–7.7 kbp, suggesting that apomorphic gene rearrangements may have also occurred in a minor proportion of species of these species-rich genera and pointing to the need of further, more detailed studies in these taxa.

Although these data refer to a limited portion of the mt genome only and rearrangements in other parts of the genomes would be ignored by this test, the results are nevertheless in agreement with the conclusions from the species of which gene order was determined in detail (fig. 1) and confirm that these species were indeed representative for their respective lineages.

No Correlation between Rates of Nucleotide Substitution and Gene Rearrangement

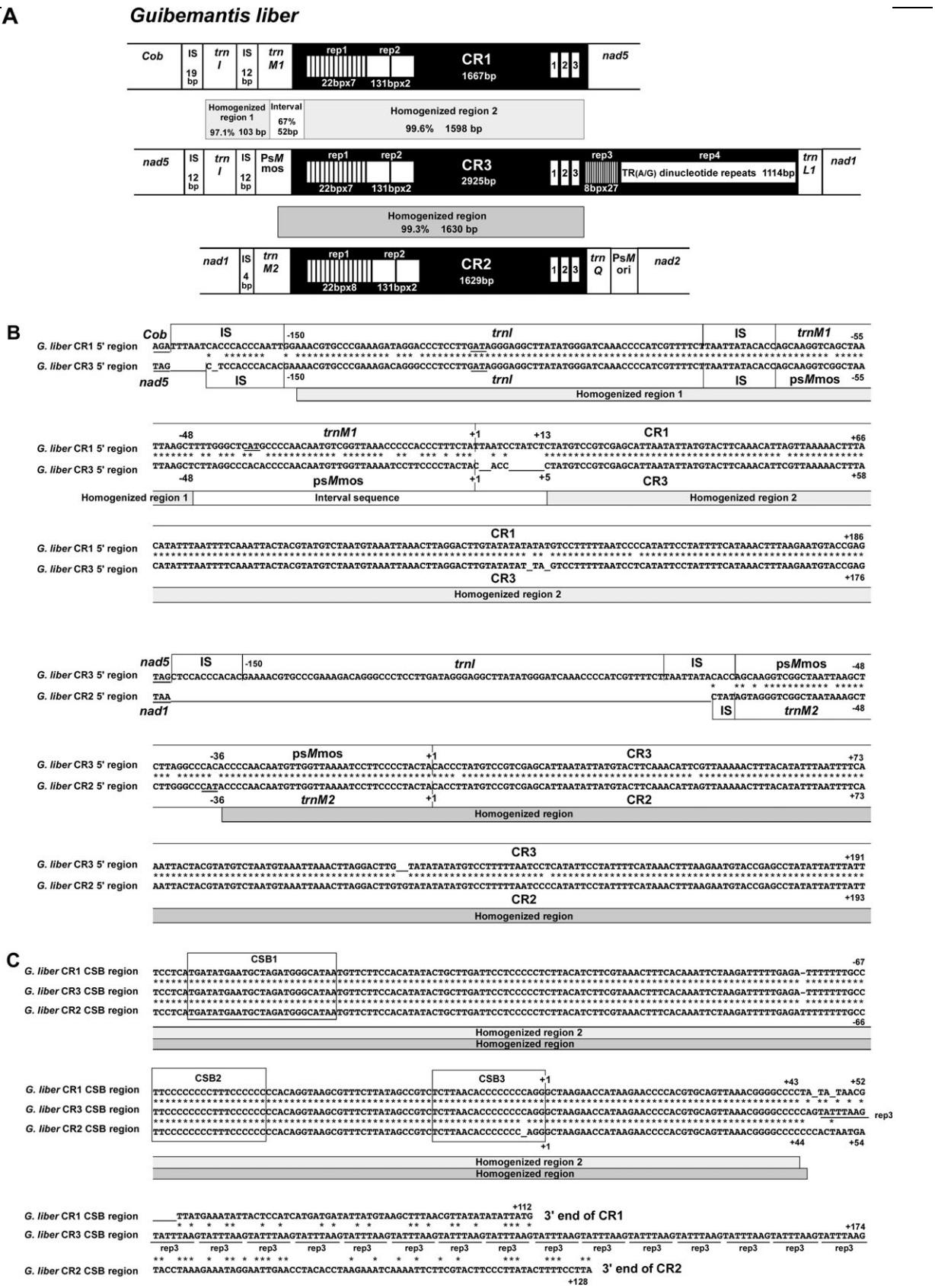
A tendency that highly rearranged mt genomes have high nucleotide substitution rate has been observed in some metazoan taxa (mollusks: Hoffmann et al. 1992; Kurabayashi and Ueshima 2000; ascidians: Yokobori et al. 2005; lampshell: Noguchi et al. 2000), and a positive correlation between the rates of nucleotide substitution and genomic rearrangement has been demonstrated in arthropod taxa (Shao et al. 2003; Xu et al. 2006). To assess this correlation in mantellid frogs, we compared the nucleotide substitution rates by relative rate tests among three mantellid groups: 1) the mostly pond-breeding mantellines (*Blommersia*, *Guibemantis*, *Mantella*, *Wakea*, and *Spinomantis*, all with highly rearranged mt genomes), 2) the mostly stream-breeding mantellines (*Boehmantis*, *Gephyromantis*, and *Mantidactylus*, without rearrangements), and 3) the remaining lineages (*Boophis*, *Aglyptodactylus*, *Laliostoma*, and *Tsingymantis*, without rearrangements). These tests did not yield any evidence for accelerated mt nucleotide substitution rate in the pond-breeding mantellines (table 4). Rather, in the full data (*rrn*s, *trn*s, and protein-coding genes) and *rrn* data sets, the pond-breeding lineage had slightly slower substitution rates than stream breeders (rate constancy rejected; $P < 0.05$) and almost identical rates as compared with the other mantellids. In protein-coding gene data, all 3 groups compared showed constant nucleotide substitution rates.

