Molecular discrimination of the European *Mesocestoides* species complex

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Abstract

Phylogenetic analysis was used to test if specimens of *Mesocestoides* spp. from carnivores at four locations in Slovakia represented two different species, *M. litteratus* and *M. lineatus* and whether these species could be delimited geographically. Three sister-taxa were used as outgroups. Sequences from three mitochondrial markers revealed the two species as distinct groups and this was confirmed by analysis of inter-taxon sequence divergence estimates. No separation by geographical location was found.

Table of Contents

Introduction

Mesocestoides Vaillant 1863 (Cestoda: Cyclophyllidea: Mesocestoididae) is a widespread genus of tapeworms commonly found in carnivores and birds of prey in the Northern hemisphere (Literák *et al.*, 2004). The family is considered distinct from other cyclophyllideans due to the presence of several unique synapormorphies: a potential three host life cycle, the position of the genital atrium and bipartite vitelline gland, and asexual reproduction of tetrathyridial metacestodes by longitudinal fission (although this latter characteristic is possibly not universal amongst species) (Crosbie *et al.*, 2000). Moreover, the identification of parasite species based on their morphological characteristics has often proved unsuccessful especially for the highly variable *Mesocestoides* spp. and the phylogenetic relationships remain unresolved (Loos-Frank, 1987; Padgett *et al.*, 2005). A limited survey of taxonomic databases reveals discrepancies in the information available with regards to the species/sub-species that constitute the genus in Europe and the USA. For example, Uniprot (The UniProt Consortium, 2009), ZipcodeZoo (Stang, 2009) and Fauna Europaea (Fauna Europaea Web Service, 2004) list 5, 9 and 12 species and subspecies, respectively. Clarification of the taxonomy of the *Mesocestoides* spp. is evidently needed.

Species are considered the fundamental biological unit of inheritance (Ridley, 2004). The mechanisms of speciation and the process of delimiting species are crucial to the understanding of evolutionary biology and have wide-ranging implications across fields such as conservation, ecology, biodiversity and assessing the risk of pathogens and disease (Hoberg, 2006). A controversial area is how species are defined and which methods are best employed to delimit them (Adams, 1998; Sites & Marshall, 2003). Traditionally morphological characters have been used and are still widely used today, but molecular techniques have become a powerful tool, particularly when trying to resolve the systematics of problematic groups where morphological features have failed to provide unambiguous phylogenetic signal by providing an independent set of characters to work with (Nadler, 1995; Wiens & Penkrot, 2002).

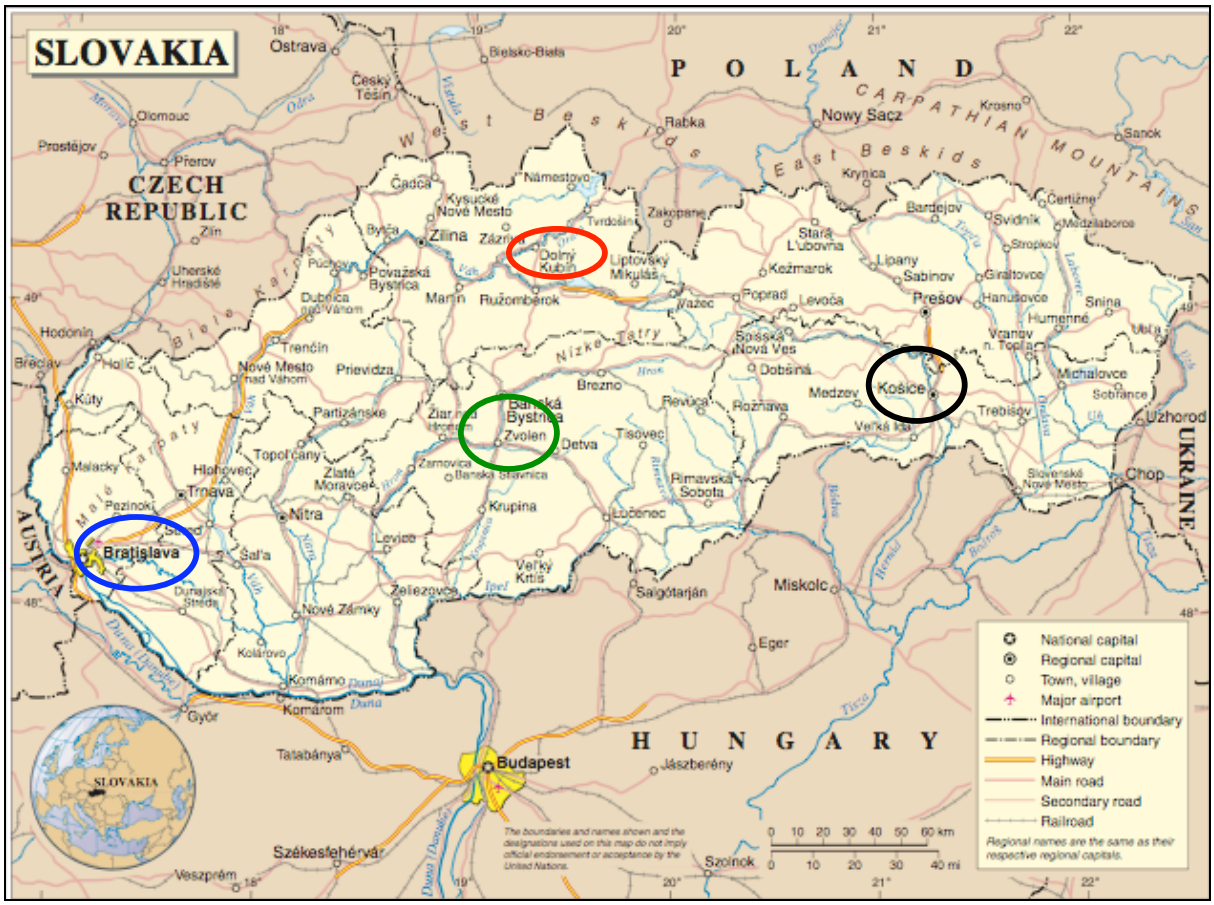
A number of molecular studies of Cestodes have been carried out to date (reviewed in Olson & Tkach, 2005). Nuclear genes, such as the commonly used 18S rDNA and

28S rDNA, have proved useful markers at ordinal and familial levels, but amongst closely related taxa they generally provide little resolution (Littlewood *et al.*, 2008). The second internal transcribed spacer (*ITS-2*) has proved beneficial in the systematic studies of helminths due to its high degree of variability (Literák *et al.*, 2006; Mariaux, 1998; Olson & Caira, 1999; Padgett *et al.*, 2005). The mitochondrial large subunit ribosomal RNA gene *rrnL* (16S) has also aided resolution at familial level (Zehnder & Mariaux, 1999). A further mitochondrial gene, cytochrome c oxidase subunit I (*cox1*), as used by Bowes and MacManus (1994), has been effective in analysing relationships between species and strains of *Echinococcus* (Cyclophyllidea), and NADH dehydrogenase subunit I (*nad1*) has been proposed for investigating population variation amongst species (Gasser *et al.*, 1999). Further progress would most likely be derived from a multi-locus approach using both mitochondrial and nuclear DNA (Hoberg, 2006). One such study has utilised this strategy for delimiting species of *Mesocestoides* present on the West Coast of the USA and has revealed a number of distinct lineages within morphologically defined 'species' (Padgett *et al.*, 2005). In Europe the situation has yet to be evaluated. Two species have been identified, *Mesocestoides litteratus* and *M. lineatus*, both parasites of foxes (*Vulpes vulpes*), although it remains controversial as to whether they should be considered distinct species (Literák *et al.*, 2006). The present study utilised modern phylogenetic methods to analyse multiple molecular markers in an attempt to determine whether distinct evolutionary lineages exist within the *Mesocestoides* species' complex based on samples previously obtained from carnivores at 4 locations in Slovakia.

Materials and Methods

Adult specimens of *Mesocestoides* spp. were collected from the intestines of foxes (*Vulpes* sp.) at 4 locations in Slovakia (fig. 1). Each specimen had been identified on the basis of morphology and the genomic DNA extracted using a QIAgen DNeasy™ tissue kit prior to use in this study. In total, 55 gDNA samples from 27 *Mesocestoides* spp. were available for analysis (Appendix 1, Table A.1).

Figure 1. Sampling locations in Slovakia



(http://www.worldofmaps.net/)

DNA amplification and gene sequencing

The polymerase chain reaction (PCR) was used to amplify two mitochondrial gene regions, *cox1* and *nad1* and 1 nuclear gene region, *ITS-2*, using primer sequences listed in table 1. The total volume (incorporating Ready-To-Go™ PCR beads) was 25 μl for the PCR reactions, comprising 5 μl of DNA template, 18 μl of sterile water, and 1 μl of each of the 2 PCR primers, in addition to 3 μl of loading buffer. The following thermocycling profile was used (program: anneITS2): 95ºC /3 min denaturation hold; 40 cycles of 95ºC /30 sec, 52ºC /30 sec and 72ºC /1 min; 72ºC /7 min extension hold. PCR products were either gel-excised using a QIAquick™ Gel Extraction Kit or purified directly using a QIAgen™ PCR Purification Kit, following standard protocols as recommended by the manufacturer.

Table 1. PCR and sequencing primers

Target gene	PCR and sequencing primers ('5-3')	Additional sequencing primers (5'-3')
nad1	Cyclo_nad1F (forward): GGNTATTSTCARTNTCGTAAGGG Cyclo_trnNR (reverse): TTCYTGAAGTTAACAGCATCA	Cyclo_nad1Fb (forward): AGGTTTGARGCKTGTTTTATG
cox1	Cyclo_cox1Fa (forward): CARCATATGTTTTGRTTTTTGG Cyclo_16SRc (reverse): AATAGATAAGAACCGACCTGGC	Cyclo_cox1Rb (reverse): CCTAAYGACATAACATAATGRAAATG
ITS-2	NC-6 (forward): ATCGACATCTTGAACGCACATTGC NC-2 (reverse): TTAGTTTCTTTTCCTCCGCT	NC-6-F1 (forward): TCTGTCCGAGCGTCGGC NC-2_R1 (reverse): GCTTAAATTCAGCGGGTCG

Nucleotide sequencing of both strands was performed by the sequencing personnel using BigDye™ v1.1 and an AB13730 automated sequencer. Complementary sequences were assembled and manually edited using Sequencher v4.6 (GeneCodes Corporation). Regions corresponding to the PCR primers were removed from the sequences prior to analysis.

Sequence alignment and phylogenetic analysis

All sequence identities were verified using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.gov/BLAST/). Sequences from three sister taxa

(*Echinococcus*, *Taenia* and *Hymenolepis*) were downloaded from GenBank and added to each gene dataset prior to analysis, to serve as outgroups. Sequences were initially aligned with ClustalX v2.0 (Thompson *et al.*, 1997), using default parameter settings: gap opening and gap penalty fixed at 10 and 0.2 respectively. The alignment was further improved by eye using MacClade v4.08 (Maddison & Maddison, 2000), with the genetic code set to flatworm mtDNA and 'codon positions' set to minimize stop codons. 12S sequences from a previous study (of the same 27 specimens) were also included in the analysis and outgroups added and the dataset realigned. In cases where two or more replicate samples of the same specimen were available, the sequences were verified as matching and just one was used in the analysis.

Phylogenetic trees were constructed using maximum parsimony, maximum likelihood and Bayesian inference. Analysis was carried out for each separate gene region and also for the combined gene regions. Where taxa had numerous missing data, i.e. where they only had data for one marker, these were removed prior to analysis of the combined dataset.

Maximum parsimony was performed using PAUP* v.4.0b (Swofford, 2003). A heuristic search (1000 replicates), random-sequence addition and tree bisection and reconnection (TBR) options were used, with all characters unweighted and unordered and gaps treated as missing data. To determine branch support 1000 bootstrap replicates were generated. All trees were rooted with outgroups.

Maximum likelihood analysis was performed using PhyML v2.4.4 (Guindon & Gascuel, 2003) with the 'best-fit' model of nucleotide substitution selected using MrModelTest v2 (Nylander, 2004). This uses likelihood scores calculated across a neighbour-joining topology with PAUP* in order to rank 24 different models of nucleotide substitution based on their Akaike information criterion (AIC) scores (Akaike, 1974). The model with the lowest AIC is deemed the 'best-fit' (Salemi & Vandamme, 2003).

Bayesian analysis was performed using MrBayes v3.2 (Robquist & Huelsenbeck, 2003). The substitution model was selected as before and a default prior probability

was used so that parameter values would be estimated (Dirichlet (1,1,1,1)). The chain length was set to 1,000,000 generations sampling every 100th generations. Two simultaneous independent runs were undertaken for each dataset starting from different random trees with 'burn-in' set to 10% (100,000 generations). To ensure convergence and an effective sample size results were verified with the Tracer v1.4 MCMC trace analysis tool (part of the BEAST package) (Drummond & Rambaut, 2007). All trees were rooted with outgroups.

Perl scripts were written to automate the phylogenetic analyses (Appendix B), apart from the concatenated dataset, where analyses were performed manually.

MEGA v4.0 (Tamura *et al.*, 2007) was used to calculate the pairwise sequence divergence between taxa. The Tamura-Nei model was used as this accounts for differences in substitution rates and unequal base frequencies and was the closest model to the 'best-fit' models selected by MrModelTest.

Results

In addition to a previously aligned 12S sequence dataset, sequences were obtained for 27 and 24 individuals for *nad1* and *cox1* mitochondrial rDNA. The *nad1* and *cox1* markers showed more variation (33% and 26%, respectively, table 2) than 12S (18%), so can be expected to have a greater impact on the tree topology. The average base composition was found to have a high T bias (40 – 50%) and very low C bias (5.7–11%) for all markers. This pattern is not uncommon and has been observed in mtDNA of other parasitic flatworms (MacManus *et al.*, 2004).

No reliable sequences were obtained for *ITS-2* – sequences contained double peaks indicating the presence of multiple products. The primers were tested along with the concentration of the PCR products and cloning of specimens revealed multiple different copies of the *ITS-2* gene. Rather than attempt to design new primers to overcome this, it was decided to exclude it from further analysis, due to time constraints.

Table 2. Summary of statistics from alignments

Target gene	Length (bp)	Parsimony informative sites	Constant sites	Base compositions			
				A	C	G	T
nad1	444	145 (33%)	234	0.2125	0.0572	0.2100	0.5010
cox1	396	103 (26%)	241	0.2290	0.0638	0.2178	0.4903
12S	378	68 (18%)	246	0.2886	0.1088	0.1986	0.4040
nad1+cox1+12S	1218	295 (24%)	732	0.2398	0.0823	0.2120	0.4660

Substitution model parameters for all datasets are given in Appendix A, Table A.2.

Trees produced independently for single markers (*nad1*, *cox1* and 12S) along with the combined markers (*nad1*+*cox1*+12S) are shown in figures 1-10. There is strong topological congruence between all trees, apart from 12S, with strong bootstrap support/clade-credibility (> 87%) for 2 groupings: (i) KE868 & KE902 and (ii) all other *Mesocestiodes* spp. One other grouping is evident from the majority of trees (> 84%

support): Taenia & KE307 & ZV643. This indicates that both KE307 and ZV643 may not be *Mesocestoides* spp. Results obtained for the 12S marker differ. This dataset differs from *nad1* and *cox1* in that it contains sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/Genbank/) for known *Mesocestoides* spp. and it also contains data from other specimens (some unidentified) that were not available for *nad1* and *cox1* analyses.

