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Extracting Species Trees From Complex Gene Trees: Reconciled Trees And Vertebrate Phylogeny

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Paralogy is a pervasive problem in trying to use nuclear gene sequences to infer species phylogenies. One strategy for dealing with this problem is to infer species phylogenies from gene trees using reconciled trees, rather than directly from the sequences themselves. In this approach, the optimal species tree is the tree that requires the fewest gene duplications to be invoked. Because reconciled trees can identify orthologous from paralogous sequences, there is no need to do this prior to the analysis. Multiple gene trees can be analysed simultaneously, however, the problem of nonuniform gene sampling raises practical problems which are discussed. In this paper the technique is applied to phylogenies for nine vertebrate genes (aldolase, a-fetoprotein, lactate dehydrogenase, rhodopsin, trypsinogen, tyrosinase, vassopressin, and Wnt-7). The resulting species tree shows much similarity with currently accepted vertebrate relationships.

INTRODUCTION

> "Regarding the analysis of nuclear genes, it is worth noting that, although the findings, in general, do not support rodent polyphly, different genes have provided dissimilar answers to the question of rodent phylogeny. Such discrepancies are often observed when using nuclear genes, perhaps because some genes evolve under different evolutionary constraints in various tree branches, or because the genes analysed might be paralogous. In contrast, mitochondrial genomes contain only orthologous single-copy genes, and can thus provide more reliable phylogenies." (D'Erchia *et al*., 1996, p. 597)

Mitochondrial and nuclear genes have different strengths and weakness when used to infer vertebrate phylogeny. There is a wealth of mitochondrial sequence data available for vertebrates, although in many taxa only one or two genes have been sequenced. Inferences made from individual mtDNA genes may differ (Cao *et al*., 1994), hence phylogenies from mtDNA are vulnerable to sampling error. This can be obviated by sequencing the complete genome (about 16,000 base pairs in vertebrates), which has been done for a small but growing number of vertebrate taxa. However, even analyses of complete mitochondrial genomes have failed to resolve key questions in vertebrate phylogeny, such as the relationships of lungfishes, tetrapods, and the coelacanth (Zardoya and Meyer, 1997). Furthermore, at the level of deep vertebrate and chordate phylogeny, analysis of mtDNA sequences fail to recover the generally accepted relationships among these taxa (Naylor and Brown, 1998).

Mitochondrial DNA has the virtues of comprising single copy genes in a genome sufficiently large to yield numerous characters yet sufficiently small for its complete sequence to be readily determined. At the same time, these virtues can be seen as limitations. In the absence of recombination mtDNA is inherited as a single unit, and hence phylogenies derived from different mtDNA genes are not independent estimates of organismal phylogeny. Furthermore, its size pales in comparison with that of the nuclear genome.

The size of the nuclear gene database is potentially enormous, especially as genome sequencing projects gain momentum (Brown, 1996). However, nuclear genes are often present in multiple, paralogous copies, making it difficult to be certain that phylogenies are based on orthologous sequences (Fitch, 1970). Paralogy is pervasive. The extent of the problem is illustrated in Fig. 1, which shows the relationship between numbers of species and numbers of sequences for a range of vertebrate mitochondrial and nuclear genes. For mitochondrial genes the relationship is 1:1; each species as a single mitochondrial genome and hence a single sequence for each mitochondrial gene. However, in nuclear genes there are almost always many more sequences than species, so each species may have several representatives of a particular gene family.

One approach to the analysis of nuclear genes is to concatenate the sequences of numerous putatively orthologous genes for the same species into one long sequence (e.g., Graur *et al*., 1996). Apart from the possibilty that such amalgamated data sets may obscure weakness in the data (Halanych, 1998), this does not directly address the problem of distinguishing orthology from paralogy — it merely hopes to overwhelm it by sheer weight of data. Phylogenies of multigene families potentially contain a wealth of information about organismal phylogeny. However, the multiplicity of sequences from the same taxa combined with uneven taxonomic sampling greatly complicates the

relationship between gene and species trees. If the nuclear database is to be fully exploited in phylogenetic studies we need appropriate analytical tools. Reconciled trees are one such tool.

RECONCILED TREES

A reconciled tree is the simplest embedding of a gene tree within a species tree. The technique has its origins in Goodman *et al.*'s (1979) study of haemoglobin gene phylogeny, where there were significant discrepancies between gene and organismal phylogenies. Suppose we have a phylogeny for four species, and four genes sampled from those species, and that the gene and species trees — which we believe to be correct — disagree (Fig. 2a). The question is, how can the trees both be true, and yet be discordant? One approach is to embed the gene tree in the species tree (Fig. 2b), which requires us to postulate a number of gene duplications and subsequent gene losses (in this instance one duplication and three losses). This embedding can also be represented using a reconciled tree (Fig. 2c), which simply takes the embedded gene tree and "unfolds" it so that it lies flat on the page. The reconciled tree depicts the complete history of the gene if there had been no gene losses. In this example, given the gene duplication we would expect species 2, 3, and 4 to each have two copies of the gene. It is the presence of only one copy of the gene in each of these species that leads us to infer the three gene losses. An alternative explanation for these "losses" is that the other copy of the gene is present in these species, but as yet undetected. Given the unevenness of the sampling of different organisms (indicated by the preponderance of a few model organisms in the sequence data banks), this may often be the case. Indeed, the "losses" indicated by the reconciled tree could be viewed as predictions

about the existence of undiscovered genes. In the example shown, further sequencing may uncover copy 1 in species 4, and copy 2 in species 2 and 3. The reconciled tree also shows that genes *b* and *c* are paralogous to gene *d*, which is not apparent from the gene phylogeny alone. This highlights the role organismal phylogeny can play in identifying homology relationships among genes. Direct evidence for paralogy is the presence of multiple genes in the same species (e.g., haemoglobin α and β in the same species). However, additional paralogous genes may be identified using reconciled trees.

Most applications of the reconciled trees in molecular systematics have been to single gene families for the purposes of illustrating the technique (e.g., Page, 1994; Page and Charleston, 1997a). To date there has been only one large-scale attempt to use reconciled trees to analyse the evolutionary history of multiple genes. Guigó, *et al*. (1996) took release 19 of the SWISS-PROT data bank (Bairoch and Apweiler, 1997) and constructed trees for 53 eukaryote genes. They then computed a species tree based on those gene trees, and counted the number of gene duplications and losses required to reconcile these trees with the best organismal tree (although this study did not actually construct reconciled trees, their measure of fit between gene and species trees is formally identical to the reconciled tree procedure, see Eulenstein *et al*., 1997). They used the resulting species tree to locate episodes of gene duplication, and suggested that the observed duplications in the 53 genes could be accounted for by five episodes of whole genome duplication. Guigó *et al*.'s pioneering study shows the potential of reconciled trees in studies of gene and genome evolution, but has several serious flaws:

1. The methods used to construct the individual gene trees were clustering techniques rather than explicitly phylogenetic methods such as parsimony, likelihood, or

neighbour-joining, and the resulting trees were midpoint rooted, which assumes a molecular clock. This assumption was not tested.

2. When more than one gene was present in the same taxon, Guigó *et al*. used the average distance between those genes and sequences from other species to construct a distance matrix. This use of "composite" sequences for some taxa almost certainly resulted in spurious trees for some genes.
3. Their search strategy for finding the optimal species tree was ineffective — Page and Charleston (1997b) found substantially more parsimonious species trees for the same data using different search strategies. These species trees different markedly from the tree found by Guigó *et al*.

My goal here is to illustrate how reconciled trees might be applied to a real systematic problem, in this case vertebrate phylogeny. I have not set out to "solve" this problem, but rather to explore the usefulness of reconciled trees when applied to a real problem. There are significant practical differences between using reconciled trees to simply depict the history of a gene family (Page and Charleston, 1997a) and using them to investigate organismal phylogeny. These problems emerged during this study and are considered below.

Searching for optimal species trees

The fit between a gene tree and a species tree can be used as an optimality criterion for choosing among competing species trees – the species tree that accommodates the gene tree with the least cost is the preferred species tree (Slowinksi and Page, in press). This approach can be generalised to more than one gene, so that we can use evidence from multiple genes. The cost of reconciling a given gene and

species tree can be computed efficiently (Eulenstein, 1997) however, the problem of finding which species tree has the optimal value of this cost is NP-complete (L. Zhang, personal communication). Hence, we rely must on heuristic searches. Page and Charleston (1997a; 1997b) showed that a combination of nearest neighbour interchanges (nni) and subtree prune and regrafting (Swofford *et al*., 1996) is effective in finding most parsimonious species trees.

Missing sequences

The extreme taxonomic bias of the sequence data bases towards a few model organisms (93% of vertebrate nucleotide sequences in GenBank come from humans, rats or mice) means it is almost certainly the case that not all genes will have been discovered (or, indeed, looked for) in all the taxa of interest. This can lead to cases where species will be grouped on the absence of genes, rather than any actual evidence of their relationship. This problem can be minimised by using only the number of duplications as the optimality criteria for selecting species trees (Page and Charleston, 1997a).

Another problem caused by missing sequences is the rapid increase in the number of species trees that are equally parsimonious explanations of the gene trees. As an example, consider the two gene trees shown in Fig. 3. Both trees have something to say about the relationships among amphibian, birds, and mammals, however genes 1 and 2 have no mammal species in common. Hence there are seven optimal species trees for these two gene trees, which correspond to the alternative placements of the horse on the subtree ((mouse,rat),(cow,human)). The practical dilemma here is whether to include gene 2. Its inclusion brings information about the

relations among the higher taxa amphibia, birds, and mammals. However, because there is no sequence for gene 1 from the horse, the relationships of this taxon are unconstrained with respect to the other mammals. There is a trade-off between adding additional or corroborating information about higher taxon relationships, versus minimising the ambiguity of multiple equally parsimonious trees due to taxa represented by few sequences "floating" with respect to other taxa.

Constrained searches

Constraint trees (Constantinescu and Sankoff, 1986) can be used to address a major problem in inferring species trees from the current sequence data bases, namely their limited taxonomic coverage. A rather extreme hypothetical example is presented in Fig. 4. Suppose that we have two genes (1 and 2), and for both genes we have a single sequence from a teleost fish, a bird and a mammal. However, in each instance the fish, bird and mammal species are different for the two genes. Given these two gene trees there are 105 six-taxon species trees that minimise the number of gene duplications and losses for these gene trees. The strict consensus of these trees is a star tree. This result is due to the lack of any shared information in the two trees that links, for example, the rat and the mouse, the salmon or the trout, or the chicken and the duck.

There are at least two strategies we could use to circumvent this problem. The simplest, but least attractive, solution is to rename each sequence with the name of the corresponding higher taxon (i.e., the rat and the mouse both become "mammals" the chicken and duck "birds," etc.). This would allow us to recover a single species tree (fish,(birds,mammals)), but has two drawbacks. The first is that it prevents us from

investigating relationships among taxa within any of these higher clades; if all mammalian sequences are simply labelled "mammals" regardless of whether they are from elephants, whales or mice, then any information about mammalian relationships from a gene with sequences from different mammals is lost. The second problem is that if we rename sequences we may infer spurious gene "duplications." For example, a clade of three mammalian sequences would require the occurrence of two duplications to explain the three sequences in the same taxon (Fig. 5). However, if the sequences are from different mammals this would be simply an artefact of relabelling the sequences. A better solution is to specify interrelationships among species using a constraint tree (Fig. 4b). This enables uncontested groupings such as birds and mammals to be specified without loosing potential information on relationships within these groups.

