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The phylogenetic position of toadfishes (order Batrachoidiformes) in the higher ray-finned fish as inferred from partitioned Bayesian analysis of 102 whole mitochondrial genome sequences

MASAKI MIYA[1]*, TAKASHI P. SATOH[2] and MUTSUMI NISHIDA[2]

[1]*Department of Zoology, Natural History Museum & Institute, Chiba, 955–2 Aoba-cho, Chuo-ku, Chiba 260–8682, Japan*
[2]*Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano-ku, Tokyo 164–8639, Japan*

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In a previous study based on 100 whole mitochondrial genome (mitogenome) sequences, we sought to provide a new perspective on the ordinal relationships of higher ray-finned fish (Actinopterygii). The study left unexplored the phylogenetic position of toadfishes (order Batrachoidiformes), as data were unavailable owing to technical difficulties. In the present study, we successfully determined mitogenomic sequences for two toadfish species (*Batrachomoeus trispinosus* and *Porichthys myriaster*) and found that the difficulties resulted from unusual gene arrangements and associated repetitive non-coding sequences. Unambiguously aligned, concatenated mitogenomic sequences (13 461 bp) from 102 higher actinopterygians (excluding the ND6 gene and control region) were divided into five partitions (1st, 2nd and 3rd codon positions of the protein-coding genes, tRNA genes and rRNA genes) and partitioned Bayesian analyses were conducted. The resultant phylogenies strongly suggest that the toadfishes are not members of relatively primitive higher actinopterygians (Paracanthopterygii), but belong to a crown group of actinopterygians (Percomorpha), as was demonstrated for ophidiiform eels (Ophidiiformes) and anglerfishes (Lophiiformes) in the previous study. We propose revised limits of major unranked categories for higher actinopterygians and a new name (Berycomorpha) for a clade comprising two reciprocally paraphyletic orders (Beryciformes and Stephanoberyciformes) based on the present mitogenomic phylogenies.

ADDITIONAL KEYWORDS: Actinopterygii – mitogenome – Paracanthopterygii – systematics – Teleostei.

INTRODUCTION

The ray-finned fish (Actinopterygii) form by far the most diversified group of all vertebrates, comprising over 23 600 species placed in 42 orders, 431 families, and 4075 genera (Nelson, 1994). Following the publication of Greenwood *et al.*'s (1966) seminal study, the advent of cladistic theory (Hennig, 1966; Wiley, 1981) and a review based on cladistic methodology (Lauder & Liem, 1983), concepts of actinopterygian phylogenies have changed from 'vertical' relationships among evolutionary grades to 'horizontal' sister-group relationships among clades (= monophyletic groups).

*Corresponding author. E-mail: miya@chiba-muse.or.jp

Numerous comparative anatomical studies have been conducted in attempts to delimit the taxa monophyletically (with concurrent breakups of paraphyletic groups; Nelson, 1989) and to resolve their inter- and intrarelationships using cladistics. However, all have aimed at more or less local resolutions of the actinopterygian phylogenies (for reviews, see Johnson & Patterson, 1993; Johnson, 1993; Helfman, Collette & Facey, 1997; Inoue & Miya, 2001; G. Nelson, 1989; J. Nelson, 1994; Stiassny, Parenti & Johnson, 1996). Consequently, the higher-level relationships, as illustrated in reference books such as *Fishes of the World* (Nelson, 1994) and *The Diversity of Fishes* (Helfman *et al.*, 1997) resemble a mosaic of local phylogenetic hypotheses based on different sets of morphological

characters. Subsequent molecular phylogenetic studies have been unable to alter the situation (e.g. see Kocher & Stepien, 1997), probably owing to shorter sequences from limited taxonomic representation (Stepien & Kocher, 1997; Miya, Kawaguchi & Nishida, 2001; Miya *et al*., 2003).

A series of recent studies based on whole mitochondrial genome (mitogenome) sequences (Inoue *et al*., 2003a, b, 2004; Ishiguro, Miya & Nishida, 2003; Miya *et al*., 2003; Saitoh *et al*., 2003) has, however, shed a new light on these relationships. Together with previous pioneering works (Tzeng *et al*., 1992; Chang, Huang & Lo, 1994; Zardoya, Garrido-Pertierra & Bautista, 1995; Johansen & Bakke, 1996; Noack, Zardoya & Meyer, 1996; Miya & Nishida, 1999, 2000b; Saitoh *et al*., 2000), they sampled all major lineages of actinopterygians (with the exception of Batrachoidiformes) at the ordinal level, with their data sets partially overlapping each other. They were, additionally, all based on the same sets of strictly homologous characters, with no potential for paralogous comparisons among sampled genes owing to ancient genome duplication (Martin & Burg, 2002) as recently suggested for actinopterygian nuclear genomes (e.g. Taylor *et al*., 2003; Van de Peer, Taylor & Meyer, 2003).

It should be noted that the resultant phylogenies were well resolved, with many basal clades receiving high statistical support irrespective of the analytical methods used (see Simmons & Miya, 2004). The mitogenome sequences have thus produced a new, comprehensive perspective of the higher-level relationships of actinopterygians, portions of which have been supported by other recent molecular phylogenetic studies using different markers (Wiley, Johnson & Dimmick, 2000; Chen, Bonillo & Lecointre, 2003).

The Batrachoidiformes (toadfishes) were excluded from our previous analysis (Miya *et al*., 2003) due to technical difficulties occurring during the DNA experiments. A taxonomic assemblage, the Batrachoidiformes contains a monotypic family, listed in Nelson (1994) as comprising three subfamilies, 19 genera, and 69 living species of marine, brackish, and freshwater actinopterygians. According to FishBase (http://www.fishbase.org/search.cfm), however, there are, as of 24 July 2004, 21 genera and 78 species.

Toadfishes are often used as experimental animals in biomedical and physiological research (e.g. Walsh, 1997; Reimschussel, 2001; Wilkie, 2002); they were one of the first animals to be used in an experiment in space (Boyle *et al*., 2001). They are all shallow-water benthic and temperate to tropical species. They are most diverse in the New World, whereas most tropical marine fishes reach their greatest levels of diversity in the Indo-Australian region (Helfman *et al*., 1997). Some members can produce audible sounds with the swim bladder and can live out of water for several hours (Nelson, 1994).

Taxonomic revisions have been published for several genera, including *Batrachoides* (see Collette & Russo, 1981) and *Porichthys* (see Walker & Rosenblatt, 1988), and undescribed species are still being discovered (e.g. Collette, 1995, 2001; Greenfield, 1997, 1998). Molecular markers have been employed to investigate phylogeography (Avise, Reeb & Saunders, 1987) and phylogeny (Freshwater *et al*., 2000) for species of the genus *Opsanus*. The Batrachoidiformes have traditionally been allied with the Lophiiformes (anglerfishes) based primarily on osteological characters of the cranium (Regan, 1912; Rosen & Patterson, 1969; Gosline, 1971) and more recently on features of the gill arches and occiput as additional synapomorphies (Patterson & Rosen, 1989). A sister-group relationship of the two groups has long been posited, and together they are termed the Pediculati (Regan, 1912; Patterson & Rosen, 1989; but see Jamieson, 1991: 162). However, Pietsch (1984: 325) has correctly stated that the relationship has has yet to be proven conclusively.

Batrachoidiformes and Lophiiformes, along with other relatively primitive higher actinopterygians (Percopsiformes, Ophidiiformes and Gadiformes), have been placed in the superorder Paracanthopterygii (Patterson & Rosen, 1989). This grouping, however, appears to exemplify the uncertainties in our knowledge of higher actinopterygian phylogenies. Rosen & Patterson (1969) first recognized it as a sister-group of the Acanthopterygii. They included within it the Polymixiiformes, Percopsiformes, Ophidiiformes, Gadiformes, Batrachoidiformes, Lophiiformes, Zeioidei, Zoarcoidei, and Gobiesocoidei (taxon names follow Nelson, 1994). Subsequently, many authors have added other higher actinopterygians to or deleted existing members from the Paracanthopterygii (see Gill, 1996; table 2 in Miya *et al*., 2003).

Interestingly, one of the original authors subsequently recanted (Rosen, 1985: 41), stating: 'ill-fated . . . unfortunate . . . the paracanthopterygians no longer can be accepted as a natural group . . .'. However, a few years later, together with Patterson, he delimited them as monophyletic (Patterson & Rosen, 1989). Later authors have followed this taxonomic treatment (e.g. Helfman *et al*., 1997; Nakabo, 2000), although Nelson (1994: 219) stated that its monophyly has not been firmly established.

Miya *et al*. (2003) demonstrated that fishes of the Lophiiformes and Ophidiiformes, the two core members of the paracanthopterygians (Rosen & Patterson, 1969; Patterson & Rosen, 1989), did not appear in relatively primitive clades within the higher actinopterygian phylogenies based on 100 whole mitogenome sequences (see also Lê, Lecointre & Perasso, 1993;

Wiley *et al*., 2000; Chen *et al*., 2003). The two groups were placed within a crown group of actinopterygians called the Percomorpha, in the belief that the name Paracanthopterygii should be retained for a clade that comprises the Polymixiiformes, Percopsiformes, Gadiformes, and Zeioidei, and placed in a position appropriate to its name as a sister group of Acanthopterygii (Acanthomorpha minus Paracanthopterygii).