Table 3. Tree statistics

Method	Statistic	*nad1*	*cox1*	*12S*	*nad1+cox1+12S*
Maximum parsimony	CI	0.80	0.71	0.83	0.76
	RI	0.63	0.71	0.85	0.71

CI: consistency index
RI: retention index

Inter-species sequence divergence within *Mesocestoides*, ranged from 17% (*cox1*) to 27% (*nad1*), while intra-species divergence ranged from up to 3% and 2% (*cox1* and *nad1*, respectively) for *M. lineatus,* and up to 4% and 1.3% (*cox1* and *nad1*, respectively) for *M. litteratus*. Between genera, sequence divergence was high: *Taenia* and *M. lineatus* was approximately 26% (*cox1*) and 44% (*nad1*); *Hymenolepis* and *M. lineatus* was up to 34% (*cox1*) and 35% (*nad1*). The full matrices of divergence values are given in Appendix A, Tables A.3 and A.4.

Trees resulting from phylogenetic analyses are shown in figures 1 – 12, apart from 12S which is included in Appendix C. Full taxon trees are shown for the combined dataset (*nad1+cox1+12S*) only. Trees for single markers are in Appendix C.

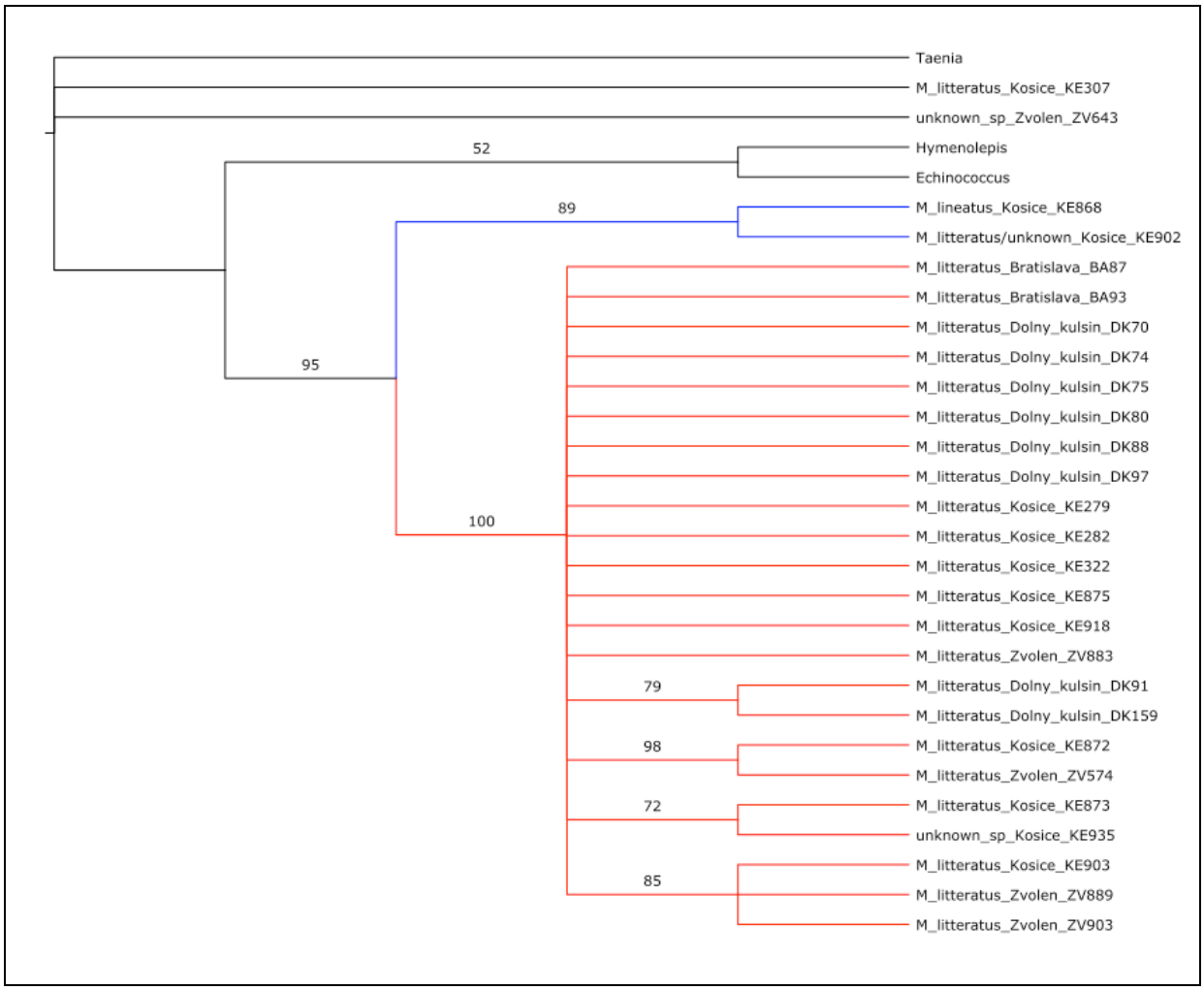


Figure 1. Maximum parsimony 50% majority rule consensus tree for the combined dataset nad1+cox1+12S. *Numbers represent bootstrap values (shown where > 50%).*

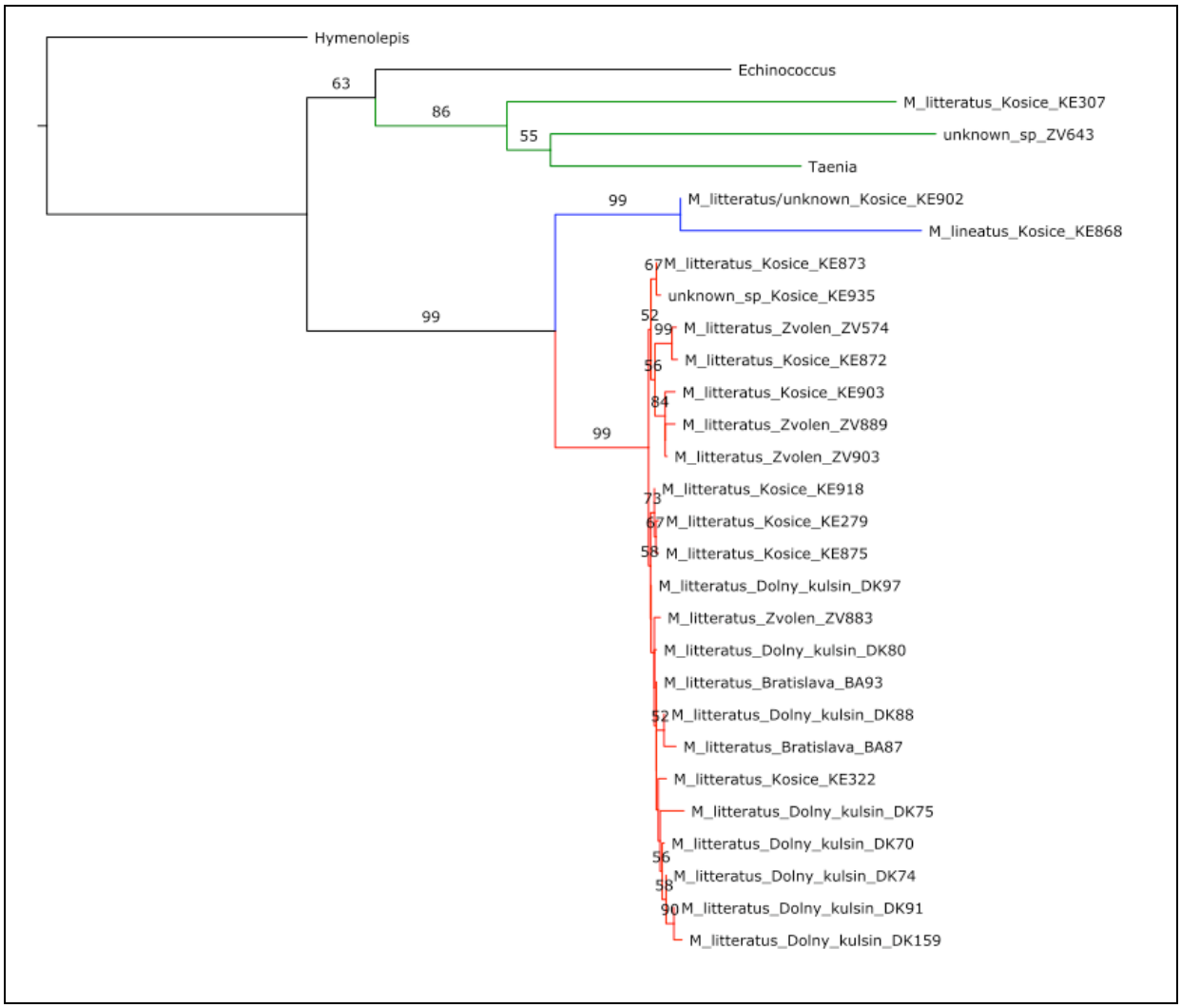


Figure 2. Maximum likelihood tree for the combined dataset nad1+cox1+12S. *Numbers represent bootstrap values (shown where > 50%).*

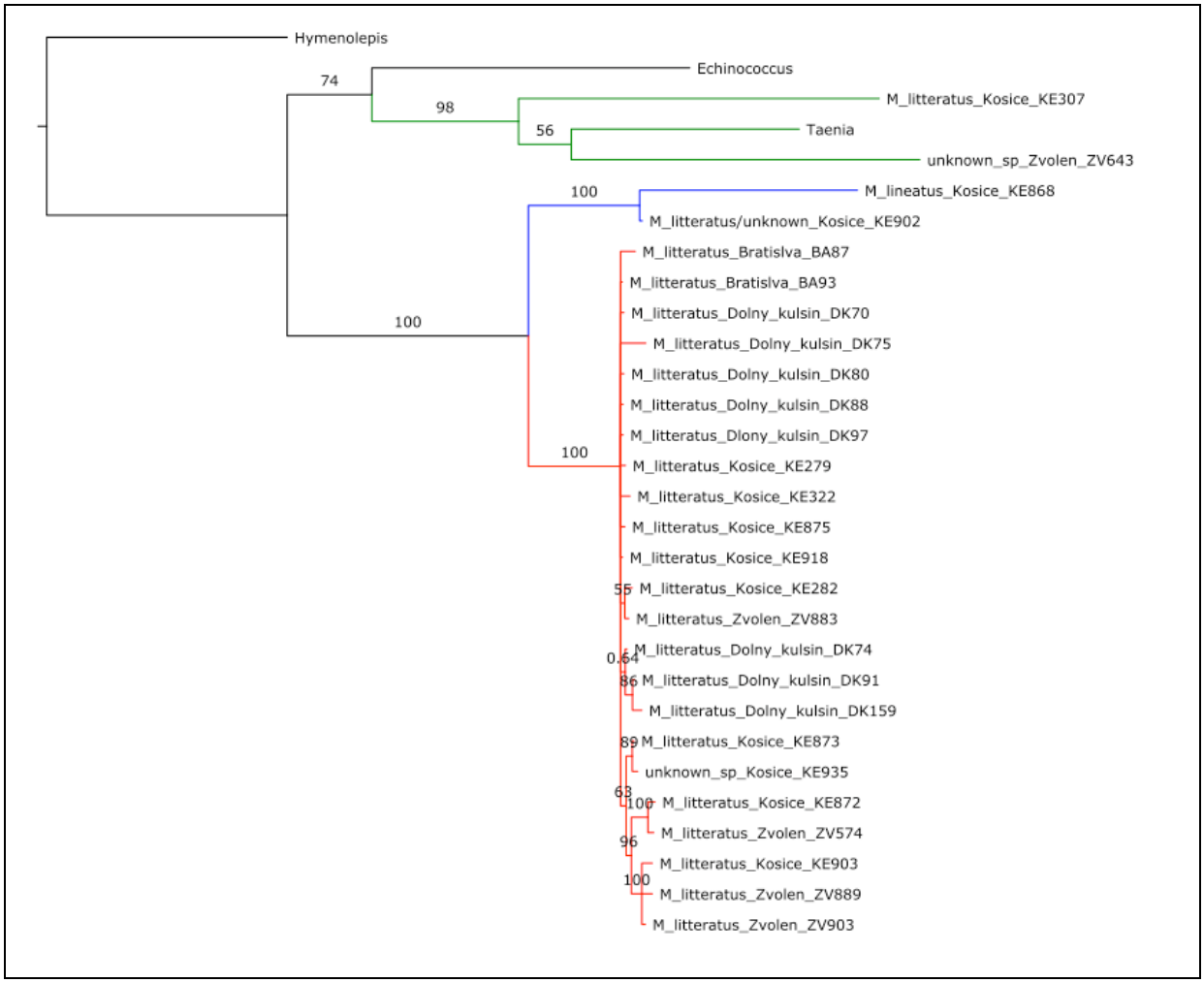


Figure 3. Bayesian inference tree for the combined dataset nad1+cox1+12S. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*

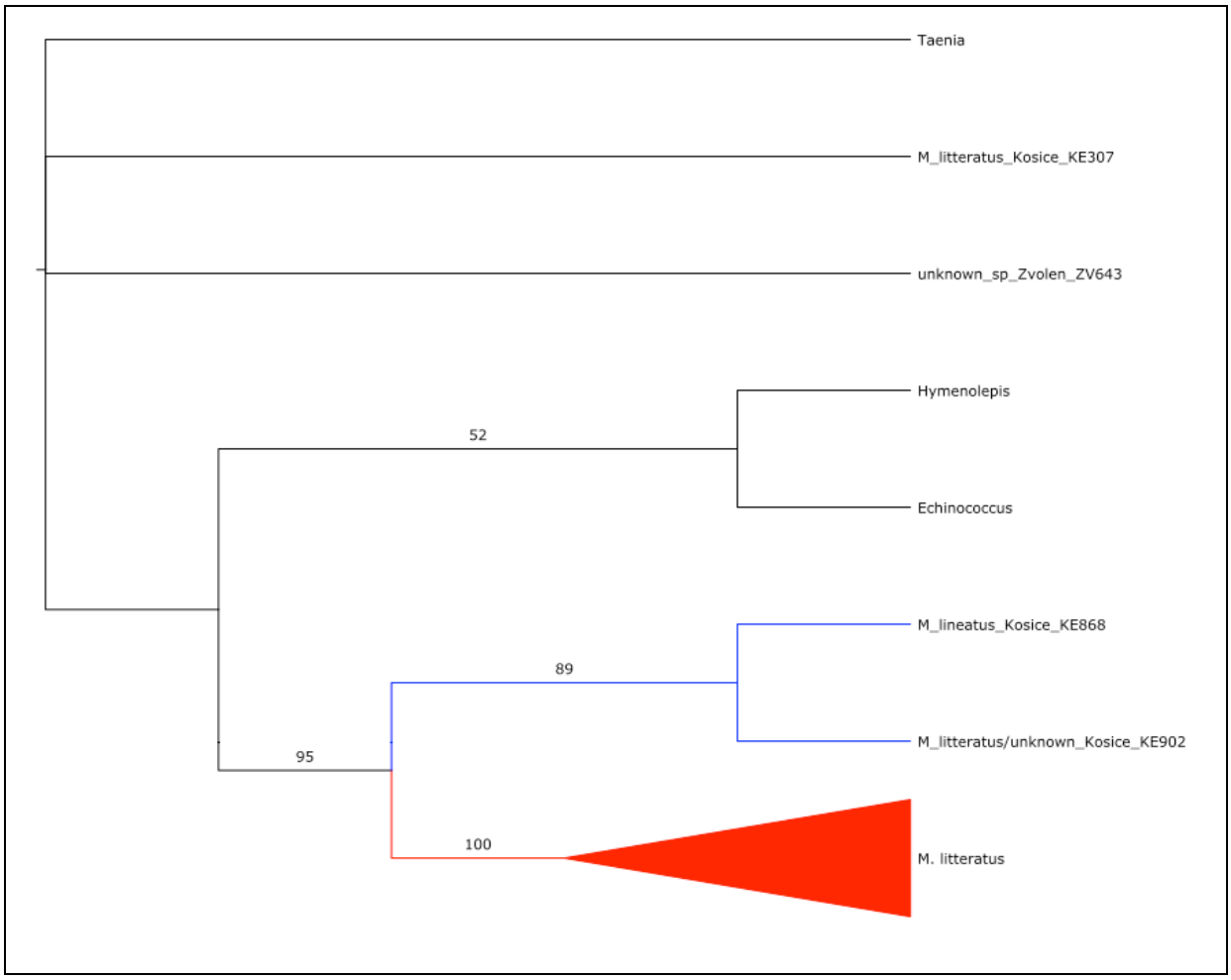


Figure 4. Maximum parsimony 50% majority rule consensus tree for the combined dataset nad1+cox1+12S, with polytomy collapsed. *Numbers represent bootstrap values (shown where > 50%).*