In the hypothesis of Shao et al. (2003), an acceleration of nucleotide changes leads to many illicit substitutions at both initiation and termination points of mt genome replication; and such illicit initiation and termination points cause frequent occurrence of tandem duplications in the mtDNA molecule, resulting in highly frequent gene rearrangements. However, the clear absence of a correlation between the substitution and rearrangement rates found here indicates that substitution rate is not relevant to the frequent gene rearrangements in pond-breeding mantellids.

Possible Mechanisms and Evolutionary Pathway of mt Genome Reorganization in Mantellids

What has been the process that has led to the reorganized genomic structures seen in mantellids? In general terms, the observed pattern is largely consistent with the "duplication and random loss model," the most general explanation for mt genomic reorganization (e.g., Boore 2000; San Mauro et al. 2006; and see supplementary fig. 2*A*, Supplementary Material online). In this model, first a portion of the genome is duplicated. Then, one of the copied genes (or CRs) turns into a pseudogene or is excised from the genome by subsequent mutational processes. Which copy is lost is determined randomly (by the first loss-of-function mutation), and therefore, a certain deletion pattern can recover the original gene arrangement but another leads to gene rearrangement. As a possible exception, Lavrov et al. (2002) reported the presence of nonrandom loss events in a specific insect taxon.

On the molecular level, it has been hypothesized that duplication in animal mt genomes is mainly caused by replication errors, such as slipped-strand mispairing or asynchrony in the points of initiation and termination (e.g., Mueller and Boore 2005; San Mauro et al. 2006; and see supplementary fig. 2*A*, Supplementary Material online). However, such replication errors only generate tandem duplications (Mueller and Boore 2005); and thus, this model (TDRL) has difficulties in explaining the many nontandem duplications observed in mantellids (fig. 1). Therefore, other duplication mechanisms seem to have acted in the mt genomic reorganization process. Two distinct recombination modes, "illegitimate recombination via minicircle" (Holt et al. 1997; Lunt and Hyman 1997; Kajander et al. 2000; Dowton and Campbell 2001; Mueller and Boore 2005) and "general (homologous) recombination" (Thyagarajan et al. 1996; Kajander et al. 2001; Ladoukakis and Zouros 2001; Sato et al. 2005) are the candidates of such an alternative mechanism. In the illegitimate recombination via minicircle, a part of the mt gene region is excised from 1 mt genome, forming a separate minicircle molecule that is then inserted into another genome, and the insertion results in nontandem-duplicated regions within the mtDNA molecule (supplementary fig. 2*B*, Supplementary Material online). The general recombination process (see e.g., Lewin 2003) exchanges DNA strands of 2 genomic portions with identical or similar nucleotide sequences between chromosomes (or within a DNA molecule) (supplementary fig. 2*C*, Supplementary Material online). When the exchanged DNA strands contain the same


A
Guibemantis liber
Cob
IS 19 bp
trn I
IS 12 bp
trn M1
rep1
rep2
22bpx7
131bpx2
CR1
1667bp
1 2 3
nad5
Homogenized region 1
97.1% 103 bp
Interval
67%
52bp
Homogenized region 2
99.6% 1598 bp
nad5
IS 12 bp
trn I
IS 12 bp
PsM mos
CR3
2925bp
rep3
rep4
8bpx27
TR(A/G) dinucleotide repeats 1114bp
trn L1
nad1
Homogenized region
99.3% 1630 bp
nad1
IS 4 bp
trn M2
CR2
1629bp
trn Q
PsM ori
nad2
B
G. liber CR1 5' region
G. liber CR3 5' region
G. liber CR2 5' region
Cob
nad5
nad1
IS
trnI
trnM1
psMmos
Homogenized region 1
Interval sequence
Homogenized region 2
CR1
CR3
Homogenized region
C
CSB1
CSB2
CSB3
G. liber CR1 CSB region
G. liber CR3 CSB region
G. liber CR2 CSB region
3' end of CR1
3' end of CR2
rep3