Wiley *et al.* (2000) observed that a batrachoidiform (*Opsanus tau*) was grouped within the Percomorpha along with an ophidiiform (*Petrotyx sanguineus*) based on combined morphological and molecular data from 25 species of the Acanthomorpha. Miya *et al*. (2003: 132) implied that batrachoidiforms were also highly derived actinopterygians, being members of the Percomorpha and that ophidiiforms, lophiiforms, and batrachoidiforms did not appear to have a close relationship with each other based on preliminary analysis of partial sequences from the several mitochondrial protein-coding genes.

To address questions regarding the phylogenetic position of the Batrachoidiformes, we determined the whole mitogenome sequences from two toadfish species (*Batrachomoeus trispinosus* and *Porichthys myriaster*) from two different subfamilies and the two sequences were aligned with those of the 100 higher actinopterygians used in Miya *et al*. (2003). The unambiguously aligned sequences (13 461 bp; excluding the ND6 gene and control region) were divided into five partitions (1st, 2nd and 3rd codon positions of the protein-coding genes; tRNA genes; and rRNA genes) and the three concatenated data sets, in which protein-coding genes were dealt with differently, were subjected to partitioned Bayesian phylogenetic analyses.

MATERIAL AND METHODS

Taxon sampling and specimens

We used 100 mitogenomic sequences (described in Miya *et al*., 2003: table 1. Consult the table for a complete list of accession numbers and citations.). We chose two species, *Batrachomoeus trispinosus* and *Porichthys myriaster*, as representatives of two of the three batrachoidiform subfamilies (Batrachoidinae and Porichthyinae). This taxon sampling strategy was based on recommendations suggested by Hillis (1998): (1) select taxa within the monophyletic group of interest that will represent the overall diversity of the group (his strategy 3), and (2) select taxa within the monophyletic group of interest that are expected (based on current taxonomy or previous phylogenetic studies) to subdivide long branches in the initial tree (his strategy 4). Hillis (1998) stated that careful addition of taxa to ensure coverage of the group of interest and to deliberately break up long branches (a combination of both strategies) appeared to be the optimal taxonomic sampling strategy.

A specimen of *B. trispinosus* was purchased from a commercial source and that of *P. myriaster* taken from coastal waters off San Francisco, California.

DNA extraction

A portion of the epaxial musculature (*c*. 0.25 g) was excised from fresh specimens of each species and immediately preserved in 99.5% ethanol. Total genomic DNA was extracted using the Qiagen QIAamp tissue kit following the manufacturer's protocol.

Mitochondrial DNA purification by long PCR

The mitogenomes of the two batrachoidiforms were amplified in their entirety using a long PCR technique (Cheng *et al*., 1994). Because of unusual gene arrangements in the two species (see Fig. 1), we had to design species-specific (Prmy-ND4-H; 5′–TYC CMA CSM TCA TAA TTA TTA CMC GMT GGG–3′) and three family specific (Toad-Trp-H; 5′–TTC YCT TAG RGY TTT GAA GGC YCT TGG TCT–3′, Toad-ND4-L(L); 5′–TYC CMA CSM TCA TAA TTA TTA CMC GMT GGG–3′; Toad-Trp-L; 5′–AGA CCA AGR GCC TTC AAA RCY CTA AGR GAA–3′) primers for long PCRs to amplify the entire mitogenomes in two or three reactions. Long PCRs were carried out as previously described (Miya *et al*., 2003), and the products diluted with TE buffer (1 : 10–20) for subsequent use as templates.

Short PCR and sequencing

Short PCRs using various combinations of 51 primers for *B. trispinosus* and 47 primers for *P. myriaster* were conducted to amplify contiguous, overlapping segments of the entire mitogenome for each of the two species (for primer locations and sequences, see Fig. 1 and Appendix 1). Species-specific primers were designed in cases where no appropriate fish-versatile primers were available (17 for *B. trispinosus* and 15 for *P. myriaster*; Appendix 1). Short PCRs were carried out as previously described (Miya *et al*., 2003).

Double-stranded PCR products, purified using a Pre-Sequencing Kit (USB), were subsequently used for direct cycle sequencing with dye-labelled terminators (Applied Biosystems). Primers used were the same as those for the long PCRs. All sequencing reactions were performed according to the manufacturer's instructions. Labelled fragments were analysed on Model 373/377/3100 DNA sequencers (Applied Biosystems).

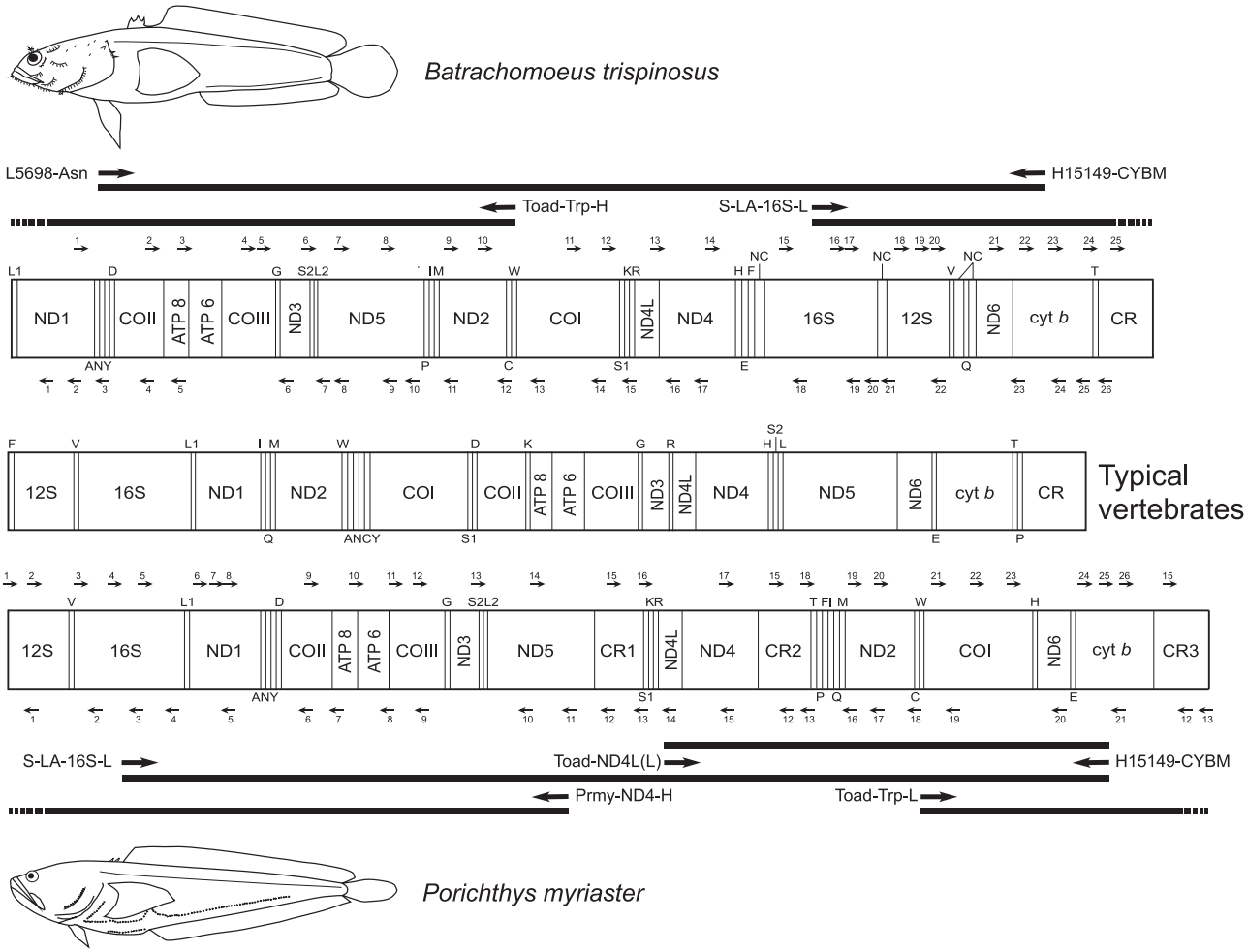


Figure 1. Linearized representation of the gene organization for the two toadfish species *Batrachomoeus trispinosus* (top) and *Porichthys myriaster* (bottom), with that of typical vertebrates shown in the middle. All protein-coding genes are encoded by the H-strand with the exception of ND6, which is coded by the L-strand. Transfer RNA (tRNA) genes are designated by single-letter amino acid codes, those encoded by the H- and L-strand being shown above and below the gene maps, respectively. Two or three pairs of long PCR primers (S-LA-16S-L + Toad-Trp-H and L5698-Asn + H15149-CYB for *B. trispinosus*; S-LA-16S-L/Toad-ND4-L(L) + H15149-CYB and Toad-Trp-L + Prmy-ND4-H for *P. myriaster*) amplify those segments that cover the entire mitogenomes. Relative position of short PCR and sequencing primers shown by small arrows, with numerals designated in Appendix 1. *Abbreviations:* 12S and 16S, 12S and 16S ribosomal RNA; ND1–6 and 4 L, NADH dehydrogenase subunits 1–6 and 4 L; COI–III, cytochrome c oxidase subunits I–III; ATPase 6 and 8, ATPase subunits 6 and 8; cyt b, cytochrome *b*; CR, putative control region; and L1, L2, S1, and S2 denote $tRNA^{Leu(UUR)}$, $tRNA^{Leu(CUN)}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Ser(AGY)}$, respectively; NC, non-coding sequences of 50 bp. Note that the putative control region in *P. myriaster* is triplicated (CR1–3).