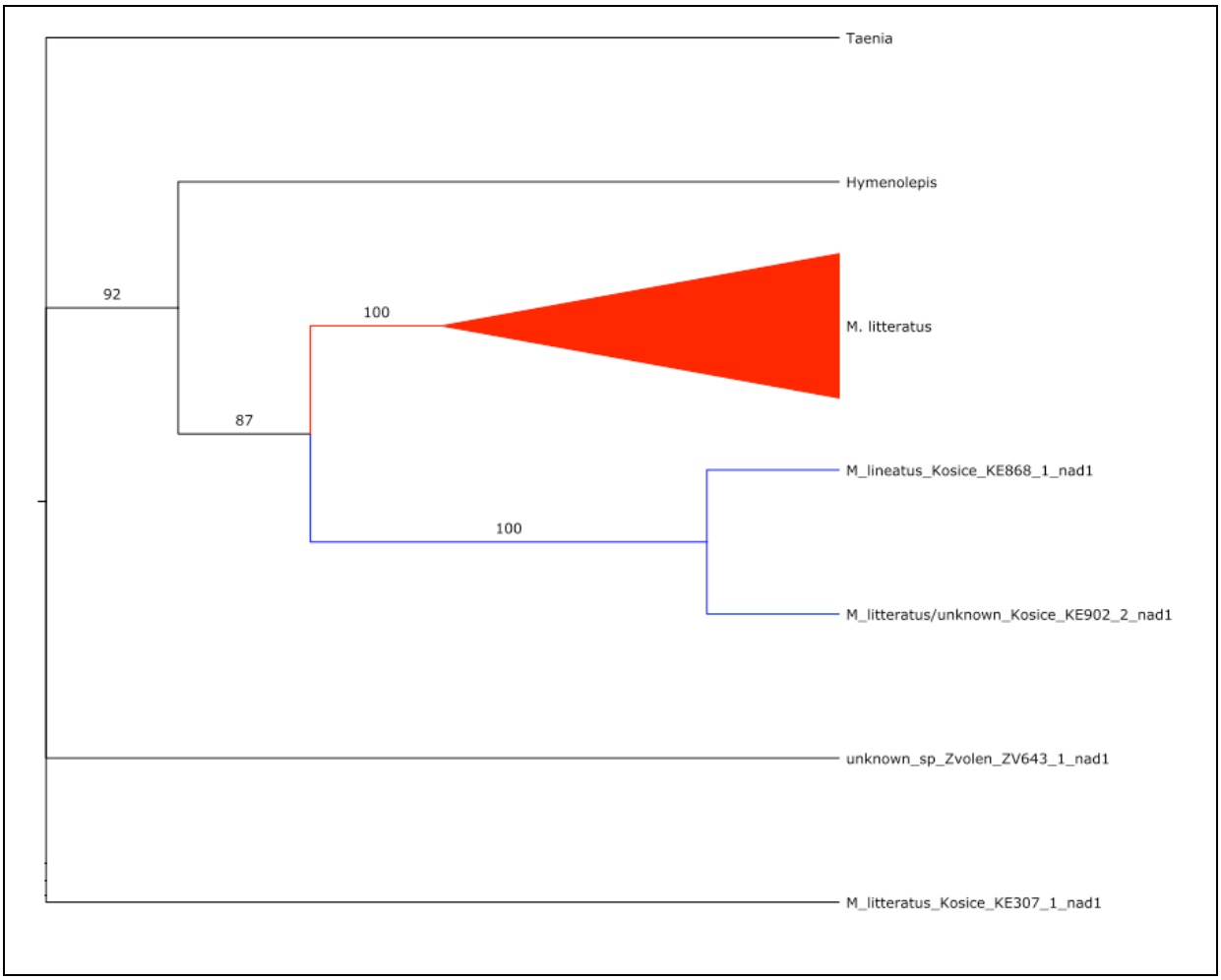


Figure 5. Maximum parsimony 50% majority rule consensus tree for nad1, with polytomy collapsed. *Numbers represent bootstrap values (shown where > 50%).*

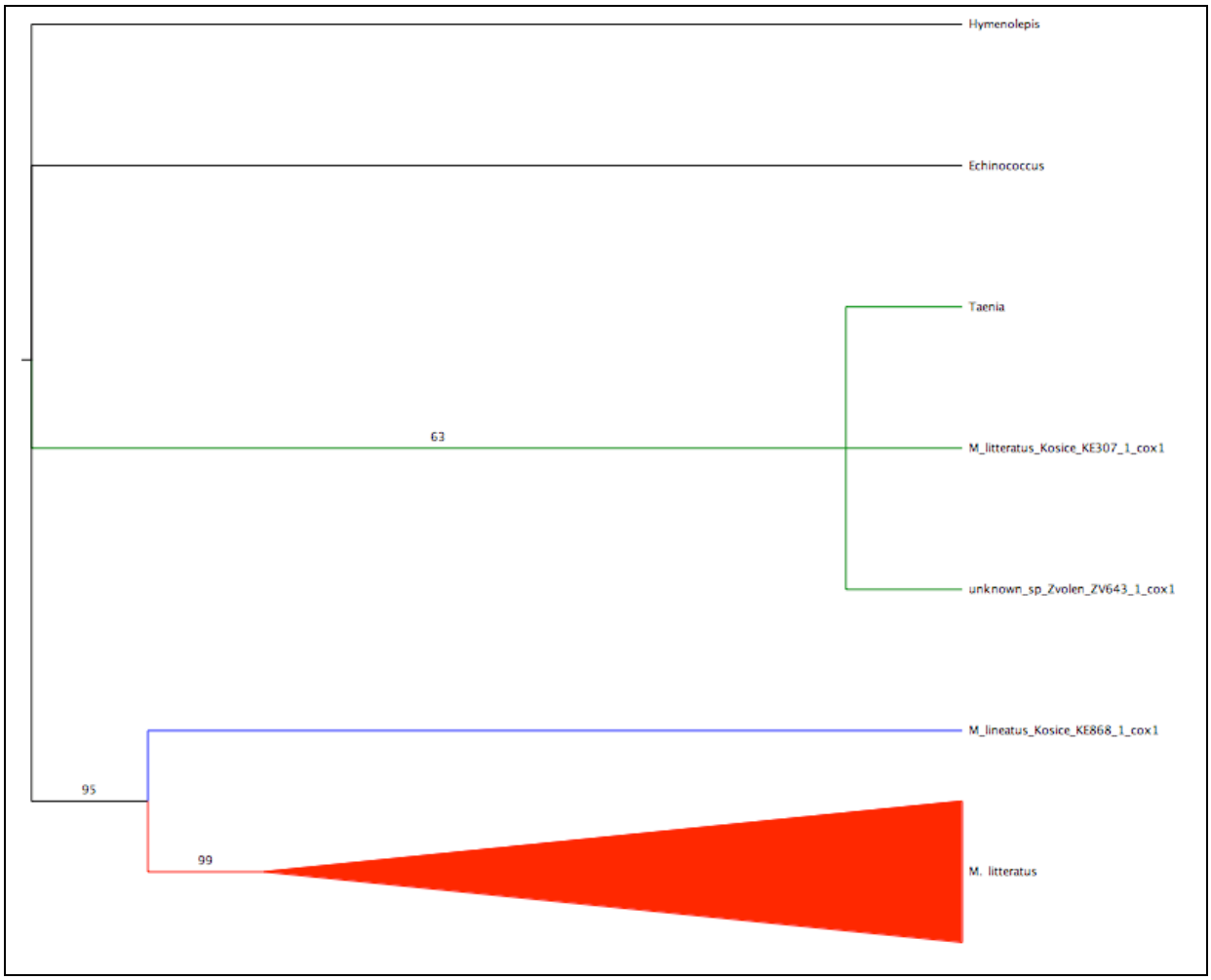


Figure 6. Maximum parsimony 50% majority rule consensus tree for cox1, with polytomy collapsed. *Numbers represent bootstrap values (shown where > 50%).*

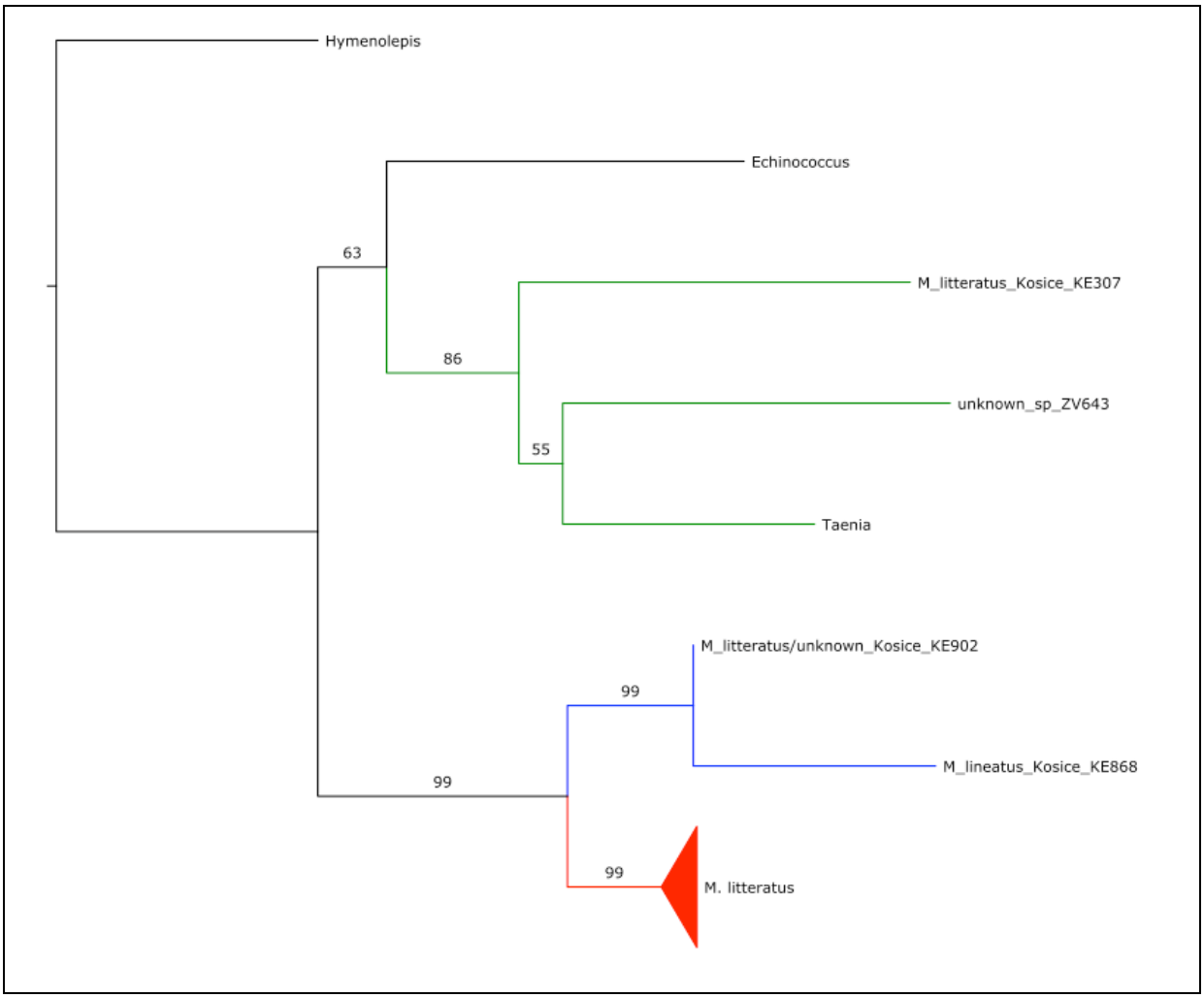


Figure 7. Maximum likelihood tree for the combined dataset nad1+cox1+12S, with polytomy collapsed. *Numbers represent bootstrap values (shown where > 50%).*

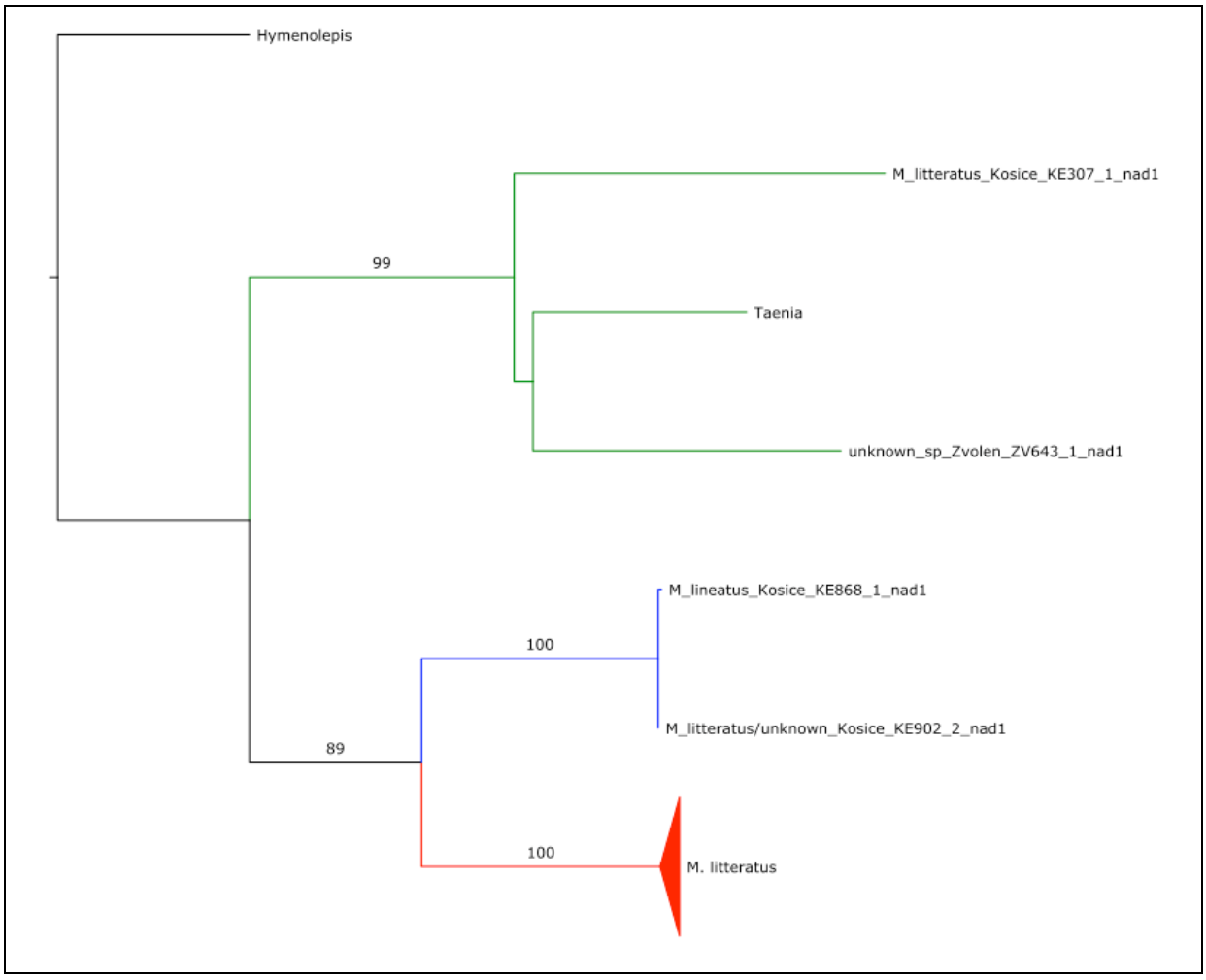


Figure 8. Maximum likelihood tree for nad1, with polytomy collapsed. *Numbers represent bootstrap values (shown where > 50%).*