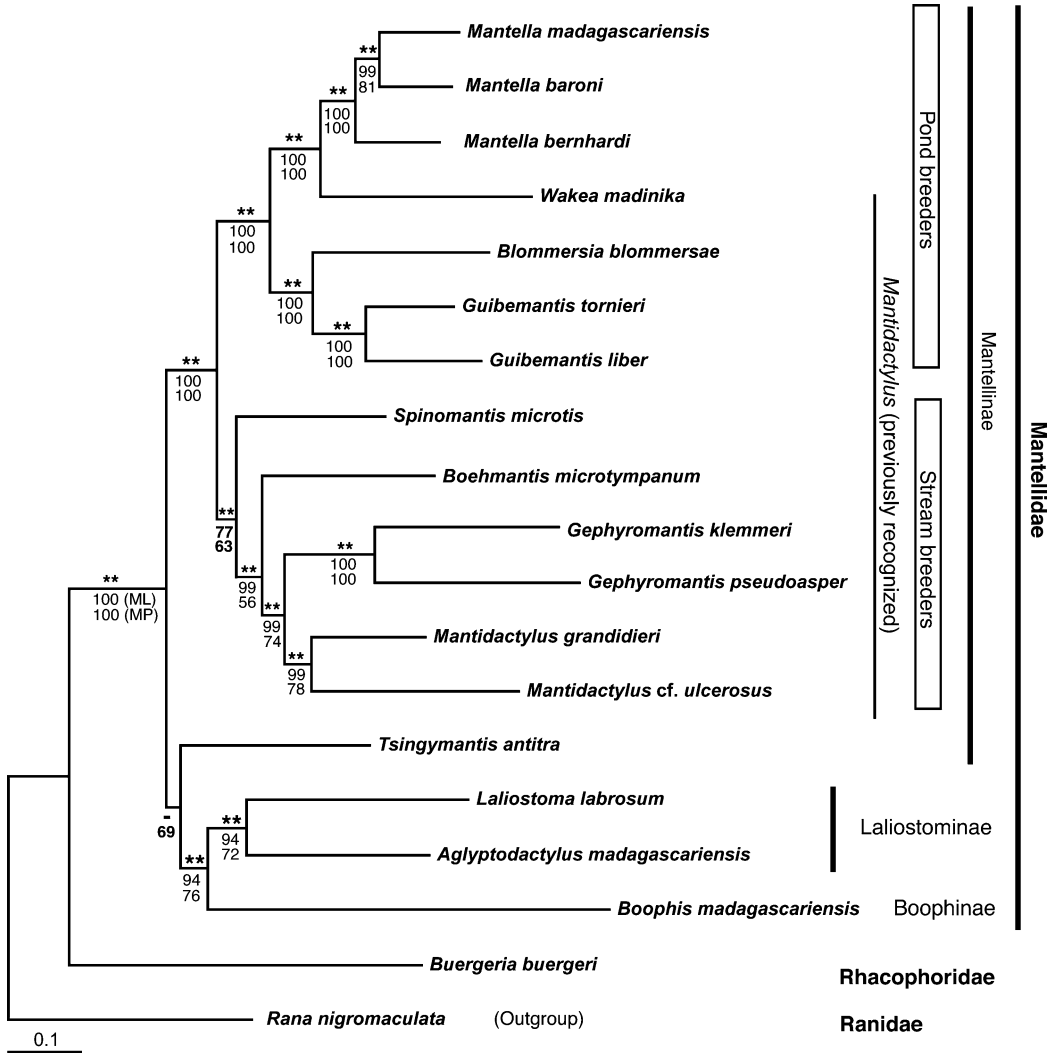


FIG. 3.—ML phylogram of mantellid frogs based on 7 265 bp of mt gene sequence. The ln *L* value of this tree is −71527.04. The BI analysis based on the same data set recovered the same topology, but the MP analysis supported a clade containing pond-breeding mantellines and *Spinomantis*. Upper and lower numbers below branches are percent nonparametric bootstrap probability from ML and MP analyses, respectively. Asterisks above branches indicate Bayesian posterior probabilities (**100).

set of genes or regions, this recombination process does not cause gene duplication but can homogenize the nucleotide sequences between the exchanged portions (gene conversion). In contrast, when the exchanged DNA strands carry unequal sets of genes, one of the resultant molecules (or genomic portions) will have an extracopied gene region from the original one (unequal crossing over).

Although any recombination had been considered to be absent in animal mt genome system (e.g., see references in Howell 1997; Shadel and Clayton 1997), many recent studies have provided experimental and circumstantial evidence of at least a limited degree of mt recombination (e.g., Thyagarajan et al. 1996; Lunt and Hyman 1997; Sato et al. 2005). Applying all these possible duplication mechanisms to explain mt genome rearrangements in mantellids results in the conclusion that one tandem duplication, followed by numerous nontandem duplications and deletions, is the most parsimonious scenario (i.e., the one minimizing the duplication and deletion events, and the number of hypothetically duplicated and deleted genes and CRs; in

←

FIG. 2.—Structure ofCRs and their flanking regions in *Guibemantis liber*. (*A*) Structure of 3 CRs and their flanking region. "rep1–4" indicates 4 distinct tandem-repeat sequences, and the length and copy number of each repeat are also denoted. The 3′ side repeats (rep-3 and rep-4) are only found in CR3 in this species. CSB1–3 are shown by open boxes. Grayed boxes denote the homogenized sequence regions between CRs. (*B*) Nucleotide sequence comparison of 5′ regions of CRs. Thin vertical lines indicate the boundaries of gene regions, and grayed boxes show the homogenized sequence regions. Positive and negative numbers indicate downstream and upstream locations from first nucleotide (=+1) of each CR, respectively. (*C*) Nucleotide sequences comparison of CSB regions. The putative CSBs are boxed. The nucleotide positions are numbered from the 3′ adjoining nucleotide (=+1) of CSB3.

