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Phylogeny and evolution of mycophagy in Drosophilidae
（キノコ食ショウジョウバエの系統関係と食性の進化）

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Abstract

Drosophilid flies feed on various substances such as fermenting fruits, mushrooms, flowers, leaves, and so on, and most of the fungus-feeding species are included in the *Zygothrica* genus group and the *quinaria* species group of the subgenus *Drosophila*. Despite numerous phylogenetic studies on the family Drosophilidae, the positions of these two groups are still equivocal. Corroborating the phylogenetic status of these two groups is important for understanding the evolution of mushroom feeding (mycophagy) in the family. In addition, mushrooms produce a defensive chemical compound, α-amanitin, to protect themselves from fungus-feeding insects. A key adaptation for these insects is the ability to cope with the toxicity of their hosts. Several fungus-feeding species from the subgenus *Drosophila*, especially the *quinaria* group, are well known to tolerate this fungal toxin. However, it is unknown whether or not species of the *Zygothrica* genus group and other related species have such tolerance. In these contexts, this study focuses on the phylogeny and evolution of mycophagy and the α-amanitin tolerance in Drosophilidae. The results are described in three chapters:

Chapter 1 focuses on the phylogeny and evolution of mycophagy in the *Zygothrica* genus group. Up to now, neither the phylogenetic position of the *Zygothrica* genus group nor the relationships within it have been resolved. Therefore, a phylogenetic analysis was conducted by assembling a DNA-sequence dataset of 24 genes from 92 species, including 42 species of the *Zygothrica* genus group mainly from the Palearctic and Oriental regions. In addition, the evolution of mycophagy in Drosophilidae is inferred by reconstructing ancestral food habits on the resulting phylogenetic tree. The resulting tree shows that the *Zygothrica* genus group is monophyletic and placed as the sister to the genus *Dichaetophora*, and that the clade *Zygothrica* genus group + *Dichaetophora* is sister to the *Siphlodora* + *Idiomyia*/*Scaptomyza* clade. Within the *Zygothrica* genus group, the genera *Mycodrosophila* and *Paramycodrosophila* are both recognized as monophyletic, while

neither the genus *Zygothrica* nor *Hirtodrosophila* is monophyletic. The ancestral reconstruction of food habits shows that fungus-feeding habits have been gained independently in two lineages. The most recent common ancestor (MRCA) of the subgenus *Drosophila* is estimated to have acquired mycophagy by expanding its ancestral feeding niche on fermenting fruits to decayed fungi, while the MRCA of the *Zygothrica* genus group may have shifted its niche from fruits to fungi as a specialist probably preferring fresh fruiting bodies.

Chapter 2 addresses a possible effect of nucleotide composition bias on the phylogenetic reconstruction of the *quinaria* species group of the subgenus *Drosophila*. In a recent phylogenetic study, the coalescent-based analysis of 43 genes has shown that the *quinaria* group is not monophyletic, while the concatenated-based analysis has shown the monophyly of it. A possible cause of incongruence may be the difference in GC content at the third codon sites among lineages, since such compositional bias is known to adversely affect the reconstruction of phylogenetic trees. In this chapter, species trees are constructed by the concatenation- and coalescent-based methods, excluding the 3rd codon sites of the nuclear genes from the dataset of the previous study. All the resulting trees represented the monophyly of the *quinaria* species group, suggesting that the nucleotide compositional bias should be taken into account in phylogenetic reconstruction.`

Chapter 3 focuses on the alpha-amanitin tolerance of Drosophilidae. Previous studies suggest the tightly correlated evolution of this tolerance with mycophagy. To test the hypothesis, the α-amanitin tolerance of adult flies is compared among 16 drosophilid species including mycophagous and non-mycophagous ones. The results are as follows: all the tested mycophagous species are tolerant of this lethal toxin, while the non-mycophagous species cannot survive on a diet containing α-amanitin with two exceptions, generalists *Drosophila hydei* and *Drosophila sternopleuralis*. This suggests that the α-amanitin tolerance may have evolved through multiple pathways of food-habit diversification in the family Drosophilidae.

General Introduction

Drosophilidae whose members are often called “fruit fly” is a speciose, cosmopolitan family, with approximately 4,500 species placed in 76 genera (Toda, 2021). In particular, this family includes the key model organism *Drosophila melanogaster* Meigen, 1830, used in biological sciences. Recent advancement in molecular research technology has opened a new era to enable not only *D. melanogaster* but also other species of this family to be studied in various disciplines of modern biology. This trend demands a reliable and well resolved phylogenetic tree of the Drosophilidae (O’Grady and DeSalle, 2018).

Throckmorton (1962, 1975) was the first to depict the family-wide phylogeny of drosophilids, based on internal morphology and ecology. He applied the concept of “radiation” to the major branches, leaving the relationships between species unsolved within each radiation. Grimaldi (1990a) conducted a cladistic analysis on 217 adult morphological characters in 120 species that represented most genera and subgenera of the family Drosophilidae. The resulting phylogeny differed substantially from the topology obtained by Throckmorton (1975). During the last 30 years, a great deal of effort has been made to clarify relationships of drosophilids using molecular phylogeny (e.g. Desalle, 1992; Kwiatowski et al., 1994; Kwiatowski and Ayala, 1999; Katoh et al., 2000, 2007; Tatarenkov et al., 2001; Remsen and O’Grady, 2002; Robe et al., 2005; Da Lage et al., 2007; O’Grady et al., 2008; van der Linde and Houle, 2008; van der Linde et al., 2010; Yassin, 2013; Russo et al., 2013, Finet et al, 2021). However, most of these studies were not comprehensive from the perspective of family-wide phylogeny, some focusing on and others biased toward particular groups. The exceptions are those of Yassin (2013) and Finet et al. (2021). Yassin (2013) reconstructed a molecular phylogenetic tree, based on seven nuclear and one mitochondrial genes (8,248 base pair, bp) from 190 species of 33 drosophilid genera, and mapped morphological characters on the resulting tree to propose a revised supra-generic classification of the Drosophilidae. More recently, Finet et al. (2021)

reconstructed a phylogeny based on 17 nuclear genes (14,961 bp) from 704 species of Drosophilidae. Even in these studies, however, some intergeneric relationships showed low statistical support. Thus, the drosophilid phylogeny is currently still far from being fully resolved, lacking enough molecular evidence for a number of groups.

An important case is of mycophagous species, whose phylogenetic positions are still equivocal. Drosophilid flies feed and/or breed on various substances such as fermenting fruits, sap fluxes, fungi, decaying plant materials, fresh plant leaves/stems, flowers, and cacti (Sturtevant, 1942; Carson, 1971; Throckmorton, 1975; Kimura et al., 1977; Shorrocks, 1982). Most of mycophagous species belong to the *Zygothrica* genus group or the *quinaria* species group of the subgenus *Drosophila* (Throckmorton, 1975; Kimura et al., 1977; Grimaldi, 1987; but see Gautério et al., 2020). Clarifying the phylogenetic status of these two groups is important for understanding the evolution of mycophagy in Drosophilidae.

To utilize mushrooms as food substance, consumers must cope with various toxins. Mushrooms produce a great variety of defense chemical compounds, such as α-amanitin and ibotenic acid, to protect themselves from mycophagous insects (Martin, 1979; Howe and Jander, 2008). The well-known α-amanitin, produced by some mushrooms of the genus *Amanita* (Wieland, 1968; Mas, 2005), binds to RNA polymerase II and inhibits transcriptional production of mRNA in eukaryotic cells (Lindell et al., 1970; Greenleaf, 1983). A key adaptation for mycophagous insects is acquirement of the ability to avoid the toxicity from their hosts (Ehrlich and Raven, 1964). It is well known that some mycophagous species belonging to the *quinaria* group have a tolerance to fungal toxins (Jaenike et al., 1983; Jaenike, 1985; Spicer and Jaenike, 1996; Tuno et al., 2007; Stump et al., 2011). However, it has never been studied whether or not species of other groups, especially the *Zygothrica* genus group, have such tolerance. Therefore, studies on the fungal toxin tolerance in the *Zygothrica* genus group and other drosophilids will shed more light on the evolution of mycophagy in the Drosophilidae.

This thesis firstly reconstructs the phylogeny of the *Zygothrica* genus group, and then the ancestral states of food habit are estimated on the resulting, well-resolved, phylogenetic tree. Secondly, the monophyly of the *quinaria* species group is discussed taking the effect of nucleotide composition bias on phylogenetic reconstruction into account. Lastly, tolerance to the fungal toxin α-amanitin is tested among mycophagous and non-mycophagous groups of drosophilids to shed light from this aspect on the evolution of mycophagy in the family Drosophilidae.

Chapter 1: Phylogeny and evolution of mycophagy in the *Zygothrica* genus group

(The content of this chapter was published in *Molecular Phylogenetics and Evolution* 163: 107257)

Introduction

The *Zygothrica* genus group (*sensu* Grimaldi, 1990a) comprises the mycophagous genera *Hirtodrosophila*, *Mycodrosophila*, *Paramycodrosophila*, and *Zygothrica*. The genera *Hirtodrosophila*, *Mycodrosophila* and *Paramycodrosophila* are distributed worldwide, while the genus *Zygothrica* is predominant in the Neotropical region (Toda, 2021).

Up to now, some studies have included this genus group in their molecular phylogenetic analyses, but with a small number of species (Da Lage et al., 2007; van der Linde et al., 2010; Russo et al., 2013; Yassin, 2013). Very recently, Gautério et al. (2020) has conducted a full-scale, phylogenetic analysis on the *Zygothrica* genus group based on four genes (*Amd*, *Ddc*, *COI* and *COII*) from 29 species of this group from the Neotropical region. However, the relationships inferred from this study were not so strongly supported by Bayesian posterior probabilities or bootstrap values on deep branches relevant to the *Zygothrica* genus group. Therefore, Gautério et al. (2020) refrained from concluding that their results were definitive, and pointed out the necessity of expanding taxon sampling to biogeographical regions other than the Neotropics and other putatively related genera/subgenera such as *Paramycodrosophila*, *Dichaetophora* and *Promycodrosophila*.

The *Zygothrica* genus group mostly comprises mycophagous specialists (Throckmorton, 1975; Kimura et al., 1977; Grimaldi, 1987; but see Gautério et al., 2020). According to the phylogenetic hypothesis by Throckmorton (1975), it is suggested that mycophagy has independently evolved on several lineages (‘radiations’) in the Drosophilidae (Kimura et al., 1977). This idea has long been accepted by drosophilid researchers (Kimura et al., 1977; Grimaldi, 1987, 1990; Powell, 1997), and supported in a recent molecular phylogenetic study by Gautério et al. (2020). However, this hypothesis has not yet been well established, since the ancestral reconstruction of character state is significantly affected by the basal tree topology and taxon sampling.

In the present study, the phylogeny was reconstructed to reveal relationships among the *Zygothrica* genus group, the probably related genus *Dichaetophora*, and three major clades of "*Drosophila*", *i.e.,* the subgenera *Sophophora*, *Drosophila*, and *Siphlodora* + *Idiomyia*/*Scaptomyza,* based on a multilocus dataset of 24 protein-coding genes. To compensate the biogeographic bias of taxon sampling in Gautério et al. (2020), as many species of the *Zygothrica* genus group from the Palearctic and Oriental regions as possible were included in the analysis. Then, the resulting phylogenetic tree is used for reconstructing ancestral states of food habit to infer the evolution of mycophagy in the Drosophilidae.

Materials and Methods

Taxon sampling

A total of 81 species were selected as ingroup taxa from non-*Sophophora* genera/subgenera of the tribe Drosophilini (*sensu* Yassin, 2013): 42 species (16 spp. of *Hirtodrosophila*, 13 spp. of *Mycodrosophila*, 11 spp. of *Zygothrica* and 2 spp. of *Paramycodrosophila*) from the *Zygothrica* genus group, 8 species from the genus *Dichaetophora*, 23 species from the subgenus *Drosophila*, and 8 species from the clade containing the subgenus *Siphlodora* (6 spp.), the genera *Idiomyia* (1 sp.) and *Scaptomyza* (1 sp.) (Tables 1 and 2). The specimens were field-collected with sweep net, obtained from the UC San Diego Drosophila Stock Center (DSSC) and Tokyo Metropolitan University (TMU), or kindly provided by Dr. Shigeyuki Koshikawa (Table 2). Based on the consistent result that, within the Drosophilini, the subgenus

Sophophora (+ the genus *Lordiphosa*) is sister to the ingroup taxa selected in this study (e.g., van der Linde et al., 2010; Gao et al., 2011; Yassin, 2013; Russo et al., 2013; Gautério et al., 2020), 11 species of *Sophophora* were selected as outgroup taxa (Tables 1 and 2).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from either fresh or alcohol-preserved specimens by the method of Boom et al. (1990) with some modifications. Polymerase chain reaction (PCR) amplifications were performed for 24 genes (22 nuclear and 2 mitochondrial genes) by using the primers listed in Table 5 (Izumitani et al., 2016; Satomura and Tamura, 2016; Finet et al., 2021; Katoh et al., 2021). Amplifications were carried out in 10 μl reaction volumes, each containing 1× ExTaq buffer (Takara Bio), 200 μM each dNTP, 0.5 μM each primer, 0.25U ExTaq polymerase (Takara Bio), and approximately 10 ng of genomic DNA.