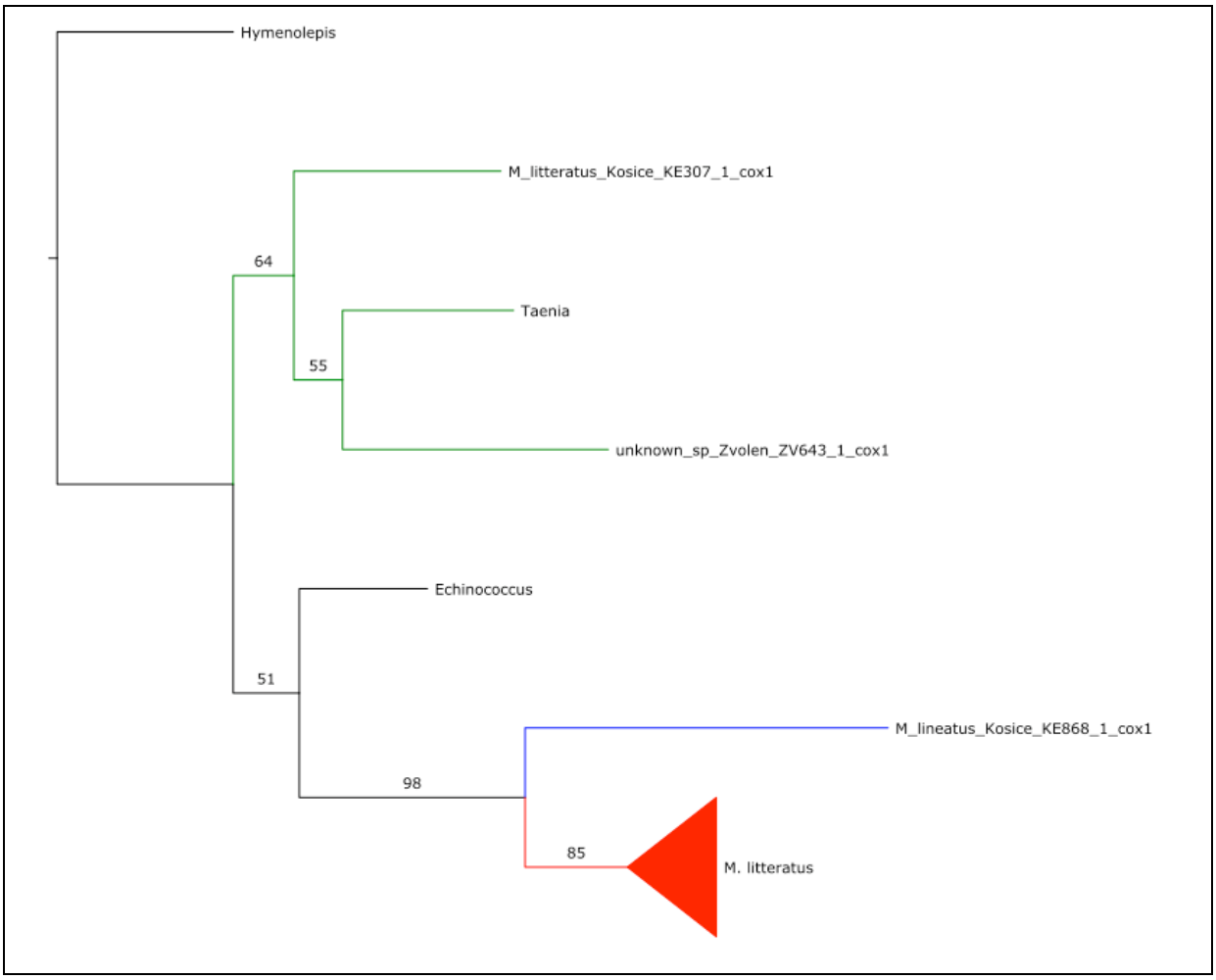


Figure 9. Maximum likelihood tree for cox1, with polytomy collapsed. *Numbers represent bootstrap values (shown where > 50%).*

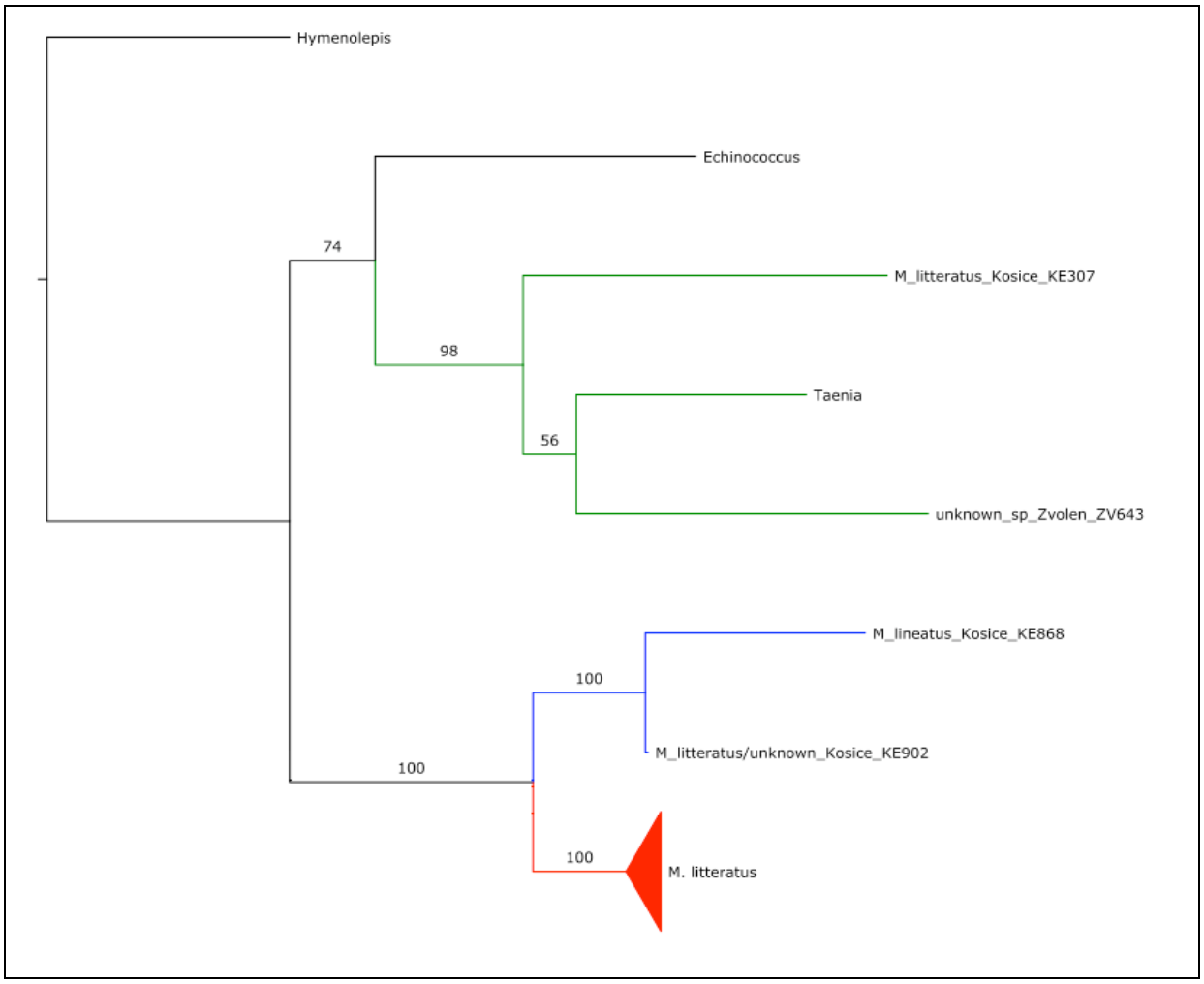


Figure 10. Bayesian inference tree for the combined dataset nad1+cox1+12S, with polytomy collapsed. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*

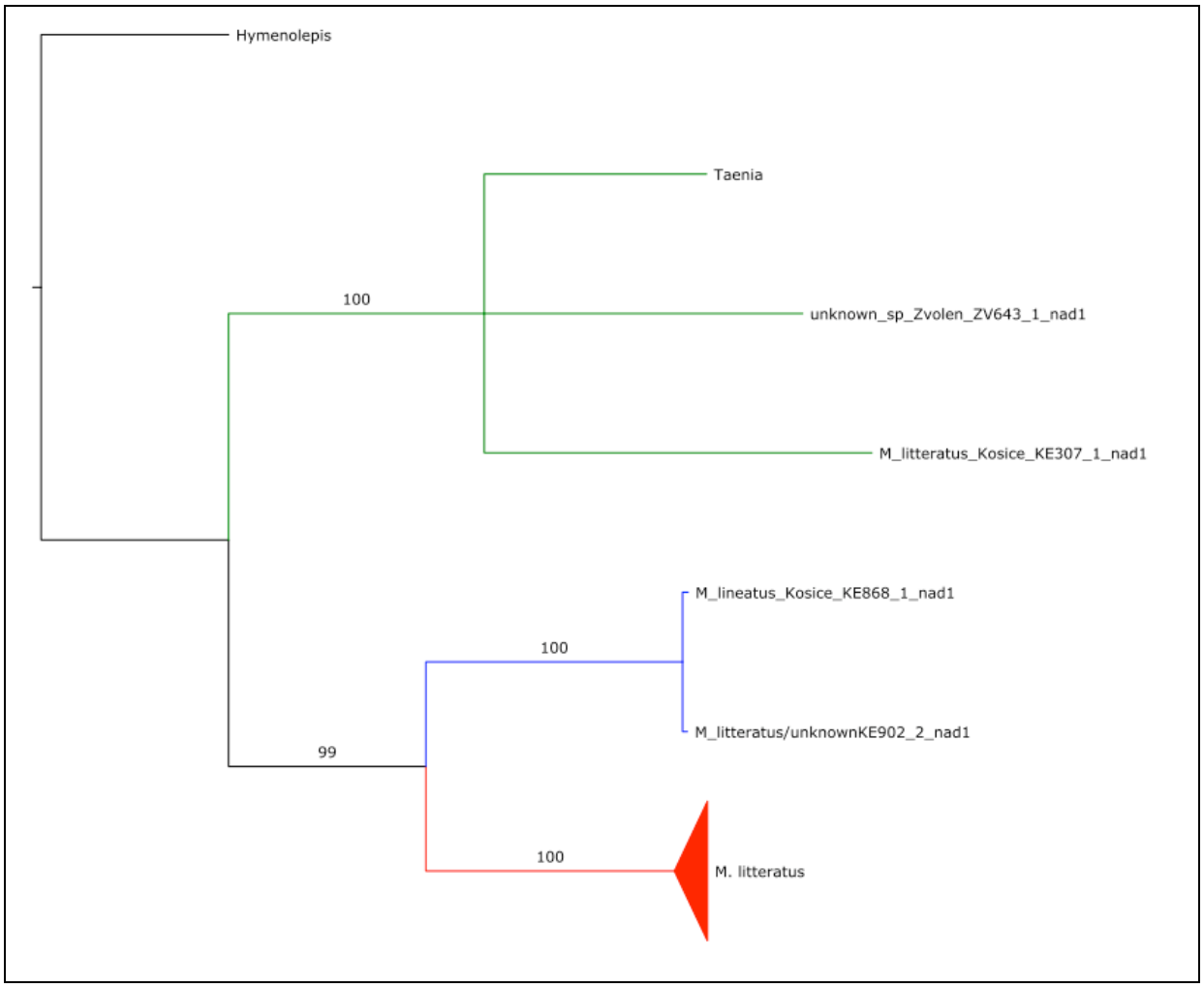


Figure 11. Bayesian inference tree for nad1, with polytomy collapsed. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*

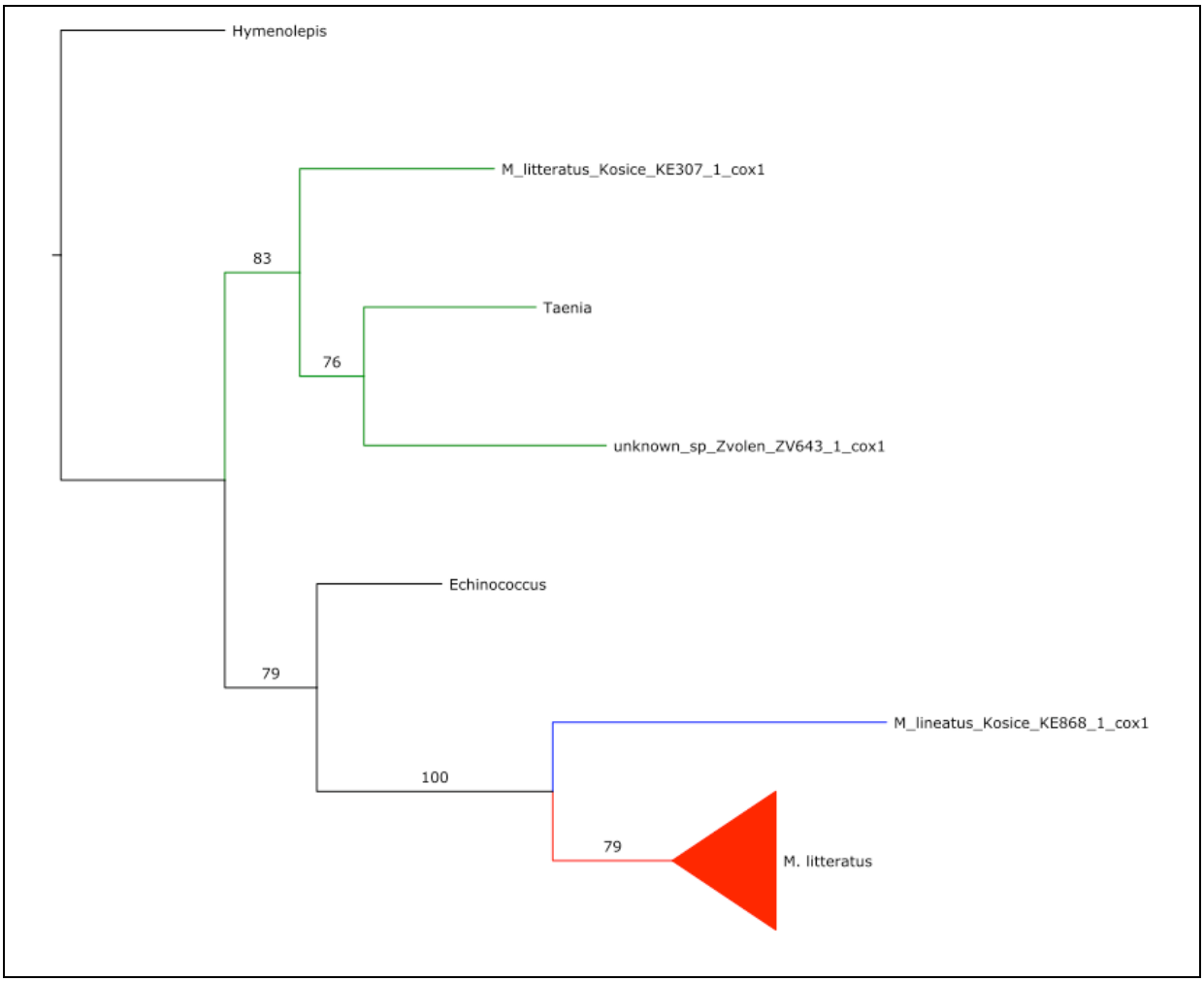


Figure 12. Bayesian inference tree for cox1, with polytomy collapsed. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*

Discussion

The results from the phylogenetic analyses and sequence divergences were found to be congruent with the identification of the specimens based on morphology (Appendix A, table A2). The extent of sequence divergence in *nad1* (up to 45%) was usually greater than that of *cox1* (up to 34%), a pattern previously observed in the sister taxon *Taenia* (Grasser *et al.*, 1999).