Table 3
Comparison of Alternative Phylogenetic Hypotheses

Alternative Hypothesis	$\Delta\ln L$[a]	AU	KH	Rejection ($P < 0.05$)
1 ML and BI tree (fig. 3)[(a)]	ML	0.803	0.606	
Alternative *Spinomantis* positions				
2 *Spinomantis* + pond-breeding mantellines[(b)]	−8.0	0.236	0.187	−/−
3 *Spinomantis* + all mantellines	−16.0	2×10^{-4}	0.012	+/+
4 *Spinomantis* + *Boehmantis*	−33.3	0.003	0.004	+/+
5 *Spinomantis* + (*Mantidactylus* + *Guibemantis*)	−36.7	10^{-5}	0.001	+/+
6 *Spinomantis* + *Guibemantis*	−80.1	4×10^{-6}	8×10^{-5}	+/+
7 *Spinomantis* + *Mantidactylus*	−80.1	4×10^{-6}	8×10^{-5}	+/+
8 *Spinomantis* + (*Boehmantis* + pond-breeding mantellines)	−69.7	2×10^{-35}	0	+/+
Alternative *Tsingymantis* positions and intersubfamilial relationships				
Mantellinae + Laliostominae[(c)]				
9 *Tsingymantis* + Boophinae	−33.8	0.003	0.008	+/+
10 *Tsingymantis* + Mantellinae	−15.3	0.008	0.046	+/+
11 *Tsingymantis* + all other mantellids	−16.5	0.009	0.042	+/+
Boophinae + Laliostominae[(d)]				
12 *Tsingymantis* + Mantellinae[(e)]	−4.8	0.243	0.183	−/−
13 *Tsingymantis* + all other mantellids	−1.6	0.577	0.394	−/−
Mantellinae + Boophinae[(f)]				
14 *Tsingymantis* + Mantellinae	−11.2	0.189	0.132	−/−
15 *Tsingymantis* + all other mantellids	−15.9	0.022	0.049	+/+
16 *Tsingymantis* + Laliostominae	−12.0	0.040	0.022	+/+

NOTE.—The results or references supporting each hypothesis are indicated using superscripted alphabets in parentheses as follows: [(a)] ML and BI trees of this study; [(b)] MP tree in this study, Vences et al. (2007); [(c)] Bossuyt and Milinkovitch (2000), figure 1 in Vences et al. (2003), figure 1 in Roelants et al. (2004), Frost et al. (2006), Vences et al. (2007); [(d)] Richards et al. (2000), figure 2 in Vences et al. (2003), figure 2 in Roelants et al. (2004); [(e)] Glaw et al. (2006), [(f)] Van der Meijden et al. (2005), Van Bocxlaer et al. (2006).

[a] Differences in the log-likelihoods (ln L) of the alternative trees from that of the ML tree.

parallel, all nontandem duplications are presumed to be caused by processes related to recombination). The preferred scenario is further elaborated in supplementary figure 4 (Supplementary Material online). According to this hypothesis, gene rearrangements among mantelline taxa were caused mainly by alternative deletions from an ancestral genome with a duplicated condition (a total of eight deletion events assumed in our scenario; i.e., del-2 to del-9 in supplementary fig. 4, Supplementary Material online) rather than by specific duplications (three duplication events; dup-4 to dup-6). The majority of these duplication events would have been nontandem (five events; dup-2 to dup-6) and only one tandem duplication (dup-1) may have occurred, indicating that recombination rather than replication slippage has probably been the main cause of mt genomic reorganization during mantellid evolution. It is also noteworthy that the downstream boundary (in the *L*-strand) of the three expected duplicated portions seems to fall into the CR in 3 cases. This supports the importance of the CRs as possible "recombination hot spot" which will be further explored in the following sections and indicates that the high frequency of mt genome reorganizations in *Spinomantis* + pond-breeding mantellines may be explained by the accidental fixation of multiple CRs in their common ancestor.

Multiple Control Regions and Their Flanking Regions with Nearly Identical Nucleotide Sequences

In this study, all taxa with multiple CRs contain putative CSB1–3 elements in each of their CRs (see fig. 2). The presence of CSBs suggests that all multiple CRs within a single mtDNA molecule have a potential to form a D-loop structure because CSBs has been hypothesized to be essential for the synthesis of D-loop DNA (Cairns and Bogenhagen 1986; Shadel and Clayton 1997).

All multiple CRs within mantellid species include portions of nearly identical sequence, except for additional numbers of 5′ repeats in certain CRs (table 2 and fig. 2). These homogenized sequences start from the 5′ end neighborhood and extend beyond the CSB3 motif. In many cases, the 5′ boundaries of the homogenized sequences are located inside CRs. However, the continuous identical sequence across the 5′ flanking genes is also observed in *Blommersia* and *Guibemantis* species (table 2 and fig. 2*B*). In *B. blommersae*, the homogenized sequence between CR1 and CR2 stretches to their 5′ flanking *trnM1* and *trnM2* along 9 bp. Similarly, in *G. tornieri*, the homogenized sequence continues across the 5′ flanking regions of CRs and 35 bp are found between the *trnM1* and ps*trnM1*. But, different from *Blommersia*, *trnM1* and ps*trnM1* are located neighboring CR1 and CR3, respectively. Most unique is the 5′ flanking region of CR3 in *G. liber* (fig. 2*A* and *B*). In this case, the sequence homogenized between CR3 and CR1 is separated into two partitions; that is, a 103-bp region (homogenized region 1 in fig. 2) spanning from the 3rd nucleotide of *trnI* to the 22nd nucleotide of ps*M*mos (corresponding to *trnM1* in CR1 upstream) and 1.6 kbp starting from the fifth nucleotide of CR3 (=13th nucleotide of CR1) and continuing to downstream of CSB3. Inbetween these homogenized regions are 47 nucleotides of 3′ ps*M*mos and 5 nucleotides of 5′ CR3 (total 52 nucleotide with 67% similarity to the relative 60 bp of the 3′ *trnM1*–5′ CR1 region). Meanwhile, the 5′ boundary of the homogenized sequence between CR3 and CR2 of this species falls into the above interval sequence (=34th nucleotide of ps*M*mos