PCR cycling conditions were 2 min at 94℃; 35 cycles of 30 s at 94℃, 1 min at annealing temperature and 90 s at 72℃; and 7 min at 72℃. Annealing temperatures were changed depending on the different primers (Table 5). PCR products were treated with Exonuclease I (Takara Bio) and Shrimp Alkaline Phosphatase (Takara Bio), and then the treated products were sequenced directly. Sequences were determined in both directions using a BigDye Terminator Sequencing Kit (Life Technologies) and an ABI 3130 or 3730 Genetic Analyzer, according to the manufacturer's protocols. The sequence data were edited by SeqMan software (Lasergene).

Of the 81 ingroup species, 52 species (32 spp. of the *Zygothrica* genus group and 20 spp. of the subgenus *Drosophila*) were newly sequenced for some of the 24 marker genes in the present study (Table 4). The determined sequence data are deposited in the DDBJ and their accession numbers are shown in Tables 1 and 2. In addition, all available Genbank data for the 24 genes in the 92 studied species used in the phylogenetic analysis are also presented in Tables 1 and 2. The total number of loci sequenced for each species and that of species analyzed for each gene are shown in Tables 1 and 2.

Phylogenetic analysis

Nucleotide sequences were aligned with the MUSCLE algorithm (Edgar, 2004) implemented in MEGAX (Kumar et al., 2018) with the default settings: gap opening = -400, gap extend = 0, max iterations = 8, clustering method = UPGMB, and minimum diagonal length = 24. Using the coding sequences of *D. melanogaster* as references, intron sequences were identified and then removed before the analysis. The alignments of the 24 genes were concatenated by using FASconCAT (Kück and Meusemann, 2010) to generate a supermatrix of 11,381 base pairs. Phylogenetic trees were constructed with concatenated sequences, using both the maximum likelihood (ML) and Bayesian inference (BI) methods. The partition scheme, including both codon and gene partitions, and their best fit substitution models were determined by using the PartitionFinder v2.1.1 (Lanfear et al., 2012, 2017) under the options Bayesian Information Criterion (BIC) “greedy” search and “models=all”.

RAxML-NG (Kozlov et al., 2019) was used to perform the ML analyses with a bootstrap (BS) analysis of 1000 replicates. Bayesian analyses were conducted by using MrBayes 3.2.7a (Ronquist et al., 2012). A Markov-Chain Monte-Carlo (MCMC) search was performed with four chains, each of which was run for 50 million generations for the concatenated dataset. Trees were sampled every 100 generations, and the first 25% of the samples were discarded as burn-in. TRACER 1.7.1 (Rambaut et al., 2018) was used to check whether the number of sampling generations and effective sample sizes (ESS) were large enough for reliable parameter estimates. A consensus of sampled trees was computed by using the “sumt” command with the “contype = allcompat” option, and the posterior probability (PP) for each interior branch was obtained to assess the robustness of inferred relationships. The consensus trees were visualized using FigTree v1.4.4 (Rambaut, 2018) or SeaView v5.0.2 (Gouy et al., 2010).

In order to evaluate the contribution of 24 genes to each clade in the phylogenetic tree, the partitioned likelihood support (PLS) was calculated by using nearest-neighbor-interchange (NNI) comparisons (Lee and Hugall, 2003).

In addition, based on the coalescent approach, a species tree was inferred using ASTRAL- III v5.6.1 (Zhang et al., 2018). First, a ML tree was constructed for each nuclear locus and one partition including two mtDNA genes. Then, a species tree based on 23 gene trees was constructed.

Ancestral state reconstruction for food habit

Food habits of the 92 species were divided into the following categories: (M) fungi, (F) fruits, (B) flowers, (T) tree sap, (L) leaves, and (?) unknown (Table 3), based on the literature (Sturtevant, 1921, 1942; Okada, 1956; Throckmorton, 1975; Kimura et al., 1977; Kimura, 1979; Shorrocks, 1982; Lacy, 1984; Toda et al., 1987; Kimura and Toda, 1989; Grimaldi, 1990b; Spicer and Jaenike, 1996; Toda and Kimura, 1997; Perlman and Jaenike, 2003; Prigent et al., 2003; Santos and Vilela, 2005; Takahashi et al., 2005; Prigent and Toda, 2006; Yorozuya, 2006, 2009; Kondo and Kimura, 2008; Markow and O’Grady, 2008; Gottschalk et al., 2009; Kadowaki, 2010; Stump et al., 2011; Morales-Hojas and Vieira, 2012; Valer et al., 2016; Machado et al., 2017; Mendes et al., 2017; Grimaldi, 2018; Santa-Brígida et al., 2019; Scott Chialvo et al., 2019; Valadão et al., 2019; Gautério et al., 2020). A number of species are specialists feeding on a single category of food, but some others use multiple types of food (Table 3). Food habits of the latter were treated as being multiple-state, without discriminating between primary (mainly used) and secondary (occasionally/subsidiarily used) food in the analysis. The possible ancestral states were reconstructed by using the Bayesian Binary MCMC (BBM) analysis implemented in RASP 4.2 (Yu et al., 2015, 2020). To take into account phylogenetic uncertainty, 10000 trees randomly selected from the post burn-in trees generated by MrBayes were used as input trees. The BBM analysis was then run on a consensus Bayesian tree. The MCMC chain was run for 5000 generations using 10 chains and sampled every 100 generations. An estimated F81 model without allowing null root distribution was used for the analysis.

Results

Phylogenetic position of the Zygothrica *genus group*

Figure 1 shows the Bayesian phylogenetic tree constructed under the partitioning scheme with the optimal substitution models (Table 6) for the concatenated dataset. The ML analysis generated a similar tree topology with only minor differences in the relationship between *M. poecilogastra* and *M. basalis* (Fig. 2), which had no influence on our conclusions. Five major clades were recognized with strong support: (1) the subgenus *Sophophora* (PP = 1.0, BS = 100); (2) the subgenus *Drosophila* (PP = 1.0, BS = 95); (3) the subgenus *Siphlodora* together with the genera *Idiomyia* and *Scaptomyza* (PP = 1.0, BS = 84); (4) the genus *Dichaetophora* (PP = 1.0, BS = 94); and (5) the *Zygothrica* genus group (PP = 1.00, BS = 94). The PLSs for the basal relationships including these major clades were summarized in Fig. 3. Except for the *Sophophora* clade and for the most basal clade of remaining species, the relationships were supported by PLSs from the majority of gene partitions.

On the other hand, the coalescent-based analysis by ASTRAL recovered a topology with weakly supported relationships among major clades (Fig. 4), although the normalized quartet score (NQS) was 0.747, indicating that the topology was less affected by incomplete lineage sorting (ILS). This may be due to uneven distribution of missing sequence data and/or less informative sites in each individual gene region (Springer and Gatesy, 2016; Blom et al., 2017; Simmons and Kessenich, 2020; Shen et al., 2021), as the topologies of individual gene trees were different from each other and these interior branches received low bootstrap supports (Fig. 5). Therefore, the following description is based on the phylogenetic trees generated from the BI and ML analyses of concatenated dataset.

Almost all the relationships between the major clades showed high PP (1.0) and BS (≥75) supports. Among these clades, the subgenus *Sophophora* was used as the outgroup in this study. Within the ingroup, the subgenus *Drosophila* was placed as the basal lineage. Then, its sister clade (PP = 1.0, BS = 98) branched into two lineages: one was comprised of the subgenus *Siphlodora* + *Idiomyia*/*Scaptomyza*; and the other of *Dichaetophora* + the *Zygothrica* genus group (PP = 1.0, BS = 75). Thus, the *Zygothrica* genus group was placed as the sister to the genus *Dichaetophora*, and this clade (the *Zygothrica* genus group + *Dichaetophora*) as the sister to the clade of *Siphlodora* + *Idiomyia*/*Scaptomyza*.

Phylogeny within the Zygothrica *genus group*

Within the *Zygothrica* genus group, the genera *Mycodrosophila* and *Paramycodrosophila* were each recognized as monophyletic with strong support (PP = 1.0, BS = 100 for both), while neither the genus *Zygothrica* nor *Hirtodrosophila* was monophyletic. Among the species of the genus *Zygothrica*, the Oriental and Australasian *Z. samoaensis* species group emerged as paraphyletic and did not show a close relationship to the Neotropical *Zygothrica* lineage. The four species of the *samoaensis* species group (*Z. aliunota*, Z. *flavofinira*, *Z. leptorostra*, and *Z. quintamaculata*) comprised, along with three species of *Hirtodrosophila* (*H. trilineata*, *H. trivittata*, and *H. sexvittata* belonging to the *trivittata* subgroup of the *quadrivittata* species group), a clade (PP = 1.0, BS = 88) basal within the *Zygothrica* genus group. Within this clade, the *samoaensis* subgroup (*Z. aliunota* and Z. *flavofinira*) was placed basally, but the *leptorostra* subgroup (*Z. leptorostra* and *Z. quintamaculata*) formed a clade (PP = 1.0, BS = 97) along with *H. trilineata* and *H. trivittata*. The sister clade of this branch was comprised of the remaining species (PP = 1.0, BS = 93). Within it, *H.*

makinoi (the *melanderi* group) and *H. histrioides* (the *confusa* subgroup of the *quadrivittata* group) were placed as basal branches, although their branching order was less resolved. The remaining species formed a clade (PP = 1.0, BS = 81), and it was divided into two subclades (PP = 1.0, BS = 99 and PP = 1.0, BS = 100, respectively). One of them was comprised of *Mycodrosophila* and four species of the *Hirtodrosophila quadrivittata* species group (PP = 1.0, BS = 100); of the latter, three species (*H. caputudis*, *H. fascipennis*, and *H. quadrivittata*) belonging to the *quadrivittata* subgroup formed a clade (PP = 1.0, BS = 98) sister to *H. alboralis* of the *trivittata* subgroup. The other subclade included seven species of *Hirtodrosophila* (five species of the *hirticornis* species group: *H. manonoensis*, *H. nokogiri*, *H. novicia*, *H. kangi*, and *H. pseudonokogiri*; and two ungrouped species: *H. apicohispida* and *H. pictiventris*), the Neotropical *Zygothrica* lineage (PP = 1.0, BS = 99), and *Paramycodrosophila*. However, relationships among these component species were less resolved.

Ancestral states of food habit

The ancestral food-habit states reconstructed by the BBM analysis were shown in Fig. 6. The most recent common ancestor (MRCA) of all the analyzed species (node 81) was estimated to be a fruit-feeder with the probability of 85.4%. This habit was inferred to have been kept in the MRCAs of the subgenus *Sophophora* (node 89) and of the ingroup taxa (node 58) with the probabilities of 97.4% and 47.0%, respectively. Fungus-feeding habit was estimated to have been acquired independently in two lineages of the ingroup taxa: the MRCA of the subgenus *Drosophila* (node 75) expanded the ancestral feeding niche on fruits to fungi as well, and the MRCA of the remaining species (node 50) shifted it from fruits to fungi, although the estimated probabilities of these ancestral states were not so high, 53.3% and 49.5%, respectively. Within the subgenus *Drosophila*, the feeding-niche specialization to fungi would have occurred in the MRCA of *D. histrio* and the *quinaria* species group (node 72, 84.5%).

On the other hand, in the sister lineage the fungus-feeding specialization was highly (98.8%) estimated in the MRCA of the *Zygothrica* genus group (node 35), but niche shifts to other food substances were estimated in the following lineages: to tree sap in the MRCA of the subgenus *Siphlodora* (node 54, 98.3%), and to plant leaves in the MRCA of *Scaptomyza*/*Idiomyia* (node 57, 97.8%).

Within the *Zygothrica* genus group, although most of the terminal species and their ancestors were fungus-feeding specialists, some species expanded their niches to other food substances (*H. alboralis*, *H. histrioides*, and *H. sexvittata*) and shifted to flower-feeding (*Z. vittimaculosa*) or fruit-feeding (*Z. orbitalis*).