Two replicates were sequenced for each specimen and included in the alignment to verify accuracy. All replicates were found to be matches, so just one of each was used in the analysis. Taxon sampling was skewed towards *M. litteratus*, with 24 out of the 27 specimens from this species, while there was just one confirmed *M. lineatus* and two unidentified specimens. However, in parasitology taxon sampling can be opportunistic, which makes it difficult to obtain a statistically representative sample (Barta, 2001). Moreover, this difference likely reflects real differences in prevalences of these two species.

Phylogenetic analysis of the single and combined datasets yielded congruent topologies with moderately strong bootstrap support (> 79%) for the 2 *Mesocestoides* groups evident: (i) *M. litteratus*, (ii) *M. lineatus*. Differences in topology were limited to weakly supported branches (< 50%). Strongest support was found with *nad1* (> 99%), whereas the weakest was with *cox1* (> 63%).

Results from 12S were incongruent with *nad1*, *cox1* and the combined dataset. As mentioned previously, this dataset was not produced during this study and contains sequences downloaded from GenBank, along with sequences for specimens of that were not available for analysis (some of unknown origin). If time had permitted, 12S would have been excluded from the combined dataset and the analysis re-run.

All specimens provisionally identified as *M. litteratus* grouped together apart from KE307, that was likely misidentified. This specimen grouped consistently with *Taenia* and anunidentified specimen, which was not a member of the genus *Mesocestoides* (ZV643). The *M. lineatus* group comprised one confirmed specimen (KE868) and one specimen (KE902) whose identification was ambiguous based on morphology.

Support for species delimitation based on geographical location was not found. The specimens were all taken from foxes, that typically have home ranges of approximately 12 km, but this can extend up to 50 km and can encompass most habitats, including mountains, forests, farmland and urban areas (Fox, 2007). Each of the four locations in Slovakia is between 60 km and 120 km apart. It is somewhat surprising that the degree of geographic structure is not evident. However, the identify and roles of intermediate hosts of *Mesocestoides* are unknown, so it is difficult to evaluate their contribution.

The multi-locus approach in phylogenetic analyses, particularly the combination of both nuclear and mitochondrial genes, is particularly useful. The two types of data are unlinked and evolving under different evolutionary constraints, which decreases the probability of mistaking the evolutionary history of a singe genetic region for that of the taxa involved. Fast evolving mitochondrial DNA is especially good when studying closely-related taxa that have diverged recently, whereas nuclear DNA typically evolves more slowly making it a better marker for resolving deeper divergences (Chung-Ping & Danforth, 2004). Resolving the amplification problems with *ITS-2* and/or the inclusion of additional regions of nuclear DNA would further enhance the analysis in this case.

Implementing the multi-locus approach can prove difficult, as the choice of evolutionary model is crucial when using a model-based approach, as genes can have differing substitution parameters. Good evolutionary models will try to accommodate as much of the process of evolution as possible, so it is imperative to select the best-fit: models with too many parameters may over-fit the data and rapidly escalate computation time whilst simultaneously decreasing statistical power, whereas those with too few parameters may be unrealistic and provide erroneous results (Soltis *et al.*, 1998).

Excluding fast evolving sites (such as the 3rd codon position) in analyses of coding sequences can reduce the effect of saturation and compositional bias and thus reduce long branch attraction (Heath *et al.*, 2008). Maximum parsimony is much more susceptible to long branch attraction than model-based approaches. However,

it is generally a much faster process and can evaluate many more trees in a given amount of time (Soltis *et al.*, 1998).

Other methods of species delimitation could be used in future studies. One recent method is the general mixed Yule coalescent (GMYC), which uses a log likelihood ratio test to determine which of two models is a better fit, using lineage sorting based on observed branching patterns (Vogler *et al.*, 2008). The method used in this study works well, nevertheless further corroboration with alternative methods would add more weight to results obtained.

Summary

This study showed that *Mesocestoides* spp. in Slovakia could be split into two separate groups: *M. litteratus* and *M. lineatus* based on phylogenetic analysis of multiple mitochondrial markers. The results were congruent with the morphology-based identification of specimens and the levels of sequence divergence between taxa. Delimitation was not found to be based on geographical location.

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References

Adams, B.J. (1998) Species concepts and the evolutionary paradigm in modern Nematology. *Journal of Nematology* **30**(1): 1 – 21.

Akaike, H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**: 716 – 723.

Barta, J.R. (2001) Molecular approaches for inferring evolutionary relationships among protistan parasites. *Veterinary Parasitology* **101**: 175 – 186.

Chung-Ping, L. & Danforth, B.N. (2004) How do insect nuclear and mitochondrial gene *substitution patterns differ? Insights from Bayesian analysis of combined datasets. Molecular Phylogeny and Evolution* **30**(3): 686 – 702.

Crosbie, P.R., Nadler, S.A., Platzer, E.G., Kerner, C., Mariaux, J. & Boyce, W.M. (2000) Molecular systematics of *Mesocestoides* spp. (Cestoda: Mesocestoididae) from domestic dogs (*Canis familiaris*) and coyotes (*Canis latrans*) *Journal of Parasitology* **86**(2): 350 – 357.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**: 214 – 221.

Fauna Europaea Web Service (2004) *Fauna Europaea version 1.1* [online]. [Available from www.faunaeur.org/] [Accessed 1 September 2009].

Fox, D. (2007) *Vulpes vulpes* [online]. Animal Diversity Web. [Available from http://animaldiversityweb.ummz.umich.edu/] [Accessed 8 September 2009].

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**(5): 696 – 704.

Hoberg, E.P. (2006) Phylogeny of *Taenia*: Species definition and origin of human parasites. *Parasitology International* **22**: S23 – S30.

Literák, I., Tenora, F., Letková, V., Goldová, M., Torres, J. & Olson, P.D. (2006) *Mesocestoides litteratus* (Batsch, 1786) (Cestoda: Cyclophyllidea: Mesocestoididae) from the red fox: morphological and 18s rDNA characterization of European isolates. *Helminthologia* **43**(4): 191 – 195.

Literák, I., Olson, P.D., Georgiev, B.B. & Spakulová, M. (2004) First record of metacestodes of *Mesocestoides* sp. in the common starling (*Sturnus vulgaris*) in Europe, with an 18S rDNA characterisation of the isolate. *Folia Parasitologica* **51**: 45 – 49.

Loos-Frank, B. Shedding of gravid proglottids and in experimental infections of foxes with *Mesocestoides leptothylacus* Loos-Frank, 1980 (Cestoda). *Journal of Helminthology* **61**: 213 – 218.

Nadler, S.A. (1995) Advantages and disadvantages of molecular phylogenetics: A case study of ascaridoid nematodes. *Journal of Nematology* **27**: 423 – 432.

McManus, D.P., Thanh, H.L. & Blair, D. (2004) Genomics of parasitic flatworms. International *Journal for Parasitology* **34**(2): 153 – 158.

Maddison, D.R. & Madison, W.P. (2000) *MacClade 4: analysis of phylogeny and character evolution, ver. 4*. Sinauer Associates, Sunderland, U.S.A. http://macclade.org.

Mariaux, J. (1998) The molecular phylogeny of the Eucestoda. *Journal of Parasitology* **84**(1): 114 – 124.

Nickisch-Rosenegk, M. von, Lucius, R., Loos_Frank, B. (1999) Contributions to the phylogeny of the Cyclophyllidea (Cestoda) inferred from mitochondrial 12S rDNA.

Journal of Molecular Evolution **48**: 586 – 596.

Nylander, J. A. A. (2004) *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Olson, P.D. & Tkach, V.V. (2005) Advances and trends in the molecular systematics of the parasitic platyhelminthes. *Advances in Parasitology* **60**: 165 – 243.

Olson, P.D. & Caira, J.N. (1999) Evolution of the Major Lineages of Tapeworms (Platyhelminthes: Cestoidea) Inferred from 18S Ribosomal DNA and Elongation Factor-1α. *Journal of Parasitology* **85**(6): 1134 – 1159.

Padgett, K.A., Nadler, S.A., Munson, L., Sacks, D. & Boyce, W.M. (2005) Systematics of *Mesocestoides* (Cestoda: Mesocestoididae): Evaluation of molecular and morphological variation among isolates. *Journal of Parasitology* **91**(6): 1435 – 1443.

Ridley, M. (2004) *Evolution*. Blackwell Publishing, Malden, U.S.A.

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**(12): 1572 – 1574.

Salemi, M. & Vandamme, A-M. (2003) *The phylogenetic handbook: A practical approach to DNA and protein phylogeny*. Cambridge University Press, Cambridge, UK.

Sites Jr., J.W. & Marshall, J.C. (2003) Delimiting species: a Renaissance issue in systematic biology. *Trends in Ecology and Evolution* **18**(9): 462 – 470.

Soltis, D.E., Soltis, P.S. & Doyle, J.J. (1998) *Molecular systematics of plants II*. Kluwer Academic Publishers, New York, U.S.A.

Stang, D. (2009) *ZipcodeZoo.com* [online]. [Available from http://zipcodezoo.com] [Accessed 1 September 2009].

Swofford, D.L. (2003) *PAUP* 4.0b phylogenetic analysis using parsimony (*and other methods).* Sinauer Associates, Massachusetts, U.S.A.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596 – 1599.

The UniProt Consortium (2009) The universal protein resource (UniProt) *Nucleic Acids Research* **37**: D169 – D174.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876 – 4882.

Voge, M. (1955) North American cestodes of the genus *Mesocestoides*. *University of California Publications in Zoology* **59**: 125 – 156.

Vogler, C., Benzie, J., Lessios, H., Barber, P. & Worheide, G. (2008) A threat to coral reefs multipled? Four species of crowm-of-thorns starfish. *Biology Letters* **4**(6): 696 – 699.

Wiens, J.J. (2003) Missing data, incomplete taxa, and phylogenetic accuracy. *Systematic Biology* **52**: 528 – 538.

Appendices

Appendix A: Supplementary Specimen Data

Table A.1. Mesocestoides sp. specimens (all hosts were foxes (Vulpes sp.)

Number	Specimen	Location	Species	Number	Specimen	Location	Species
1	DK91-1	Dolny kulsin	*M. litteratus*	21	KE322-1	Kosice	*M. litteratus*
2	DK91-2	Dolny kulsin	*M. litteratus*	22	KE322-2	Kosice	*M. litteratus*
3	DK88-1	Dolny kulsin	*M. litteratus*	23	KE307-1	Kosice	*M. litteratus*
4	DK88-2	Dolny kulsin	*M. litteratus*	24	KE307-2	Kosice	*M. litteratus*
5	DK75-1	Dolny kulsin	*M. litteratus*	25	KE873-1	Kosice	*M. litteratus*
6	DK75-2	Dolny kulsin	*M. litteratus*	26	KE873-2	Kosice	*M. litteratus*
7	DK97-1	Dolny kulsin	*M. litteratus*	27	KE902-1	Kosice	*M. litteratus (?)*
8	DK97-2	Dolny kulsin	*M. litteratus*	28	KE902-2	Kosice	*M. litteratus (?)*
9	DK159-1	Dolny kulsin	*M. litteratus*	29	KE935-1	Kosice	?
10	DK159-2	Dolny kulsin	*M. litteratus*	30	KE935-2	Kosice	?
11	DK74-1	Dolny kulsin	*M. litteratus*	31	KE918-1	Kosice	*M. litteratus*
12	DK74-2	Dolny kulsin	*M. litteratus*	32	KE918-2	Kosice	*M. litteratus*
13	DK80-1	Dolny kulsin	*M. litteratus*	33	KE868-1	Kosice	*M. lineatus*
14	DK80-2	Dolny kulsin	*M. litteratus*	34	KE868-2	Kosice	*M. lineatus*
15	DK70-1	Dolny kulsin	*M. litteratus*	35	KE872-1	Kosice	*M. litteratus*
16	DK70-2	Dolny kulsin	*M. litteratus*	36	KE872-2	Kosice	*M. litteratus*
17	KE279-1	Kosice	*M. litteratus*	37	KE903-1	Kosice	*M. litteratus*
18	KE279-2	Kosice	*M. litteratus*	38	KE903-2	Kosice	*M. litteratus*
19	KE282-1	Kosice	*M. litteratus*	39	KE875-1	Kosice	*M. litteratus*
20	KE282-2	Kosice	*M. litteratus*	40	KE875-2	Kosice	*M. litteratus*

Table A.1 (continued)

Number	Specimen	Location	Species
41	BA87-1	Bratislava	*M. litteratus*
42	BA87-2	Bratislava	*M. litteratus*
43	BA93-1	Bratislava	*M. litteratus*
44	BA93-2	Bratislava	*M. litteratus*
45	ZV574-1	Zvolen	*M. litteratus*
46	ZV574-2	Zvolen	*M. litteratus*
47	ZV889-1	Zvolen	*M. litteratus*
48	ZV889-2	Zvolen	*M. litteratus*
49	ZV883-1	Zvolen	*M. litteratus*
50	ZV883-2	Zvolen	*M. litteratus*
51	ZV903-1	Zvolen	*M. litteratus*
52	ZV903-2	Zvolen	*M. litteratus*
53	ZV643-1	Zvolen	*M. litteratus*
54	ZV643-2	Zvolen	*M. litteratus*
55	DK91-3	Dolny kulsin	*M. litteratus*

Table A.2. Substitution model parameters

Parameter	*nad1*	*cox1*	*12S*
model	GTR+G+I	HKY+G	GTR+G
r (A⇔C)	0.6337	N/A	0.00003
r (A⇔G)	8.9271	N/A	2.5290
r (A⇔T)	2.5284	N/A	1.2854
r (C⇔G)	1.8765	N/A	0.1151
r (C⇔T)	4.0239	N/A	1.8143
r (G⇔T)	1.0 (fixed)	N/A	1.0 (fixed)
gamma	1.906	0.232	0.453
lnL	-2000.80	-1838.34	-1378.94

Table A.3. Inter-taxon sequence divergences for nad1

TAXA	TAXA																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Taenia	1																												
Hymenolepis	2	0.42																											
DK70_1_nad1	3	0.35	0.40																										
DK74_1_nad1	4	0.35	0.40	0.00																									
DK75_1_nad1	5	0.36	0.41	0.01	0.01																								
DK80_1_nad1	6	0.35	0.40	0.00	0.00	0.01																							
DK88_2_nad1	7	0.35	0.40	0.00	0.00	0.01	0.00																						
DK91_1_nad1	8	0.35	0.40	0.00	0.00	0.01	0.00	0.00																					
ZV574_2_nad1	9	0.36	0.42	0.01	0.01	0.02	0.01	0.01	0.01																				
ZV643_1_nad1	10	0.30	0.45	0.41	0.41	0.42	0.41	0.41	0.41	0.43																			
ZV883_1_nad1	11	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41																		
ZV889_1_nad1	12	0.34	0.40	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.41	0.01																	
ZV903_1_nad1	13	0.34	0.40	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.41	0.01	?																
KE279_1_nad1	14	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01															
KE282_1_nad1	15	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00														
KE307_1_nad1	16	0.38	0.50	0.40	0.40	0.41	0.40	0.40	0.40	0.42	0.42	0.40	0.40	0.40	0.40	0.40													
KE322_2_nad1	17	0.36	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.02	0.41	0.00	0.01	0.01	0.00	0.00	0.41												
KE868_1_nad1	18	0.44	0.35	0.27	0.27	0.28	0.27	0.27	0.27	0.28	0.43	0.27	0.27	0.27	0.27	0.27	0.44	0.27											
KE872_1_nad1	19	0.36	0.42	0.01	0.01	0.02	0.01	0.01	0.01	0.00	0.43	0.01	0.02	0.02	0.01	0.01	0.42	0.02	0.28										
KE873_1_nad1	20	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01									
KE875_2_nad1	21	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00								
KE903_1_nad1	22	0.34	0.40	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.41	0.01	?	?	0.01	0.01	0.40	0.01	0.27	0.02	0.01	0.01							
KE918_1_nad1	23	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00	0.00	0.01						
BA87_1_nad1	24	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00	0.00	0.01	0.00					
BA93_1_nad1	25	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00	0.00	0.01	0.00	0.00				
DK159_2_nad1	26	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00	0.00	0.01	0.00	0.00	0.00			
DK97_2_nad1	27	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00		
KE902_2_nad1	28	0.44	0.35	0.27	0.27	0.28	0.27	0.27	0.27	0.28	0.43	0.27	0.27	0.27	0.27	0.27	0.44	0.27	0.00	0.28	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	
KE935_2_nad1	29	0.35	0.40	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.41	0.00	0.01	0.01	0.00	0.00	0.40	0.00	0.27	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.27