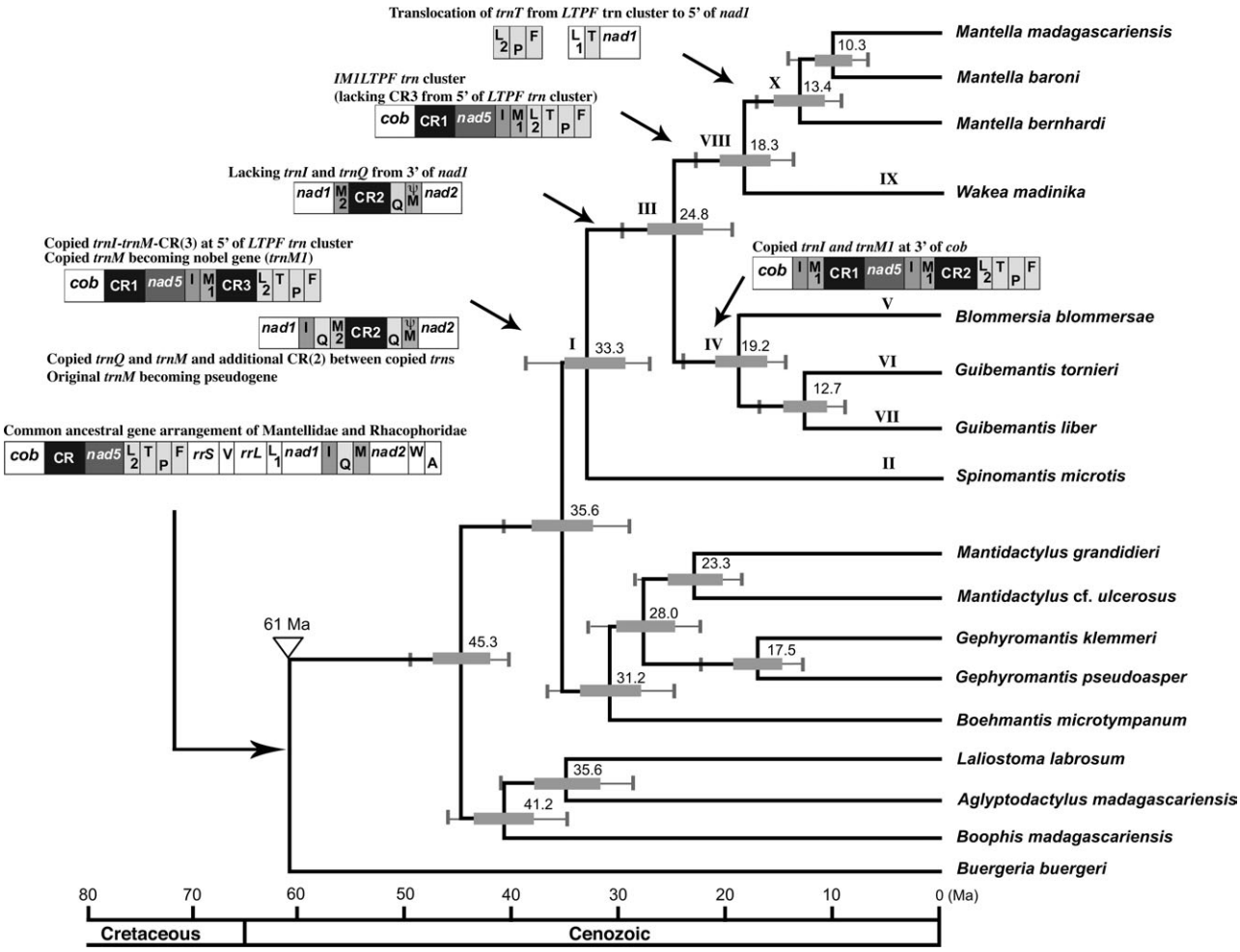


FIG. 4.—Divergence times and synapomorphic mt genomic characters of mantellid taxa (a color version of this figure is provided as a Supplementary Material online). Estimated divergence times with standard deviation (gray boxes) and 95% confidence intervals (gray lines) are shown for each node. Inset figures show probable synapomorphic mt characters for each clade. The roman numbers above the nodes indicate the lineages of gene rearrangement events described in supplementary figure 4 (Supplementary Material online).

and *trnM2*). Thus, the 5′ flanking region of CR3 has a nested homologous sequence with respect to CR1 and CR2.

Homogenized sequences between two copied CRs are often observed in animal mt genomes. But, the presence of 3 CRs found in several mantellines is rare (only known from a *Plethodon* salamander thus far; Mueller and Boore 2005). It is also relevant that homogenized sequences along 5′ flanking genes are found between different CR combinations (i.e., CR1 vs. CR2 in *Blommersia*, CR1 vs. CR3 in *G. tornieri*). This strongly suggests that sequence homogenization of multiple CRs occurs not between a specific pair of CRs but between random pairs, also meaning that the process of sequence homogenization is not related to particular genomic portions or to relative distance of CRs.

Possible Roles and Mechanisms of General Recombination in Concerted Sequence Evolution

As in mantellids, duplicated CRs have been reported from a number of animal mt genomes (octopuses: Yokobori et al. 2004; ticks: Black and Roehrdanz 1998, Campbell and Barker 1999; sea fire-fly: Ogoh and Ohmiya 2007; sea cucumbers: Arndt and Smith 1998; fishes: Lee et al. 2001, Inoue et al. 2003; salamanders: Mueller and Boore 2005; rhacophorid frogs: Sano et al. 2005; snakes: Kumazawa et al. 1996; birds: e.g., Mindell et al. 1998, Eberhard et al. 2001). These multiple CRs within species (and occasionally their upstream regions) have nearly identical nucleotide sequence with few exceptions. To explain such homogenized sequences between CRs, 2 alternative mechanisms of concerted evolution have been postulated: one is the tandem-duplication model by replication slippage (this model is based on the strand-asynchronous replication model) and another is the gene conversion model by general recombination (Kumazawa et al. 1998). Of these alternatives, the gene conversion model is strongly supported by the nested homologous region observed at the 5′ flanking region of CR3 found in *G. liber*. If the homogenized sequences between CRs were maintained by tandem duplication via replication slippage, the sequences of the duplicated region would need to be sequentially homogenized (see fig. 7a of Kumazawa et al. 1998); and thus, this mechanism alone