Kinds of duplications

Gene duplications identified in reconciled trees can be classified into two categories (Goodman *et al.*, 1979): those that are based on direct physical evidence such as sequencing more than one copy in the same species, and those that are inferred from incongruence between gene and species trees. Estimates of gene trees are subject to uncertainty, but current algorithms for computing reconciled trees do not take this into account — any incongruence no matter how weakly supported will result in duplications being inferred. It would be desirable to incorporate some measure of the robustness of the gene tree when constructing reconciled trees (see *Discussion* below) but no method for doing this is presently available. The approach used in this paper to assess the degree of support for an inferred duplication is based on the close

relationship between the number of duplications required to fit a gene tree into a species tree and the nni distance between the two trees (Ma *et al*., 1998). Given two trees the nni distance between them is the number of nni's required to transform on tree into the other (Waterman and Smith, 1978) (Fig. 6a). Consider the gene and species trees shown in Fig. 6b. Gene tree 1 is incongruent with the species trees, hence a duplication is inferred. However, if the gene tree were really (((a,b),c),d) instead of (((a,c),b),d), there would be no incongruence and hence no duplication. The trees (((a,b),c),d) and (((a,c),b),d) are similar to each other, differing in a single nearest neighbour interchange. Hence, a fairly trivial modification to the gene tree would undermine the evidence for the inferred duplication. In contrast, gene tree 2 is more dissimilar to the species tree, differing by 2 nni's and requiring two duplications to fit the species tree. Given the greater mismatch between these two trees we might have more confidence in the existence of those duplications — it would take a more drastic rearrangement of the gene tree to make it concordant with the species tree. However, using nni's alone as a measure of incongruence ignores the degree of support for the nodes in the tree. If the bootstrap values are as shown in Fig. 6, then although only a single nni will suffice to eliminate the duplication required by gene tree 1, the nni is across a very well supported node (bootstrap value of 95%). By comparison, the two nni's required to eliminate the duplications implied by gene tree 2 involve relatively weakly supported nodes. Hence we might have rather less confidence in these duplications. A crude measure of confidence in duplication would be the average bootstrap value of the edges in the tree taking involved in the nni's required to "undo" the duplication. For the example in Fig. 6, the duplication required by gene tree 1 has a score of 0.95 (a single nni with a bootstrap value of 95), whereas gene tree 2 has a score of 0.30 (2 nni's of with bootstrap values of 40 and 20, respectively). This

measure has limitations (among them the non-independence of bootstrap values), but allows a quick assessment of the degree of support for inferred duplications.

Supertrees

Using reconciled trees to infer species trees from multiple gene trees is superficially similar to methods for assembling "supertrees" (Sanderson *et al*., 1998) from suites of smaller trees. However, apart from the differences discused earler (Page, 1994), reconciled trees use an explicitly biological criterion (numbers of gene duplications and losses) to choose the optimal species tree (Mirkin *et al*., 1995), in contrast to consensus supertree methods which do not. Furthermore, a supertree approach to constructing a species tree from complex gene trees will encounter the problem of multiple occurrences of genes from the same species in the gene tree.

MATERIALS AND METHODS

Genes were selected for this study by surveying recent publications on molecular phylogeny of vertebrates and gene families (e.g., Caspers *et al*., 1996; Mannen *et al*., 1997; Stock *et al*., 1997), and by browsing the HOVERGEN (Duret *et al*., 1994) and SWISS-PROT data banks, supplemented by BLAST (Altschul *et al*., 1990) searches. The primary selection criteria were breadth of taxonomic coverage (which eliminated the vast majority of gene families) followed by robustness of the inferred gene trees. Protein sequences were aligned and neighbour joining trees were inferred using CLUSTALX (Thompson *et al*., 1997). The alignments are available from the author's Web site (http://taxonomy.zoology.gla.ac.uk/rod/data/vertebrates/).

Robustness of the gene trees was assessed using bootstrapping (Felsenstein, 1985). Throughout this paper a sequence is referred to by a data base identifier (such as an accession number or locus name) and the name of the species from which the sequence was obtained.

The reconciled tree algorithm requires rooted gene trees, so the trees were rooted using one of two criteria. Firstly, where possible the gene tree was rooted using one or more obviously paralogous sequences. For example, the tree for prolactin sequences was rooted using somatotropin sequences. Alternatively, a sequence from a closely related non-vertebrate taxon, such as a tunicate or amphioxous, was used as the outgroup (see lactate dehydrogenase below).

A total of nine gene families were selected for analysis. Table 1 lists the species for which sequences were available. Most taxa are represented by only a few genes. In five cases ("whale", "turtle", "frog," "*Scleropus*", and "goose") a higher taxon was represented by different species in different genes, but none of these species were sequenced for the same gene. In these instances I combined the taxa.

Optimal species trees were inferred using GENETREE (PAGE, 1998). The optimality criterion was number of duplications. The constraint tree used (Fig. 7) enforced the monophyly of the Vertebrata, Amphibia (frogs and salamanders), Squamata (lizards and snakes), Aves (birds) and the Actinopterygii (bony fish). Because of the very patchy coverage of the Actinopterygii within this clade the constraint tree is moderately resolved. Other constraints involved terminal taxa which are obviously closely related (e.g., ducks and geese) but for which there were no sequences from the same gene available. Although the constraint tree imposes some structure on the result, note that it does not specify any relationships among the vertebrate classes.

An initial search was undertaken to find a good starting point for more extensive branch swapping. This preliminary search was performed using 20 different random start trees. The heuristic search employed alternating nearest neighbour interchanges (nni) and subtree pruning and regrafting (spr) (Swofford *et al.*, 1996) rearrangements, with the constraint tree shown in Fig. 7 enforced, equally good trees were not retained. This last option was chosen to avoid spending time rearranging what would eventually prove to be suboptimal trees. As discussed above (see Fig. 3), even moderate numbers of missing sequences can result in huge numbers of equally good species trees. Rather than spend search time trapped in local "branch swapping eddies" (Novacek, 1992) due to missing data I preferred to survey the search landscape (Charleston, 1995) more thoroughly from multiple starting points. The best tree from this initial search was rearranged with all equally good trees retained (up to GENETREE's limit of 1000 trees). Consensus trees were computed using COMPONENT 2.0 (Page, 1993).

RESULTS

Gene trees

Trees for each of the nine genes are shown in Fig. 8. Aldolase sequences were taken from Nikoh et al. (1997) supplemented by additional sequences from the data banks. The vertebrate genes were rooted using a sequence from the amphioxus *Branchistoma belcheri*. Alpha-fetoprotein sequences were obtained from HOVERGEN and rooted using rat vitamin D-binding protein precursor. Lactate dehydrogenase sequences were obtained by combining those from papers by Stock et al. (1997) and Mannen et al. (1997). The tree was rooted using the tunicate *Styela*

plicata. Prolactin sequences from SWISS-PROT were rooted with the related gene somatotropin. The rhodopsin data comes from Yokohama (1994), supplemented with sequences from the data banks. The tree was rooted using invertebrate sequences. Roach et al. (1997) was the source for the trypsinogen sequences, which were rooted using the tunicate *Boltenia villosa*. Tyrosinase is among the genes used by Caspers et al. (1996) in their study of turtle relationships. The tree was rooted using "tyrosinase-related proteins" 1 and 2. The vassopressin gene family was extracted from the HOVERGEN data base, with further sequences obtained from SWISS-PROT. The tree was rooted using the vasopressin-related peptide Lys-conopressin from the pond snail *Lymnaea stagnalis* (van Kesteren *et al*., 1995). Deeper branches within the tree show poor bootstrap values, but the gene tree was included in this analysis because of the breadth of taxa included (including lungfish and hagfish). Wnt-7 is part of a larger gene family (Sidow, 1992). This gene was chosen for its relatively broad taxonomic coverage relative to other Wnt genes. The tree was rooted with echinoderm sequences.

Species tree

The initial search found two trees with 87 duplications. Rearranging these trees found 1000 trees with the same cost. The strict consensus of these trees is shown in Fig. 9. It should be emphasised that the topology of this species tree depends entirely on the topology of the nine gene trees (and the constraint tree), no reference is made to the underlying sequence data. Much of the lack of resolution in the consensus tree is due to lack of shared information among the gene trees (see Fig. 3), rather than conflicts among the gene trees.

The species tree shows a basal split between hagfish and the rest of the vertebrates, with lampreys as the sister taxa to the Gnathostomes (jawed vertebrates). Within the Gnathostomes the basal split is between chondrichthyans (sharks and rays) and actinopterygians (ray-finned fish) on one side and lungfish and tetrapods on the other. Because of the poor taxonomic overlap in sequences from actinopterygians the constraint tree imposed considerable structure on this clade, with only the relationships among the Gadiformes, Salmoniformes, Ostariophysi, and Acanthopterygii left unspecified. The consensus tree groups the Acanthopterygii and the Salmoniformes together. Relationships between lungfish, amphibians, and amniotes are unresolved. The amniotes are divided into mammals and the diapsids (turtles, crocodilians, snakes + lizards, and birds). The four diapsid groups form an unresolved clade. Mammalian relationships are reasonably resolved with myomorph rodents (rats, mice and their relatives) being basal to the remaining sampled placental mammals.

Duplications

The numbers of duplications for each gene are listed in Table 2. The duplications are divided into those based on direct physical evidence and those that are inferred from incongruence between gene and species trees. For the latter the total number of nni's required to "undo" the duplications (Fig. 6), and the mean cost of those nni's in terms of bootstrap values are listed. The degree of support for individual inferred duplications spans the range from very weak to bootstrap values of 100% (Fig. 10). This suggests that some inferred duplications are likely to be artefacts of erroneous gene trees. For example, the apparent duplications among mammalian prolactin genes have either low or very weak bootstrap support, suggesting that the sequence data

support alternative gene trees that do not require duplications. In contrast, the duplications inferred for aldolase have strong (> 80%) bootstrap support.

DISCUSSION

Vertebrate phylogeny

Although the broad outlines of vertebrate relationships are generally agreed, some aspects are subjects of considerable debate (Janvier, 1998; Patterson *et al*., 1993). Relationships among major vertebrate clades inferred from the nine gene trees (Fig. 11) agree in many respects with the currently accepted view of vertebrate phylogeny. The paraphyly of the agnatha (hagfishes and lampreys) agrees with Forey and Janvier's (1993) morphological study, and with Rasmussen et al.'s (1998) analysis of complete mitochondrial genomes, although not with trees inferred from nuclear ribosomal genes (Mallatt and Sullivan, 1998; Stock and Whitt, 1992). The grouping of sharks and teleost fish is unconventional; the current consensus is that teleosts are more closely related to lungfish and tetrapods than to chondrichthyians. None of the genes (lactate dehydrogenase, rhodopsin, trypsinogen, Wnt-7) that have both chondrichthyian and teleost sequences support this conventional relationship. Interestingly, trees inferred from complete mitochondrial genomes place chondrichthyians within the teleosts (Rasmussen and Arnason, 1999).

The close relationship between lungfish and tetrapods is supported by reconciled tree analysis, although the monophyly of the tetrapods is neither supported nor contradicted. The uncertainty in lungfish relationships reflects the contradictory

evidence offered by the two genes in this study for which lungfish have been sequenced. Lungfish prolactin is sister to a monophyletic clade of tetrapod prolactins, whereas the two lungfish vassopressin sequences group with mammals and amphibians, respectively. The grouping of turtles with other diapsids agrees with recent morphological (Rieppel and deBraga, 1996) and molecular (Caspers *et al.*, 1996) studies. This is not surprising as two of the genes included here (prolactin and tyrosinase) were also studied by Caspers *et al.* (1996). Although the widely accepted sister grouping of birds and crocodilians is supported by prolactin and rhodopsin, this relationship is contradicted by lactate dehydrogenase, consequently the strict consensus tree is unresolved for these taxa. Mammalian phylogeny is somewhat uncertain (de Jong, 1998). The species relationships recovered here are not unreasonable for most taxa, although the grouping of carnivores and the pig is unorthodox.