ALIGNMENT

The DNA sequences were edited and analysed with EditView v. 1.0.1, AutoAssembler v. 2.1 (Applied Biosystems), and DNASIS v. 3.2 (Hitachi Software Engineering). Protein-coding gene sequences for the two toadfish species were aligned manually using DNASIS, based on the previously aligned sequences of 100 higher actinopterygians (Miya *et al*., 2003). Amino acids were used for alignments of the protein-coding genes and secondary structure models (Kumazawa & Nishida, 1993) for alignment of tRNA genes.

Since strictly secondary structure-based alignment for the two rRNA genes as used in Miya & Nishida (1998, 2000a) and Yamaguchi *et al*. (2000) was impractical for the large data set, we employed machine alignment that would minimize erroneous assessment of positional homology of the rRNA molecules. The 12S and 16S rRNA sequences were initially aligned using ClustalX v. 1.83 (Thompson *et al*., 1997) with default

gap penalties and subsequently adjusted by eye using MacClade v. 4.05 (Maddison & Maddison, 2000). The sequences were realigned using ProAlign v. 0.5 (Löytynoja & Millinkovitch, 2003) and those regions with posterior probabilities (PP) of 90% were used in phylogenetic analyses.

ND6 was not used in the phylogenetic analyses because of its heterogeneous base composition, consistently poor phylogenetic performance (Zardoya & Meyer, 1996; Miya & Nishida, 2000b), and difficulties in unambiguous alignment across all the taxa examined. Ambiguous alignment regions (such as the 5′ and 3′ ends of several protein-coding genes and loop regions of several tRNA genes) and all positions including gaps were excluded, leaving a total of 13 461 available nucleotide positions (10 512, 1342 and 1607 positions for protein-coding, tRNA, and rRNA genes, respectively) for phylogenetic analysis.

Those regions that could not be sequenced owing to technical difficulties (e.g. *Lampris guttatus* $tRNA^{Thr}$ and $tRNA^{Pro}$ genes and *Synbranchus marmoratus* ND1 gene; see Miya *et al*., 2001, 2003) were coded as missing. The three sets of unambiguously aligned sequences (the 12 protein-coding, 22 tRNA and two rRNA genes) were combined into a single data matrix using MacClade and the protein-coding sequences were handled so as to construct three different data sets (all of which included the tRNA and rRNA sequences) as follows: data set #1, without 3rd codon positions (total positions 9957); data set #2, with 3rd codon positions (13 461); and data set #3, triplets converted into amino acid sequences (6453).

Phylogenetic analysis

In the earlier stages of our mitogenomic studies, we employed maximum parsimony (MP) and maximum likelihood (ML) methods for analysing the data set in order to detect topological congruence between the two approaches (Inoue *et al*., 2001b; Miya *et al*., 2001; Ishiguro *et al.*, 2003). Following Hillis' (1998) strategies, the resulting tree topologies from the two methods were virtually congruent, with minor differences restricted to internal branches with low statistical support and no statistical differences observed between the two topologies.

As the taxon sampling had become more extensive, tree searches using ML along with bootstrapping (or jackknifing) became impracticable. In our earlier study (Miya *et al*., 2003) we relied upon weighted MP as an alternative to ML, but no bootstrapping (or jackknifing) was feasible for the weighted data sets. The advent of Bayesian inference (Ranala & Yang, 1996; Larget & Simon, 1999; Mau, Newton & Larget, 1999; see also Huelsenbeck *et al*., 2001) has altered the situation. It is both a faster character-based method when used with large data sets, and can also incorporate the power of complex statistical models (Holder & Lewis, 2003). Mitogenomic data were analysed using this approach by Inoue *et al*. (2003b, 2004) and Simmons & Miya (2004) .

In the present study, partitioned Bayesian phylogenetic analyses were conducted with a parallel version of MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003) using the above three data matrices. The program was run using two separate clusters of four Linux nodes, each equipped with Pentium 4 (3.0 GHz). We set four (data set #1: 1st and 2nd codon positions, tRNA and rRNA), five (data set #2: 1st, 2nd and 3rd codon positions, tRNA and rRNA), and three (data set #3: amino acids, tRNA and rRNA) partitions depending on the data sets, assuming that functional constraints on sequence evolution are more similar within codon positions (or types of molecules) across genes than across codon positions (or types of molecules) within genes, at least for a set of mitochondrial genes.

The general time reversible model with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (GTR + I + Γ; Yang, 1994) was consistently selected as the best-fit model of nucleotide substitution by ModelTest v. 3.06 (Posada & Crandall, 1998) for each partition. We set GTR + I + Γ in MrBayes as follows: 'lset nst = 6' (GTR), and 'rates = invgamma' (I + Γ). We assumed that all of the model parameters were unlinked and the rate multipliers were variable across partitions: 'unlink revmat = (all) pinvar = (all) shape = (all) statefreq = (all)' (unlinking substitution rates of the GTR model, proportion of invariable sites, gamma shape parameters, and base frequency across all partitions), and 'prset ratepr = variable' (rate multipliers variable across partitions).

We used the default settings for the priors on the proportion of invariable site (0–1) and the gamma shape parameter (0.1–50.0). A Dirichlet distribution was assumed for the rate matrix and base frequency, and every tree topology was assumed to be equally probable, except for data set #2. For data set #2, we had to use a starting tree (one of the best likelihood trees for data set #1) as a prior in the final two independent runs for quickly reaching stationarity with higher likelihood scores (for details, see 'Higher actinopterygian relationships', below). For data set #3, we assumed that 10 alternative models of amino acid substitutions (for details, see manual of MrBayes 3.0b4; Ronquist & Huelsenbeck, 2003) were equally probable and set the prior in MrBayes as follows: 'aamodelpr = mixed'. The mtREV model (Adachi & Hasegawa, 1996) was consistently selected during all runs of MrBayes using data set #3.

The Markov chain Monte Carlo (MCMC) process was set so that four chains (three heated and one cold)

ran simultaneously. On the basis of 2–4 preliminary runs with varying cycles ($1.0–3.0 \times 10^6$), we estimated average log likelihood scores at stationarity (data set #1 ≈ –246 230; data set #2 ≈ –593 140; data set #3 ≈ –223 940) and subsequently conducted two independent runs for each data set. After reaching stationarity in the two runs, we continued the runs for 3.0×10^6 cycles to confirm lack of improvement in the likelihood scores, with one in every 100 trees being sampled.

Parameters of the model of sequence evolution for the two sets of 3.0×10^6 cycles (30 000 trees) after reaching stationarity were in excellent agreement for each of the three data sets (see Appendices 2–5). Thus we determined posterior probabilities of the phylogeny and its branches based on the 60 000 trees pooled from the two runs for the three data sets. An important aspect of Bayesian analysis relates to evaluation of the alternative hypotheses using the collection of trees (Miller, Buckley & Manos, 2002). By counting the number of times a particular hypothesis has appeared in the simulation, we quantitatively assessed the strength of support for it.

RESULTS AND DISCUSSION

Genome organization

The complete L-strand nucleotide sequences from the mitogenomes of the two batrachoidiforms have been deposited in DDBJ/EMBL/GenBank under the accession numbers of AP006738 for *Batrachomoeus trispinosus* and AP006739 for *Porichthys myriaster*. The total lengths of *B. trispinosus* and *P. myriaster* mitogenomes were 17 086 bp (except for a portion of the control region that could not be sequenced owing to technical difficulties) and 18 910 bp, respectively. The genome contents of the two species included two rRNA, 22 tRNA, and 13 protein-coding genes, plus the putative control region(s), as found in other vertebrates. Also, as in other vertebrates, most genes of the two species were encoded on the H-strand, except for ND6 and eight tRNA genes.

The gene arrangements of the two species, however, differed greatly not only from those of typical vertebrates (Fig. 1), but also from each other. This appeared to be a potential cause of the technical difficulties incurred during the DNA experiments. Regions common to all of the three arrangements were limited, with only three blocks (COII–ND3, ND4L–ND4, and ND6–cyt *b*) being found, even if the tRNA genes were excluded from the comparisons. Such common gene blocks increase in number and size when we compare those of the two toadfishes (ND1–ND5, ND2–COI, ND4L–ND4, and ND6–cyt *b*) and the ND1–ND5 block is unique among the known actinopterygian mitogenomes (see Inoue *et al*., 2003b), suggesting a common origin of these unusual gene arrangements.

Another notable feature of the two mitogenomes was repetitive non-coding sequences. In *B. trispinosus*, four stretches of non-coding sequences of > 50 bp (76–117 bp) were found in and around a region of the 16S/12S rRNA and ND6 genes other than the putative control region. In *P. myriaster*, three major non-coding sequences were observed (924–1131 bp) and one of them (designated CR3 in Fig. 1) located in a position similar to the putative control region of *B. trispinosus* and the other two regions (CR1 and 2 in Fig. 1) shared completely identical sequences of 903 bp. Such duplicated control region-like sequences have also been found in two species of fish, including a live bearer *Rivulus marmoratus* (see Lee *et al*., 2001) and a gulper eel *Saccopharynx pelecanoides* (see Inoue *et al*., 2003b), as well as in a snake (Kumazawa *et al*., 1996).