Table A.4. Inter-taxon sequence divergences for cox1

TAXA	TAXA																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		
Hymenolepis	1																												
Echinococcus	2	0.22																											
Taenia	3	0.27	0.22																										
BA87_1_cox1	4	0.27	0.23	0.30																									
BA93_1_cox1	5	0.26	0.21	0.27	0.01																								
DK70_1_cox1	6	0.26	0.21	0.28	0.02	0.00																							
DK74_1_cox1	7	0.26	0.21	0.28	0.02	0.00	0.00																						
DK80_2_cox1	8	0.25	0.20	0.27	0.02	0.00	0.01	0.01																					
DK91_3_cox1	9	0.27	0.20	0.27	0.02	0.01	0.01	0.01	0.01																				
DK159_1_cox1	10	0.26	0.21	0.27	0.02	0.01	0.01	0.01	0.01	0.00																			
KE868_1_cox1	11	0.34	0.23	0.29	0.21	0.19	0.19	0.19	0.18	0.18	0.18																		
KE872_1_cox1	12	0.27	0.20	0.26	0.04	0.03	0.03	0.03	0.03	0.02	0.03	0.18																	
KE873_1_cox1	13	0.28	0.21	0.26	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.18	0.02																
KE875_1_cox1	14	0.26	0.20	0.26	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.18	0.02	0.01															
KE279_1_cox1	15	0.26	0.20	0.26	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.18	0.02	0.01	0.00														
KE282_1_cox1	16	0.26	0.21	0.27	0.02	0.01	0.01	0.01	0.01	0.01	0.02	0.18	0.03	0.02	0.01	0.01													
KE307_1_cox1	17	0.25	0.22	0.18	0.27	0.25	0.24	0.24	0.26	0.24	0.24	0.28	0.23	0.23	0.24	0.24	0.26												
KE322_2_cox1	18	0.26	0.21	0.28	0.02	0.01	0.00	0.00	0.01	0.01	0.01	0.18	0.03	0.02	0.01	0.01	0.01	0.24											
KE902_1_cox1	19	0.26	0.20	0.26	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.18	0.02	0.01	0.00	0.00	0.01	0.24	0.01										
KE903_1_cox1R	20	0.27	0.21	0.26	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.17	0.02	0.02	0.01	0.01	0.02	0.25	0.02	0.01									
KE918_1_cox1	21	0.26	0.20	0.26	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.18	0.02	0.01	0.00	0.00	0.01	0.24	0.01	0.00	0.01								
KE935_2_cox1	22	0.28	0.21	0.26	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.18	0.02	0.00	0.01	0.01	0.02	0.23	0.02	0.01	0.02	0.01							
ZV574_1_cox1	23	0.27	0.20	0.25	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.17	0.01	0.01	0.01	0.02	0.03	0.23	0.03	0.01	0.01	0.01	0.01						
ZV643_1_cox1F	24	0.27	0.23	0.19	0.27	0.26	0.25	0.25	0.25	0.24	0.24	0.31	0.25	0.25	0.25	0.24	0.27	0.22	0.25	0.25	0.24	0.25	0.25	0.24					
ZV883_2_cox1	25	0.25	0.21	0.27	0.02	0.00	0.01	0.01	0.01	0.01	0.01	0.18	0.03	0.02	0.01	0.01	0.00	0.26	0.01	0.01	0.02	0.01	0.02	0.02	0.26				
ZV889_1_cox1	26	0.27	0.21	0.26	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.17	0.02	0.02	0.01	0.01	0.02	0.25	0.02	0.01	0.00	0.01	0.02	0.01	0.24	0.02			
ZV903_1_cox1	27	0.27	0.21	0.26	0.03	0.02	0.02	0.02	0.02	0.01	0.02	0.17	0.02	0.02	0.01	0.01	0.02	0.25	0.02	0.01	0.00	0.01	0.02	0.01	0.24	0.02	0.00		

Appendix B: Perl Scripts

Maximum Parsimony Script

```
#########################################################################
#
# Script written by Anne O'Connor August 2009 for MRes project 3
# Runs on Mac OSX using command line Paup* 4.0 (windows version also available)
#
#########################################################################

#!/usr/local/bin/perl
use warnings;
use strict;

#########################################################################
# To run this script type: perl mp_paup.pl inputfilename.nex
# Input file must be in nexus format
# Paup executable must be in the same folder as the perl script and the input nexus file
#########################################################################

my $nex_file = $ARGV[0];                    # get input file name from command line
my $nex_file_no_ext = $nex_file . "_out";   # copy of input file

$nex_file_no_ext =~ s/\..*//;               # remove .nex from input file name

open (IN, $nex_file) || die "can't open file";
open (OUT, ">$nex_file_no_ext") || die "can't open file";

my @lines = <IN>;
seek IN,0,0;

foreach my $lines (@lines)
{
        print OUT $lines;           # write nexus input file to the new input file
};
print OUT "\n";                     # start writing paup block to end of new input file
print OUT "begin paup;";
print OUT "\n";
print OUT "set autoclose=yes warntree=no warnreset=no notifybeep=no pause=no errorbeep=no;";
print OUT "\n";
print OUT "log start file=", $nex_file_no_ext . ".log", " replace;";
print OUT "\n";
print OUT "set criterion=parsimony maxtrees=10000 increase=auto autoinc=100;";
print OUT "\n";
print OUT "bootstrap nreps=1000 search=heuristic treefile=", $nex_file_no_ext . ".trees", " replace brlen=yes
/ start=stepwise addseq=random nreps=100 savereps=no randomize=addseq hold=1 swap=tbr
multrees=yes;";
print OUT "\n";
print OUT "savetrees from=1 to=1 file=", $nex_file_no_ext . ".savetrees", " replace format=altnexus
brlens=yes savebootp=nodelabels maxdecimals=0;";
print OUT "\n";
print OUT "contree all /strict=no majrule=yes le50=yes usetreewts=no showtree=yes treefile=",
$nex_file_no_ext . "_contree_file.trees", " replace grpfreq=yes;";
print OUT "\n";
print OUT "log stop;";
print OUT "\n";
print OUT "end;";
print OUT "\n";

max_pars($nex_file_no_ext);

close IN;
close OUT;
```

```
#########################################################################
# Sub-routine to run parsimony bootstrap in Paup
#########################################################################

sub max_pars()
{
        system("./paup4b10-ppc-macosx -n $nex_file_no_ext");       # -n quits paup once processing
                                                                   # complete
};
```

Bayesian Inference Script

```
#########################################################################
#
# Script written by Anne O'Connor August 2009 for MRes project 3
# Runs on Mac OSX using command line Paup* 4.0 and MrBayes 3.2 (windows version also available)
#
#########################################################################

#!/usr/local/bin/perl
use warnings;
use strict;

#########################################################################
# To run this script type: perl modeltest_mrbayes.pl filename.nex
# Input file must be in nexus format
# mrbayes and MrModelTest executables must be in the same folder as the perl script and the following files:
# input nexus file and mrmodeltest.txt
#########################################################################

my $nex_file = $ARGV[0];                              # input nexus file
my $nex_file_no_ext = $nex_file . "_out";             # copy of input nexus file
$nex_file_no_ext =~ s/\..*//;                         # remove .nex from input file name
my $outfile1 = $nex_file_no_ext . "_modeltest.out"; # concatenation of input nexus file and mrmodeltest paup
block
my $outfile2 = $nex_file_no_ext . ".out";             # concatenation of input nexus file and mrbayes paup
block

open (IN1, $nex_file) || die "can't open file";
open (IN2, "mrmodeltest.txt") || die "can't open file";                  # file containing mrmodeltest paup
block
open (OUT1, ">$outfile1") || die "can't open file";
open (OUT2, ">$outfile2") || die "can't open file";

my @lines1 = <IN1>;
my @lines2 = <IN2>;
seek IN1,0,0;
seek IN2,0,0;

foreach my $lines1 (@lines1)
{
        print OUT1 $lines1;        # write nexus input file to the new input file
};

foreach my $lines2 (@lines2)
{
        print OUT1 $lines2;        # write mrmodeltest paup block to new input file
};

close IN1;
close IN2;
close OUT1;
```

```
mod_test($outfile1, $nex_file);      # subroutine to find best fit substitution model using mrmodeltest.exe

######################################################################
# Need to get the MrModelMest MrBayes block from the mrmodel.out file and use this in paup block
# line looking for is one that starts with the 4th occurrence of "Lset" - need whole line for paup block
######################################################################

my $outfile3 = $nex_file_no_ext . "_paupblock.out";          # file with paup block
open (IN3, "mrmodel.out") || die "can't open file";          # file to search for "Lset"
open (OUT3, ">$outfile3") || die "can't open file";

my @lines3 = <IN3>;
my $AIC_Lset;
my $AIC_Prset;
seek IN3,0,0;

my $found_Lset = 0;                  # will record number of times Lset string found
my $found_Prset = 0;                 # will record number of times Pset string found

foreach my $lines3 (@lines3)         # read file line by line
{
        if ($lines3 =~ m/Lset/)      # search for string in file
        {
                $found_Lset++;
        }
        if ($found_Lset == 4)        # found 4th occurrence - AIC model settings are on this line
        {
                print OUT3 $lines3;
                chomp($lines3);
                $AIC_Lset = $lines3;
                $found_Lset = 0; # set found counter back to zero, so only print out the 1 line I want
        }
};

foreach my $lines3 (@lines3)         # read file line by line
{
        if ($lines3 =~ m/Prset/)     # search for string in file
        {
                $found_Prset++;
        }
        if ($found_Prset == 2)       # found 2nd occurrence - AIC model settings are on this line
        {
                print OUT3 $lines3;
                chomp($lines3);
                $AIC_Prset = $lines3;
                $found_Prset = 0;          # set found counter back to zero, so only print out the line I want
        }
};

close IN3;
close OUT3;

######################################################################
# Add mrbayes paup block to nexus infile
######################################################################

foreach my $lines1 (@lines1)
{
        print OUT2 $lines1;                  # write nexus input file to the new input file
};
print OUT2 "\n";                     # start writing ml paup block to new input file
print OUT2 "begin mrbayes;";
print OUT2 "\n";
print OUT2 "set autoclose=yes nowarn=yes;";
print OUT2 "\n";
print OUT2 "log start file=", $nex_file_no_ext . "_bi.log", " replace;";
print OUT2 "\n";
print OUT2 "outgroup Taenia;";       # currently has to be set manually – had no time to automate this part!
```

```
print OUT2 "\n";
print OUT2 $AIC_Lset;               # add in AIC ml Lset settings from mrmodeltest
print OUT2 "\n";
print OUT2 $AIC_Prset;              # add in AIC ml Pset settings from mrmodeltest
print OUT2 "\n";
print OUT2 "mcmc nruns=1 ngen=1000000 samplefreq=10 file=", $nex_file_no_ext . "1.bayes;";
print OUT2 "\n";
print OUT2 "mcmc file=", $nex_file_no_ext . "2.bayes;";
print OUT2 "\n";
print OUT2 "sumt burnin=100000;";
print OUT2 "\n";
print OUT2 "log stop;";
print OUT2 "\n";
print OUT2 "end;";
print OUT2 "\n";

mr_bayes($outfile2);                # call subroutine to run Bayesian inference with MrBayes

close OUT2;


##########################################################################
# Sub-routine to find substitution model using MrModelTest
##########################################################################

sub mod_test()
{
        system("./paup4b10-ppc-macosx execute $nex_file");                  # -n quits paup once
                                                                            # processing complete
        system("./paup4b10-ppc-macosx -n $outfile1");
        system("./mrmodeltest2 < mrmodel.scores > mrmodel.out");            # mrmodel.out has best
                                                                            # models in it along with
                                                                            # MrBayes parameter settings
};

##########################################################################
# Sub-routine to run bayesian analysis in MrBayes
##########################################################################

sub mr_bayes()
{
        system("./mb $outfile2");
};
```

Appendix C: Supplementary Trees

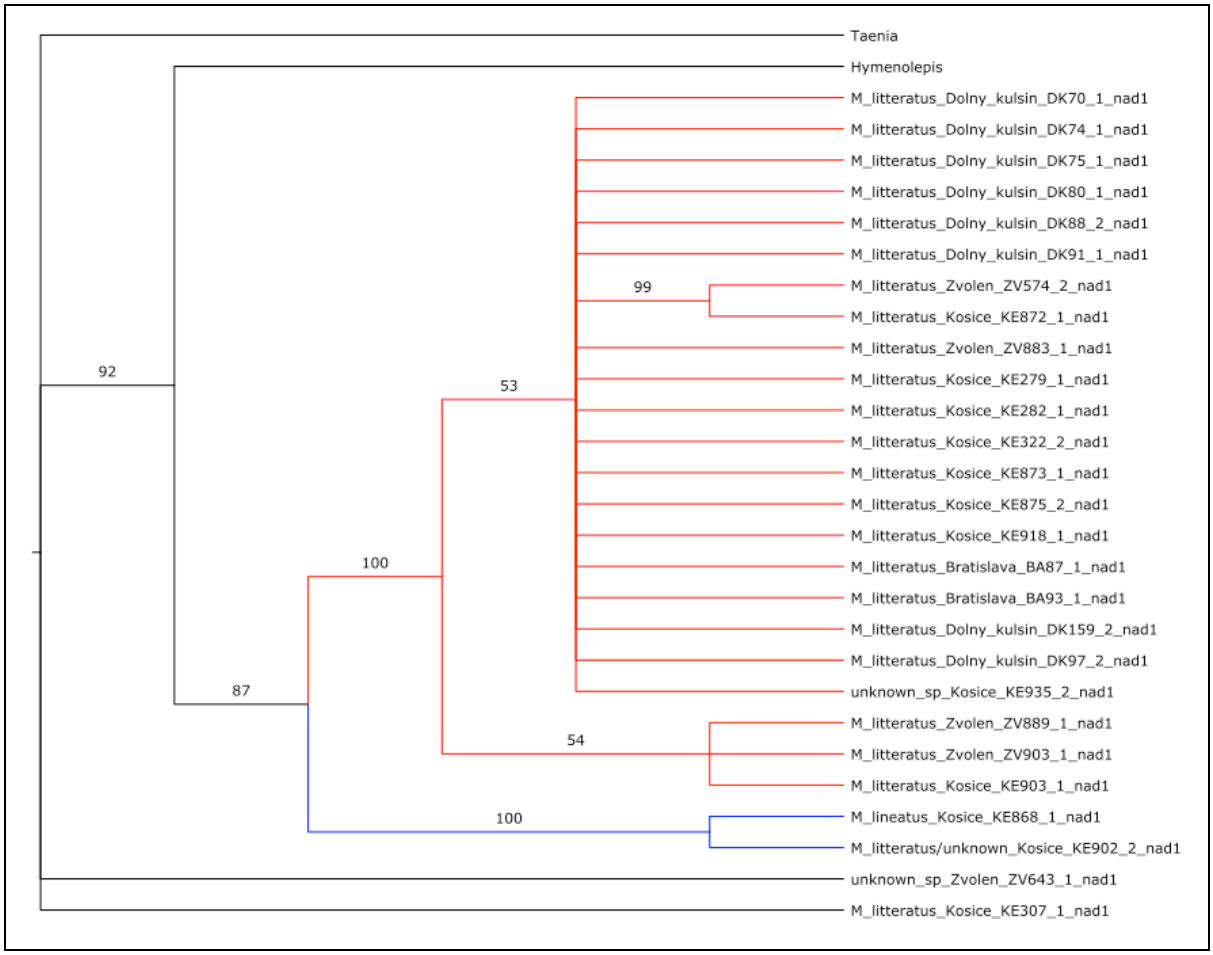

Figure C.1. Maximum parsimony 50% majority rule consensus tree for nad1. *Numbers represent bootstrap values (shown where > 50%).*