Limitations and future directions

Perhaps the two greatest limitations of the method used here are its reliance on fully resolved gene trees, and its inability to distinguish among nodes within a tree based on their degree of support — weakly supported nodes may have as much influence on the result as those that are much more robust. This problem could be addressed in a number of ways, which are currently being investigated. One solution would be to incorporate some measure of node support, such as bootstrap values (Felsenstein, 1985). In this study I've attempted to measure the relative support for inferred duplications using bootstrap values and nni's (Table 2 and Fig. 10), given a species tree inferred under the assumption that all nodes in the gene trees have equal

weight. While this approach can be used to identify weakly supported (and hence possibly erroneous) gene duplications at the end of the analysis, it could be improved by taking the bootstrap values into account during the search for the optimal species tree, such that duplications and associated losses inferred from weakly supported nodes would be down-weighted. In this way poorly supported gene tree nodes would have less influence on the inferred species tree.

Another approach would be to consider a set of gene trees for each gene, such as those comprising a "confidence interval" around the optimal gene tree (Page, 1996; Sanderson, 1989). The cost of a given species tree would be computed for all gene trees within the confidence interval, and one value (such as the minimum cost for all gene trees) would be assigned to the species tree. Indeed, the fit between gene and species tree could be used as an additional criterion for selecting among competing gene trees that cannot be discriminated amongst on the basis of nucleotide or protein sequences data alone. Goodman *et al.* (1979) suggested such a strategy in their pioneering work on reconciled trees in which they preferred less parsimonious haemoglobin gene trees which had better fit to accepted species trees than most parsimonious trees that required more duplications and losses. Their approach assigned to a gene tree a total score based on the length of the tree in terms of number of nucleotide substitutions plus the number of gene duplications and losses, where the each type of event had the same cost. This drew immediate criticism from Fitch (1979), who argued that there was no obvious way of determining the relative cost of a nucleotide substitution versus a gene duplication. A likelihood framework may provide one solution to this problem, as has been suggested in the context of coalescence models by Maddison (1997). However, while reasonable models of nucleotide substitution exist, there are none for gene duplication. Furthermore, any

model would need to incorporate the extreme sampling bias that exists in the sequence databases (and hence that many gene "losses" are sampling artefacts).

The optimality criterion being minimised in this study is the number of gene duplications. A key assumption made is that gene duplications in different genes are mutually independent, and hence can be minimised independently. Given that vertebrate genes have been duplicated in blocks of various sizes, up to entire genomes (Holland *et al*., 1994; Pébusque *et al*., 1998), it might be more appropriate to minimise episodes of multiple duplication, rather than the individual duplications themselves. However, this greatly increases the computational complexity of the problem. Minimising individual gene duplications can be done in linear time (Eulenstein, 1997), whereas minimising episodes of multiple duplication is NP-complete (Fellows *et al*., 1998). Even if the frequency of block duplications has been overestimated (Hughes, 1998), there is a need to develop techniques that incorporate this process.

Given the reasonable success reconciled trees had in recovering vertebrate phylogeny from a small number of gene trees of variable quality, I think the method merits further study and application. Other taxa that are good candidates for study are angiosperms, and eukaryotes as a whole. Another obvious extension is to apply the method to much larger sets of gene trees. In this study I have restricted my attention to a few genes that have good coverage of the vertebrate classes. Given the large (and ever increasing) number of gene families available for analysis there is considerable scope for automating the analysis. For example, it would be useful to be able to extract gene trees from data bases like HOVERGEN and input this directly into software such as GENETREE. Hence it would be possible, in principle, to obtain the best estimates of species phylogeny based on simultaneous analysis of thousands of gene families.

ACKNOWLEDGMENTS

Access to the HOVERGEN database was kindly provided by the UK Human Genome Mapping Project Resource Centre, Hinxton, Cambridge. I thank Michael Fellows and Louxin Zhang for sending preprints of their work.

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TABLE CAPTIONS

Table 1 Taxonomic distribution of sequences for the nine genes included in this study. Gene abbreviations: ALD = aldolase, AFP = α-fetoprotein, LDH = lactate dehydrogenase, PRL = prolactin, OPS = rhodopsin, TRYP = trysinogen, TYR= tyrosinase, VASS = vassopressin.

Table 2 Number of gene duplications for each gene. Duplications are divided into those for which there is the direct evidence of multiple sequences from the same taxon ("observed") and duplications inferred from mismatches between gene and species tree ("inferred"). The total number of number of nni's required to make the gene tree congruent with species tree by and the mean bootstrap value for the edges involved in these nni's (see Fig. 6) are listed.

Table 1

Taxon	Scientific name	ALD	AFP	LDH	PRL	OPS	TRYP	TYR
human	*Homo sapiens*	•	•	•	•	•	•	•
macaque	*Macaca mulatta*				•			
marmoset	*Callithrix jacchus*					•		
mouse	*Mus musculus*	•	•	•	•	•	•	•
rat	*Rattus norvegicus*	•	•	•	•	•	•	
hamster	*Mesocricetus auratus*				•	•		
guinea pig	*Cavia porcellus*							
rabbit	*Oryctolagus cuniculus*	•	•	•	•	•		
cow	*Bos taurus*		•	•	•	•	•	
sheep	*Ovis aries*	•	•		•	•		

pig	*Sus scrofa*					•		•				•		
goat	*Capra hircus*							•						
fox	*Vulpes vulpes*					•								
dog	*Canis familiaris*			•						•		•		•
cat	*Felis silvestris catus*							•						
horse	*Equus caballus*			•				•						
whale	*Balaenoptera borealis*							•						
	Balaenoptera physalus													
chicken	*Gallus gallus*	•		•		•		•		•		•		•
duck	*Anas platyrhynchos*					•								
goose	*Anser anser*													
	Anser caerulescens													
pigeon	*Columba livia*					•				•				
quail	*Coturnix coturnix*													•

turkey	*Meleagris gallopavo*				•			
ostrich	*Struthio camelus*							
Scleropus	*Sceloporus undulatus*			•				
	Sceloporus occidentalis							
anole	*Anolis carolinensis*					•		
gecko	*Gecko gecko*					•		
alligator	*Alligator mississippiensis*			•	•	•		
crocodile	*Crocodylus novaeguineae*				•			
turtle	*Trachemys scripta*			•				
	Trionyx sinensis							•
	Chelonia mydas caranigra				•			
cobra	*Naja naja*		•					
Xenopus	*Xenopus laevis*	•	•	•		•		
bull frog	*Rana catesbeiana*		•			•		

Japanese toad	*Bufo japonicus*			•			
frog	*Rana pipiens*				•		
	Rana esculenta						
	Rana nigromaculata						•
tiger salamander	*Ambystoma tigrinum*				•		
salamander	*Plethodon jordani*						
newt	*Pleurodeles waltl*						
lungfish	*Protopterus aethiopicus*			•			
	Neoceratodus forsteri						
killifish	*Fundulus heteroclitus*		•				
goldfish	*Carassius auratus*	•			•		
sea bream	*Sparus aurata*	•					
Sphoeroides	*Sphoeroides nephelus*	•					
fugu	*Takifugu rubripes*					•	

cod	*Gadus morhua*					•	
plaice	*Pleuronectes platessa*					•	
atlantic salmon	*Salmo salar*	•		•		•	
cherry salmon	*Oncorhynchus masou*						
white sucker	*Catostomus commersoni*						
chum salmon	*Oncorhynchus keta*			•			
medeka fish	*Oryzias latipes*				•		•
cave fish	*Astyanax fasciatus*				•		
eel	*Anguilla anguilla*			•	•		
Bailkal omul	*Coregonus autumnalis*			•	•		
goby	*Pomatoschistus minutus*						
mosquito fish	*Gambusia affinis*				•		
guppy	*Poecilia reticulata*				•		
zebrafish	*Brachydanio rerio*				•		

carp	*Cyprinus carpio*					•				
catfish	*Ictalurus punctatus*					•				
trout	*Oncorhynchus mykiss*					•				
tilipia	*Oreochromis mossambicus*					•				
sea bass	*Dicentrarchus labrax*					•				
dogfish	*Squalus acanthias*				•					•
Alopias	*Alopias vulpinus*									
electric ray	*Torpedo marmorata*									
skate	*Raja erinacea*							•		
Japanese lamprey	*Lampetra japonica*		•					•		
lamprey	*Petromyzon marinus*			•	•			•		•
Pacfic hagfish	*Eptatretus stouti*									
inshore hagfish	*Eptatretus burgeri*									

Table 2

Gene	Duplications			nni	Mean bootstrap
	Observed	Inferred	Total		
aldolase	4	2	6	3	0.84
α-fetoprotein	5	2	7	4	0.78
lactate dehydrogenase	6	5	11	6	0.54
prolactin	4	4	8	5	0.43
rhodopsin	9	7	16	9	0.54
trypsinogen	16	3	19	3	0.24
tyrosinase	0	0	0	0	-
vassopressin	8	6	14	7	0.51
Wnt-7	3	3	6	4	0.61

FIGURE CAPTIONS

Fig. 1. Number of sequences plotted against number of species for vertebrate gene families in release 29 (March 17, 1998) of the HOVERGEN (Duret *et al.*, 1994) data base. Note that usually each species has a single mitochondrial sequence for a given gene (hence the mitochondrial genes fall along the 1:1 line), whereas most nuclear genes are present in multiple copies. Due to redundancy in species names (for example, "human" and "*Homo sapiens*" being used to describe the source of different genes in the same family), some gene families appear to have fewer sequences than species.

Fig. 2. (a) Incongruent gene and species trees. This incongruence can be explained by hypothesising a gene duplication (❑) at the base of the gene tree (b). The presence of only a single gene (*a*-*d*) extant each of the present day species (1-4) requires postulating three gene losses (✞). (c) The corresponding reconciled tree.

Fig. 3. Two gene trees containing sequences from amphibia, birds, and mammals. Whereas the amphibian and bird are the same in the two genes, no mammals are shared between the two genes. Hence, any species tree that is consistent with these two gene trees will be a parsimonious explanation of the data. In this example, there are seven such trees, which correspond to the alternative placements of the horse on the subtree ((mouse,rat),(cow,human)) indicated by •.

Fig. 4. (a) Trees for two different genes (1 and 2) for which sequences have been obtained from a teleost fish, a bird, and a mammal. The sequences have been obtained from different representatives of these higher taxa. For these two gene trees there are 105 equally parsimonious species trees, which yield an unresolved strict consensus tree. This is because there is no information in the gene trees that the salmon and the

trout are, for example, both teleost fishes (and that there are two birds and two mammals). One solution is to enforce constraints on the set of possible optimal species trees by accepting only those trees that preserve the higher taxon relationships among the sequences (b).

Fig. 5. An example where relabelling a set of sequences with the corresponding higher taxon (in this instance "mammals") implies false gene duplications.

Fig. 6. (a) An unrooted tree for four objects. Interchanging any pair of nodes either side of the internal edge *e* results in one of two trees, hence these trees are one nearest neighbour interchange (nni) away from the original tree. (b) Two gene trees which are both incongruent with the species tree and hence require gene duplications to be postulated. For each gene the number of nni's required to make the gene tree match the species are shown. Gene tree 1 requires only a single nni, whereas gene tree 2 requires two nni's. However, the nni in gene tree 1 is across a well-supported node (bootstrap value of 95) whereas the two nni's in gene tree 2 are across poorly supported nodes (bootstrap values of 20 and 40). Hence the duplications inferred from the mismatch between gene tree 2 and the species tree are more likely to be artefacts of an incorrect gene tree than the duplication inferred from gene tree 1.