Although we were unable to find $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$, $tRNA^{Arg}$, ND4, and ND4L in *P. myriaster* during earlier stages of the study, subsequent searches using various combinations of the PCR primers revealed the presence of the five genes between CR1 and 2 (Fig. 1), which have completely identical sequences of 919 bp. It should be noted that the automatic sequence assembler erroneously connected two identical sequences from the two different regions before the entire mitogenome was sequenced. Use of species-specific PCR primer pairs designed for these regions – e.g. a forward primer in the upstream region of CR1 (#15 in Fig. 1) and a reverse primer in the more downstream region of CR2 (#12 in Fig. 1) – selectively amplified the within-regions (i.e. CR1–3) but not between-regions (i.e. CR1 and 2, in which unobserved genes were located) area, resulting in unexpectedly shorter PCR products and apparent absence of the intervening genes.

Details of the molecular/evolutionary aspects of the mitogenomes will be dealt with elsewhere, along with those of other representative toadfishes including *Opsanus tau* (Batrachoidinae) and *Thalassophryne amazonica* (Thalassophryninae).

Stationarity

We conducted two to four preliminary Bayesian analyses for each data set to estimate average log likelihood scores at stationarity and to ensure that our analyses were not trapped on local optima on the basis of those values. Stationarity was reached at $0.9–1.2 \times 10^6$ generations in most runs, although our analyses were trapped on a local optimum three times for data set #2 (Fig. 2A) that included 3rd codon positions of the protein-coding genes.

Subsequent attempts revealed that employing a starting tree effectively avoided being trapped on the

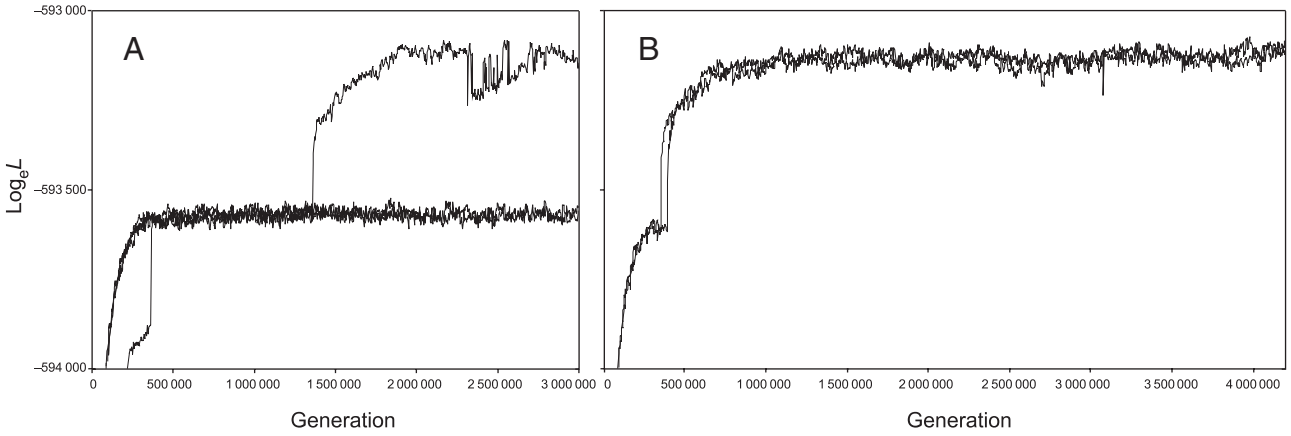


Figure 2. Changes of the log likelihood scores through time for data set #2. A, four preliminary runs started from different random trees. Note that only one of the four chains reached stationarity with the higher likelihood. B, two independent runs started from a user-specified tree topology (the best likelihood tree in the analyses of data set #1). Note that the two chains were still trapped at a local optimum during a short period of time and thereafter quickly reached stationarity with the higher likelihood scores.

local optimum. Accordingly, we employed a tree with the best likelihood score in two analyses for data set #1 as a starting tree and stationarity at higher likelihood scores was quickly reached in the two independent runs (Fig. 2B). It should be noted, however, that these runs were still trapped on the local optimum for a short period of time before reaching stationarity (Fig. 2B).

As there is no noticeable difference in estimates of the parameters between those runs with lower (Appendix 3) and higher likelihood scores (Appendix 4), we envisage that inclusion of the 3rd codon positions makes the tree space more complicated (existence of multiple near-optimal tree; Rogers & Swofford, 1999; Chor *et al*., 2000; Salter, 2001) than the other two analyses possibly because of the extremely rapid evolutionary rate of those positions. Indeed, relative evolutionary rates estimated from the rate multipliers at 1st, 2nd and 3rd codon positions of the protein-coding, tRNA, and rRNA genes (Appendix 3) were 3.28, 1.00, 54.97, 0.90, and 0.97, respectively. We therefore suggest that the tree space should be extensively explored when the 3rd codon positions from the mitochondrial protein-coding genes are included in Bayesian phylogenetic analysis with the large data set.

Higher actinopterygian relationships

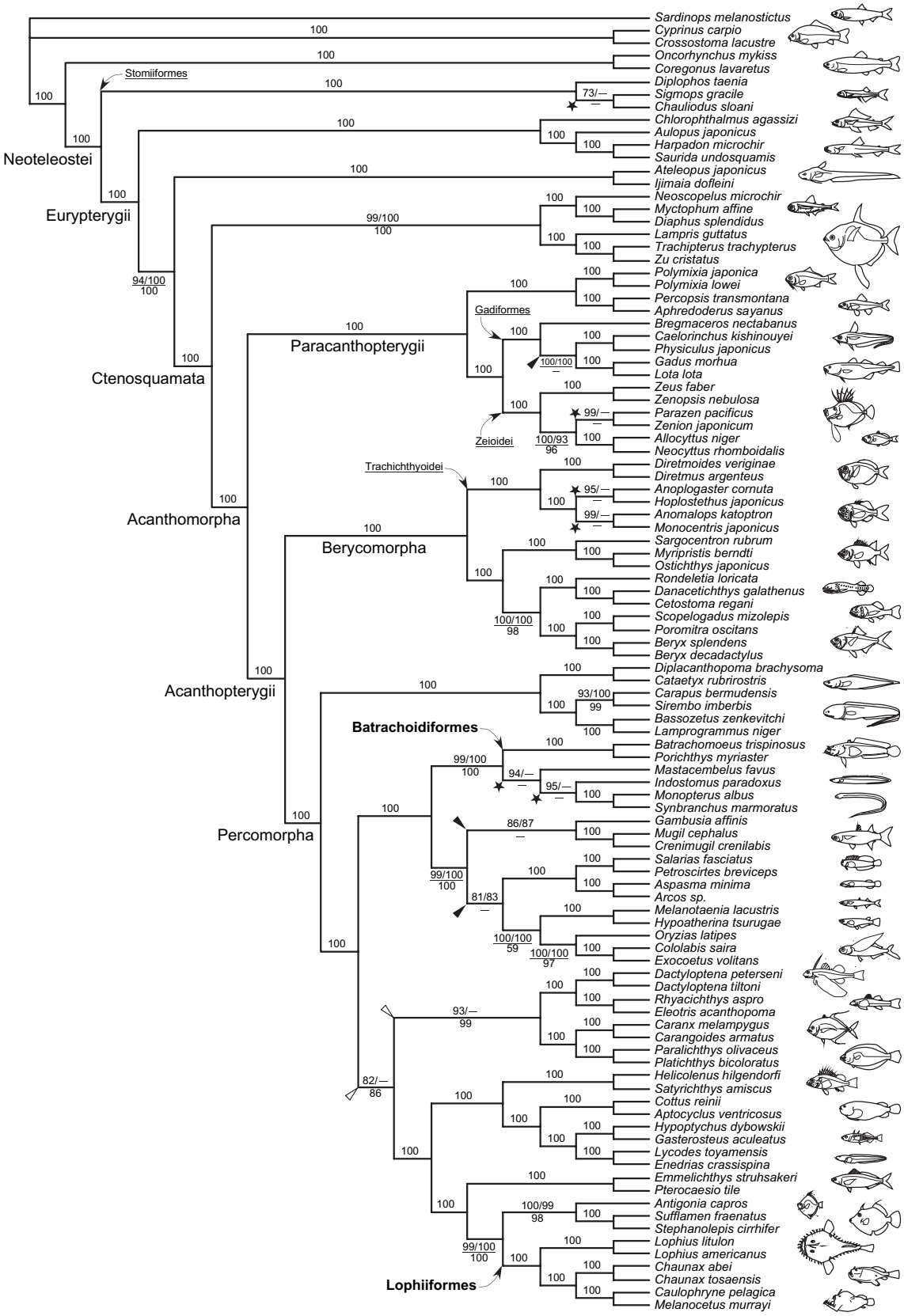
Figure 3 shows a 50% majority rule consensus tree of the 60 000 pooled samples from the two independent Bayesian analyses for data set #1. Topological differences among the three data sets were minor (denoted by symbols in Fig. 3) and restricted to relatively shallow internal branches either outside or inside the Percomorpha (five and six branches, respectively).

Outside the Percomorpha, the least comprehensive monophyletic groups that contained such topological incongruities were all supported by 100% PP and corresponded either to orders (Stomiiformes and Gadiformes) or suborders (Zeioidei and Trachichthyoidei) in which taxon sampling at family levels appeared to be insufficient to resolve their intrarelationships.

Inside the Percomorpha, the situation was the same, with sparse taxon sampling (51 species in 39 families and 48 genera) compared to the tremendous taxonomic diversity (≈ 14 000 species in 251 families and 2332 genera; calculated from Nelson, 1994). In either case, denser taxon sampling that deliberately bisects long branches within those clades might in future resolve such incongruities.