Table 4
Results of Relative Rate Tests among Lineages of Mantellids

Comparison	Lb1	Lb2	*Z*
All genes			
A/B	0.1261	0.1352	2.92116*
A/C	0.1392	0.1379	0.40742
B/C	0.1473	0.1368	2.98889*
Protein-coding genes			
A/B	0.1623	0.1702	1.76642
A/C	0.1792	0.1804	0.23794
B/C	0.1853	0.1785	1.31056
rrns			
A/B	0.07018	0.08721	3.72420*
A/C	0.07643	0.08108	1.06295
B/C	0.09228	0.0799	2.48744*

NOTE.—A, pond-breeding lineage + *Spinomantis*; B, stream-breeding lineage; C, other mantellids. *Rana nigromaculata* and *Buergeria buergeri* were used as outgroups. Lb1 and Lb2, average branch lengths of first and second group in analysis. Z, parameter calculated by Phyltest (Kumar 1996). Analyses were carried out under Kimura-2-parameter distances. *Significantly different substitution rates ($P < 0.05$).

cannot maintain the nested homogenized portions. Furthermore, the tandem-duplication model cannot produce the nested homogenized sequence in *Guibemantis*. In contrast, the general recombination mechanism can easily create and maintain the observed pattern (see supplementary figs. 3*A* and 4, Supplementary Material online).

Another remarkable observation from this study is that almost all extracopied genes (with only one exception of two copied *trnI*s in *G. liber*) are deleted or become pseudogenes or ISs; at least 15 duplicated genes have apparently been deleted (supplementary fig. 4, Supplementary Material online). In contrast, many of the copied CRs have been maintained with nearly identical sequences, and only two CR deletion events need to be invoked. Similar situations have been reported from other animal mt genomes with multiple CRs (Kumazawa et al. 1996; Mueller and Boore 2005). These observations indicate that multiple CRs are particularly prone to homogenization compared with other genomic portions, suggesting that CRs could be considered as "hot spots" of recombination.

Why do CRs have such a high recombination frequency? To answer this question, a novel scheme for vertebrate mtDNA replication may be suggested here. Recent studies based on the technique of 2-dimensional agarose gel electrophoresis have challenged the strand-asynchronous model, the conventional replication scheme for animal mtDNA (Clayton 1982), and suggested that usual leading-ragging replication is a major mode of vertebrate mtDNA replication (Holt et al. 2000; Yang et al. 2002; Bowmaker et al. 2003; Reyes et al. 2005; Yasukawa et al. 2006): replication of vertebrate mtDNA is initiated from a wide genomic portion without any specific site, in a region of approximately 4 kbp across *cob* to the 5′ region of the CR in mammals (Bowmaker et al. 2003) and throughout the mt genome (only excluding the replication fork barrier [RFB] region in the CR) in birds (Reyes et al. 2005). The 3′ side of the CR (D-loop region) corresponds to a RFB rather than the replication origin of *H*-strand (O_H) so far considered. Furthermore, because replication fork stalls were observed from many stretches within the D-loop region, it has been postulated that the D-loop itself assumes the role of RFB (Bowmaker et al. 2003) rather than specific *cis*-elements. Based on this replication scheme, the 3′ end of the nascent *L*-strand is suspected to remain free at the RFB region until replication restarts (or replication is completed by the backward directional replication fork; see supplementary fig. 3*A*, Supplementary Material online). According to the Holliday (1964) model of recombination, this process is initiated by formation of free 3′ ends of two homologous DNAs by single-strand nicking; subsequently strand exchange takes place between each free end and the complementary strand in the opposite homologue (also see supplementary fig. 3*A*, Supplementary Material online). Therefore, the long time exposure of the free 3′ end of the nascent strand is thought to lead to a high frequency of recombination. Especially, strand exchange of the nascent strands will take place very easily when replication forks are arrested at RFB regions in CRs of two mtDNA molecules (or within a taxon, if two independent replication forks occur in a single mtDNA) (supplementary fig. 3*A*, Supplementary Material online). It should be noted that, based on the strand-asynchronous replication model, the same concept for a high recombination potential of the free 3′ end of the nascent *H*-strand in light-strand replication origin in human mtDNA has been proposed by Kraytsberg et al. (2004). These authors also suggested that the high recombination rate at the CR region is caused by recombination of D-loop DNA itself. However, this explanation does not seem to match the expected recombination in mantellid CRs. In fact, the D-loop DNA initiates from upstream neighbor of or within CSB motifs and does not extend to the 5′ flanking genes, but nevertheless, the region from the 5′ flanking genes to the 3′ portion of CSB3 seems to be included in the recombination unit in mantellid CRs (see below).

The frequent recombination mediated by RFBs of distinct CRs also becomes the explanation for the concerted evolution observed between multiple CRs because successive recombination processes initiated from strand exchange of the nascent *L*-strand will cause gene conversion, resulting in homogenized CRs (and 5′ flanking genes with similar nucleotide sequence; see supplementary fig. 3*A*, Supplementary Material online). Furthermore, in this concerted evolution model, gene conversion only occurs at the upstream region from the 3′ end of the nascent *L*-strand. In mantellid mtDNAs, the 3′ boundary of the homogenized region falls into the 30–71st nt portion from CSB3, and only the region upstream from this portion is homogenized. These observations match with predictions of the concerted evolution model and suggest that synthesis of the nascent *L*-strand often arrests at the CSB3 downstream portion in mantellid mtDNAs.