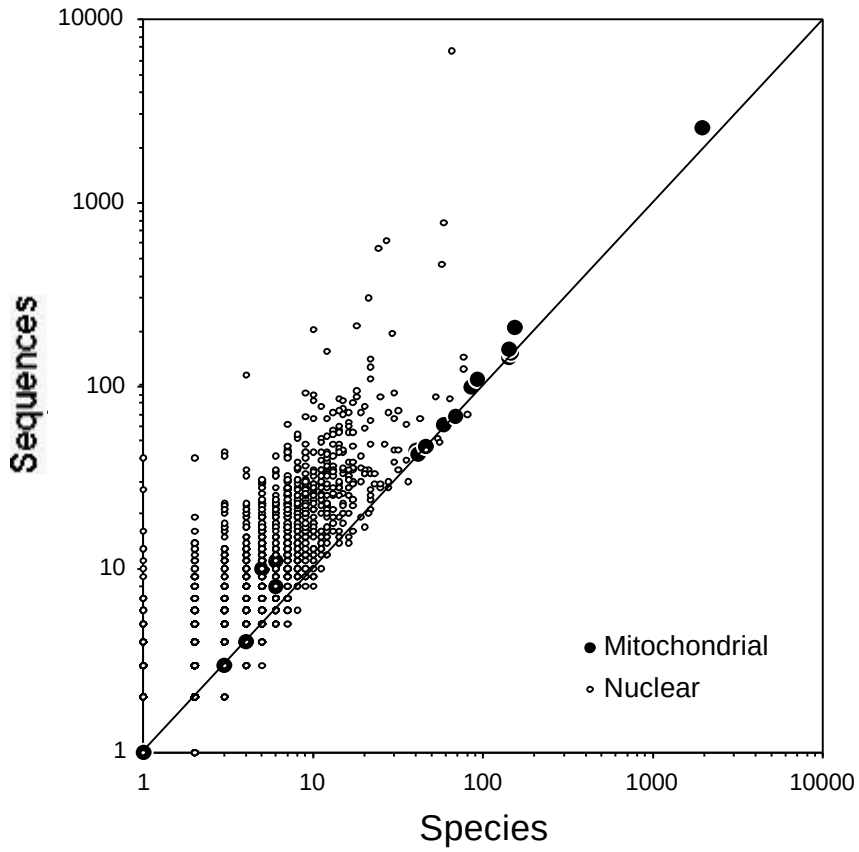
Fig. 7. Constraint tree used in this analysis. Only species trees compatible with this constraint were accepted. The tree specifies the monophyly of some vertebrate classes, but does not specify any relationship among those classes.

Fig. 8. Neighbour-joining trees for the vertebrate genes included in this study. Numbers above branches are bootstrap values. Scale bar represents 0.1 amino acid replacements per amino acid site.

Fig. 9. Strict consensus tree of 1000 equally parsimonious species trees for the gene trees shown in Fig. 8. Nodes that were defined in the constraint tree (Fig. 7b) are indicated by •.

Fig. 10. Distribution of bootstrap values for each edge across which a nni must be made to make the gene trees shown in Fig. 8 congruent with the species tree in Fig. 9. The mean bootstrap value for each gene is shown in Table 2.

Fig. 11. Summary of the relationships among major vertebrate groups suggested by the reconciled trees for nine gene familes.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Sequences
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10
100
1000
10000
Species
Mitochondrial
Nuclear

Fig. 1

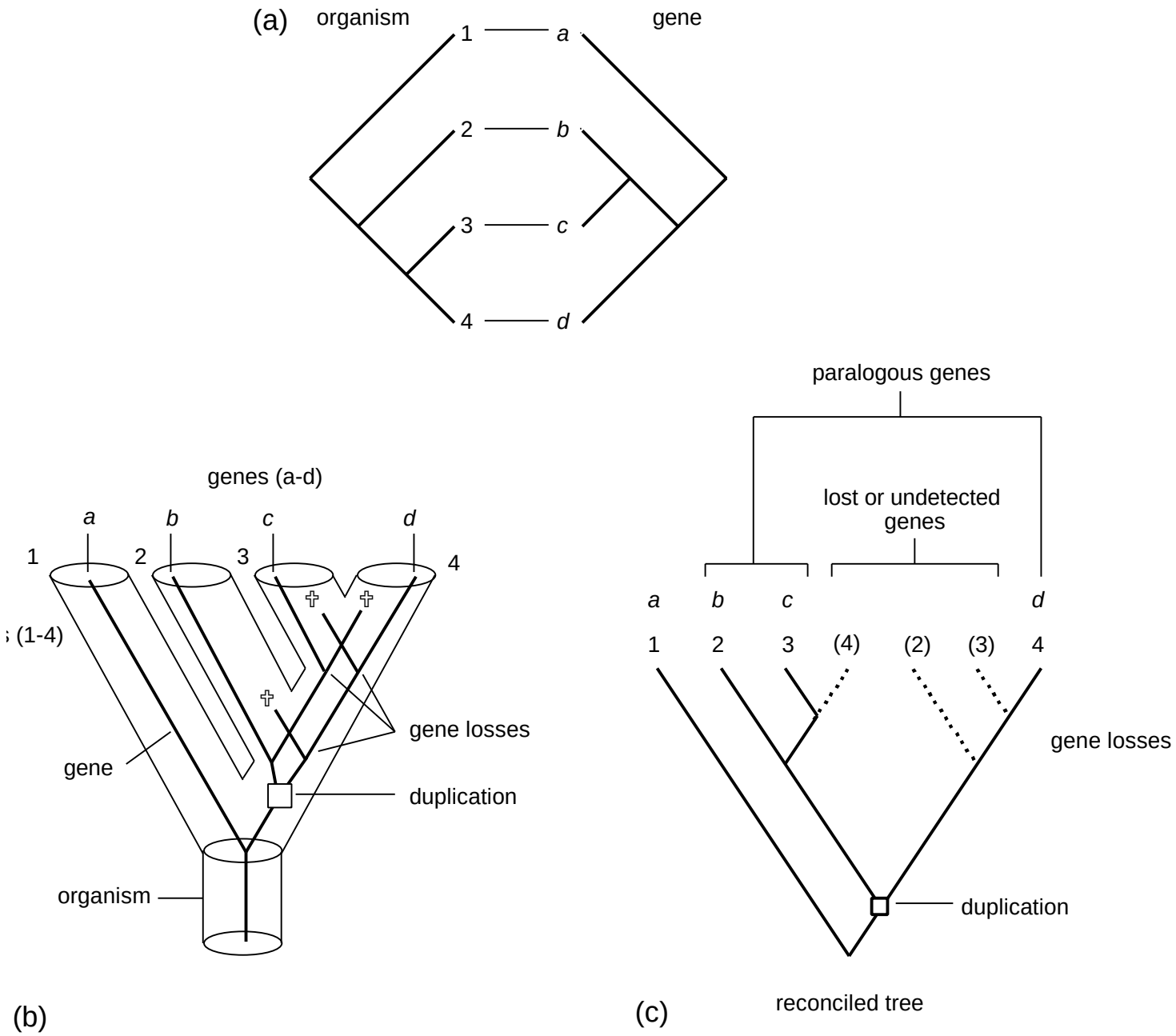
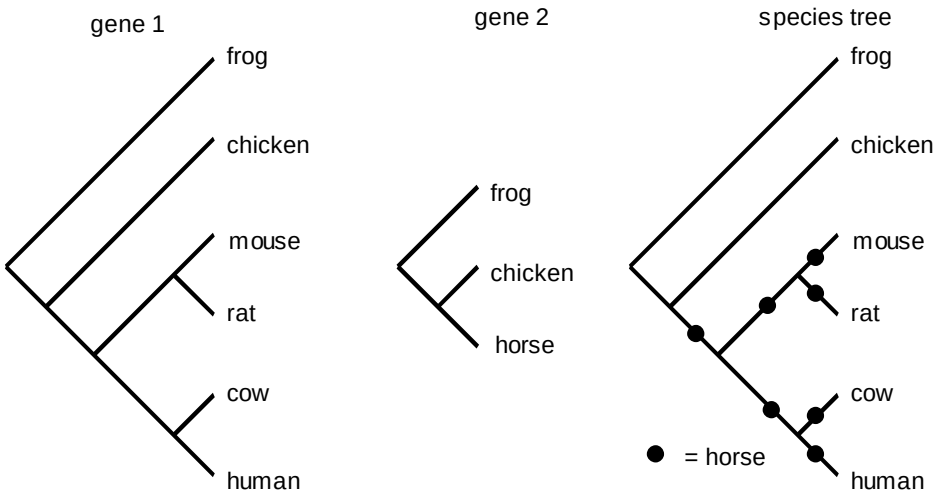

(a)
organism
gene
1
2
3
4
a
b
c
d
genes (a-d)
a
b
c
d
1
2
3
4
; (1-4)
gene losses
gene
duplication
organism
(b)
paralogous genes
lost or undetected genes
a
b
c
d
1
2
3
(4)
(2)
(3)
4
gene losses
duplication
reconciled tree
(c)

Fig. 2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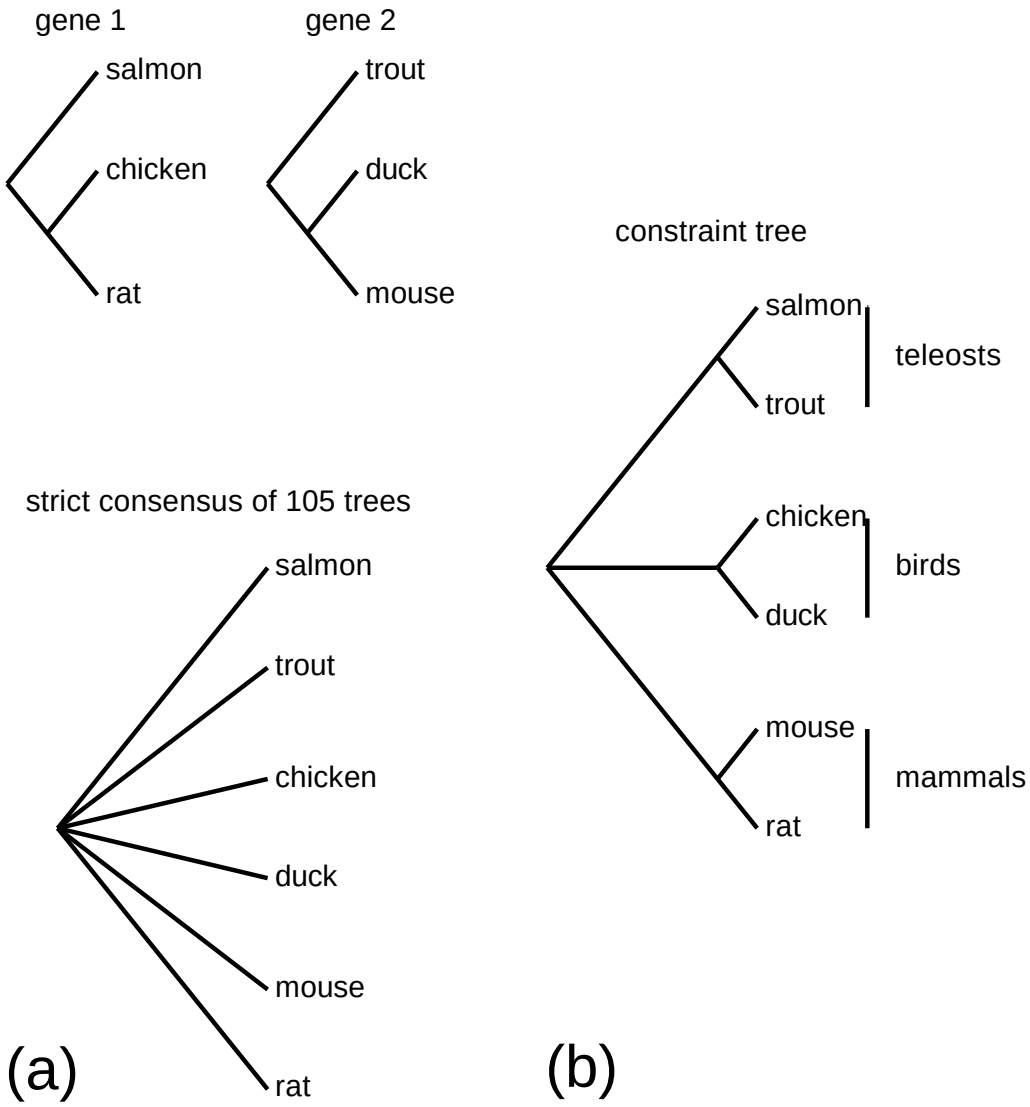
frog
chicken
mouse
rat
cow
human
gene 2
frog
chicken
horse
species tree
frog
chicken
mouse
rat
cow
human
= horse