It should be noted that, with the exception of 11 internal branches that exhibited these incongruities, most (77/88) were supported by 100% PP irrespective of data set. The 11 branches also received high statistical support (between 93% and 100% PP) except for the one that comprised five species of the Atherinomorpha (59%; Fig. 3).

The partitioned Bayesian phylogenetic analyses definitely resolved some of the ambiguous basal relationships obtained in the unweighted and weighted MP analyses in Miya *et al*. (2003). For example, although monophyly of the Berycomorpha (the proposed new name, Fig. 3) was supported by 100% PP for all three data sets (Fig. 3), it was either reproduced or not reproduced in the weighted (transition/transversion = 1/2 and 1/3) and unweighted MP analyses, respectively. Note that statistical support for the internal branch in the two weighted MP analyses was not available owing to prohibitive computation time (Miya


Sardinops melanostictus
Cyprinus carpio
Crossostoma lacustre
Oncorhynchus mykiss
Coregonus lavaretus
Diplophos taenia
Sigmops gracile
Chauliodus sloani
Chlorophthalmus agassizi
Aulopus japonicus
Harpadon microchir
Saurida undosquamis
Ateleopus japonicus
Ijimaia dofleini
Neoscopelus microchir
Myctophum affine
Diaphus splendidus
Lampris guttatus
Trachipterus trachypterus
Zu cristatus
Polymixia japonica
Polymixia lowei
Percopsis transmontana
Aphredoderus sayanus
Bregmaceros nectabanus
Caelorinchus kishinouyei
Physiculus japonicus
Gadus morhua
Lota lota
Zeus faber
Zenopsis nebulosa
Parazen pacificus
Zenion japonicum
Allocyttus niger
Neocyttus rhomboidalis
Diretmoides veriginae
Diretmus argenteus
Anoplogaster cornuta
Hoplostethus japonicus
Anomalops katoptron
Monocentris japonicus
Sargocentron rubrum
Myripristis berndti
Ostichthys japonicus
Rondeletia loricata
Danacetichthys galathenus
Cetostoma regani
Scopelogadus mizolepis
Poromitra oscitans
Beryx splendens
Beryx decadactylus
Diplacanthopoma brachysoma
Cataetyx rubrirostris
Carapus bermudensis
Sirembo imberbis
Bassozetus zenkevitchi
Lamprogrammus niger
Batrachomoeus trispinosus
Porichthys myriaster
Mastacembelus favus
Indostomus paradoxus
Monopterus albus
Synbranchus marmoratus
Gambusia affinis
Mugil cephalus
Crenimugil crenilabis
Salarias fasciatus
Petroscirtes breviceps
Aspasma minima
Arcos sp.
Melanotaenia lacustris
Hypoatherina tsurugae
Oryzias latipes
Cololabis saira
Exocoetus volitans
Dactyloptena peterseni
Dactyloptena tiltoni
Rhyacichthys aspro
Eleotris acanthopoma
Caranx melampygus
Carangoides armatus
Paralichthys olivaceus
Platichthys bicoloratus
Helicolenus hilgendorfi
Satyrichthys amiscus
Cottus reinii
Aptocyclus ventricosus
Hypoptychus dybowskii
Gasterosteus aculeatus
Lycodes toyamensis
Enedrias crassispina
Emmelichthys struhsakeri
Pterocaesio tile
Antigonia capros
Sufflamen fraenatus
Stephanolepis cirrhifer
Lophius litulon
Lophius americanus
Chaunax abei
Chaunax tosaensis
Caulophryne pelagica
Melanocetus murrayi
Stomiiformes
Neoteleostei
Eurypterygii
Ctenosquamata
Gadiformes
Paracanthopterygii
Zeioidei
Trachichthyoidei
Acanthomorpha
Berycomorpha
Acanthopterygii
Batrachoidiformes
Percomorpha
Lophiiformes

et al., 2003). Similarly, ambiguous relationships between two major lineages within the Paracanthopterygii (Polymixiiformes and Percopsiformes) in Miya *et al*. (2003) were fully resolved in the present analyses (Fig. 3), indicating that incorporation of explicit models of sequence evolution in Bayesian analyses can accommodate superimposed changes (i.e. multiple changes at the same sites) that have been minimized and underestimated in MP analyses (Huelsenbeck *et al*., 2001).

Phylogenetic position of the Batrachoidiformes

As observed in Wiley *et al.* (2000) and implied in Miya *et al*. (2003: 132), the Batrachoidiformes could be confidently placed within a crown group (Percomorpha, as delimited by Miya *et al*., 2003: fig. 3) rather than within a relatively primitive group (Paracanthopterygii) of the acanthopterygians. More specifically, it was nested within a clade comprising three species of the Synbranchiformes plus the enigmatic *Indostomus paradoxus* (Gasterosteiformes: Indostomidae; see Britz & Johnson, 2002 for a recent view of its phylogenetic position based on morphology), which was itself nested within a more comprehensive clade which included two species of the Mugilliformes, six species of the Atherinomorpha, two species of the Gobiesocidae and two species of the Blennidae (Fig. 3).

Interestingly, some members of this clade, such as the Synbranchiformes, Mugilliformes, Gasterosteiformes (only *I. paradoxus*), and Atherinomorpha are characterized by having the first epineural on the parapophysis, a morphological synapomorphy based on which Johnson & Patterson (1993) established an unranked higher taxon named Smegmamorpha. As far as we know, no fish systematist has yet proposed such a novel phylogenetic affinity and there appears to be no unambiguous morphological synapomorphy suggesting such relationships up to now (see Johnson & Patterson, 1993).

On the other hand, the Lophiiformes, a putative sister-group of the Batrachoidiformes, form a sister-group relationship with a clade comprising two species of the Tetraodontiformes plus *Antigonia capros* (Zeiformes: Caproidae), which was supported by 99–100% PP (Fig. 3). In addition, the least comprehensive monophyletic group that includes both the Batrachoidiformes and Lophiiformes encompasses nearly the entire Percomorpha (Percomorpha minus Ophidiiformes; Fig. 3), strongly suggesting that the two groups have diverged relatively basally within the Percomorpha.

Finally, Bayesian analyses of the three data sets found no tree topology congruent with the above two hypotheses, indicating that the probability of the Batrachoidiformes being both a member of the Paracanthopterygii and a sister-group of the Lophiiformes was less than 1/60 000, or 0.00002 in the Bayesian context. Of course, we acknowledge that more extensive taxon sampling within the Percomorpha, together with analysis of independent nuclear markers, is necessary to corroborate this novel hypothesis.

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Figure 3. The 50% majority rule consensus tree of the 60 000 pooled trees from the two independent Bayesian analyses of data set #1. The data set comprises unambiguously aligned nucleotide sequences of 9957 bp from 102 higher actinopterygians; we set four partitions (1st and 2nd codon positions from 12 protein-coding genes plus 22 tRNA and two rRNA genes). Partitioned Bayesian analyses were conducted using MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003), with the best-fit model of sequence evolution (GTR + I + Γ; Yang, 1994) being set for each partition and all model parameters variable and unlinked across partitions. Numerals beside internal branches indicate Bayesian posterior probabilities (shown as percentages) for the data set #1 (upper left), #2 (upper right), and #3 (lower middle). Single numerals are given when analyses for all the data sets have shown the same values. Topological incongruities among the data sets are denoted by stars (data set #1 vs. #2 vs. #3), open arrowheads (data set #1 vs. #2), and filled arrowheads (data set #1 vs. #3), with those found in outside the Percomorpha denoted by names of the least comprehensive monophyletic groups (orders or suborders) with underlines. Six major, unranked higher taxa were assigned sequentially to those clades with the highest posterior probabilities (100%) depending on their limits. Only limits of the Ctenosquamata were different from those of Miya *et al*. (2003) because of ambiguous phylogenetic position of the Ateleopodiformes (compare Miya *et al*., 2003; Inoue *et al*., 2003b and Simmons & Miya, 2004). The Berycomorpha is newly proposed for a clade comprising two reciprocally paraphyletic orders (Beryciformes and Stephanoberyciformes).