Consequently, if we accept the novel replication model for animal mtDNA as outlined above, the high frequency of recombination among CRs and the long maintenance of homogenized CRs (and of their 5′ flanking genes) can be explained without any contradiction. Until now, for the mechanism of concerted evolution of CRs in animal mt genomes, two alternative hypotheses, recombination or replication, have been discussed (Kumazawa et al. 1998; and see Ogoh and Ohmiya 2007). In our model, however, both

recombination and replication contribute to the concerted evolution, because the mechanism of concerted evolution is likely recombination, but recombination occurs during the period of replication fork pausing.

In our data set, there are two short regions having identical sequence with other genomic portions of the same mtDNA molecule: *trnI*–ps*M*mos (103 bp in length) has identical sequence with *trnI–trnM1* in *G. liber*, and a region of *Spinomantis* rCR3 (ca. 280 bp) shows a homogenized sequence with those of other CRs of this species (table 2). These short homogenized regions would have been involved in the region of high frequent recombination but they are now discrete from the recombination region (rCR3 lacks CSBs and thus does not appear to be able to form a D-loop). Short recombinant mtDNA fragments occurring at unrelated portion from CR (50- to 100-bp fragments in *nad1*, *nad2*, and *cox1*) have been shown by using mito-mouse, which artificially carries two distinct mtDNA molecules in an individual; but mito-mouse analysis also suggested that such recombination occurs at low frequency (Sato et al. 2005).

Possible Roles of General Recombination in the Process of Gene Rearrangement

Generally, animal mtDNA contains only one set of single copied genes and a single CR; and homoplasmy, mediated by strict maternal inheritance, is a common feature of almost all metazoans (e.g., Wolstenholme 1992). In such a situation, homologous recombination does not seem to occur within a mtDNA molecule, and the possible recombination between molecules will not result in any changes of neither nucleotide sequence nor gene arrangement (Sato et al. 2005). In those mantellid mt genomes that possess several copied CRs and gene regions, however, general recombination seems to have an important influence on gene rearrangement events. At least, general recombination has the potential to cause gene replacement between genes with high nucleotide similarity (ca. 80%) by gene conversion events, as in the cases of *trnM1* and *M2* of *Blommersia* and *Guibemantis*.

General recombination in the mt genome also seems to be responsible for unequal crossing over. First, in mantellids, the copy numbers of 5′ side tandem repeats occasionally differ among multiple CRs within species (see fig. 2). The different repeat numbers can be interpreted as a result of the unequal crossing over because unequal crossing over is frequently caused by simple repetitive sequences during the recombination process (e.g., Lewin 2003).

Second, the duplication event of the *trnI–trnM1* block into the region upstream of CR1 in the common ancestor of *Guibemantis* and *Blommersia* (supplementary fig. 4, Supplementary Material online) seems to be an example of unequal crossing over as well. This nontandem duplication is difficult to explain by illegitimate recombination via minicircle (see supplementary fig. 3*B*, Supplementary Material online) because a minicircle insertion inevitably changes the nucleotide sequence at the insertion site (i.e., the boundary of newly copied region), and/or the insertion boundary must have an IS sequence corresponding to the remnant of the extra sequence involved in the minicircle. In contrast, in *Blommersia* and *Guibemantis*, continuous identical sequences across the boundaries (*trnM*–CR) of the original and the copied region are observed. These homologous sequences suggest that this duplication event has been caused by an accidental involvement of 5′ flanking genes in a recombination process between CRs.

The recombination process mediated by RFB lying in CR might have played a role in the other duplication events during mantellid evolution. If the 3′ end of the nascent *L*-strand arrested at RFB misprimes to another mtDNA molecule and illegitimate replication starts from the mispriming site, one of the resulting mtDNA molecules will contain a duplicated region corresponding to the nascent *L*-strand (supplementary fig. 3*B*, Supplementary Material online). The 3′ boundaries of two nontandem duplicated regions predicted in mantellid evolution are near to the CSB region of CR (dup-2 and dup-3 in supplementary fig. 4, Supplementary Material online). This might indicate the occurrence of duplication events caused by the nascent strand mispriming process. A similar replication mode, recombination-dependent DNA replication, is known from genome systems of *E. coli* and other organisms and organelles; and this illicit replication mode is thought to play important roles in DNA repair and replication restart under inhibited condition of replication fork progression (Kogoma 1997).

Finally, a model of minicircle formation via two RFB (*Ter*) sites has been postulated for the *E. coli* genome (Bierne et al. 1997). Because the region between two RFBs forms a minicircle in this model, the upstream boundaries of the nontandem duplications expected in mantellid mtDNAs do not match for this model. However, this minicircle formation probably would lead to highly frequent duplication of the region between RFBs and therefore may be one possible explanation for the highly frequent gene rearrangements that are often found between duplicated CRs (Kumazawa and Endo 2004).