Discussion

Phylogenetic position of the Zygothrica *genus group*

The phylogenetic position of the *Zygothrica* genus group in Drosophilidae has been controversial according to the previous studies. Throckmorton (1975) placed the genera *Hirtodrosophila*, *Zygothrica*, *Mycodrosophila*, and *Paramycodrosophila* of the current *Zygothrica* genus group, together with Hawaiian drosophilids (*Idiomyia*/*Scaptomyza*) and the genus *Nesiodrosophila*, a junior synonym of *Dichaetophora* (Hu and Toda, 2002), in the “Old World *Hirtodrosophila*” radiation, which branched off from the large “*immigrans-Hirtodrosophila*” radiation comprised mainly of taxa currently assigned to the subgenus *Drosophila* (Fig. 7A). Grimaldi (1990a) proposed a phylogenetic classification partly consistent with Throckmorton’s (1975) hypothesis. The *Zygothrica* genus group was placed closest to *Idiomyia*, and classified together as the *Hirtodrosophila* genus complex. However, the *Hirtodrosophila* genus complex was placed as the sister to the largest *Drosophila* genus complex comprised of the whole genus *Drosophila* and many other drosophiline genera including *Nesiodrosophila* and *Scaptomyza* (Fig. 7B).

Even some recent molecular phylogenetic studies have not attained to a consistent conclusion about the phylogenetic position of the *Zygothrica* genus group (e.g., Yassin, 2013; Russo et al., 2013; Gautério et al., 2020). Yassin (2013) showed that almost all the species analyzed for the *Zygothrica* genus group formed a clade together with *Dichaetophora* and some minor genera as the sister to a large clade including the subgenus *Drosophila*, “Hawaiian Drosophilidae”, and the subgenus *Siphlodora* (Fig. 7C). Yassin (2013) included another *Hirtodrosophila* species, *H. actinia*, in his analysis and inferred that it was placed as the sister to the genus *Lissocephala*. This relationship was reconfirmed by Fu et al. (2016), and when they established the new genus *Impatiophila* sister to *Lissocephala* within the tribe Colocasiomyini (*sensu* Yassin, 2013), they transferred this species along with two other *Hirtodrosophila* species, *H. limbicostata* and *H. yapingi*, to this new genus; but Yassin’s (2013) *H. actinia* was a misidentification of *Impatiophila pipa* (Fu et al., 2016). On the other hand, Russo et al. (2013) showed a different relationship, where the *Zygothrica* genus group emerged as polyphyletic: *H. sexvittata* and *H. trilineata* were sister to *Dichaetophora*, while the remaining *Hirtodrosophila* and *Mycodrosophila* species formed a clade sister to the subgenus *Drosophila* (Fig. 7D). However, the inferred relationship was less supported with PP or BS values in either of these studies. Most recently, Gautério et al. (2020) conducted molecular phylogenetic analyses using sequences of four genes with denser taxon sampling of the *Zygothrica* genus group from the Neotropical region, and inferred the monophyly of the *Zygothrica* genus group and its placement as the sister to the clade of *Siphlodora* + *Idiomyia/Scaptomyza* (Fig. 7E).

The present study conducted phylogenetic analyses based on the sequences of 24 genes with the increased taxon sampling of the *Zygothrica* genus group and *Dichaetophora* from the Palearctic and Oriental regions. Both trees resulting from the BI and ML analyses showed that the *Zygothrica* genus group was monophyletic and placed as the sister to *Dichaetophora*, and that this clade of the *Zygothrica* genus group + *Dichaetophora* was regarded as sister to the *Siphlodora* + *Idiomyia*/*Scaptomyza* clade (Fig. 7F). This topology is partly consistent with previous hypotheses. The monophyly of the *Zygothrica* genus group was also supported in Grimaldi (1990a). The close relationship between the *Zygothrica* genus group and *Dichaetophora* was suggested by Throckmorton (1975), Yassin (2013), and partly by Russo et al. (2013) as well. However, none of these previous hypotheses is compatible in its topology as a whole with the tree of the present study, specifically with respect to relationships of the *Zygothrica* genus group to two major lineages of *Drosophila*, *i.e.*, the subgenera *Siphlodora* (the so-called, traditional "*virilis-repleta*" radiation) and *Drosophila* (the "*immigrans-tripunctata*" radiation). Grimaldi (1990a) and Yassin (2013) suggested the basal position of the *Zygothrica* genus group to these two lineages, while Throckmorton (1975) and Russo et al. (2013) suggested its close relationship to the subgenus *Drosophila*. The result of the present study, *i.e.*, the close relationship of the *Zygothrica* genus group with the subgenus *Siphlodora*, was also supported in Gautério et al. (2020). The topology of their tree was wholly compatible with that in this study, except for the lack of *Dichaetophora* from taxon sampling in their study. However, the relationships inferred from their analyses were significantly supported only in terms of PP value, while the topology of the present tree was strongly supported with both PP and BS values (Fig. 7). Thus, the phylogeny reconstructed in the present study provides a well-resolved and reliable hypothesis for the phylogenetic position of the *Zygothrica* genus group, probably due to the increased taxon sampling and the large sequence dataset of 24 genes.

Phylogeny within the Zygothrica *genus group*

The current classification of genera, subgenera, species groups and/or subgroups of the *Zygothrica* genus group is shown with different colors on the phylogenetic tree of Fig. 1. The present study has inferred the monophyly of the genus *Paramycodrosophila*, and confirmed that of the genus *Mycodrosophila*. Based on the morphological homogeneity, the monophyly of *Mycodrosophila* has been previously suggested in several studies (Wheeler and Takada, 1963; Okada, 1986; McEvey and

Polak, 2005), and inferred from a few molecular phylogenetic studies (Yassin, 2013; Russo et al., 2013; Gautério et al., 2020), but always with a limited taxon sampling of two or three species. The present study provides robust molecular evidence for the monophyly of this genus with a substantial taxon sampling of 13 species including one species (*M. separata*) of the subgenus *Promycodrosophila*. However, the phylogenetic relationships within *Mycodrosophila*, especially the status of the subgenus *Promycodrosophila*, have remained to be challenged in future studies under a broader taxon sampling as suggested by Gautério et al. (2020). Up to now, a total of 129 *Mycodrosophila* species (74 spp. assigned to the subgenus *Mycodrosophila*, 17 spp. to *Promycodrosophila*, but the remaining 38 spp. unassigned to either) have been recorded from all biogeographic regions (except Antarctica) of the world (Toda, 2021). Some representatives of these subgeneric groups from each biogeographic region should be included in future phylogenetic analyses. For the genus *Paramycodrosophila* currently with 16 species known from the world (Toda, 2021), the taxon sampling should be expanded as well in future studies.

On the contrary, the genera *Hirtodrosophila* and *Zygothrica* were inferred to be non-monophyletic. *Hirtodrosophila* is a speciose genus, currently with 173 known species (Toda, 2021). In addition, a large number of undescribed species have been found from the Old and New World tropics (Machado et al. 2017; O’Grady and DeSalle, 2018; Junges et al. 2019). Its polyphyletic nature has been repeatedly pointed out in previous studies (Da Lage et al., 2007; van der Linde et al., 2010; Yassin, 2013; Russo et al., 2013), although the taxon sampling from this genus was quite limited. Recently, Gautério et al. (2020) has suggested that *Hirtodrosophila* is paraphyletic with respect to *Zygothrica*, based on the increased taxon sampling of Neotropical species from these genera (6 spp. and 19 spp., respectively). The present study also increased the taxon sampling of *Hirtodrosophila* from the Old World (15 spp. from the Palearctic, Oriental, and Australasian regions), and showed that those species were scattered among different lineages across the *Zygothrica* genus group. For example, three species (*H. trilineata*, *H. trivittata*, and *H. sexvittata*) of the *trivittata* subgroup formed a clade together with four species of the *Zygothrica samoaensis* group, while *H. alboralis* of the *trivittata* subgroup formed another clade together with the three species of the *quadrivittata* subgroup. And, five species of the *hirticornis* group were paraphyletic to a clade including the Neotropical *Zygothrica* lineage, the genus *Paramycodrosophila*, and two ungrouped species of *Hirtodrosophila* (*H. apicohispida* and *H. pictiventris*). Thus, *Hirtodrosophila* was regarded as non-monophyletic at either level of the whole genus, species group or subgroup. Therefore, revision of the classification system is necessary for both of the Old and New World species of this genus (O'Grady and DeSalle, 2018).

In this study, *Zygothrica* was also regarded as non-monophyletic, because the Oriental and Australasian *Z. samoaensis* group emerged as paraphyletic and did not show a close relationship to the Neotropical *Zygothrica* lineage. The *samoaensis* group formed the basal clade, along with the three species of the *Hirtodrosophila trivittata* subgroup, within the *Zygothrica* genus group, while the Neotropical *Zygothrica* was inferred to be monophyletic within another clade including the *Hirtodrosophila hirticornis* group and *Paramycodrosophila*. However, Grimaldi (1987) regarded the genus *Zygothrica* as monophyletic, based on a cladistic analysis of morphological characteristics: the *samoaensis* group was placed as one of the five polytomous earliest-branching lineages of *Zygothrica*. Gautério et al. (2020) indicated that the Neotropical *Zygothrica* is not monophyletic with respect to *Hirtodrosophila*. However, Gautério et al. (2020) suggested that three nested *Hirtodrosophila* species are to be assigned to *Zygothrica* because of sharing a synapomorphy with all *Zygothrica* species, with implication of the monophyly of the Neotropical *Zygothrica*. Although the present study is insufficient in taxon sampling from the Neotropical *Zygothrica* for comparison with these previous studies, some inferred relationships are concordant with Grimaldi's (1987) classification system based on a morphological, cladistic analysis: *Z. vittimaculosa*, *Z. vitticlara* and *Z. sectipoeyi* forming a clade (but with low support values) belong to the "*vittimaculosa* species-group" (Clade 1.2.2.2.B; Grimaldi, 1987), and *Z. nigropleura* and *Z. microeristes* forming another clade (PP =

1.0, BS = 100) belong to the "*aldrichii* species-subgroup" (Clade 1.2.1.2.B.2; Grimaldi, 1987).

Evolution of mycophagy

The idea that mycophagy evolved independently in different lineages of the family Drosophilidae has long been accepted by drosophilid researchers (Sturtevant, 1942; Throckmorton, 1975; Kimura et al., 1977;Grimaldi, 1987, 1990a; Powell, 1997), and supported in a recent molecular phylogenetic study by Gautério et al. (2020). The ancestral state reconstruction analysis of food habits in the present study also inferred independent evolution of mycophagy in two lineages of the studied ingroup species. The MRCAs of the *Zygothrica* genus group and of *D. histrio* and the *quinaria* species group were estimated to be fungus-feeding specialists with high probability. However, some differences in host selection were observed between these two lineages of fungus-feeders: members of the *Zygothrica* genus group preferred the fresh fruiting bodies of fungi such as Pleurotaceae and Polyporaceae (bracket fungi), although the Neotropical *Zygothrica* species were more labile, while fungus-feeders of the subgenus *Drosophila* preferred the decayed fungi (Kimura, 1976, 1980; Grimaldi, 1987; Kimura and Toda, 1989; Toda and Kimura, 1997; Valer et al., 2016; Gautério et al., 2020). The preference of the latter mycophagous lineage would have come from the MRCA of the subgenus *Drosophila*, which was estimated to have acquired mycophagy by expanding its ancestral feeding niche depending on fermenting fruits to include decayed fungi. Thus, these differences in fungus-host preference would reflect the independent acquisition of mycophagy between these two lineages.

Chapter 2: Effect of nucleotide composition bias on the phylogenetic reconstruction of the *quinaria* species group in the subgenus *Drosophila*

Introduction

The subgenus *Drosophila*, which has been revised to represent the *immigrans-tripunctata* radiation (*sensu* Throckmorton, 1975) by Yassin (2013), includes 21 species groups (Toda, 2021). This lineage has been intensively studied from various aspects in relation to mycophagy, such as the toxin tolerance (Jaenike et al., 1983; Jaenike, 1985; Spicer and Jaenike, 1996; Tuno et al., 2007; Stump et al., 2011), parasite prevalence and resistance (Perlman and Jaenike, 2003; Perlman et al., 2003) and so on. To better understand the evolution in these aspects, it is critical to establish a robust phylogenetic framework for this lineage. Up to now, a number of studies have challenged to reconstruct the phylogeny of this lineage (Perlman et al., 2003; Hatadani et al., 2009; Morales-Hojas and Vieira, 2012; Izumitani et al., 2016; Scott Chialvo et al., 2019; Finet et al., 2021). However, the inferred relationships among and within species groups are still controversial.