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APPENDIX 1

PCR and sequencing primers for *Batrachomoeus trispinosus* (upper) and *Porichthys myriaster* (lower). For locations of primers, see Figure 1

	L Primers	Sequences (5′→3′)	H primers	Sequences (5′→3′)
1	L4166-ND1[a]	CGATATGATCAACTMATKCA	Batr-ND1-H[f]	ATGCCATCTGCAAATGGTTG
2	L8202-CO2[f]	TGYGGAGCWAATCAYAGCTT	Batr-ND1-H2[f]	CCACATCATAGCAAGAGGTC
3	L8894-ATP[a]	TTGGACTACTWCCSTATAC	Batr-ND1-H3[f]	TAAGTGAAGTCTTGTGGACC
4	L9655-CO3[b]	GTAACWTGGGCTCATCACAG	H7892-CO2[f]	TCGTAGCTTCAGTATCATTG
5	L9916-CO3[a]	CACCATTTTGGCTTTGAAGC	Batr-CO2H[f]	GGTCAAGTTGAGGCATGTGC
6	L12321-Leu[c]	GGTCTTAGGAACCAAAAACTCT TGGTGCAA	H10019-Gly[a]	CAAGACKGKGTGATTGGAAG
7	Batr-ND5-L(L)[f]	GTATCATGGGCAATTTTAGAAT ATACTCTG	H12293-Leu[c]	TTGCACCAAGAGTTTTTGGTTC CTAAGACC
8	Batr-ND5-L[f]	GTCAATCTAACCTTTCCACG	H12632-ND5[a]	GATCAGGTTACGTAKAGKGC
9	L4633-ND2[a]	CACCACCCWCGAGCAGTTGA	H13396-ND5[c]	CCTATTTTTCGGATGTCTTG
10	L5261-ND2[c]	CWGGTTTCRTRCCWAAATGA	H13727-ND5[a]	GCGATKATGCTTCCTCAGGC
11	L6730-CO1[a]	TATATAGGAATRGTMTGAGC	Batr-ND2-H[f]	TAGTAGATGTTTCATTGTCC
12	Batr-CO1-L[f]	ACGAACACCCTCAAACCTAG	H5334-ND2[a]	CGKAGGTAGAAGTAHAGGCT

APPENDIX 1 *Continued*

	L Primers	Sequences (5′→3′)	H primers	Sequences (5′→3′)
13	L10681-ND4[c]	GCKTTTTCKGCKTGTGAAGC	H5937-CO1[c]	TGGGTGCCAATGTCTTTGTG
14	Batr-ND4-L[f]	CCAGCCACATAATCTACCCG	Batr-CO1-H[f]	TATGCACGAGTATCAACATC
15	L1803-16S[a]	AGTACCGCAAGGGAAAGCTGAAA	H7447-Ser[f]	AWGGGGGTTCRATTCCTYCCT TTCTC
16	L2510-16S[a]	CGCCTGTTTACCAAAAACAT	Batr-ND4-H[f]	TGGGTAGGAGGATTTTTAGC
17	Batr-16S-L[f]	AAAAGACCCCGTGGAGCTTTAGG ACGCCTC	H11618-ND4[b]	TGGCTGACKGAKGAGTAGGC
18	L701-12S[e]	TAGCTCAACTTACACATGCAAG	H1903-16S[a]	GTAGCTCGTYTAGTTTCGGG
19	Batr-ND1?-L[f]	AGCTGGTTTAGCATGAAGCC	H2590-16S[a]	ACAAGTGATTGCGCTACCTT
20	L1374-12S[d]	GAAGAAATGGGCTACATTTTCTA	Batr-16S-H[f]	TGGCTCTTGATTTAGGTGTGT GTACCATGC
21	L14504-ND6[b]	GCCAAWGCTGCWGAATAMGCAAA	H3084-16S[a]	AGATAGAAACTGACCTGGAT
22	L14850-CYB[a]	GCCTGATGAAACTTTGGCTC	H1358-12S[a]	CGACGGCGGTATATAGGC
23	L15369-CYB[c]	ACAGGMTCAAAYAACCC	Batr-ND6-H[f]	GTGGTAGTTGTAGCTAATCC
24	Batr–CYB-L	GACCCGTACATTACTTTAGG	H15149-CYB[b]	GGTGGCKCCTCAGAAGGACAT TTGKCCTCA
25	Batr-CYB-L2	AATAATTCAAACAGGCCAAC	H15560-CYB[a]	TAGGCRAATAGGAARTATCA
26			H15915-Thr[f]	ACCTCCGATCTYCGGATTACA AGAC
1	L708-12S[f]	TTAYACATGCAAGTMTCCGC	H885-12S[f]	TAACCGCGGYGGCTGGCACGA
2	L1083-12S[a]	ACAAACTGGGATTAGATAC	H1903-16S[a]	GTAGCTCGTYTAGTTTCGGG
3	L1803-16S[a]	AGTACCGCAAGGGAAAGCTGAAA	H2590-16S[a]	ACAAGTGATTGCGCTACCTT
4	L2510-16S[a]	CGCCTGTTTACCAAAAACAT	H3084-16S[a]	AGATAGAAACTGACCTGGAT
5	L2949-16S[a]	GGGATAACAGCGCAATC	H3976-ND1[b]	ATGTTGGCGTATTCKGCKAGG AA
6	L3686-ND1[b]	TGAGCMTCWAATTCMAAATA	H7892-CO2[f]	TCGTAGCTTCAGTATCATTG
7	Prmy-ND1-L[f]	TAACGTAGAATATGCAGGCG	H8168-CO2[a]	CCGCAGATTTCWGAGCATTG
8	L4166-ND1[a]	CGATATGATCAACTMATKCA	H8650-ATP[f]	ATKCCKAGGWGKGWGGGGCT
9	L8202-CO2[f]	TGYGGAGCWAATCAYAGCTT	H9267-ATP[f]	ACAAAKACGTAKGCTTGAAT
10	L8894-ATP[a]	TTGGACTACTWCCSTATAC	Prmy-ND5-H[f]	ATAGAATCTGGGGCACTAGG
11	L9220-CO3[b]	AACGTTTAATGGCCCACCAAGC	H13396-ND5[c]	CCTATTTTTCGGATGTCTTG
12	L9655-CO3[b]	GTAACWTGGGCTCATCACAG	Toad-NC-H[f]	TGTACTARTACATACCCASG
13	L12329-Leu[a]	CTCTTGGTGCAAMTCCAAGT	Toad-NC-H2[f]	TATGATACRTGCCTCKAAGC
14	Prmy-ND5-L[f]	GCAGCTGCCGGAAAGTCTGC	Toad-ND4L-H[f]	GARGTGTGTWMGGTGAACTGT
15	Toad-NC-L[f]	TCTCTCTAAAGTRCCTGGGG	Prmy-ND4-H[f]	GGCAGATAGAGCTGGTTATAAT GATGCCCC
16	L8343-Lys[c]	AGCGTTGGCCTTTTAAGCTAAW GATWGGTG	H4432-Met[c]	TTTAACCGWCATGTTCGGGGT ATG
17	Toad-ND4-L[f]	CCCACKATTAACMTTRTAGC	H4866-ND2[a]	AAKGGKGCKAGTTTTTGTCA
18	L15927-Thr[c]	AGAGCGTCGGTCTTGTAAKCCG	H5334-ND2[a]	CGKAGGTAGAAGTAHAGGCT
19	L4633-ND2[a]	CACCACCCWCGAGCAGTTGA	Prmy-CO1-H[f]	ACTGTTCAGCCGGTTCCAAC
20	L5260-ND2[a]	CTGGSTTTATGCCMAARTG	Prmy-ND6-H[f]	CTGCTGATTCTTACCCGGAG
21	L6199-CO1[a]	GCCTTCCCWCGAATAAATAA	H15149-CYB[b]	GGTGGCKCCTCAGAAGGACAT TTGKCCTCA
22	Prmy-CO1-L[f]	CTCATCATATGTTTACTGTG		
23	Prmy-CO1-L2[f]	TTCCAACTAAGCTCTATAGG		
24	L14850-CYB[a]	GCCTGATGAAACTTTGGCTC		
25	Prmy-CYB-L1[f]	TGGTTACTATGCTTACAGCC		
26	Prmy-CYB-L2[f]	AGGACGCATCTCCACTGCCG		

[a]Miya & Nishida (1999); [b]Inoue *et al*. (2000); [c]Miya & Nishida (2000b); [d]Inoue *et al*. (2001a); [e]Kawaguchi *et al.* (2001); [f]this study.

APPENDIX 2

Estimates of the parameters of the substitution model (GTR + I + Γ) for the two independent Bayesian analyses of data set #1. The instantaneous rate change from nucleotide i to j is denoted r_{ij}, and is measured relative to the rate of change between G and T ($r_{GT} = 1$). The frequency of nucleotide i is denoted π, the gamma shape parameter is α, the proportion of invariant sites is p_{inv}, and the rate multiplier for a partition is m. The numbers in each column give the mean of the marginal posterior probability distribution and the 95% credible interval (in parentheses) for the parameter