Fig. 3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chicken
rat
gene 2
trout
duck
mouse
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salmon
trout
chicken
duck
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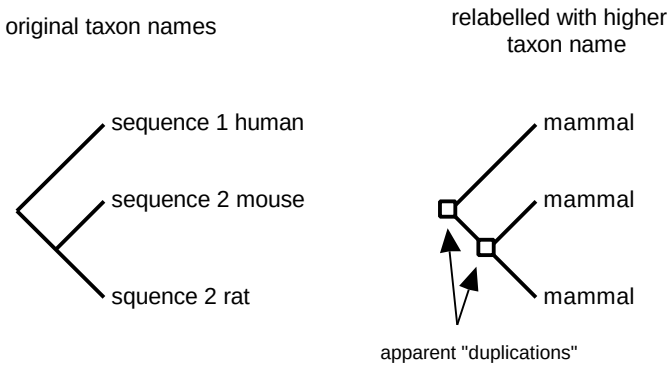
constraint tree
salmon
trout
teleosts
chicken
duck
birds
mouse
rat
mammals
(b)

Fig. 4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relabelled with higher taxon name
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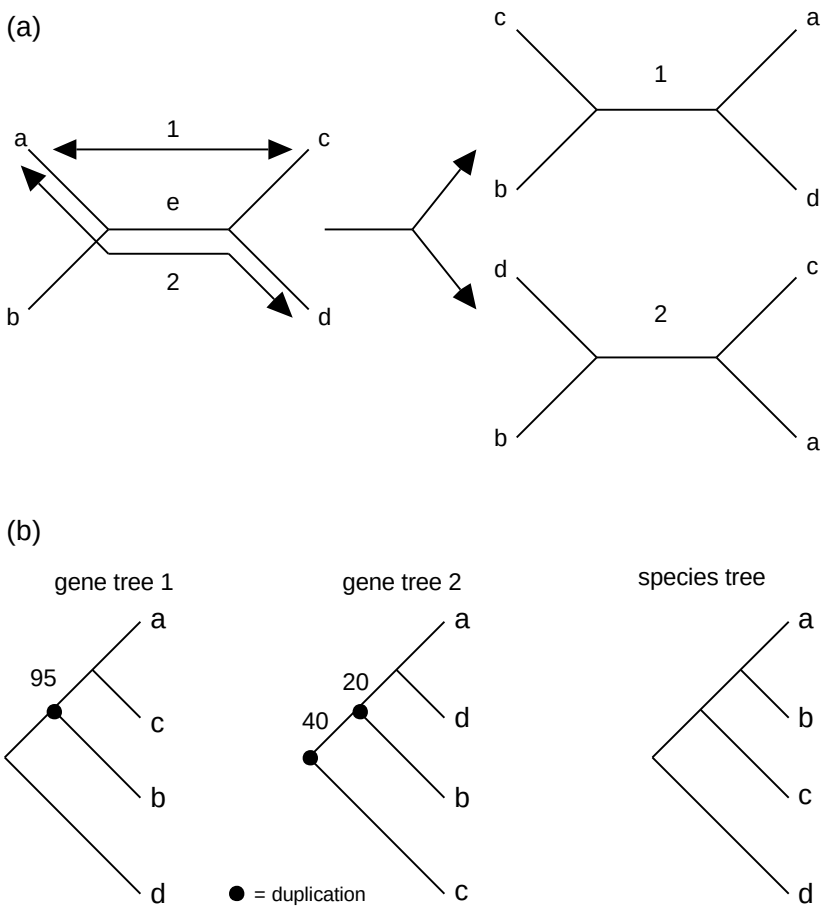
sequence 2 mouse
squence 2 rat
mammal
mammal
mammal
apparent "duplications"

Fig. 5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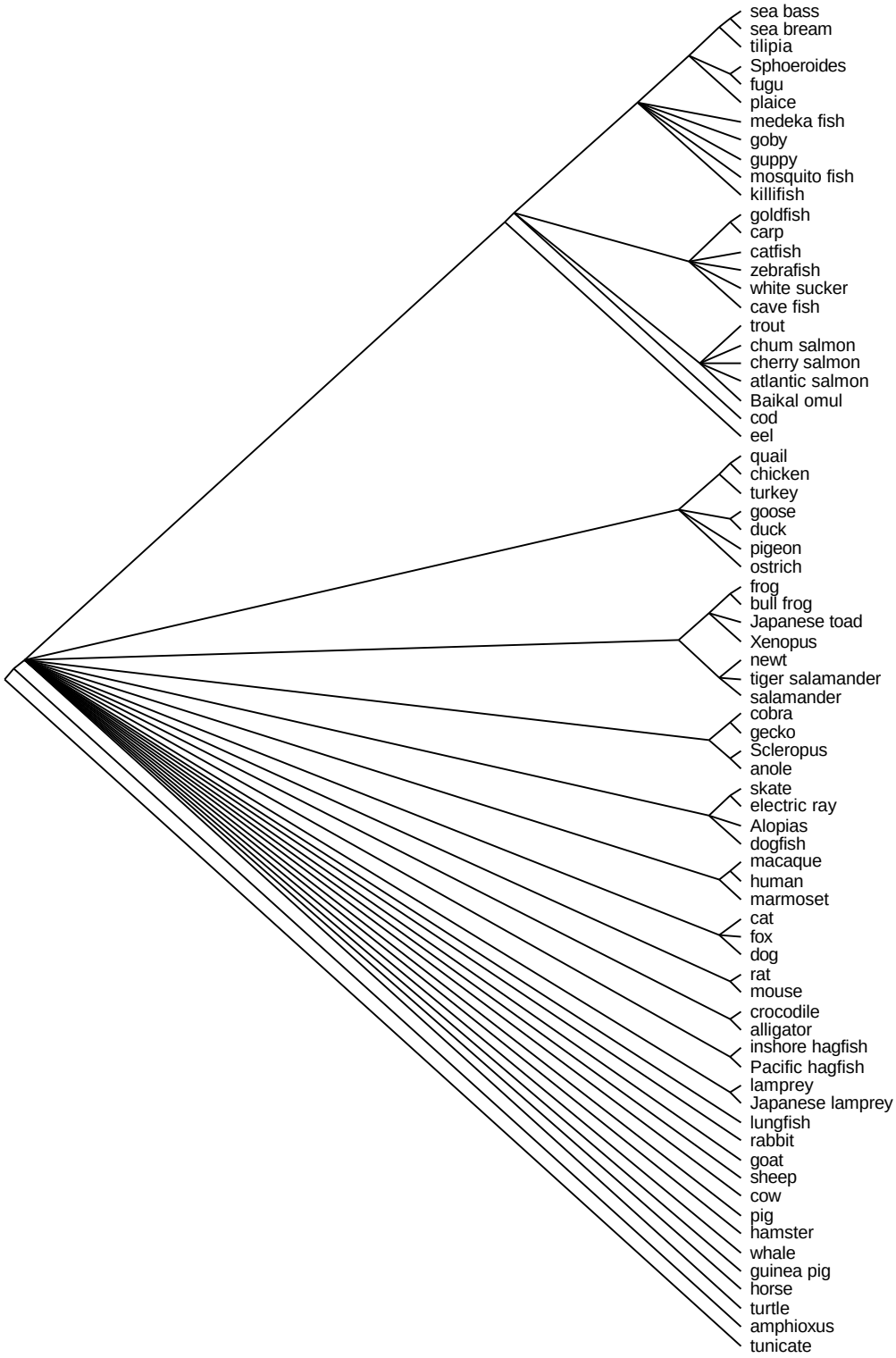
a
1
c
e
2
b
d
c
a
1
b
d
d
c
2
b
a
(b)
gene tree 1
95
a
c
b
d
gene tree 2
20
40
a
d
b
c
= duplication
species tree
a
b
c
d

Fig. 6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tilipia
Sphoeroides
fugu
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mosquito fish
killifish
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zebrafish
white sucker
cave fish
trout
chum salmon
cherry salmon
atlantic salmon
Baikal omul
cod
eel
quail
chicken
turkey
goose
duck
pigeon
ostrich
frog
bull frog
Japanese toad
Xenopus
newt
tiger salamander
salamander
cobra
gecko
Scleropus
anole
skate
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Alopias
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macaque
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rat
mouse
crocodile
alligator
inshore hagfish
Pacific hagfish
lamprey
Japanese lamprey
lungfish
rabbit
goat
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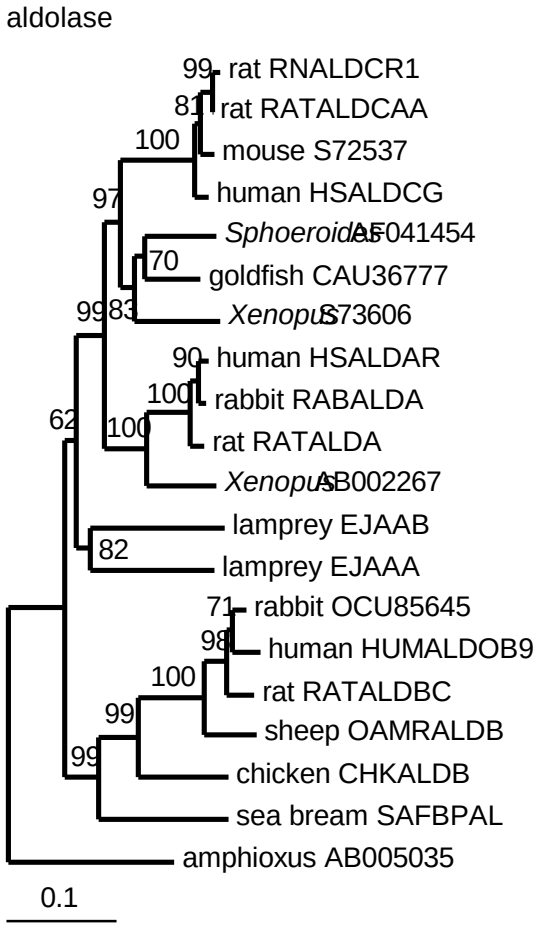
cow
pig
hamster
whale
guinea pig
horse
turtle
amphioxus
tunicate