An important species group of this lineage is the *quinaria* group comprised of 31 species (Toda, 2021). Most of previous phylogenetic studies, based on concatenated sequence datasets of multiple genes, have resulted in the consistent inference that the *quinaria* group is monophyletic and that the group is further divided into two subclades (Perlman et al., 2003; Hatadani et al., 2009; Morales-Hojas and Vieira, 2012; Izumitani et al., 2016; Finet et al., 2021). However, Scott Chialvo et al. (2019) recently questioned the monophyly of the *quinaria* group, comparing species trees inferred from different approaches: ML and BI analyses of a concatenated sequence dataset and ASTRAL coalescent-based model analysis of gene trees of 43 loci.

The coalescent-based approach is used to reconstruct the species phylogeny by considering the potential incomplete lineage sorting (ILS) in individual gene trees (Kingman, 1982; Rannala and Yang, 2003; Liu and Pearl, 2007; Edwards, 2009; Shen et al., 2021). This approach relies on inference of individual gene trees, and therefore the final tree topology could be affected by the errors from individual gene trees. These errors can be caused by the uneven distribution of missing sequence data and/or less informative sites (Springer and Gatesy, 2016; Blom et al., 2017; Simmons and Kessenich, 2020; Shen et al., 2021). In addition, it should be noted that a probable source of gene tree estimation error could come from variation in the nucleotide composition among taxa. Tarrio et al. (2001) pointed out large compositional variation in the GC content, especially at the third codon site, among species of the family Drosophilidae. The heterogeneous GC contents can seriously mislead phylogenetic reconstruction as the sequences of similar base compositions could become mistakenly clustered (Galtier and Gouy, 1995; Tarrio et al., 2001, Da Lage et al., 2007).

Given the above, to account for effects of the compositional heterogeneity among taxa, the present study excludes the 3rd codon sites, where the significant heterogeneity is detected, from the dataset of Scott Chialvo et al. (2019) and analyzes the modified dataset by the concatenation- and coalescent-based methods. As a result, the present study obviously improves the accuracy of phylogeny reconstruction and supports the monophyly of the *quinaria* group. Thus, the nucleotide composition bias seems responsible for the non-monophyly of the *quinaria* group inferred from the coalescent-based analysis in Scott Chialvo et al. (2019).

Materials and Methods

DNA sequences

The dataset of 43 genes (40 nuclear and 3 mitochondrial genes) from Scott Chialvo et al. (2019) was re-aligned with the MUSCLE algorithm (Edgar, 2004) implemented in MEGAX (Kumar et al., 2018). Gaps and incompletely determined columns were deleted and a locus (*per*) with bad alignment was removed before the analysis.

Base composition

The GC contents (percentage) of the 1st, 2nd and 3rd codon sites of each gene were counted in MEGAX (Kumar et al., 2018). The nucleotide composition homogeneity between taxa was tested by the chi-square (X^2) test implemented in PAUP* 4.0b10 (Swofford, 2002)and the relative compositional variability (RCV) proposed by Phillips and Penny (2003).

Phylogenetic analyses

Firstly to assess effects of the uneven distribution of missing sequence data on the species phylogeny, datasets of 25 genes (22 nuclear and 3 mitochondrial genes), each of which covered more than 90% of the 27 species studied by Scott Chialvo et al. (2019), were selected and analyzed by the coalescent-based approach. The coalescent-based model implemented in ASTRAL-III v5.6.1 (Zhang et al., 2018) was used. First, using RAxML-NG (Kozlov et al., 2019), the gene tree along with 1000 bootstrap replicates was obtained for each nuclear locus. The three mtDNA loci were concatenated and analyzed as a single locus. Then, a species tree was reconstructed from the gene trees of the 22 nuclear loci plus the mtDNA tree.

Secondly to test effects of compositional heterogeneity, for the entire dataset, the 3rd codon positions were excluded from 39 nuclear genes where the significant compositional heterogeneity was detected, although those of mtDNA genes were remained because for the nucleotide composition of three mtDNA genes, all species are shown GC-poor (Table 8). The sequence dataset of the 39 modified nuclear genes and 3 unmodified mitochondrial ones was subjected to the species-tree reconstruction analyses. ASTRAL-III v5.6.1 (Zhang et al., 2018) was used to estimate the species tree based on the coalescent model. First, the gene tree was obtained for each nuclear locus with only the 1st and 2nd codon positions using RAxML-NG (Kozlov et al., 2019). The three mtDNA loci were concatenated and analyzed as a single locus. Then, the species tree was reconstructed from the individual gene trees of the 39 nuclear loci and the mtDNA tree. In addition to the coalescent-based approach, a concatenation-based ML analysis was performed. The alignments of the 39 nuclear and 3 mitochondrial genes were concatenated into a single dataset using FASconCAT (Kück and Meusemann, 2010). The partition scheme including both codon and gene partitions and their best fit substitution models were determined by using the PartitionFinder v2.1.1 (Lanfear et al., 2012, 2017) under the options Bayesian Information Criterion (BIC) "greedy" search and "models=all". The ML tree was then constructed along with a bootstrap (BS) analysis of 1000 replicates using RAxML-NG (Kozlov et al., 2019).

The resulting trees were visualized using FigTree v1.4.4 (Rambaut, 2018) or SeaView v5.0.2 (Gouy et al., 2010), and were rooted with *D. immigrans* as well as in Scott Chialvo et al. (2019).

Results

In order to facilitate comparison with the results of Scott Chialvo et al. (2019), the same labels referring to major clades are used in this study as well (see Tables 7 and 8), and the resulting trees are shown alongside the corresponding ones of Scott Chialvo et al. (2019).

Nucleotide compositional heterogeneity

The X^2 test detected the significant heterogeneity of GC content ($P < 0.05$) at the 3rd codon positions of 8 nuclear genes (Table 9). In addition, RCV detected the heterogeneity of GC content (RCV > 0.05: Phillips and Penny, 2003; Phillips et al., 2021) at the 3rd codon positions of all the nuclear genes except *kl3*, the 1st and 2rd codons of *amy*, and the 1st codon of *desatII* (Table 9).

Phylogeny of the quinaria *species group*

Fig. 8A shows the species tree inferred by the coalescent-based method with 25 genes (22 nuclear and 3 mitochondrial genes), each of which exceeds 90% in the taxon coverage. The tree did not support the monophyly of the *quinaria* group, but its normalized quartet score (NQS) was 0.816, indicating that this topology was less affected by incomplete lineage sorting (ILS). The NQS represents the proportion of quartets in the input gene trees that are congruent with the given species tree, and thus serves as a measure of the presence of ILS (Sayyari and Mirarab, 2016; Maiet al., 2017; Zhang et al., 2018). Comparing with the "Fig. 1C" in Scott Chialvo et al. (2019) (Fig. 8B), the similar topology were recovered in the present study: Clade A (BS = 100) comprised of the *tripunctata* and *cardini* species groups, Clade B (BS = 100) of the *bizonata* and *testacea* species groups, and two Clades C1 (BS = 100) and C2 (BS = 100) of the *quinaria* species group. There is only minor difference in branch support value for these major clades between the inferred tree and the tree ("Fig. 1C") of Scott Chialvo et al. (2019). Therefore, the number of species analyzed for each gene has less influence on the monophyly of the *quinaria* group in the study of Scott Chialvo et al. (2019).

Fig. 9A shows the species tree reconstructed by the coalescent-based approach with the modified dataset, where the *per* gene and the 3rd codon sites of the remaining 39 nuclear genes are excluded from the original dataset of Scott Chialvo et al. (2019). Its NQS is 0.631, indicating that this topology was less affected by the effect of ILS (Sayyari and Mirarab, 2016; Maiet al., 2017; Zhang et al., 2018). Comparing with the "Fig. 1C" in Scott Chialvo et al. (2019), the same four major clades with the same component species were recovered in the present study: Clade A (BS = 91) comprised of the *tripunctata* and *cardini* species groups, Clade B (BS = 100) of the *bizonata* and *testacea* species groups, and two Clades C1 (BS = 100) and C2 (BS = 83) of the *quinaria* species group. However, Fig. 9A shows that the sister relationship between Clades C1 and C2 is highly supported (BS = 96), representing the monophyly of the *quinaria* group, while the *quinaria* group was paraphyletic to the Clade A + B in the "Fig. 1C" in Scott Chialvo et al. (2019) (Fig. 9B).

On the other hand, the ML topologies constructed by the concatenation-based analysis were almost identical, regardless of exclusion (Fig. 10A) or inclusion (Fig. 10B) of nucleotides at the third codon positons, representing the relationship among the four major clades as follows: (Clade A, (Clade B, (Clade C1, C2))).

Discussion

Concatenation- and coalescent-based methods sometimes result in incongruent topologies, but the causes are difficult to determine (Shen et al., 2021). The coalescent-based approach is used to reconstruct the species phylogeny while accounting for the potential ILS in individual gene trees (Kingman, 1982; Rannala and Yang, 2003; Liu and Pearl, 2007; Edwards, 2009; Shen et al., 2021). The major weakness of this approach is that the tree topology can be affected by errors of individual gene trees. Such errors can be caused by the uneven distribution of missing sequence data and/or less informative sites in each individual gene region (Springer and Gatesy, 2016; Blom et al., 2017; Simmons and Kessenich, 2020; Shen et al., 2021). Thus, the coalescent-based method strongly relies on the inference of individual gene trees, and has a higher requirement for the taxon coverage analyzed for each gene and the accuracy of individual gene trees.

Another notable factor affecting the accuracy of phylogenetic reconstruction is the base composition heterogeneity. When estimating phylogenetic trees, it is generally assumed that all the nucleotides are equally used and that the GC content is expected to be around 50%. However, this assumption is sometimes violated when the homologous genome region is compared among different lineages. Some phylogenetic studies have suggested that the variation of GC content among organisms could have adverse effects on the reconstruction of evolutionary history (Mooers and Holmes, 2000; Tarrio et al., 2001), because even the unrelated species with similar GC contents tend to be clustered with each other (Mooers and Holmes, 2000; Ishikawa et al., 2012).

The composition heterogeneity sometimes could give wrong phylogenetic signals in studies of the drosophilid phylogeny. It is known that large compositional variation exists among species of Drosophilidae, especially in the third codon positions (Rodriguez-Trelles et al., 1999, 2000a, 2000b; Tarrio et al., 2001). For example, within the subgenus *Sophophora*, the *melanogaster* and *obscura* species groups have high GC contents, while the *willistoni* species group is GC-poor. Due to this heterogeneity, the *willistoni* group was clustered with other genera with low GC contents in some previous studies (Tarrio et al., 2001; Da Lage et al., 2007).

In the present study, when the nucleotide sites showing compositional heterogeneity was removed from the sequence dataset, both concatenation- and coalescent-based approaches recovered the monophyly of the *quinaria* species group with high branch supports. This implies that the base compositional bias could be responsible for the non-monophyly of the *quinaria* group in the species tree reconstructed by the coalescent-based analysis in Scott Chialvo et al. (2019), and that the concatenation-based approach seems less affected by the nucleotide compositional heterogeneity.

Apart from excluding codon sites heterogeneous in nucleotide composition from protein-coding gene sequences, there are other methods to reduce the compositional bias, such as RY-coding of purines (A and G) as R and pyrimidines (C and T) as Y (Phillips and Penny, 2003; Ishikawa et al., 2012; Phillips et al., 2021) and non-homogeneous maximum likelihood (NHML) method to improve the ML inference (Galtier and Gouy, 1998). Although Gao et al. (2011) and Phillips et al. (2021) pointed out limited success of these methods in some cases, it would be worthy to compare all possible methods for solving the issue of nucleotide compositional bias in further studies.

Chapter 3: Alpha-amanitin tolerance of adult flies of Drosophilidae

Introduction

The family Drosophilidae is highly diverse in various traits of the morphology, physiology, behavior, and ecology. Evolutionary changes in those traits have allowed drosophilid flies to become distributed world-wide, inhabit various environments, exploit various food resources, or specialize to particular niches. So, the Drosophilidae is one of the most attractive model groups for evolutionary genetic studies on adaptive radiations.

Drosophilid flies use a wide range of resources including fruits, sap, leaves/stems, flowers, cacti, and fungi (Sturtevant, 1942; Carson, 1971; Throckmorton, 1975; Kimura et al., 1977; Shorrocks, 1982). In evolutionary processes of these food habits, various traits of hosts and consumers have been acquired through interactions between them. As plants produce a great variety of defense chemical compounds to protect themselves from herbivores, mushrooms have various toxins against fungivores. Alpha-amanitin is one of such toxins produced by *Amanita* mushrooms (Wieland 1968; Mas, 2005). This cyclopeptide compound is toxic to most multi-cellular organisms by binding to RNA polymerase II (RNAP II) and thereby inhibiting RNA transcription, which leads to cell death (Lindell et al., 1970; Greenleaf, 1983). However, some mycophagous *Drosophila* species have evolved to be tolerant of this mycotoxin. It is suggested that this ability facilitates such *Drosophila* species by allowing their larvae to grow under less competitive condition on ephemeral food resource and preventing the infection of parasitic nematodes (Grimaldi and Jaenike, 1984; Jaenike, 1985; Jaenike and Perlman, 2002).