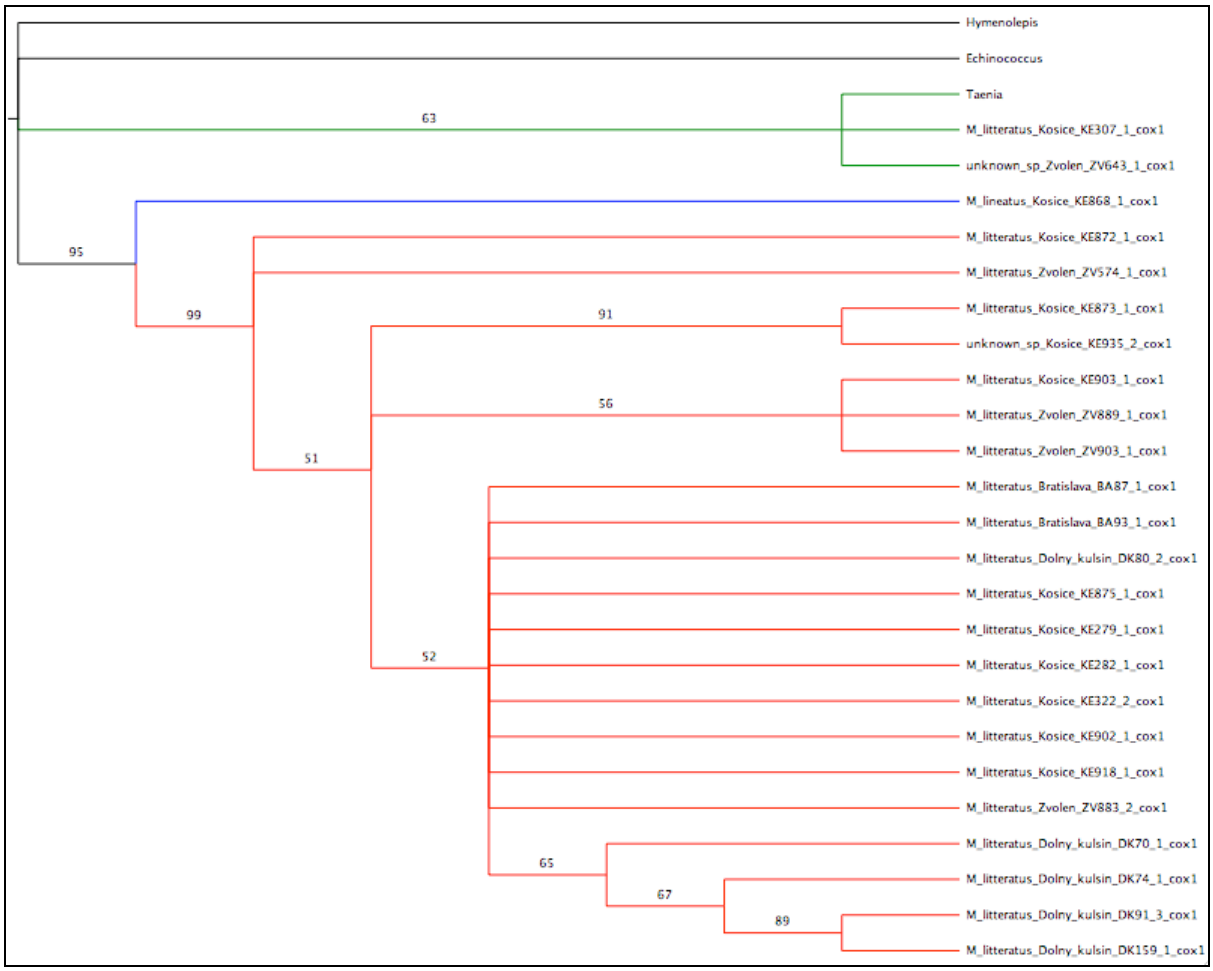

Figure C.2. Maximum parsimony 50% majority rule consensus tree for cox1. *Numbers represent bootstrap values (shown where > 50%).*

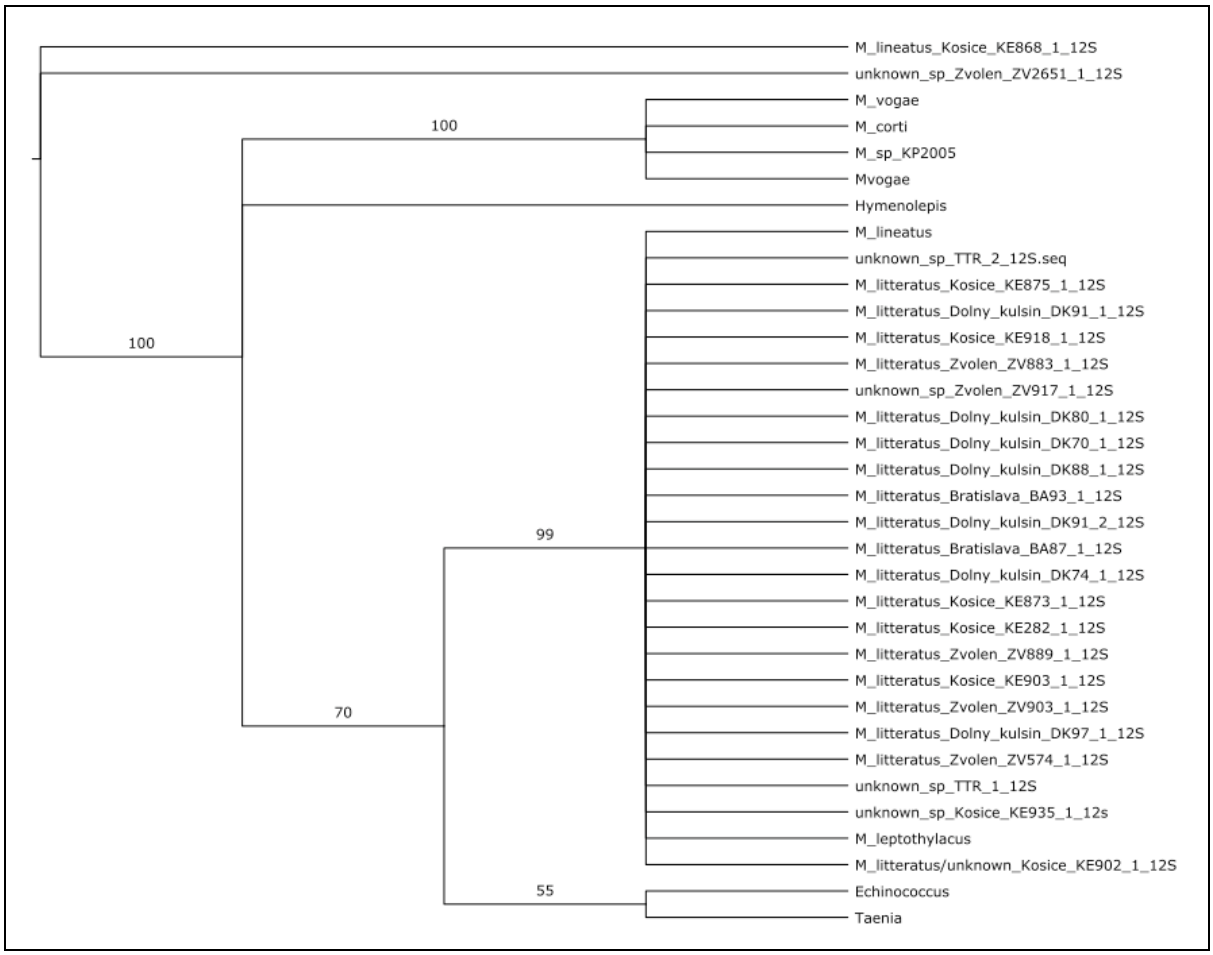

Figure C.3. Maximum parsimony 50% majority rule consensus tree for 12S. *Numbers represent bootstrap values (shown where > 50%).*

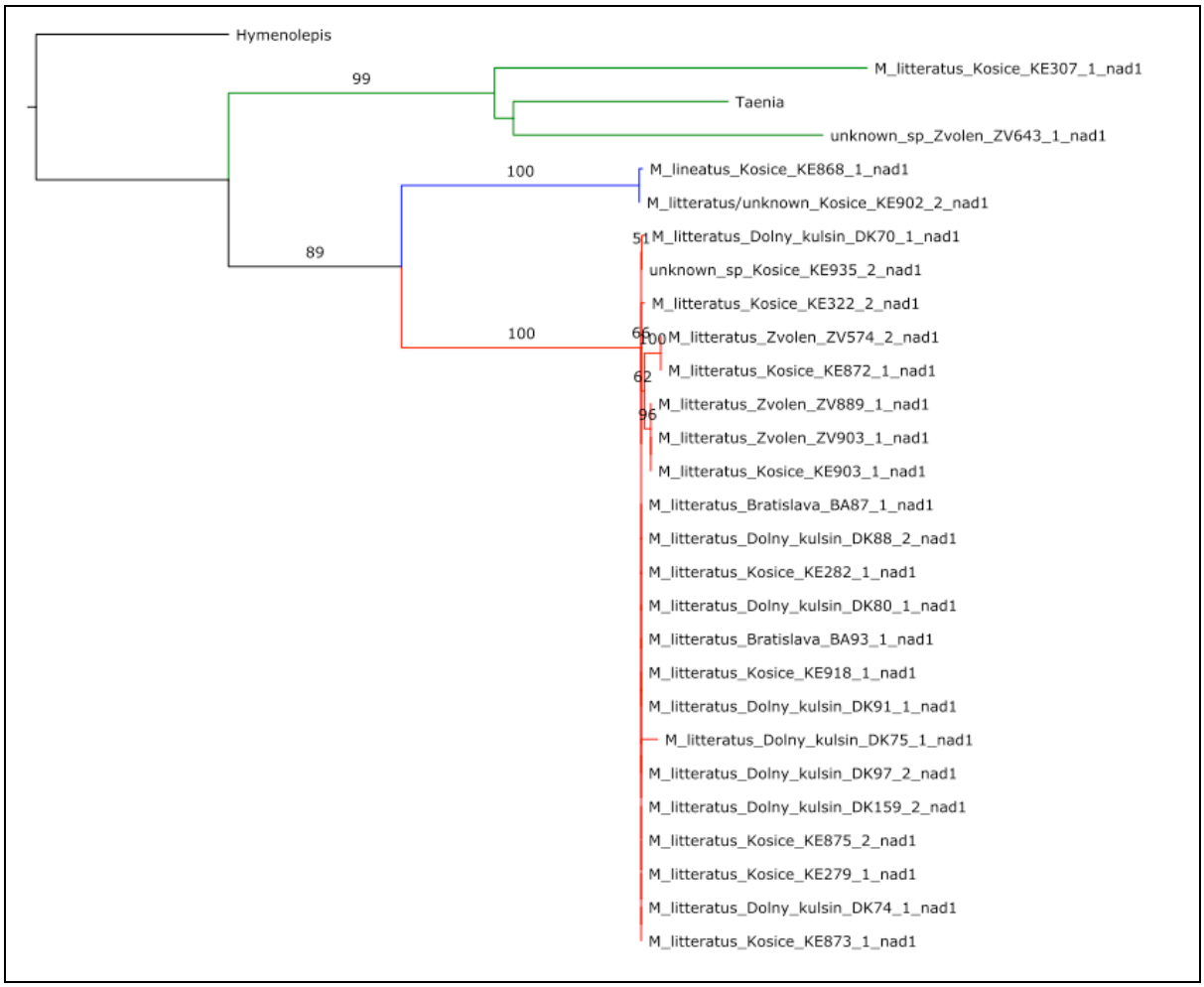

Figure C.4. Maximum likelihood tree for the combined dataset nad1. *Numbers represent bootstrap values (shown where > 50%).*

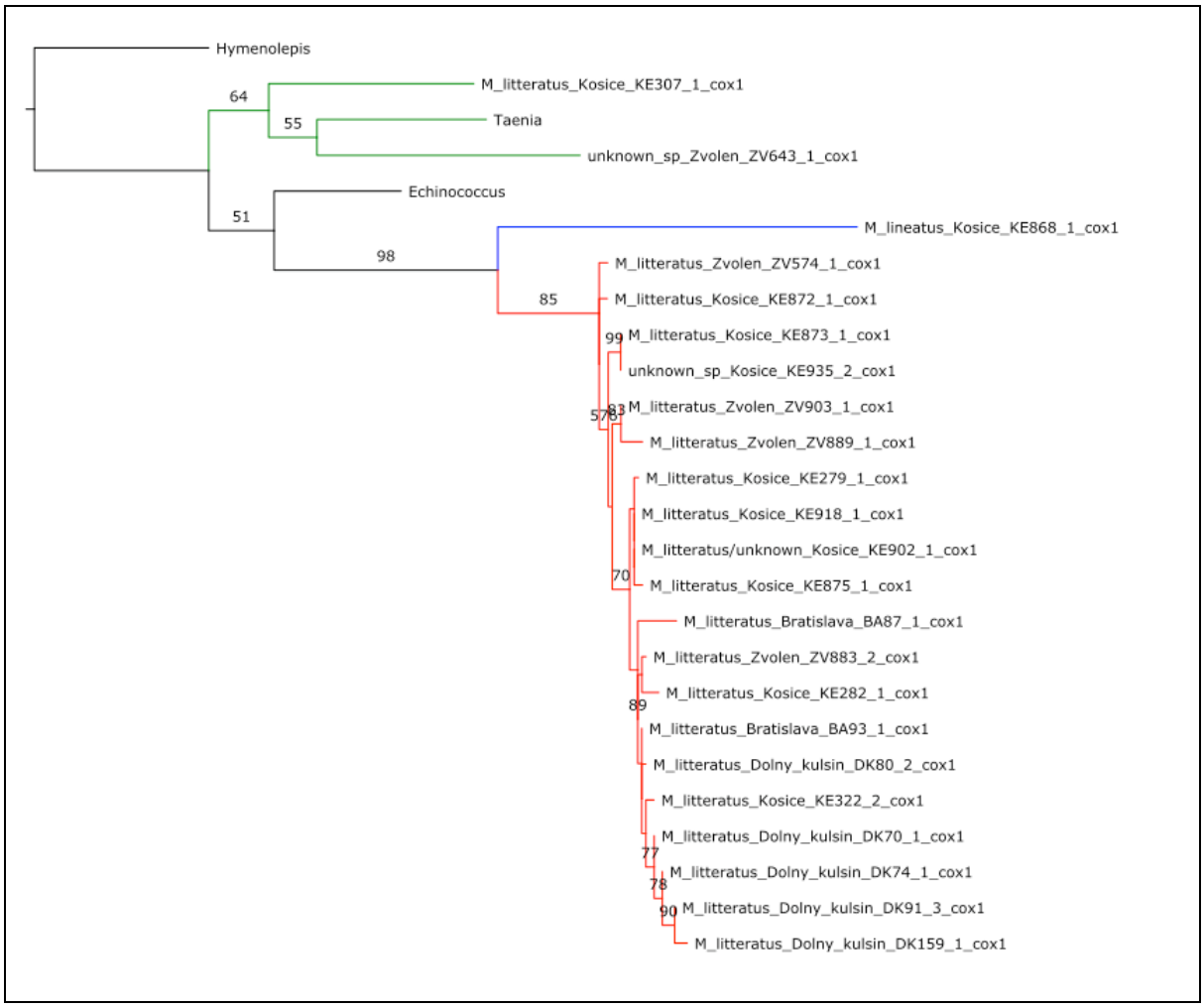

Figure C.5. Maximum likelihood tree for cox1. *Numbers represent bootstrap values (shown where > 50%).*