Fig. 7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goldfish CAU36777
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Xenopus S73606
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human HSALDAR
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rabbit RABALDA
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rat RATALDA
Xenopus AB002267
lamprey EJAAB
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lamprey EJAAA
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rabbit OCU85645
98
human HUMALDOB9
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sea bream SAFBPAL
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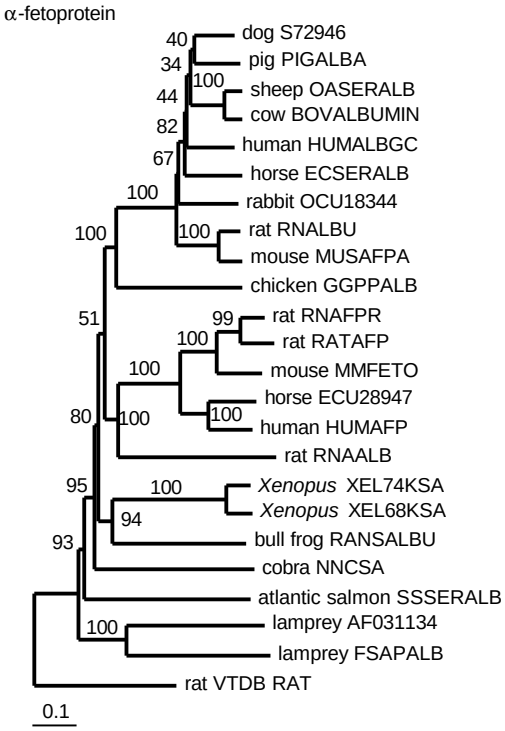
Fig. 8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sheep OASERALB
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horse ECSERALB
rabbit OCU18344
rat RNALBU
mouse MUSAFPA
chicken GGPPALB
rat RNAFPR
rat RATAFP
mouse MMFETO
horse ECU28947
human HUMAFP
rat RNAALB
Xenopus XEL74KSA
Xenopus XEL68KSA
bull frog RANSALBU
cobra NNCSA
atlantic salmon SSSERALB
lamprey AF031134
lamprey FSAPALB
rat VTDB RAT
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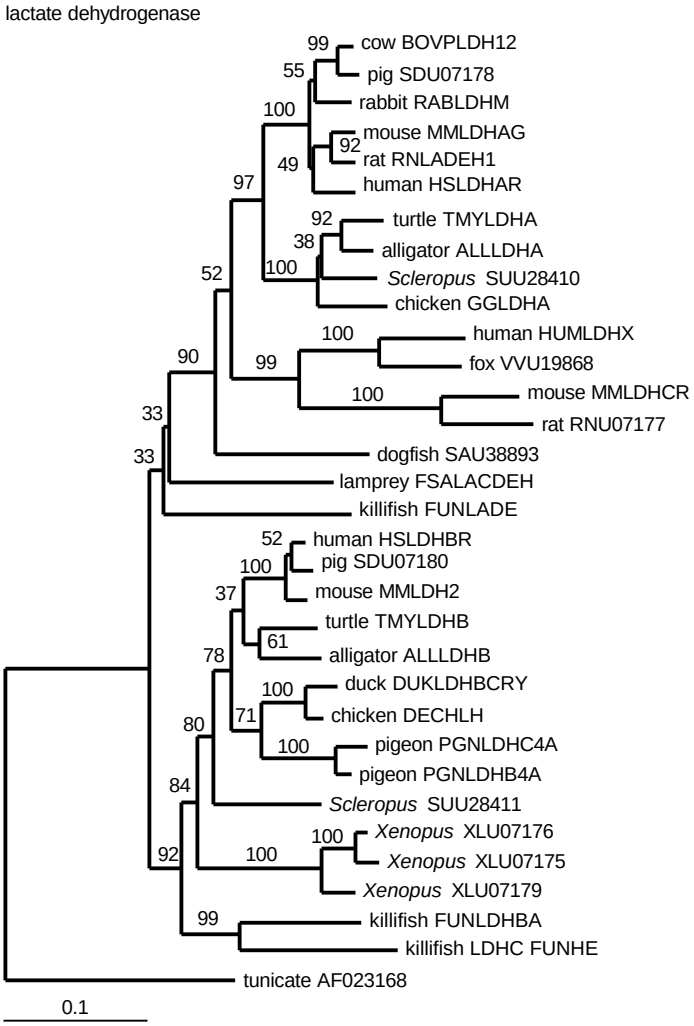
0.1

Fig. 8 (cont'd)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rat RNLADEH1
human HSLDHAR
turtle TMYLDHA
alligator ALLLDHA
Scleropus SUU28410
chicken GGLDHA
human HUMLDHX
fox VVU19868
mouse MMLDHCR
rat RNU07177
dogfish SAU38893
lamprey FSALACDEH
killifish FUNLADE
human HSLDHBR
pig SDU07180
mouse MMLDH2
turtle TMYLDHB
alligator ALLLDHB
duck DUKLDHBCRY
chicken DECHLH
pigeon PGNLDHC4A
pigeon PGNLDHB4A
Scleropus SUU28411
Xenopus XLU07176
Xenopus XLU07175
Xenopus XLU07179
killifish FUNLDHBA
killifish LDHC FUNHE
tunicate AF023168
0.1

Fig. 8 (cont'd)

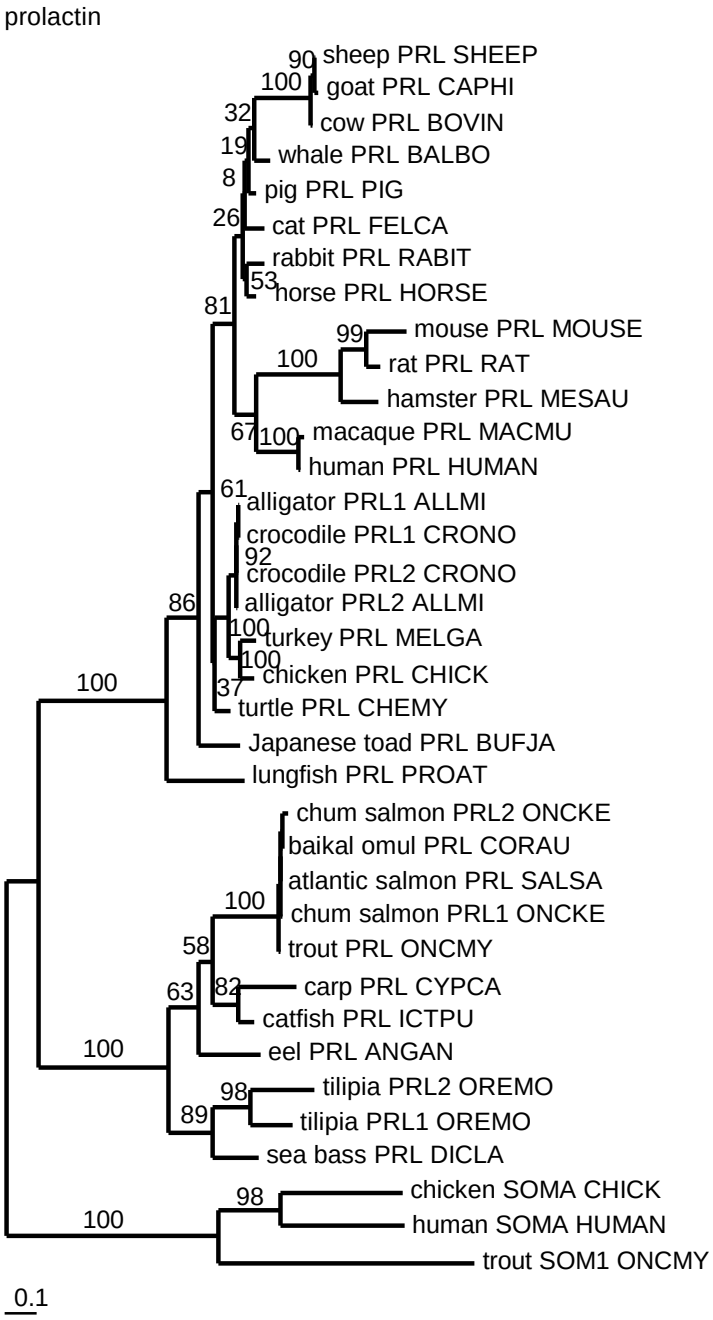
prolactin
sheep PRL SHEEP
goat PRL CAPHI
cow PRL BOVIN
whale PRL BALBO
pig PRL PIG
cat PRL FELCA
rabbit PRL RABIT
horse PRL HORSE
mouse PRL MOUSE
rat PRL RAT
hamster PRL MESAU
macaque PRL MACMU
human PRL HUMAN
alligator PRL1 ALLMI
crocodile PRL1 CRONO
crocodile PRL2 CRONO
alligator PRL2 ALLMI
turkey PRL MELGA
chicken PRL CHICK
turtle PRL CHEMY
Japanese toad PRL BUFJA
lungfish PRL PROAT
chum salmon PRL2 ONCKE
baikal omul PRL CORAU
atlantic salmon PRL SALSA
chum salmon PRL1 ONCKE
trout PRL ONCMY
carp PRL CYPCA
catfish PRL ICTPU
eel PRL ANGAN
tilipia PRL2 OREMO
tilipia PRL1 OREMO
sea bass PRL DICLA
chicken SOMA CHICK
human SOMA HUMAN
trout SOM1 ONCMY
0.1

Fig. 8 (cont'd)

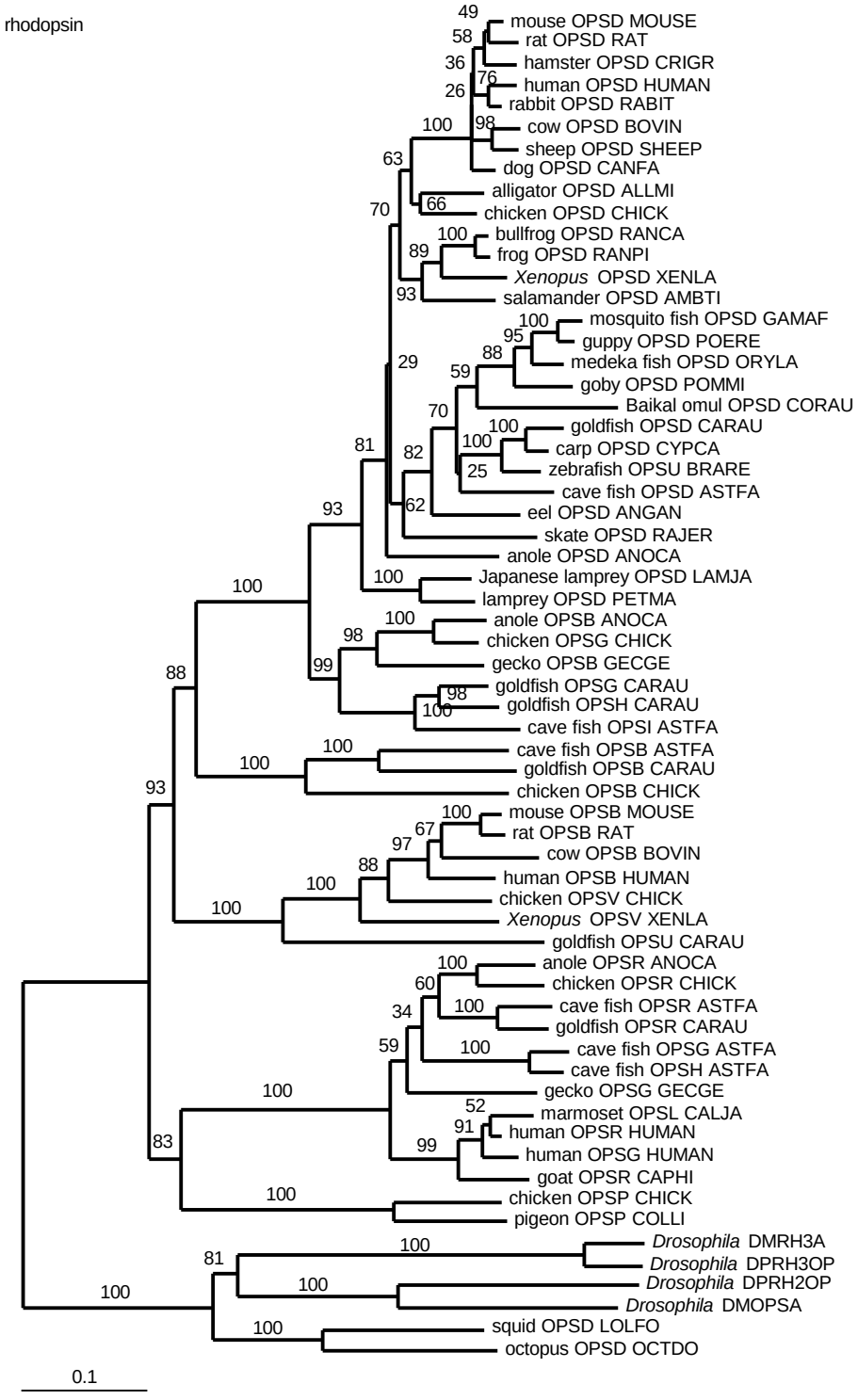
rhodopsin
mouse OPSD MOUSE
rat OPSD RAT
hamster OPSD CRIGR
human OPSD HUMAN
rabbit OPSD RABIT
cow OPSD BOVIN
sheep OPSD SHEEP
dog OPSD CANFA
alligator OPSD ALLMI
chicken OPSD CHICK
bullfrog OPSD RANCA
frog OPSD RANPI
Xenopus OPSD XENLA
salamander OPSD AMBTI
mosquito fish OPSD GAMAF
guppy OPSD POERE
medeka fish OPSD ORYLA
goby OPSD POMMI
Baikal omul OPSD CORAU
goldfish OPSD CARAU
carp OPSD CYPCA
zebrafish OPSU BRARE
cave fish OPSD ASTFA
eel OPSD ANGAN
skate OPSD RAJER
anole OPSD ANOCA
Japanese lamprey OPSD LAMJA
lamprey OPSD PETMA
anole OPSB ANOCA
chicken OPSG CHICK
gecko OPSB GECGE
goldfish OPSG CARAU
goldfish OPSH CARAU
cave fish OPSI ASTFA
cave fish OPSB ASTFA
goldfish OPSB CARAU
chicken OPSB CHICK
mouse OPSB MOUSE
rat OPSB RAT
cow OPSB BOVIN
human OPSB HUMAN
chicken OPSV CHICK
Xenopus OPSV XENLA
goldfish OPSU CARAU
anole OPSR ANOCA
chicken OPSR CHICK
cave fish OPSR ASTFA
goldfish OPSR CARAU
cave fish OPSG ASTFA
cave fish OPSH ASTFA
gecko OPSG GECGE
marmoset OPSL CALJA
human OPSR HUMAN
human OPSG HUMAN
goat OPSR CAPHI
chicken OPSP CHICK
pigeon OPSP COLLI
Drosophila DMRH3A
Drosophila DPRH3OP
Drosophila DPRH2OP
Drosophila DMOPSA
squid OPSD LOLFO
octopus OPSD OCTDO
0.1