Based on observations that many adult flies of some *Drosophila* species emerged from poisonous *Amanita* mushrooms (Jaenike, 1978a,b; Jaenike and Selander, 1979), Jaenike et al. (1983, 1985) conducted an experiment for the first time to quantitatively compare the α-amanitin tolerance among mycophagous and non-mycophagous

Drosophila species, and found that mycophagous species of the *quinaria*, *testacea* and *tripunctata* species groups tolerated a high concentration (0.05 mg/ml) of α-amanitin, while none of frugivorous species survived. Following this experiment, several studies have challenged this problem by expanding tested taxa. Up to now, 17 mycophagous species all belonging to the subgenus *Drosophila* have proved to be tolerant of 0.05 mg/ml α-amanitin, but this concentration was lethal for all non-mycophagous species tested (Jaenike et al., 1983; Jaenike, 1985; Spicer and Jaenike, 1996; Tuno et al., 2007; Stump et al., 2011).

It is hypothesized that mycophagy has evolved independently on separate lineages of Drosohilidae (Sturtevant, 1942; Throckmorton, 1975; Kimura et al., 1977;Grimaldi, 1987, 1990a; Powell, 1997). Chapter 1 reconfirmed this and mentioned some differences in host selection between two lineages: mycophagous species of the subgenus *Drosophila* preferred decayed fungi, while members of the *Zygothrica* genus group exhibited a tendency to prefer fresh fruiting bodies of fungi such as Pleurotaceae and Polyporaceae (Kimura, 1976, 1980; Grimaldi, 1987; Kimura and Toda, 1989; Toda and Kimura, 1997; Valeret al., 2016; Gautério et al., 2020). In contrast to rich evidence for the α-amanitin tolerance of the former lineage, it has never been studied whether species of the *Zygothrica* genus group are tolerant of α-amanitin or not. And, all the previous studies tested the tolerance of pre-imaginal stages (egg to pupa) but never of adult flies. In the present study, therefore, effects of α-amanitin on adult survival are compared among mycophagous species of the subgenus *Drosophila* and the genus *Hirtodrosophila* (one of the genera comprising the *Zygothrica* genus group) and non-mycophagous species of the genus *Drosophila*.

Materials and Methods

Flies

The α-amanitin tolerance of adult flies was assessed for the following groups of drosophilids: (i) mycophagous species of the subgenus *Drosophila* — *D.* (*D.*) *brachynephros*, *D.* (*D.*) *unispina*, *D.* (*D.*) *orientacea*, *D.* (*D.*) *cardini*, *D.* (*D.*) *histro*, and *D.* (*D.*) *multispina*; (ii) mycophagous species of the genus *Hirtodrosophila* — *H. trilineata*, *H. trivittata*, *H. sexvittata*, and *H. histrioides*; and (iii) non-mycophagous species of the genus *Drosophila* — *D.* (*D.*) *deflecta*, *D.* (*D.*) *palustris*, *D.* (*D.*) *sternopleuralis*, *D.* (*Siphlodora*) *hydei*, and *D.* (*Sophophora*) *melanogaster*. Laboratory stocks from Tokyo Metropolitan University (TMU) or kindly provided by Dr. Shigeyuki Koshikawa and Mr. Hiroyuki F. Izumitani were used for *D. cardini*, *D. multispina*, *D. deflecta*, *D. palustris*, *D. sternopleuralis*, *D. hydei*, and *D. melanogaster* (strain Canton-S). For the remaining species, isofemale lines were newly established from females collected by sweeping or aspirating from wild fungal fruiting-bodies in Sapporo, northern Japan. The non-mycophagous species were reared on standard medium containing dry yeast, malt powder, cornmeal, sugar, agar, and propionic acid as a preservative, and the mycophagous species were cultured by adding some pieces of fresh mushroom (*Agaricus bisporus*) to the standard medium. Before the test, all the flies were maintained in the laboratory for several generations.

Experiments of α-amanitin tolerance

The experimental design was the same as that of Jaenike (1985), except for the tested developmental stages: Jaenike (1985) examined the egg-to-pupa survival (i.e. larval development), while the present study examined the adult survival. Five experimental flies including both female and male individuals of each species were put into a vial (20 × 75 mm) containing experimental (α-amanitin+) or control (α-amanitin–) diet, and their survival was checked everyday for two weeks. The control diet was made of 0.25 g Formula 4-24 instant *Drosophila* medium and 1.0 ml deionized water (D.W.), and the experimental diet of 0.25 g instant *Drosophila* medium and 1.0 ml α-amanitin solution in the concentration of 0.05mg/ml. This concentration was lethal for non-mycophagous species but not for mycophagous ones in the previous experiments by Jaenike et al. (1983) and Jaenike (1985). In addition to this α-amanitin concentration, lower ones of 0.005, 0.01 and 0.025 mg/ml were also tested for α-amanitin susceptible species. Each treatment was replicated five times. The effect of α-amanitin on the survival was analyzed using Analysis of Variance (ANOVA) in R 4.1.2 (R Core Team, 2021).

Results

The survival rates (the number of individuals having survived after two weeks) were compared between the experimental (α-amanitin+) and control (α-amanitin–) treatments for each species (Figs 11, 12 and Table 11). The significant differences were detected in three non-mycophagous species. *D. melanogaster* absolutely survived on the control diet but died on the experimental diet. In *D. deflecta* and *D. palustris*, although neither the survival nor the mortality was absolute, the survival rate significantly reduced on the experimental diet. However, even these susceptible species could survive rather well on diets with α-amanitin in low (≤0.01 mg/ml) concentrations. The remainder species including all the fungivores (*D. brachynephros*, *D. unispina*, *D. orientacea*, *D. cardini*, *D. histro*, *D. multispina*, *H. trilineata*, *H. trivittata*, *H. sexvittata,* and *H. histrioides*) and two non-fungivores (*D. hydei* and *D. sternopleuralis*) survived almost wholly on both diets.

Discussion

The present study reconfirmed the results of previous studies (Jaenike et al., 1983; Jaenike, 1985; Spicer and Jaenike, 1996; Stump et al., 2011); that is, the α-amanitin tolerance is quite strongly associated with mycophagy. Despite of the difference in the tested developmental stage, i.e. adult *vs*. larva, five commonly studied species exhibited consistent natures between the present and previous studies: two mycophagous species, *D. brachynephros* and *D. cardini*, were tolerant, and three non-mycophagous ones, *D. melanogaster*, *D. deflecta* and *D. palustris*, were susceptible (Table 12). In addition, the present study newly confirmed the α-amanitin tolerance in eight mycophagous species (4 spp. of the subgenus *Drosophila* and 4 spp. of the genus *Hirtodrosophila*; Table 12). The most noticeable new finding of the present study is that two non-mycophagous species, generalists *D. hydei* and *D. sternopleuralis*, have the α-amanitin tolerance. The food habits and α-amanitin tolerance/susceptibility of the studied species are mapped on a hypothetical phylogeny (Fig. 13) inferred from Chapter 1. The α-amanitin tolerance is observed in two large mycophagous lineages of the subgenus *Drosophila* and the *Zygothrica* genus group, which are different from each other in fungal host selection, and further a few non-mycophagous species as well. This suggests that the α-amanitin tolerance has been acquired more or less independently from the food-habit evolution. However, it is difficult to estimate evolutionary trajectories of α-amanitin tolerance and food habits from this tree, due to its limited taxon-sampling.

It should be noted that the present study examined the tolerance or susceptibility of adult flies. Of the three susceptible species, *D. deflecta* and *D. palustris* belong to the *quinaria* species group, of which most species are fungivores and tolerant of α-amanitin (Jaenike et al., 1983; Jaenike, 1985; Spicer and Jaenike, 1996; Stump et al., 2011). On the other hand, the two species breed exclusively on decayed leaves/stems of herbaceous plants (Shorrocks, 1982; Stump et al., 2011) and are susceptible to α-amanitin at both larval and adult stages. However, while the larval mortality is absolute at 0.05 mg/ml concentration of α-amanitin (Stump et al., 2011), some (24 or 48% on average) of the experimental adult flies can survive for two weeks at the same α-amanitin concentration (the present study). Thus, larvae seem to be more sensitive to α-amanitin than adult flies. Therefore, the present study should be followed up by further experiments on the larval tolerance, especially for the non-mycophagous species, such as *D. sternopleuralis* and *D. hydei*, newly found to be absolutely tolerant at the adult stage.

Until now, the mechanisms of α-amanitin tolerance are not fully known. Jaenike et al. (1983) found that RNAP II of mycophagous species is as susceptible to α-amanitin as that of non-mycophagous species, suggesting that the tolerance is not based on mutational alteration of the molecular structure of RNAP II, which would inhibit α-amanitin from binding. Stump et al. (2011) confirmed this by finding two non-synonymous mutations outside of the α-amanitin binding site of RNAP II from both mycophagous and non-mycophagous species. They further tested effects of other detoxification enzymes on the α-amanitin tolerance and suggested that cytochrome P450 gene families play an important role in rendering some but not all mycophagous species tolerant. The enzyme cytochrome P450s are known to be involved in the tolerance of cactus toxins in cactophilic species of the subgenus *Siphlodora* of *Drosophila* (Stump et al., 2011). The *D.* (*Siphlodora*) *hydei* adult tolerance to α-amanitin detected in the present study may be attributed to this enzyme. Griffin and Reed (2020) assessed effects of gut microbiome on the α-amanitin tolerance of *D. tripunctata*, and showed that the microbiome composition is irrelevant to the toxic tolerance of the fly host. *Amanita* mushrooms produce some toxins other than α-amanitin. Tuno et al. (2007) demonstrated that three mycophagous species, *D. bizonata*, *D. angularis*, and *D. brachynephros*, with the α-amanitin tolerance (Spicer and Jaenike, 1996; Stump et al., 2011) were also tolerant of ibotenic acid and its derivative compound, muscimol, found in *Amanita* mushrooms. All these taken together, multiple mechanisms would act on the α-amanitin tolerance. So in the future, genome-wide research will give new insights into better understanding of mechanisms for the evolution of mycophagy and α-amanitin tolerance in Drosophilidae.

General Conclusions

The family Drosophilidae is highly diverse and exploits various food resources to increase its fitness and specialization. Mycophagy is an important adaptation for some drosophilid species. A large number of mycophagous species belong to the *Zygothrica* genus group or the *quinaria* species group of the subgenus *Drosophila* (Throckmorton, 1975; Kimura et al., 1977; Grimaldi, 1987; but see Gautério et al., 2020). Clarifying the phylogenetic status of these two groups is important for understanding the evolution of mycophagy in Drosophilidae. This thesis focused on the phylogeny and evolution of mycophagy and α-amanitin tolerance in Drosophilidae.

Chapter 1 addresses the phylogeny and evolution of mycophagy in the *Zygothrica* genus group. Up to now, some studies have included the *Zygothrica* genus group in their molecular phylogenetic analyses, but with a small number of species (Da Lage et al., 2007; van der Linde et al., 2010; Russo et al., 2013; Yassin, 2013). Very recently, Gautério et al. (2020) has conducted a phylogenetic analysis on the *Zygothrica* genus group from the Neotropical region. However, the relationships inferred from their study were not so strongly supported for deep branches relevant to the *Zygothrica* genus group. To compensate the biogeographic bias of taxon sampling in Gautério et al. (2020), as many species of the *Zygothrica* genus group from the Palearctic and Oriental regions as possible were included in the present study. The increased taxon sampling and the large sequence dataset of 24 genes improved the phylogenetic reconstruction, providing a well-established hypothesis: (i) the *Zygothrica* genus group is monophyletic and placed as the sister to the genus *Dichaetophora*, and the clade *Zygothrica* genus group + *Dichaetophora* is sister to the *Siphlodora* + *Idiomyia*/*Scaptomyza* clade; and (ii) within the *Zygothrica* genus group, the genera *Mycodrosophila* and *Paramycodrosophila* are both monophyletic, while neither the genus *Zygothrica* nor *Hirtodrosophila* is monophyletic, suggesting the necessity of taxonomic revision for these genera. Based on this robust phylogenetic hypothesis, the ancestral state reconstruction of food habits was conducted, and reconfirmed a traditional view that fungus-feeding habit has independently evolved on several lineages of Drosophilidae: specifically the *Zygothrica* genus group and the subgenus *Drosophila* in the present study. However, some differences in host selection were estimated between these two lineages: the MRCA of the *Zygothrica* genus group may have been a specialist preferring fresh fungal fruiting bodies, while the MRCA of the subgenus *Drosophila* may have been a generalist depending on fermenting fruits and decayed fungi.