It is generally recognized that the CR neighborhood is one of gene rearrangement hotspot in metazoan mt genomes (e.g., Mindell et al. 1998; Boore 1999); and, as in mantellids, divergent genomic structures have been commonly observed from many animal lineages with multiple CRs (e.g., Kumazawa et al. 1996, 1998; Inoue et al. 2003). The recombination mediated gene rearrangement modes proposed here could be a general explanation for this phenomenon. On the other hand, other rearrangement hot spots, that is, WANCY and IQM *trn* clusters, are also known from vertebrates (see Macey et al. 1997; Inoue et al. 2003; San Mauro et al. 2006). For these CR extraneous regions, our rearrangement models seem not to be relevant directly. Indeed, even in pond-breeding mantellids, the WANCY *trn* cluster retains the typical vertebrate condition (Kurabayashi et al. 2006; and A.K. unpublished data). Furthermore, almost all rearrangements of these *trn* clusters in vertebrates can be explained by only a single tandem duplication (e.g., San Mauro et al. 2006). Thus, at present, the TDRL model would be a suitable explanation for gene rearrangements in these regions. It should be also noted that the IQM *trn* cluster appears to be a particularity of a duplicated CR formation. Adding to pond-breeding

mantellids, several vertebrate lineages possess a second CR in this region (snakes, Kumazawa et al. 1996; a gulper eel, Inoue et al. 2003; a hyperoliid frog, A.K. unpublished data). Furthermore, several species of these taxa retain duplicated copies or remnants (=pseudogenes) of these *trn*s around the second CR (see fig. 1 and Kumazawa et al. 1996). These may suggest that the first step of 2nd CR formation is duplication of the *trn* cluster by tandem duplication; and then, a CR is inserted in the previously duplicated region by recombination (see dup-1 to dup-2 in supplementary fig. 4, Supplementary Material online).

Does Recombination Discount the Phylogenetic Utility of mt Gene Arrangement?

Gene rearrangements of animal mtDNA are considered to be effective phylogenetic markers (Brown 1985; Wolstenholme 1992; Smith et al. 1993; Boore and Brown 1994, 1998; Boore et al. 2005). In fact, rearrangements supposedly occur only at low frequency and homoplasy is rare in animal mt genomes (e.g., Boore and Brown 1998; Kurabayashi et al. 2006); and the lack of recombination has been considered as one of the reasons for this (Brown 1985; Boore and Brown 1994). In this study, however, we found evidence for gene duplication events that can best be explained by general or illegitimate recombination (dup-2 to dup-6 in supplementary fig. 4, Supplementary Material online), and the resultant duplicated condition seems to be a main cause of the high frequency of subsequent gene rearrangements in pond-breeding mantellines. But at the same time, we show the good congruence of the molecular phylogenetic reconstructions with gene order synapomorphies, indicating that recombination by itself did not lead to homoplasious gene rearrangements.

To explain this pattern, several hypotheses can be drawn. 1) Recombination in mtDNA mainly begins inside CRs (RFB region), proceeds to the upstream side, and stops at a region with less sequence similarity (see supplementary fig. 3*A*, Supplementary Material online). Thus, gene replacement (gene conversion) occurs only exceptionally and is limited to cases where two genes with high nucleotide similarity are located at 5′ sides of duplicated CRs. 2) Occurrence of unequal crossing over as an illegitimate result of homologous recombination is also limited to 5′ sides of duplicated CRs and occurs at only low frequency. 3) Nontandem duplication can emerge from processes of illegitimate recombination or from mispriming on the nascent strand. However, because almost all boundaries of animal mt genes have very few intergenic nucleotides, integration of a minicircle or mispriming of a nascent DNA strand, will cause destruction of a certain single copied gene in the resulting mtDNA molecule (Boore and Brown 1994; and see supplementary fig. 2*C*, Supplementary Material online). Thus, such integration and mispriming events occur rarely and only at the noncoding regions with significant length (i.e., CR), previously copied regions (like dup-2 in supplementary fig. 4, Supplementary Material online), or rarely at the precise gene boundary between single-copy genes.

Although recombination by itself will thus not lead to a high degree of gene rearrangements, it can possibly become a remote cause of parallel occurrence of the same rearrangement pattern. Boore and Brown (1998) and Boore et al. (2005) pointed out that the occurrence of identical duplications followed by identical deletions is unlikely, but homoplasious rearrangement would occur if a duplicated genomic condition persisted through several lineage splits. In this context, several descendent lineages would inherit the identically duplicated genes; subsequently, independent events of identical deletions may cause the parallel occurrence of the same derived gene arrangement with relative ease in remotely related groups. Several genomic conditions reported here almost certainly correspond to this situation. For example, two copied *trnI–trnM1*–CR blocks seem to have been maintained through the splits of *Spinomantis*, *Blommersia*, and *Guibemantis*, and the *trnI* upstream of CR3 was deleted independently in each lineage leading to *S. microtis* and *G. tornieri* (supplementary fig. 4, Supplementary Material online). Thus, except for the ISs between *nad5* and *trnM1*, these two species have the same derived *nad5–trnM1*–CR3 (rCR3 in *Spinomantis*) arrangement (fig. 1) despite their appartenances to rather distant phylogenetic lineages. An important conclusion from our study is that such processes can extend over very long time spans. Boore et al. (2005) mentioned that duplicated gene conditions in animal mt genomes are usually short lived. In mantellids, the copied CRs and their 5′ flanking genes have been maintained by recombination for a long span through lineage splits (>33 MYA; fig. 3). There is no doubt that mt genomic rearrangements are in most cases appropriate markers to resolve ancient and fast divergence processes, but a careful estimation of the rearrangement pathway is especially required where derived arrangements consisting of CRs and their 5′ genes are to be interpreted as synapomorphic characters.

Supplementary Material

Supplementary tables 1 and 2, figures 1–4, and color versions of figures 1 and 4 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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