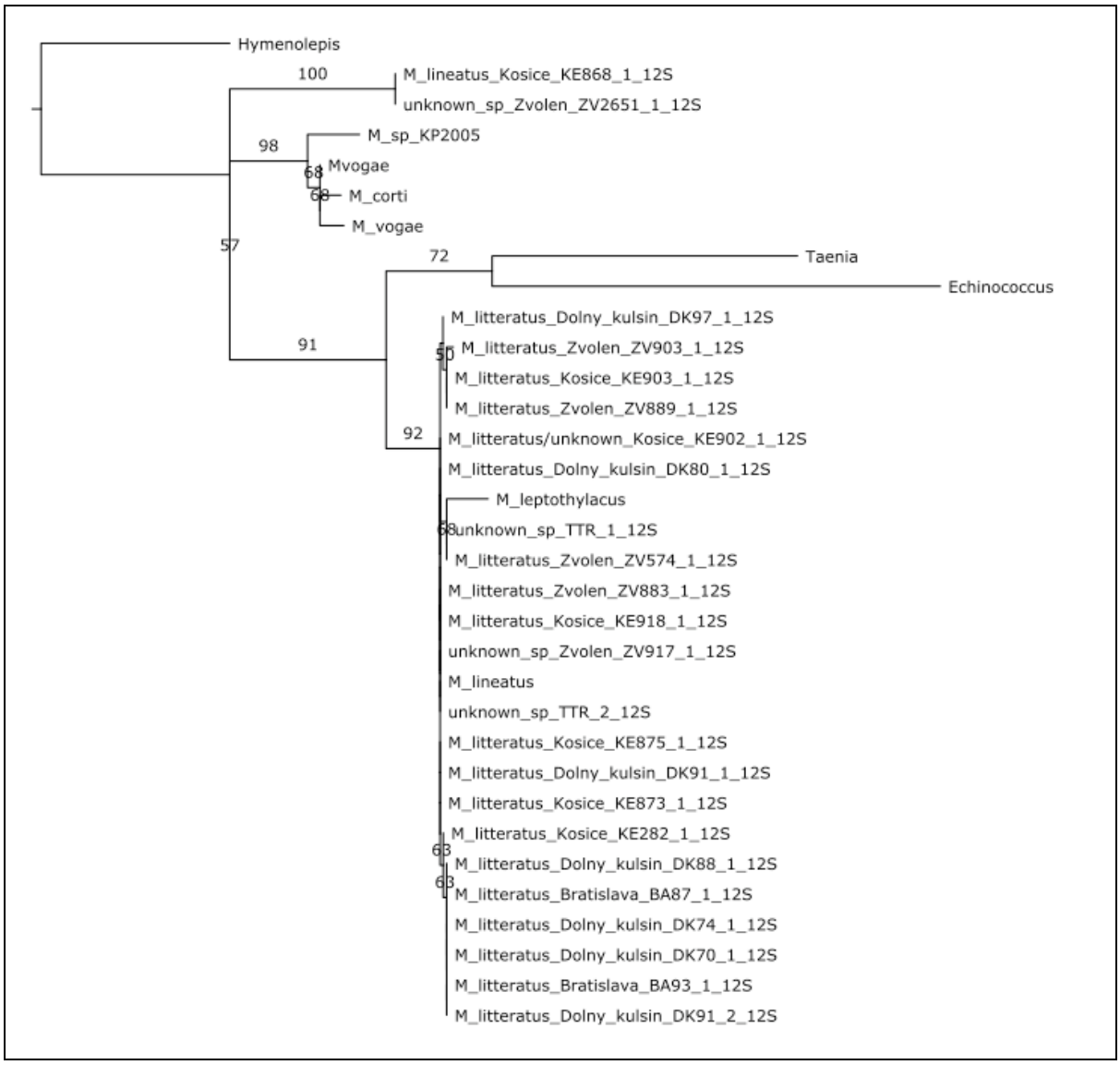

Figure C.6. Maximum likelihood tree for the combined dataset 12S. *Numbers represent bootstrap values (shown where > 50%).*

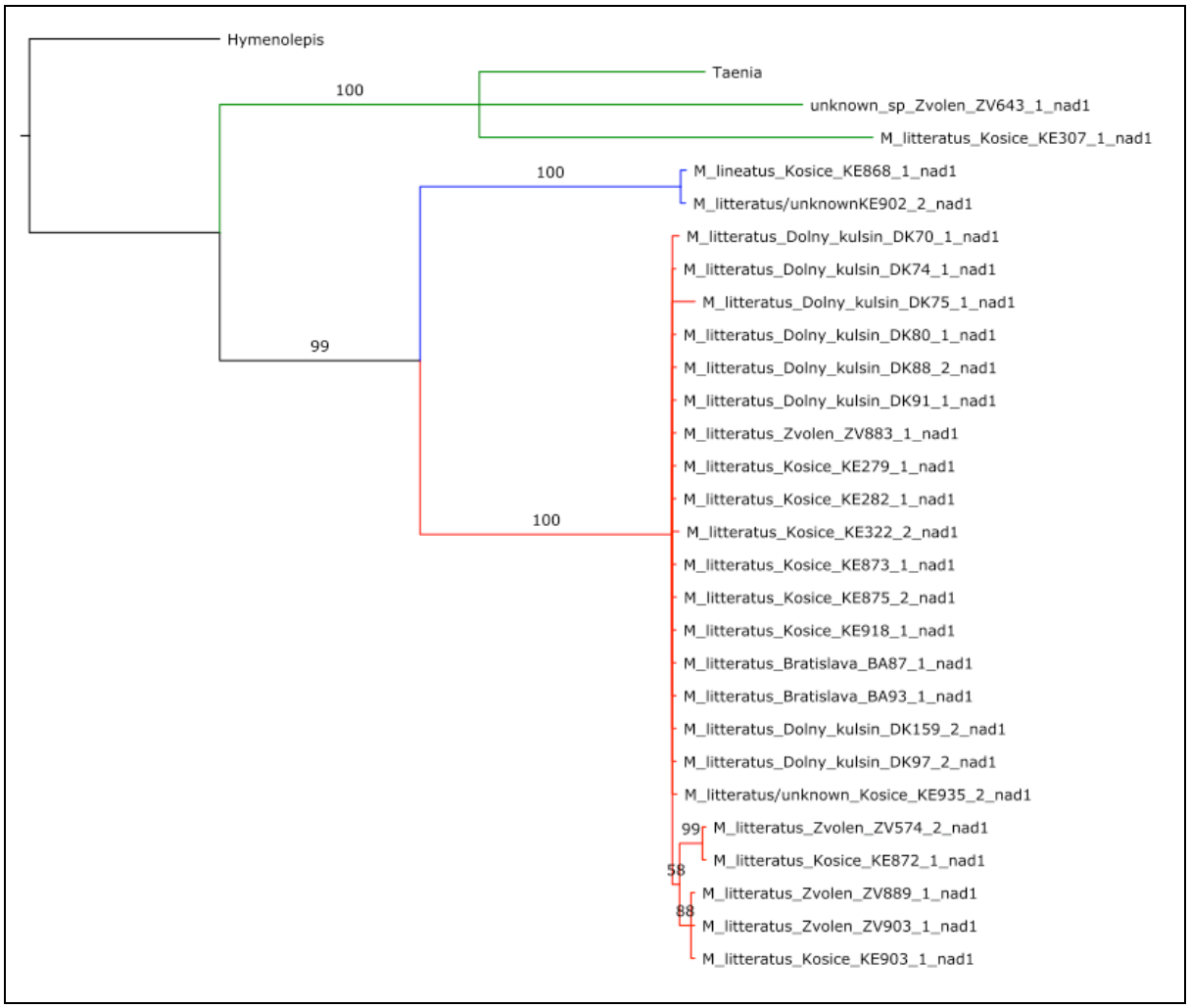

Figure C.7. Bayesian inference tree for the combined dataset nad1. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*

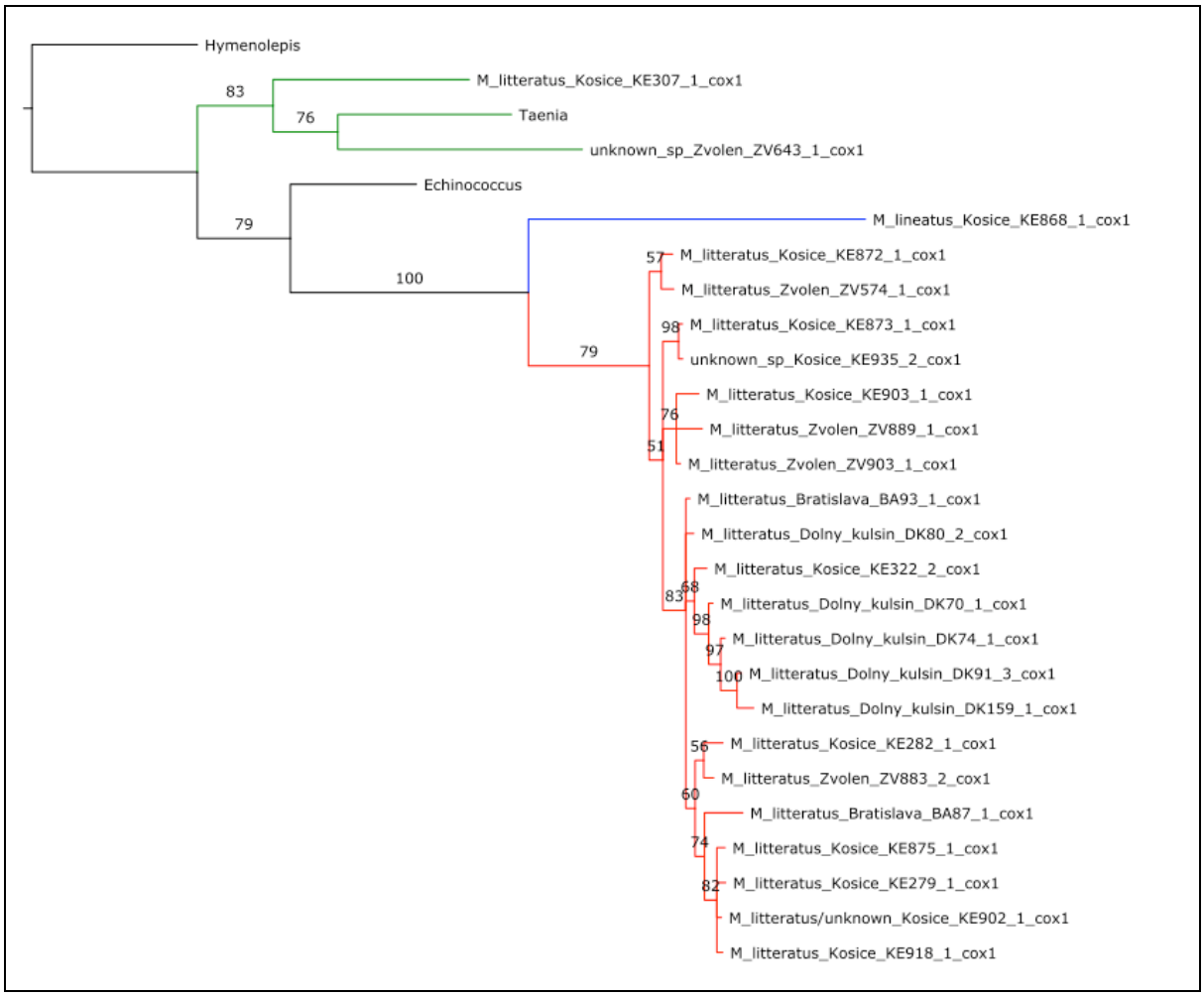

Figure C.8. Bayesian inference tree for the combined dataset cox1. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*

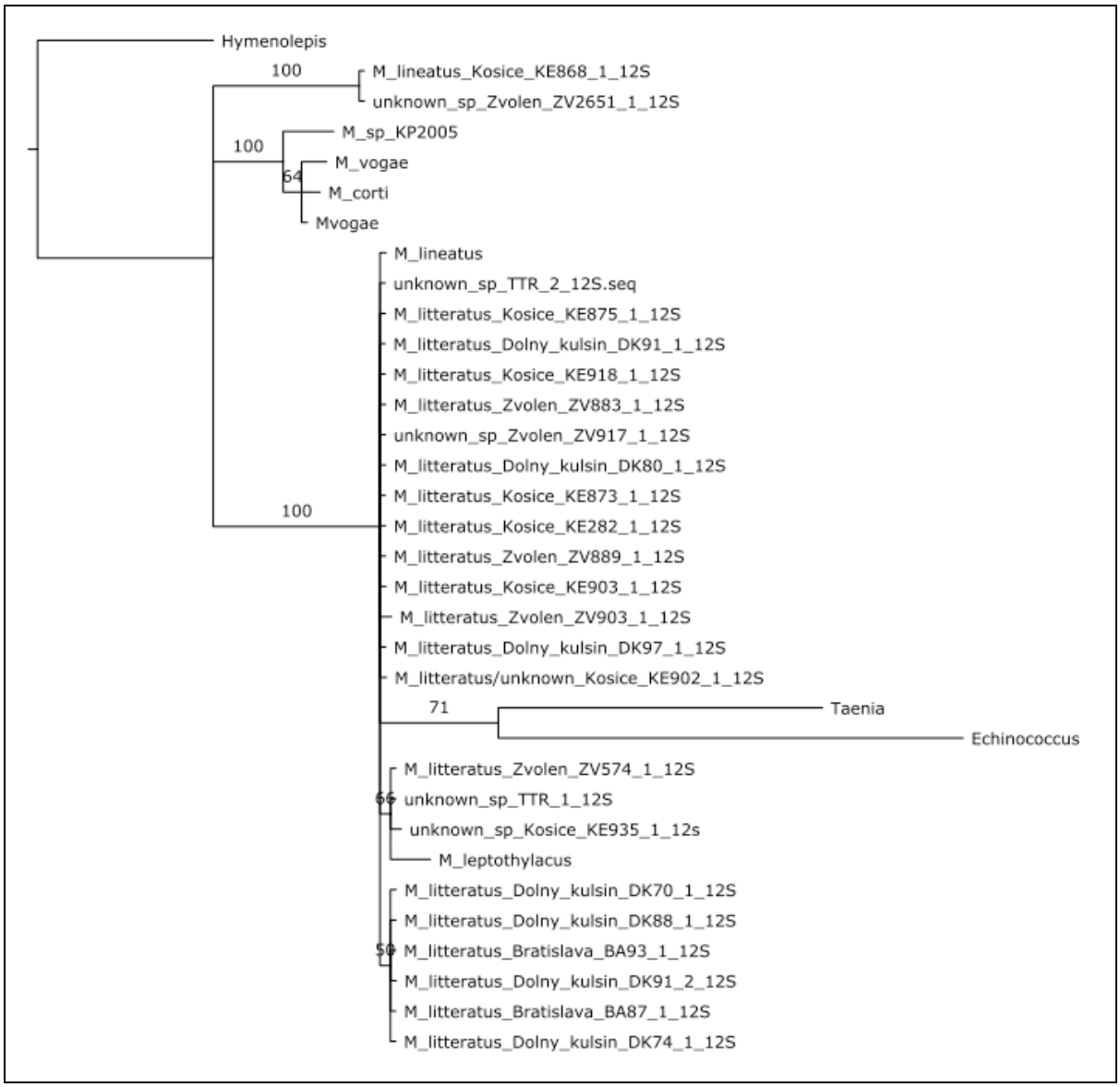

Figure C.9. Bayesian inference tree for the combined dataset 12S. *Numbers represent posterior-probability (clade-credibility) values (shown where > 50%).*