In spite of the consistent inference that the *quinaria* species group of the subgenus *Drosophila* is monophyletic, based on concatenated sequence datasets, in most of previous phylogenetic studies (Perlman et al., 2003; Hatadani et al., 2009; Morales-Hojas and Vieira, 2012; Izumitani et al., 2016; Finet et al., 2021), Scott Chialvo et al. (2019) recently questioned its monophyly based on a phylogenetic tree constructed by the coalescent-based method. However, it has been pointed out that the coalescent-based tree topology is vulnerable to errors of individual gene trees (Springer and Gatesy, 2016; Blom et al., 2017; Simmons and Kessenich, 2020; Shen et al., 2021). One of such errors is caused by the nucleotide compositional heterogeneity, especially at 3rd codon positions, among studied taxa (Rodriguez-Trelles et al., 1999, 2000a, 2000b; Tarrio et al., 2001). In Chapter 2, to assess the effect of compositional heterogeneity, species trees were reconstructed with the modified dataset where 3rd codon sites significantly heterogeneous in nucleotide composition were excluded from the dataset of Scott Chialvo et al. (2019). Both trees resulting from concatenation- and coalescent-based methods consistently recovered the monophyly of the *quinaria* group, suggesting that the compositional heterogeneity is responsible for the non-monophyly of the *quinaria* group inferred in Scott Chialvo et al. (2019). Thus, it is necessary to pay attention to the influence of compositional heterogeneity on phylogenetic reconstruction.

Mushrooms have various toxins, such as α-amanitin produced by *Amanita* mushrooms (Wieland, 1968; Mas, 2005), against fungus-feeders. There is rich evidence of the α-amanitin tolerance for mycophagous species of the subgenus

Drosophila. It is, however, unknown whether species of the *Zygothrica* genus group are tolerant of α-amanitin or not. And, all the previous studies tested the tolerance of pre-imaginal stages (egg to pupa) but never of adult flies. In Chapter 3, the α-amanitin tolerance was tested for adult flies, extending taxon sampling not only from the subgenus *Drosophila* but also to the *Zygothrica* genus group and further some non-mycophagous species. Despite the difference in the tested developmental stage, the α-amanitin tolerance was observed in association with mycophagy. Furthermore, two non-mycophagous species, generalists *D. hydei* and *D. sternopleuralis,* also had the α-amanitin tolerance, suggesting a possibility that the α-amanitin tolerance has been acquired in some cases independently from the evolution of mycophagy.

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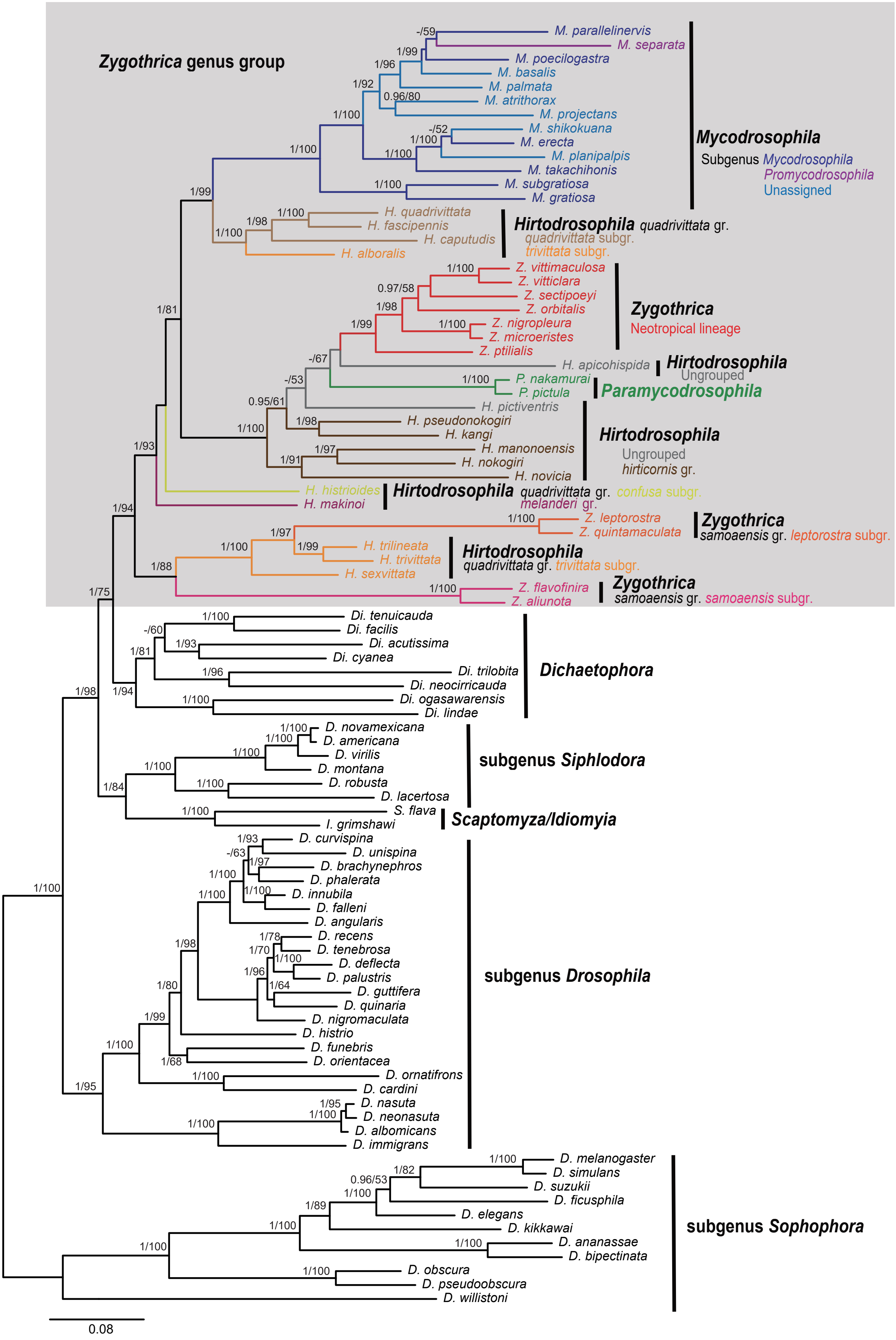


Fig. 1. Phylogenetic tree constructed by the Bayesian analysis of the concatenated sequence dataset of 24 genes. Branch support values are indicated by the Bayesian posterior probability (≥0.9) followed by the bootstrap value (≥50%). The *Zygothrica* genus group clade is shaded gray, with indication of genera, subgenera, species groups and/or subgroups in different colors.

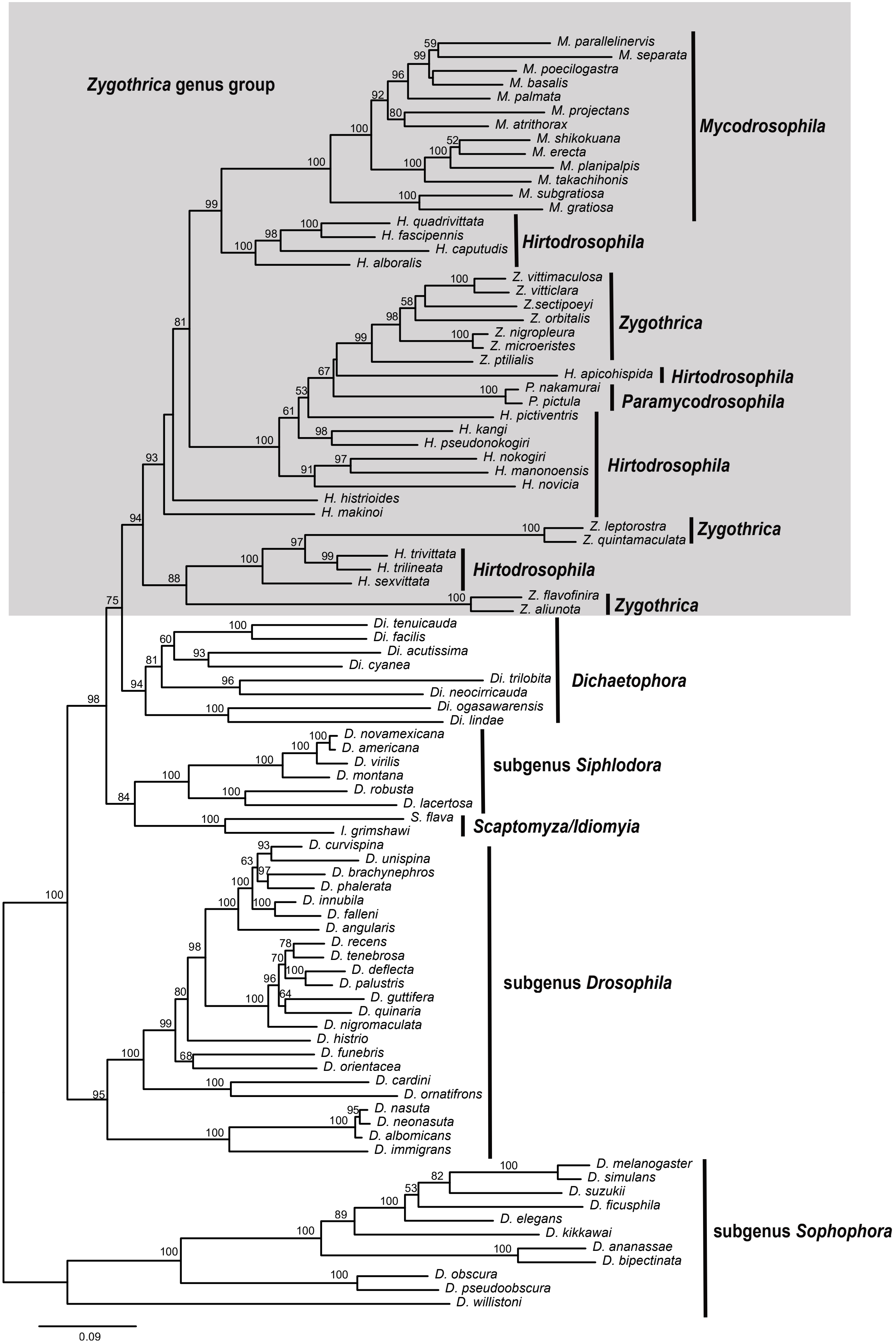


Fig. 2. Phylogenetic tree constructed by the maximum likelihood (ML) analysis of the concatenated sequence dataset of 24 genes. Branch supports are indicated by bootstrap values (≥50%).