		Parameter (θ)	
Partition		1st analysis	2nd analysis
1st position	r_{CT}	2.85 (2.64–3.06)	2.85 (2.62–3.10)
	r_{CG}	0.15 (0.12–0.17)	0.15 (0.12–0.17)
	r_{AT}	0.75 (0.69–0.83)	0.76 (0.69–0.83)
	r_{AG}	2.34 (2.15–2.52)	2.35 (2.19–2.54)
	r_{AC}	0.33 (0.29–0.36)	0.33 (0.29–0.36)
2nd position	r_{CT}	6.44 (5.40–7.62)	6.51 (5.47–7.83)
	r_{CG}	6.43 (5.40–7.55)	6.50 (5.45–7.85)
	r_{AT}	1.65 (1.35–1.99)	1.67 (1.38–2.03)
	r_{AG}	9.51 (7.99–11.28)	9.65 (8.14–11.77)
	r_{AC}	2.59 (2.10–3.17)	2.62 (2.13–3.25)
tRNA	r_{CT}	19.78 (16.78–23.55)	19.89 (16.70–23.86)
	r_{CG}	0.78 (0.57–1.03)	0.79 (0.57–1.07)
	r_{AT}	1.78 (1.46–2.16)	1.80 (1.46–2.22)
	r_{AG}	15.50 (13.16–18.48)	15.83 (13.20–18.92)
	r_{AC}	1.87 (1.50–2.34)	1.89 (1.49–2.36)
rRNA	r_{CT}	20.27 (16.37–24.61)	19.99 (16.00–25.22)
	r_{CG}	0.49 (0.33–0.68)	0.48 (0.33–0.69)
	r_{AT}	2.83 (2.26–3.49)	2.77 (2.21–3.55)
	r_{AG}	8.45 (6.78–10.11)	8.26 (6.84–10.20)
	r_{AC}	2.91 (2.27–3.61)	2.91 (2.24–3.67)
1st position	π_A	0.32 (0.31–0.33)	0.32 (0.31–0.33)
	π_C	0.32 (0.31–0.33)	0.32 (0.31–0.33)
	π_G	0.17 (0.16–0.17)	0.17 (0.16–0.17)
	π_T	0.19 (0.19–0.20)	0.19 (0.19–0.20)
2nd position	π_A	0.18 (0.17–0.19)	0.18 (0.17–0.19)
	π_C	0.31 (0.30–0.32)	0.31 (0.30–0.32)
	π_G	0.10 (0.09–0.11)	0.10 (0.09–0.11)
	π_T	0.40 (0.39–0.42)	0.40 (0.39–0.42)
tRNA	π_A	0.30 (0.28–0.31)	0.30 (0.28–0.31)
	π_C	0.19 (0.18–0.20)	0.19 (0.18–0.20)
	π_G	0.21 (0.20–0.23)	0.21 (0.20–0.23)
	π_T	0.30 (0.29–0.32)	0.30 (0.28–0.31)
rRNA	π_A	0.34 (0.32–0.36)	0.34 (0.32–0.36)
	π_C	0.23 (0.22–0.25)	0.23 (0.22–0.25)
	π_G	0.20 (0.19–0.22)	0.20 (0.19–0.22)
	π_T	0.23 (0.21–0.24)	0.22 (0.21–0.24)
1st position	α	0.68 (0.66–0.71)	0.68 (0.66–0.71)
2nd position	α	0.54 (0.50–0.59)	0.54 (0.50–0.59)
tRNA	α	0.68 (0.62–0.74)	0.68 (0.62–0.74)
rRNA	α	0.58 (0.53–0.63)	0.58 (0.53–0.63)
1st position	p_{inv}	0.32 (0.30–0.33)	0.32 (0.30–0.33)
2nd position	p_{inv}	0.42 (0.39–0.44)	0.42 (0.39–0.44)
tRNA	p_{inv}	0.13 (0.10–0.16)	0.13 (0.10–0.16)
rRNA	p_{inv}	0.32 (0.29–0.35)	0.32 (0.29–0.35)
1st position	m	1.56 (1.52–1.60)	1.57 (1.52–1.61)
2nd position	m	0.44 (0.42–0.47)	0.44 (0.42–0.47)
tRNA	m	1.06 (1.00–1.13)	1.06 (1.00–1.13)
rRNA	m	0.93 (0.87–0.99)	0.93 (0.87–0.99)

APPENDIX 3

Estimates of the parameters of the substitution model (GTR + I + Γ) for the three independent Bayesian analyses of data set #2 in the partitioned Bayesian analyses that reached stationarity at lower likelihood scores ($\log_e L \approx -593\,600$; see Fig. 1). For details, see legend of Appendix 2

		Parameter (θ)		
Partition		1st analysis	2nd analysis	3rd analysis
1st position	r_{CT}	2.90 (2.66–3.13)	2.90 (2.68–3.10)	2.89 (2.67–3.13)
	r_{CG}	0.15 (0.13–0.18)	0.16 (0.13–0.18)	0.16 (0.13–0.18)
	r_{AT}	0.81 (0.73–0.89)	0.81 (0.73–0.89)	0.80 (0.73–0.88)
	r_{AG}	2.32 (2.11–2.50)	2.35 (2.18–2.60)	2.34 (2.16–2.53)
	r_{AC}	0.38 (0.33–0.42)	0.38 (0.34–0.43)	0.38 (0.34–0.43)
2nd position	r_{CT}	6.33 (5.37–7.57)	6.70 (5.39–8.27)	6.33 (5.42–7.48)
	r_{CG}	6.07 (5.10–7.23)	6.39 (5.12–7.93)	6.05 (5.17–7.24)
	r_{AT}	1.70 (1.43–2.04)	1.80 (1.39–2.23)	1.71 (1.43–2.06)
	r_{AG}	9.21 (7.79–10.96)	9.65 (7.70–11.54)	9.24 (7.83–11.24)
	r_{AC}	2.64 (2.20–3.19)	2.81 (2.12–3.60)	2.66 (2.18–3.20)
3rd position	r_{CT}	13.50 (9.52–18.45)	13.55 (11.03–16.42)	13.90 (10.78–16.26)
	r_{CG}	0.40 (0.18–0.71)	0.39 (0.21–0.58)	0.42 (0.23–0.61)
	r_{AT}	1.14 (0.71–1.63)	1.12 (0.87–1.36)	1.17 (0.87–1.40)
	r_{AG}	33.83 (23.16–45.35)	34.12 (28.41–40.81)	34.96 (27.42–41.42)
	r_{AC}	0.42 (0.30–0.56)	0.43 (0.35–0.53)	0.44 (0.33–0.54)
tRNA	r_{CT}	18.69 (15.66–22.42)	18.50 (15.35–21.09)	18.55 (15.76–22.09)
	r_{CG}	0.79 (0.58–1.03)	0.79 (0.58–1.03)	0.78 (0.57–1.03)
	r_{AT}	1.83 (1.52–2.20)	1.81 (1.46–2.14)	1.81 (1.49–2.19)
	r_{AG}	15.35 (13.04–17.95)	15.22 (12.67–18.02)	15.24 (12.65–18.00)
	r_{AC}	1.91 (1.50–2.36)	1.88 (1.46–2.31)	1.89 (1.50–2.34)
rRNA	r_{CT}	19.51 (16.31–23.29)	19.52 (15.88–23.89)	19.82 (16.05–24.41)
	r_{CG}	0.48 (0.33–0.66)	0.49 (0.34–0.68)	0.49 (0.33–0.69)
	r_{AT}	2.95 (2.44–3.58)	2.99 (2.35–3.64)	3.00 (2.39–3.74)
	r_{AG}	8.15 (6.90–9.47)	8.36 (6.82–10.13)	8.31 (6.77–9.98)
	r_{AC}	2.91 (2.39–3.58)	2.94 (2.36–3.71)	2.97 (2.32–3.72)
1st position	π_A	0.32 (0.31–0.33)	0.32 (0.31–0.33)	0.32 (0.31–0.33)
	π_C	0.31 (0.30–0.32)	0.31 (0.30–0.32)	0.31 (0.30–0.32)
	π_G	0.18 (0.17–0.18)	0.17 (0.17–0.18)	0.17 (0.17–0.18)
	π_T	0.19 (0.19–0.20)	0.20 (0.19–0.20)	0.20 (0.19–0.20)
2nd position	π_A	0.18 (0.17–0.19)	0.18 (0.17–0.19)	0.18 (0.17–0.19)
	π_C	0.31 (0.30–0.33)	0.31 (0.30–0.32)	0.31 (0.30–0.33)
	π_G	0.10 (0.09–0.11)	0.10 (0.09–0.11)	0.10 (0.10–0.11)
	π_T	0.40 (0.39–0.42)	0.40 (0.39–0.41)	0.40 (0.39–0.42)
3rd position	π_A	0.33 (0.33–0.33)	0.33 (0.33–0.33)	0.33 (0.32–0.33)
	π_C	0.36 (0.36–0.36)	0.36 (0.36–0.36)	0.36 (0.36–0.36)
	π_G	0.08 (0.08–0.08)	0.08 (0.08–0.08)	0.08 (0.08–0.08)
	π_T	0.23 (0.23–0.23)	0.23 (0.23–0.23)	0.23 (0.23–0.23)
tRNA	π_A	0.29 (0.28–0.31)	0.29 (0.28–0.31)	0.29 (0.28–0.31)
	π_C	0.19 (0.18–0.20)	0.19 (0.18–0.20)	0.19 (0.18–0.20)
	π_G	0.21 (0.20–0.23)	0.21 (0.20–0.23)	0.21 (0.20–0.23)
	π_T	0.31 (0.29–0.32)	0.31 (0.29–0.32)	0.31 (0.29–0.32)
rRNA	π_A	0.34 (0.32–0.35)	0.33 (0.32–0.35)	0.34 (0.32–0.35)
	π_C	0.23 (0.22–0.25)	0.24 (0.22–0.25)	0.23 (0.22–0.25)
	π_G	0.21 (0.19–0.22)	0.21 (0.19–0.22)	0.21 (0.19–0.22)
	π_T	0.22 (0.21–0.24)	0.22 (0.21–0.24)	0.22 (0.21–0.24)
1st position	α	0.73 (0.70–0.75)	0.73 (0.70–0.76)	0.73 (0.70–0.75)
2nd position	α	0.56 (0.52–0.61)	0.56 (0.52–0.61)	0.56 (0.52–0.61)
3rd position	α	0.49 (0.47–0.51)	0.48 (0.46–0.51)	0.49 (0.46–0.51)
tRNA	α	0.71 (0.65–0.77)	0.71 (0.65–0.77)	0.71 (0.65–0.78)