Fig. 8 (cont'd)

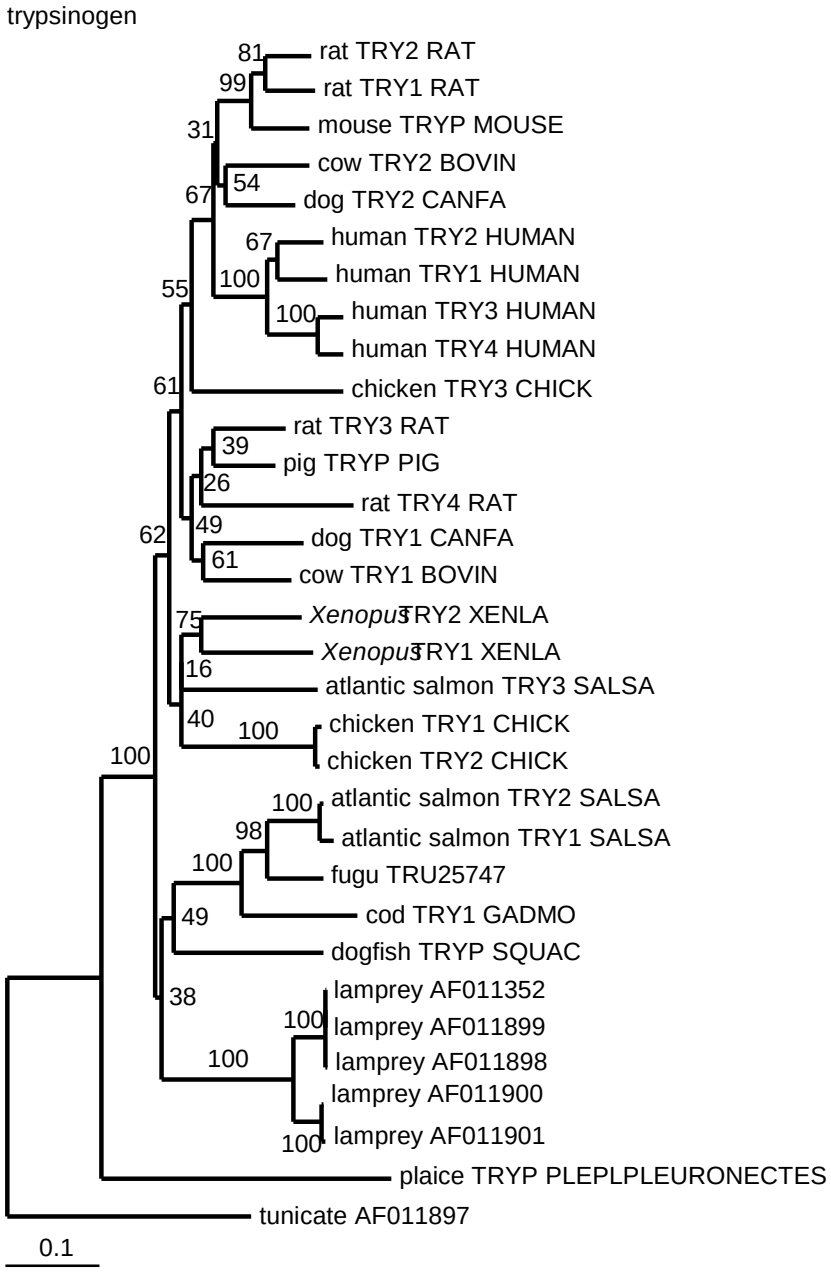
trypsinogen
81
rat TRY2 RAT
99
rat TRY1 RAT
mouse TRYP MOUSE
31
cow TRY2 BOVIN
67
54
dog TRY2 CANFA
67
human TRY2 HUMAN
100
human TRY1 HUMAN
55
100
human TRY3 HUMAN
human TRY4 HUMAN
61
chicken TRY3 CHICK
rat TRY3 RAT
39
pig TRYP PIG
26
rat TRY4 RAT
62
49
dog TRY1 CANFA
61
cow TRY1 BOVIN
75
Xenopus TRY2 XENLA
Xenopus TRY1 XENLA
16
atlantic salmon TRY3 SALSA
40
100
chicken TRY1 CHICK
100
chicken TRY2 CHICK
100
atlantic salmon TRY2 SALSA
98
atlantic salmon TRY1 SALSA
100
fugu TRU25747
49
cod TRY1 GADMO
dogfish TRYP SQUAC
38
lamprey AF011352
100
lamprey AF011899
100
lamprey AF011898
lamprey AF011900
100
lamprey AF011901
plaice TRYP PLEPLPLEURONECTES
tunicate AF011897
0.1

Fig. 8 (cont'd)

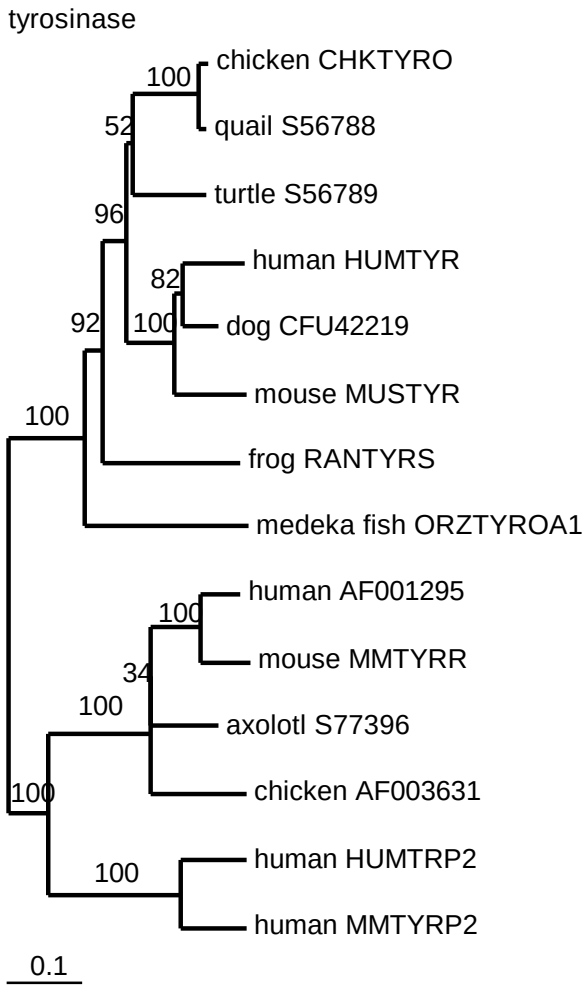
tyrosinase
chicken CHKTYRO
quail S56788
turtle S56789
human HUMTYR
dog CFU42219
mouse MUSTYR
frog RANTYRS
medeka fish ORZTYROA1
human AF001295
mouse MMTYRR
axolotl S77396
chicken AF003631
human HUMTRP2
human MMTYRP2
100
52
96
82
92
100
100
100
34
100
100
100
0.1

Fig. 8 (cont'd)

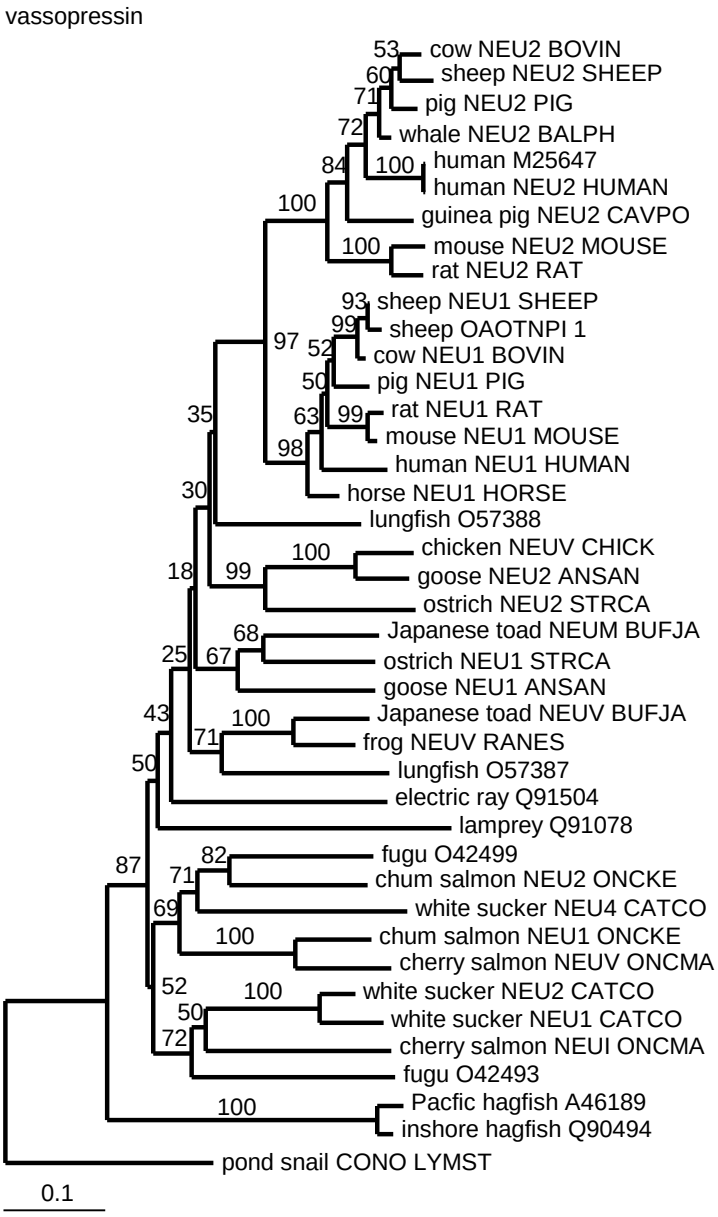
vassopressin
53
cow NEU2 BOVIN
60
sheep NEU2 SHEEP
71
pig NEU2 PIG
72
whale NEU2 BALPH
84
100
human M25647
human NEU2 HUMAN
100
guinea pig NEU2 CAVPO
100
mouse NEU2 MOUSE
rat NEU2 RAT
93
sheep NEU1 SHEEP
99
sheep OAOTNPI 1
97
52
cow NEU1 BOVIN
50
pig NEU1 PIG
35
63
99
rat NEU1 RAT
mouse NEU1 MOUSE
98
human NEU1 HUMAN
30
horse NEU1 HORSE
lungfish O57388
100
chicken NEUV CHICK
18
99
goose NEU2 ANSAN
ostrich NEU2 STRCA
68
Japanese toad NEUM BUFJA
25
67
ostrich NEU1 STRCA
goose NEU1 ANSAN
43
100
Japanese toad NEUV BUFJA
71
frog NEUV RANES
50
lungfish O57387
electric ray Q91504
lamprey Q91078
82
fugu O42499
87
71
chum salmon NEU2 ONCKE
69
white sucker NEU4 CATCO
100
chum salmon NEU1 ONCKE
cherry salmon NEUV ONCMA
52
100
white sucker NEU2 CATCO
50
white sucker NEU1 CATCO
72
cherry salmon NEUI ONCMA
fugu O42493
100
Pacfic hagfish A46189
inshore hagfish Q90494
pond snail CONO LYMST
0.1