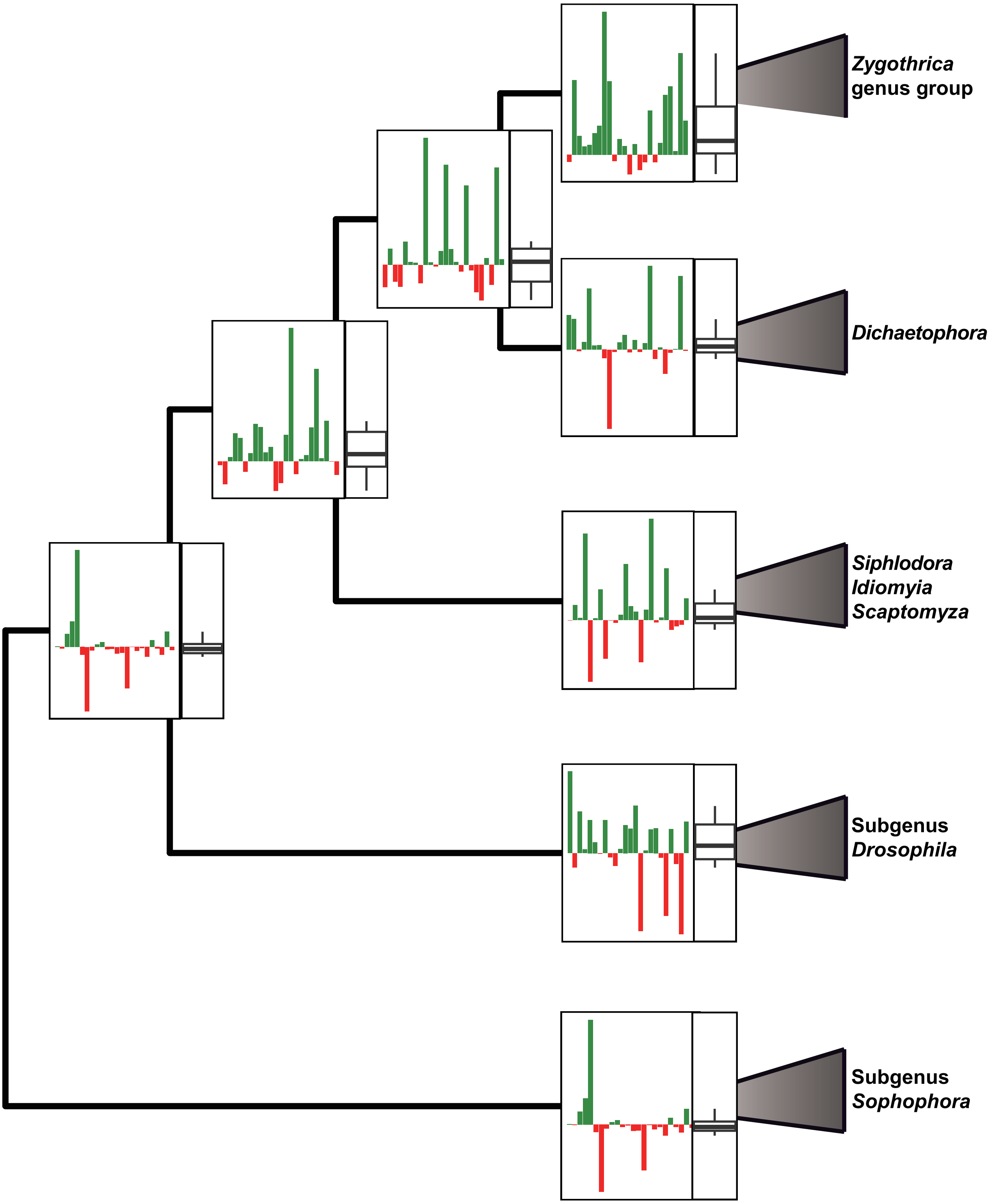


Fig. 3. Partition likelihood support (PLS) for each clade. Green bars indicate genes favor ML tree over alternative NNI tree, red bars indicate genes favor alternative NNI tree over ML tree. The order of genes from left to right is *ATPsynB, AdSS, Adh, COI, COII, ERp60, Gpdh, Pdi, Pgi, RpL3, RpS17, VhaSFD, bur, ced-6, Ddc, Dll, eb, eIF3-S8, eve, hh, not, ptc, sina and wg.*

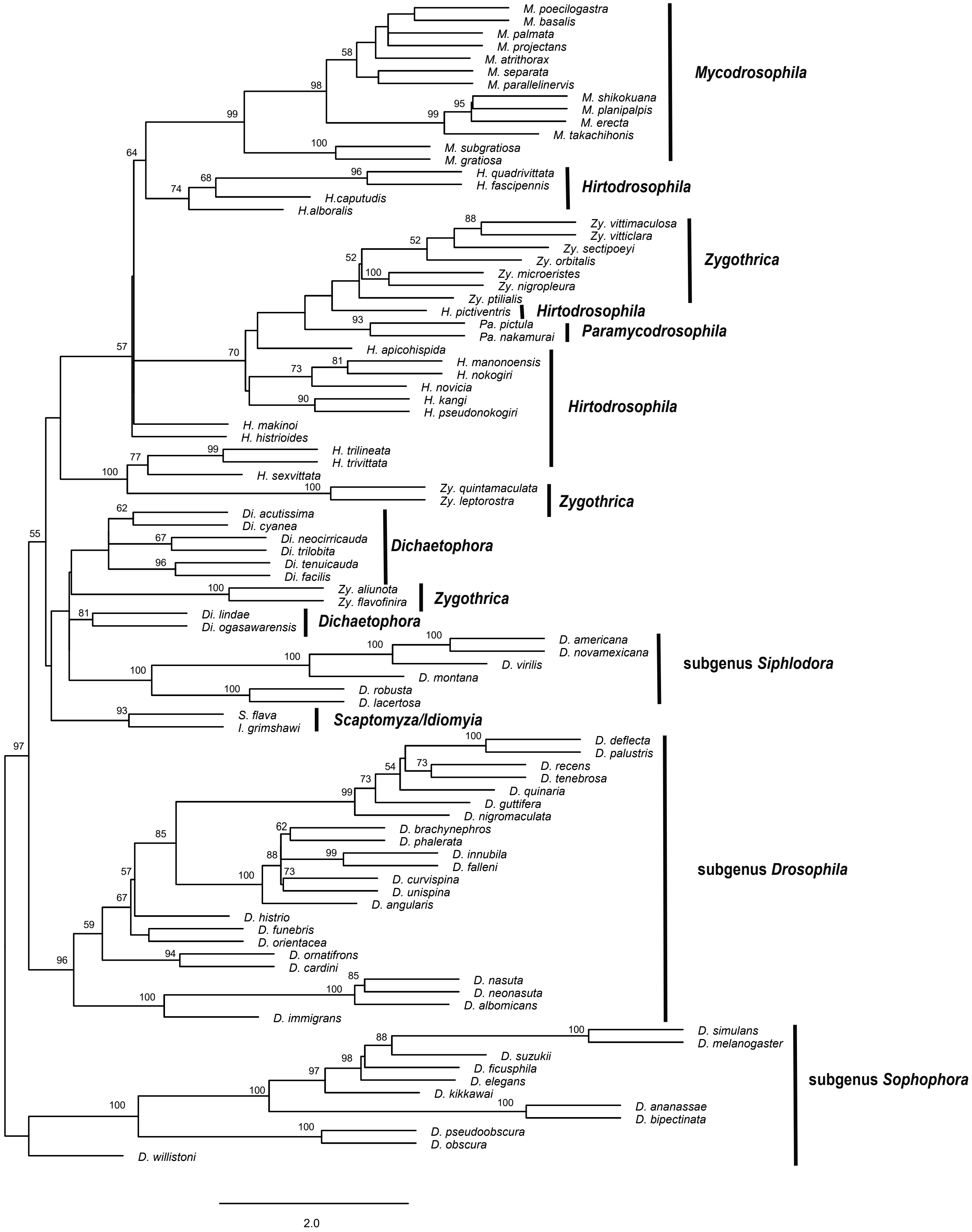
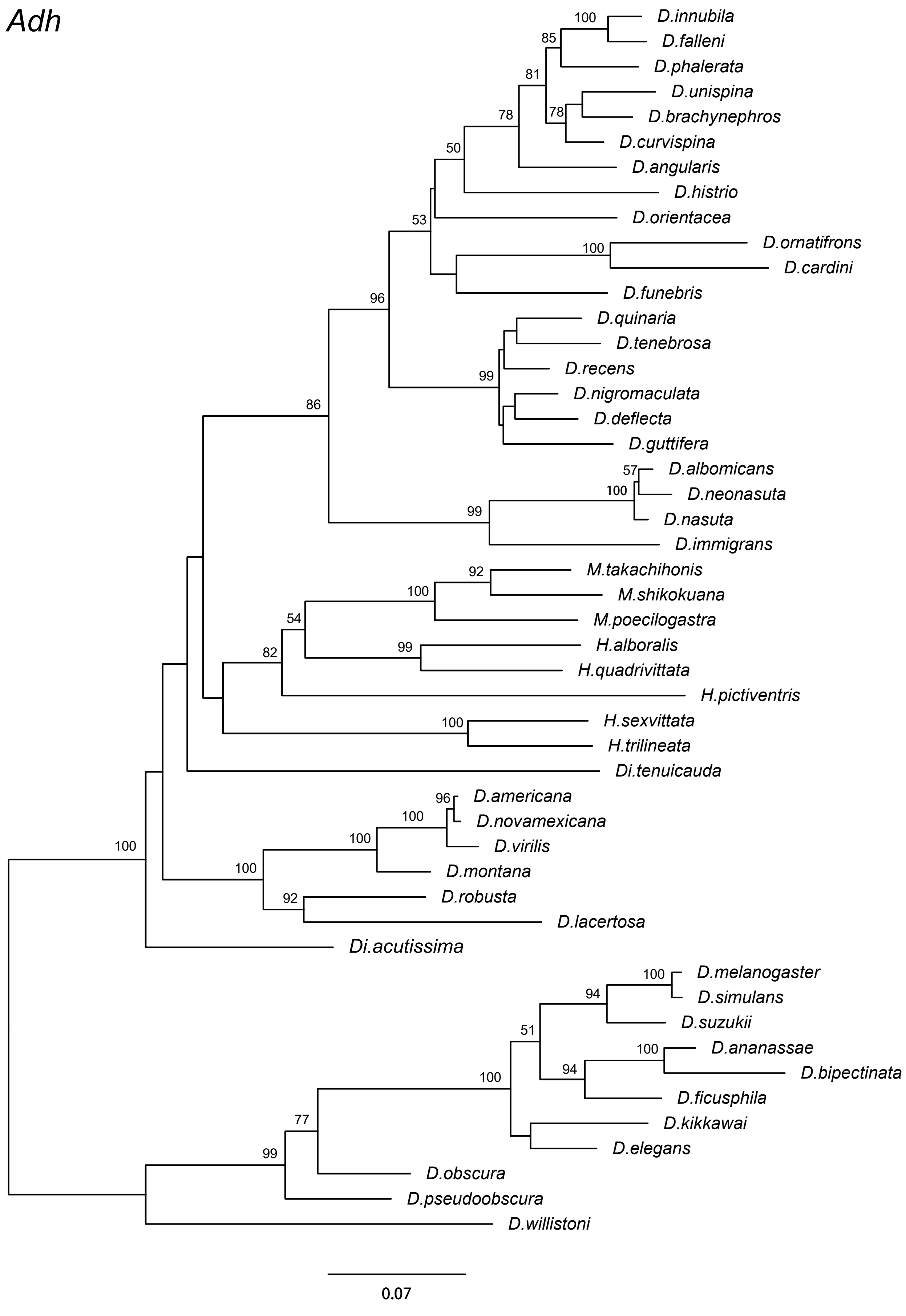


Fig. 4. Species tree resulted from the ASTRAL analysis of 22 nuclear and one concatenated mitochondrial gene trees. Branch supports are indicated by bootstrap values (≥50%).



Adh

Fig. 5. Phylogenetic tree constructed by the maximum likelihood (ML) analysis of each of 23 gene partitions (22 nuclear and one concatenated mitochondrial regions). Branch supports are indicated by bootstrap values (≥ 50%).