APPENDIX 3 *Continued*

Partition		Parameter (θ) 1st analysis	2nd analysis	3rd analysis
rRNA	α	0.61 (0.56–0.66)	0.61 (0.56–0.66)	0.61 (0.56–0.66)
1st position	p_{inv}	0.32 (0.31–0.34)	0.32 (0.31–0.34)	0.32 (0.30–0.34)
2nd position	p_{inv}	0.42 (0.39–0.45)	0.42 (0.40–0.45)	0.42 (0.40–0.45)
3rd position	p_{inv}	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
tRNA	p_{inv}	0.14 (0.11–0.17)	0.14 (0.11–0.17)	0.14 (0.11–0.17)
rRNA	p_{inv}	0.33 (0.29–0.36)	0.32 (0.29–0.36)	0.33 (0.30–0.36)
1st position	m	0.28 (0.26–0.29)	0.27 (0.25–0.28)	0.27 (0.26–0.29)
2nd position	m	0.08 (0.08–0.09)	0.08 (0.08–0.09)	0.08 (0.08–0.09)
3rd position	m	3.33 (3.31–3.35)	3.34 (3.32–3.36)	3.33 (3.32–3.35)
tRNA	m	0.19 (0.18–0.21)	0.19 (0.18–0.21)	0.19 (0.18–0.21)
rRNA	m	0.17 (0.16–0.19)	0.17 (0.16–0.19)	0.17 (0.16–0.19)

APPENDIX 4

Estimates of the parameters of the substitution model (GTR + I + Γ) for the two independent Bayesian analyses of data set #2 in the partitioned Bayesian analyses that reached stationarity at higher likelihood scores ($\log_e L \approx -593\,140$; see Fig. 1). For details, see legend of Appendix 2

Partition		Parameter (θ) 1st analysis	2nd analysis
1st position	r_{CT}	2.91 (2.71–3.13)	2.87 (2.68–3.10)
	r_{CG}	0.16 (0.13–0.18)	0.15 (0.13–0.18)
	r_{AT}	0.82 (0.74–0.89)	0.80 (0.73–0.89)
	r_{AG}	2.34 (2.12–2.56)	2.31 (2.13–2.54)
	r_{AC}	0.38 (0.34–0.43)	0.37 (0.33–0.43)
2nd position	r_{CT}	6.34 (5.15–7.66)	6.54 (5.49–7.64)
	r_{CG}	6.05 (4.97–7.18)	6.23 (5.31–7.27)
	r_{AT}	1.71 (1.38–2.07)	1.76 (1.44–2.10)
	r_{AG}	9.18 (7.66–10.93)	9.41 (7.93–11.03)
	r_{AC}	2.64 (2.11–3.24)	2.74 (2.28–3.26)
3rd position	r_{CT}	11.10 (8.45–14.68)	10.19 (8.48–12.44)
	r_{CG}	0.42 (0.22–0.67)	0.36 (0.21–0.55)
	r_{AT}	0.97 (0.68–1.34)	0.88 (0.67–1.13)
	r_{AG}	31.31 (22.72–41.09)	28.70 (23.66–35.85)
	r_{AC}	0.28 (0.20–0.37)	0.26 (0.21–0.34)
tRNA	r_{CT}	18.63 (15.67–21.99)	19.03 (15.78–22.11)
	r_{CG}	0.80 (0.58–1.05)	0.82 (0.59–1.06)
	r_{AT}	1.83 (1.48–2.21)	1.88 (1.51–2.23)
	r_{AG}	15.30 (12.46–18.18)	15.64 (12.93–18.35)
	r_{AC}	1.91 (1.51–2.38)	1.96 (1.53–2.39)
rRNA	r_{CT}	19.64 (16.11–24.18)	19.59 (15.96–24.72)
	r_{CG}	0.49 (0.34–0.68)	0.49 (0.33–0.69)
	r_{AT}	2.99 (2.38–3.76)	2.99 (2.41–3.78)
	r_{AG}	8.29 (6.67–10.18)	8.30 (6.73–10.28)
	r_{AC}	2.96 (2.31–3.79)	2.95 (2.33–3.71)
1st position	π_A	0.32 (0.31–0.33)	0.32 (0.31–0.33)
	π_C	0.31 (0.30–0.32)	0.31 (0.30–0.32)
	π_G	0.18 (0.17–0.18)	0.17 (0.17–0.18)
	π_T	0.20 (0.19–0.20)	0.19 (0.19–0.20)

APPENDIX 4 *Continued*

Partition		Parameter (θ) 1st analysis	2nd analysis
2nd position	π_A	0.18 (0.17–0.19)	0.18 (0.17–0.19)
	π_C	0.31 (0.31–0.33)	0.31 (0.30–0.33)
	π_G	0.10 (0.10–0.11)	0.10 (0.10–0.11)
	π_T	0.40 (0.39–0.41)	0.40 (0.39–0.42)
3rd position	π_A	0.32 (0.32–0.33)	0.32 (0.32–0.33)
	π_C	0.37 (0.36–0.37)	0.37 (0.36–0.37)
	π_G	0.08 (0.08–0.08)	0.08 (0.08–0.08)
	π_T	0.23 (0.23–0.24)	0.23 (0.23–0.23)
tRNA	π_A	0.29 (0.28–0.31)	0.29 (0.28–0.31)
	π_C	0.19 (0.18–0.20)	0.19 (0.18–0.20)
	π_G	0.21 (0.20–0.23)	0.21 (0.20–0.23)
	π_T	0.31 (0.29–0.32)	0.31 (0.29–0.32)
rRNA	π_A	0.33 (0.32–0.35)	0.33 (0.32–0.35)
	π_C	0.23 (0.22–0.25)	0.23 (0.22–0.25)
	π_G	0.21 (0.19–0.22)	0.21 (0.19–0.22)
	π_T	0.22 (0.21–0.24)	0.22 (0.21–0.24)
1st position	α	0.73 (0.70–0.76)	0.73 (0.70–0.76)
2nd position	α	0.56 (0.52–0.61)	0.57 (0.52–0.61)
3rd position	α	0.90 (0.88–0.92)	0.90 (0.88–0.92)
tRNA	α	0.72 (0.66–0.78)	0.72 (0.65–0.78)
rRNA	α	0.61 (0.56–0.66)	0.61 (0.56–0.66)
1st position	p_{inv}	0.32 (0.31–0.34)	0.32 (0.31–0.34)
2nd position	p_{inv}	0.42 (0.40–0.45)	0.42 (0.40–0.45)
3rd position	p_{inv}	0.00 (0.00–0.01)	0.00 (0.00–0.01)
tRNA	p_{inv}	0.14 (0.11–0.17)	0.14 (0.11–0.17)
rRNA	p_{inv}	0.33 (0.30–0.36)	0.33 (0.29–0.36)
1st position	m	0.21 (0.20–0.22)	0.21 (0.20–0.22)
2nd position	m	0.06 (0.06–0.07)	0.06 (0.06–0.07)
3rd position	m	3.46 (3.44–3.48)	3.45 (3.44–3.47)
tRNA	m	0.15 (0.14–0.16)	0.15 (0.14–0.16)
rRNA	m	0.13 (0.12–0.14)	0.13 (0.12–0.14)

APPENDIX 5

Estimates of the parameters for the two independent Bayesian analyses of data set #3 (mtREV model for amino acid and GTR + I + Γ for nucleotide substitution models). For details, see legend of Appendix 2

Partition		Parameter (θ) 1st analysis	2nd analysis
tRNA	r_{CT}	20.08 (16.94–23.61)	20.42 (16.89–24.49)
	r_{CG}	0.76 (0.55–1.01)	0.76 (0.56–1.01)
	r_{AT}	1.82 (1.49–2.20)	1.83 (1.47–2.23)
	r_{AG}	15.60 (12.75–18.65)	15.50 (13.12–18.12)
	r_{AC}	1.84 (1.47–2.27)	1.85 (1.49–2.28)
rRNA	r_{CT}	20.29 (16.58–24.81)	20.35 (16.77–24.42)
	r_{CG}	0.47 (0.32–0.65)	0.48 (0.32–0.67)
	r_{AT}	2.84 (2.28–3.49)	2.83 (2.23–3.51)
	r_{AG}	8.16 (6.60–10.00)	8.21 (6.71–10.10)
	r_{AC}	2.81 (2.22–3.50)	2.81 (2.25–3.49)

APPENDIX 5 *Continued*

Partition		Parameter (θ) 1st analysis	2nd analysis
tRNA	π_A	0.29 (0.28–0.31)	0.30 (0.28–0.31)
	π_C	0.19 (0.18–0.20)	0.19 (0.18–0.20)
	π_G	0.22 (0.20–0.23)	0.22 (0.20–0.23)
	π_T	0.30 (0.28–0.32)	0.30 (0.28–0.31)
rRNA	π_A	0.34 (0.33–0.36)	0.34 (0.33–0.36)
	π_C	0.23 (0.22–0.25)	0.23 (0.22–0.25)
	π_G	0.21 (0.19–0.22)	0.20 (0.19–0.22)
	π_T	0.22 (0.21–0.23)	0.22 (0.21–0.23)
amino acid	α	0.65 (0.63–0.67)	0.65 (0.63–0.67)
tRNA	α	0.67 (0.62–0.73)	0.67 (0.61–0.73)
rRNA	α	0.57 (0.52–0.62)	0.57 (0.52–0.62)
amino acid	p_{inv}	0.26 (0.24–0.28)	0.26 (0.24–0.28)
tRNA	p_{inv}	0.13 (0.10–0.16)	0.13 (0.10–0.16)
rRNA	p_{inv}	0.32 (0.29–0.35)	0.32 (0.29–0.35)
amino acid	m	1.21 (1.18–1.23)	1.21 (1.18–1.23)
tRNA	m	0.81 (0.77–0.86)	0.81 (0.77–0.87)
rRNA	m	0.70 (0.66–0.75)	0.70 (0.66–0.75)