Fig. 8 (cont'd)

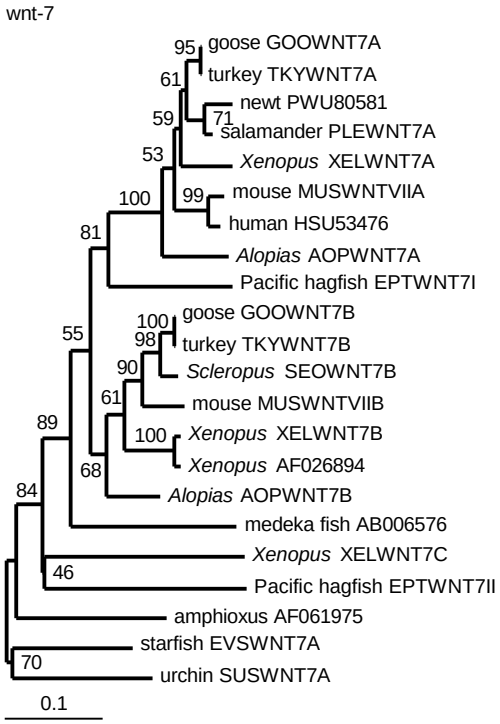
wnt-7
goose GOOWNT7A
turkey TKYWNT7A
newt PWU80581
salamander PLEWNT7A
Xenopus XELWNT7A
mouse MUSWNTVIIA
human HSU53476
Alopias AOPWNT7A
Pacific hagfish EPTWNT7I
goose GOOWNT7B
turkey TKYWNT7B
Scleropus SEOWNT7B
mouse MUSWNTVIIB
Xenopus XELWNT7B
Xenopus AF026894
Alopias AOPWNT7B
medeka fish AB006576
Xenopus XELWNT7C
Pacific hagfish EPTWNT7II
amphioxus AF061975
starfish EVSWNT7A
urchin SUSWNT7A
95
61
59
71
53
100
99
81
100
98
55
90
61
89
100
68
84
46
70
0.1

Fig. 8 (cont'd)

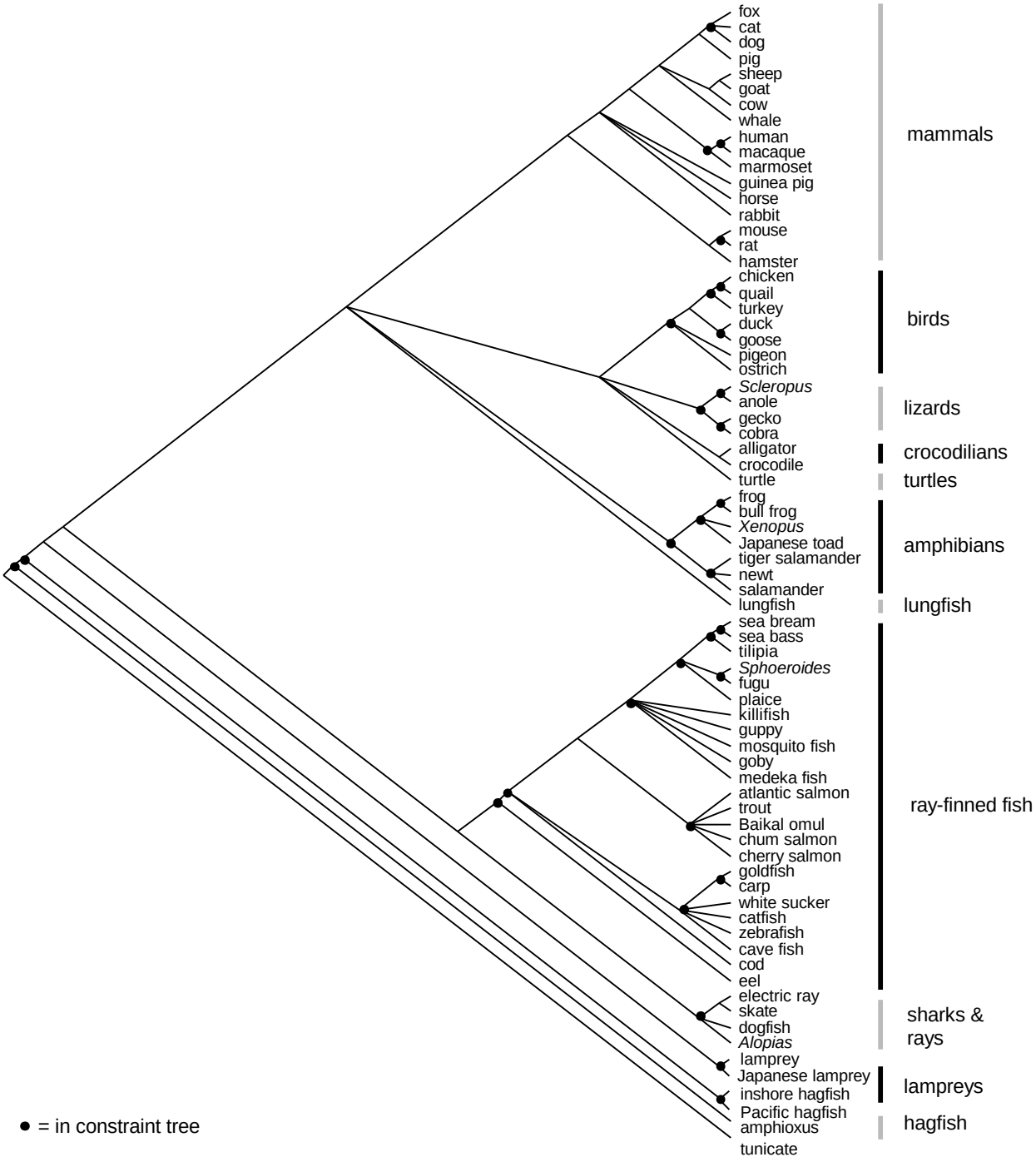
fox
cat
dog
pig
sheep
goat
cow
whale
human
macaque
marmoset
guinea pig
horse
rabbit
mouse
rat
hamster
chicken
quail
turkey
duck
goose
pigeon
ostrich
Scleropus
anole
gecko
cobra
alligator
crocodile
turtle
frog
bull frog
Xenopus
Japanese toad
tiger salamander
newt
salamander
lungfish
sea bream
sea bass
tilipia
Sphoeroides
fugu
plaice
killifish
guppy
mosquito fish
goby
medeka fish
atlantic salmon
trout
Baikal omul
chum salmon
cherry salmon
goldfish
carp
white sucker
catfish
zebrafish
cave fish
cod
eel
electric ray
skate
dogfish
Alopias
lamprey
Japanese lamprey
inshore hagfish
Pacific hagfish
amphioxus
tunicate
mammals
birds
lizards
crocodilians
turtles
amphibians
lungfish
ray-finned fish
sharks & rays
lampreys
hagfish
● = in constraint tree

Fig. 9

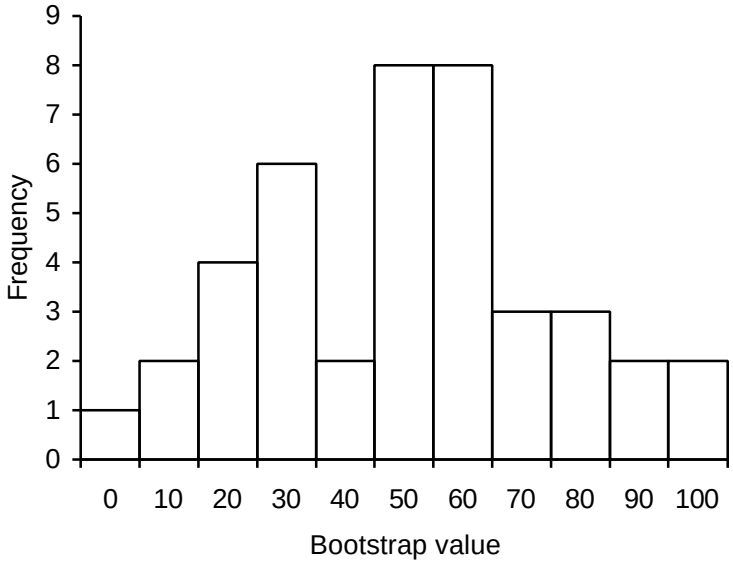
Frequency
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
80
90
100
Bootstrap value

Fig. 10

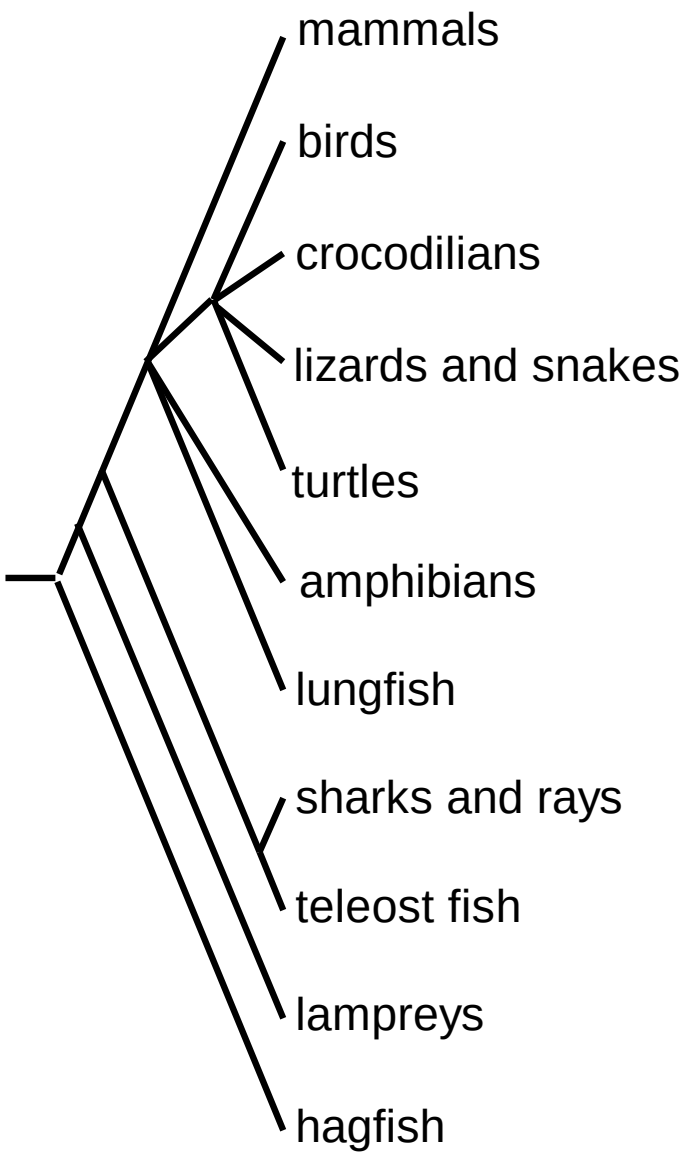
mammals
birds
crocodilians
lizards and snakes
turtles
amphibians
lungfish
sharks and rays
teleost fish
lampreys
hagfish

Fig. 11