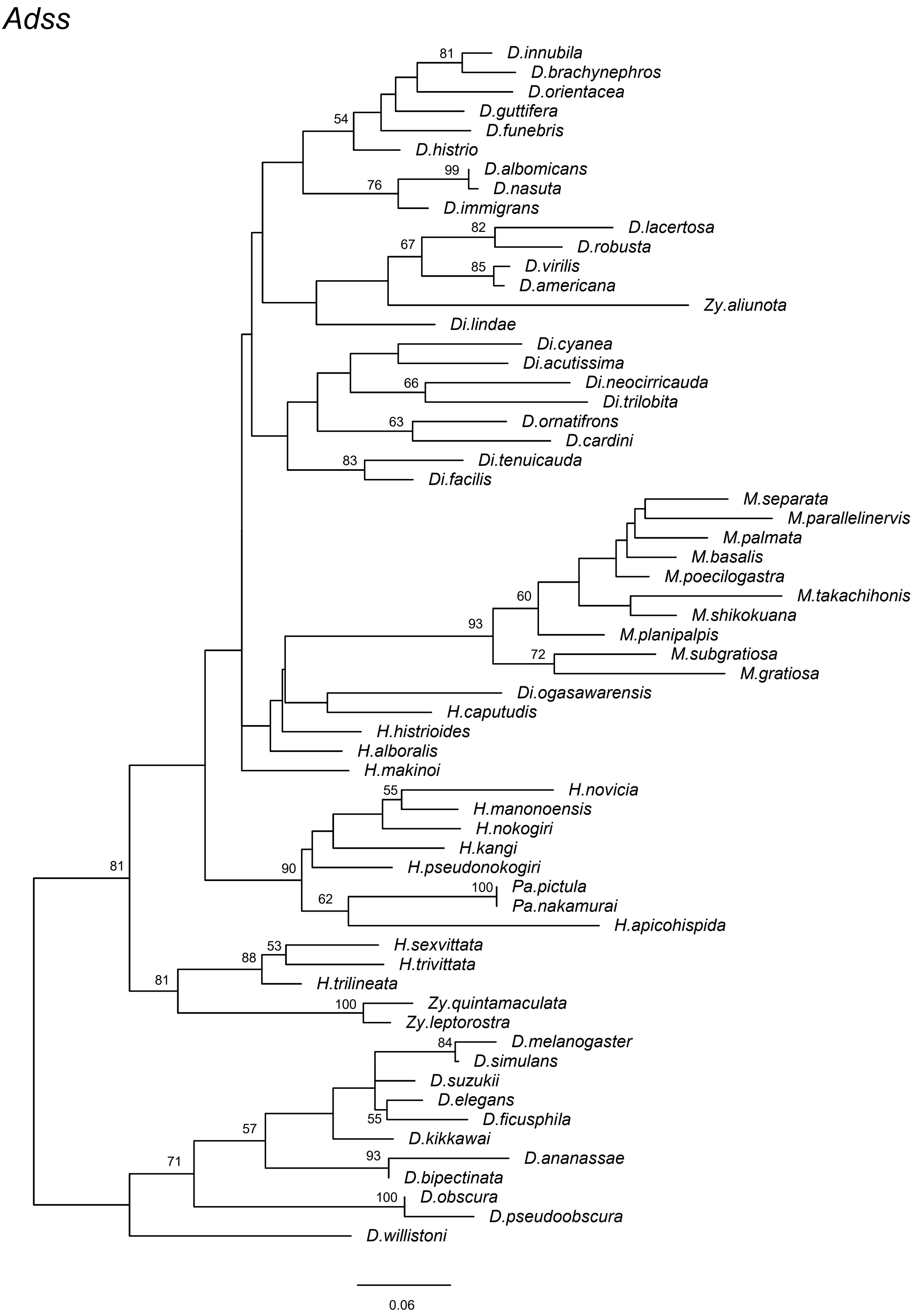
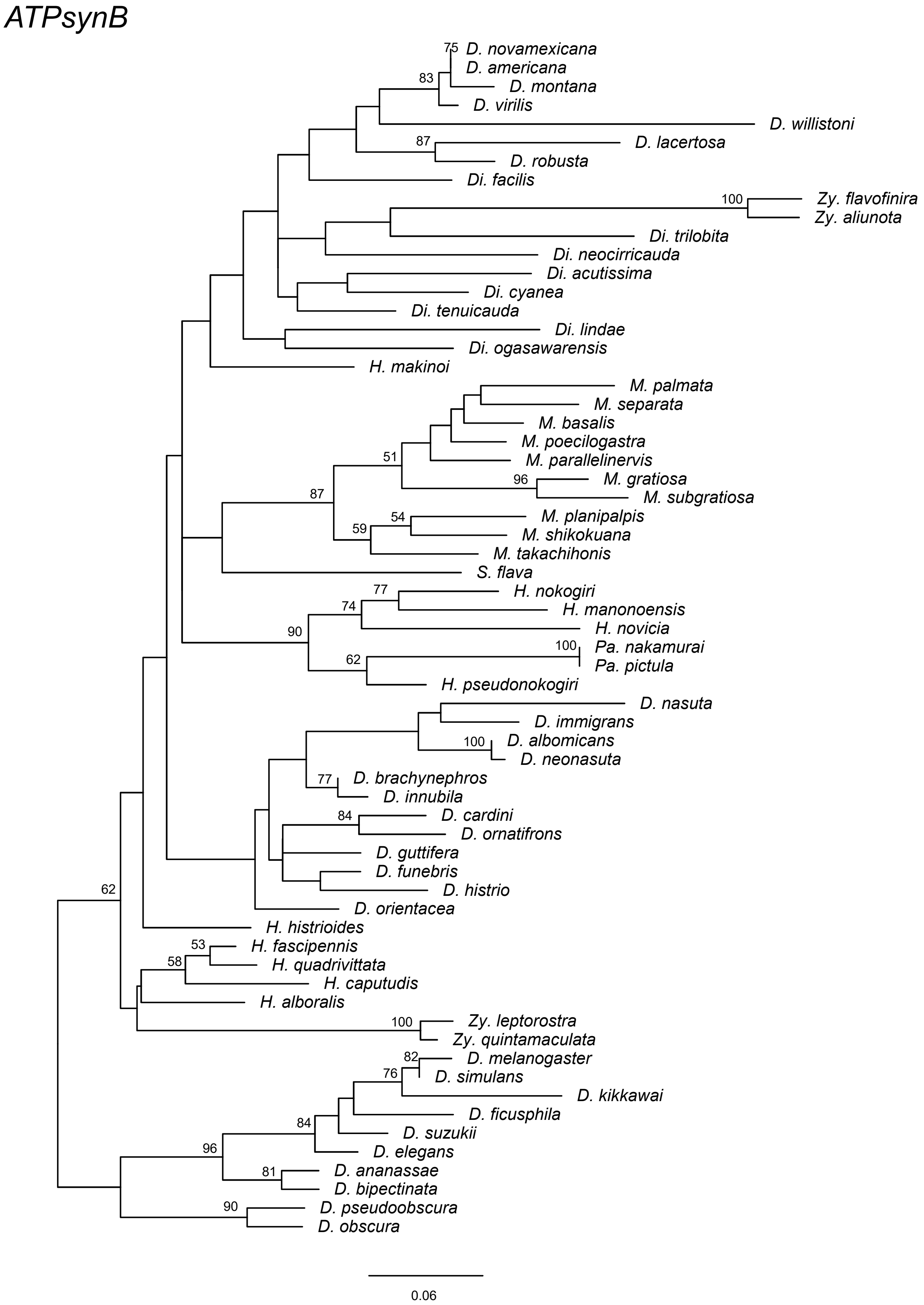


Fig. 5. (*continued*)



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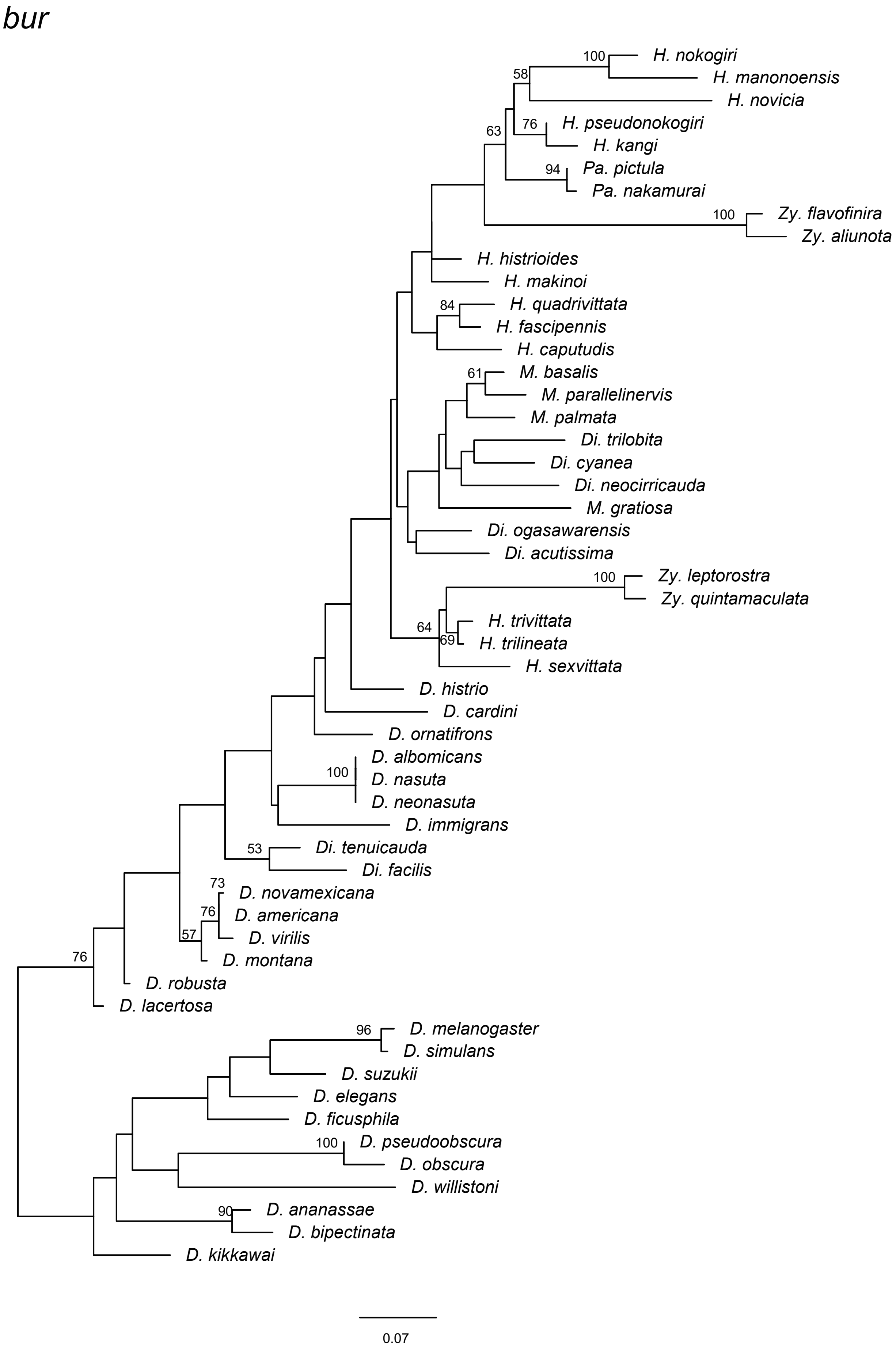
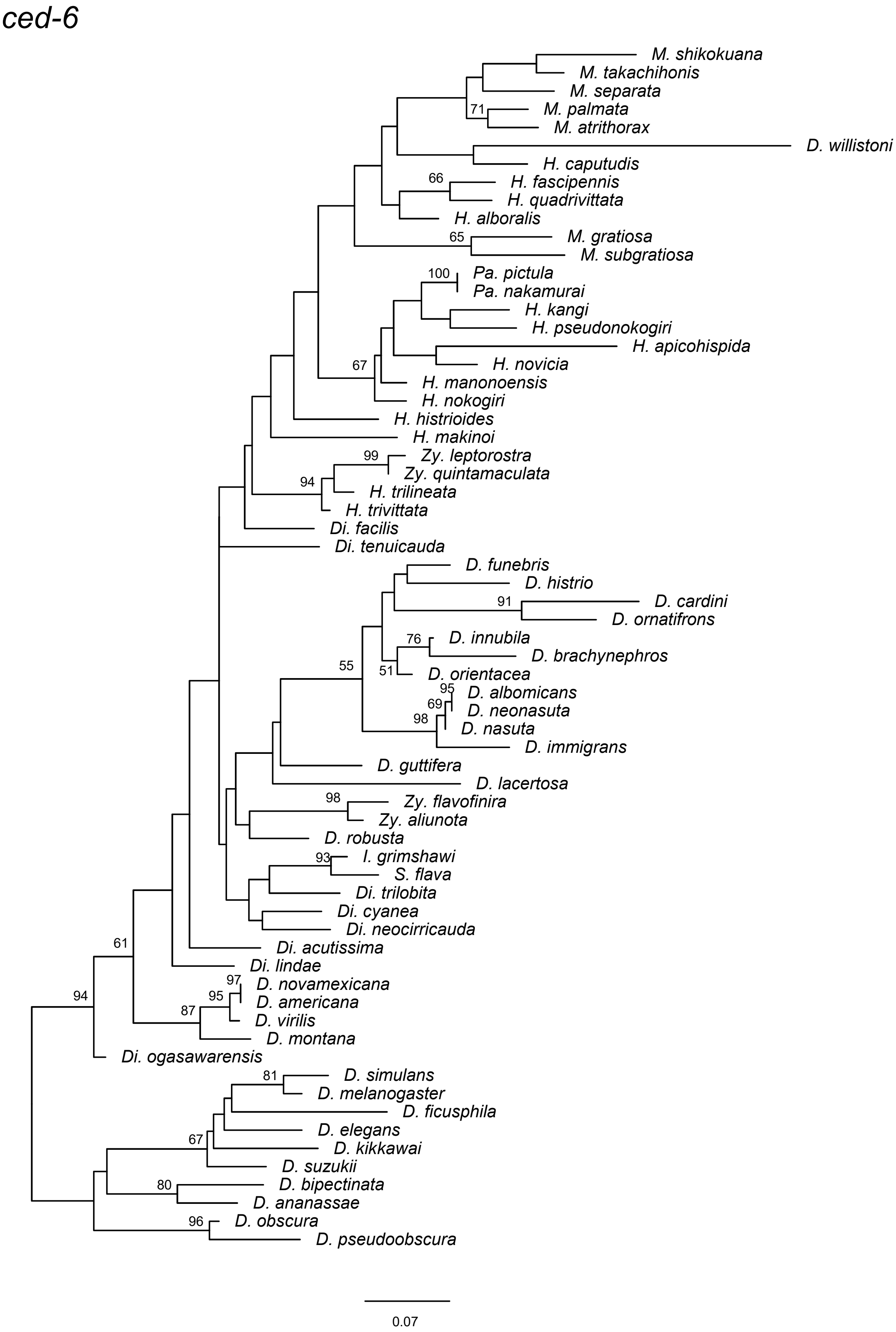

bur

Fig. 5. (*continued*)



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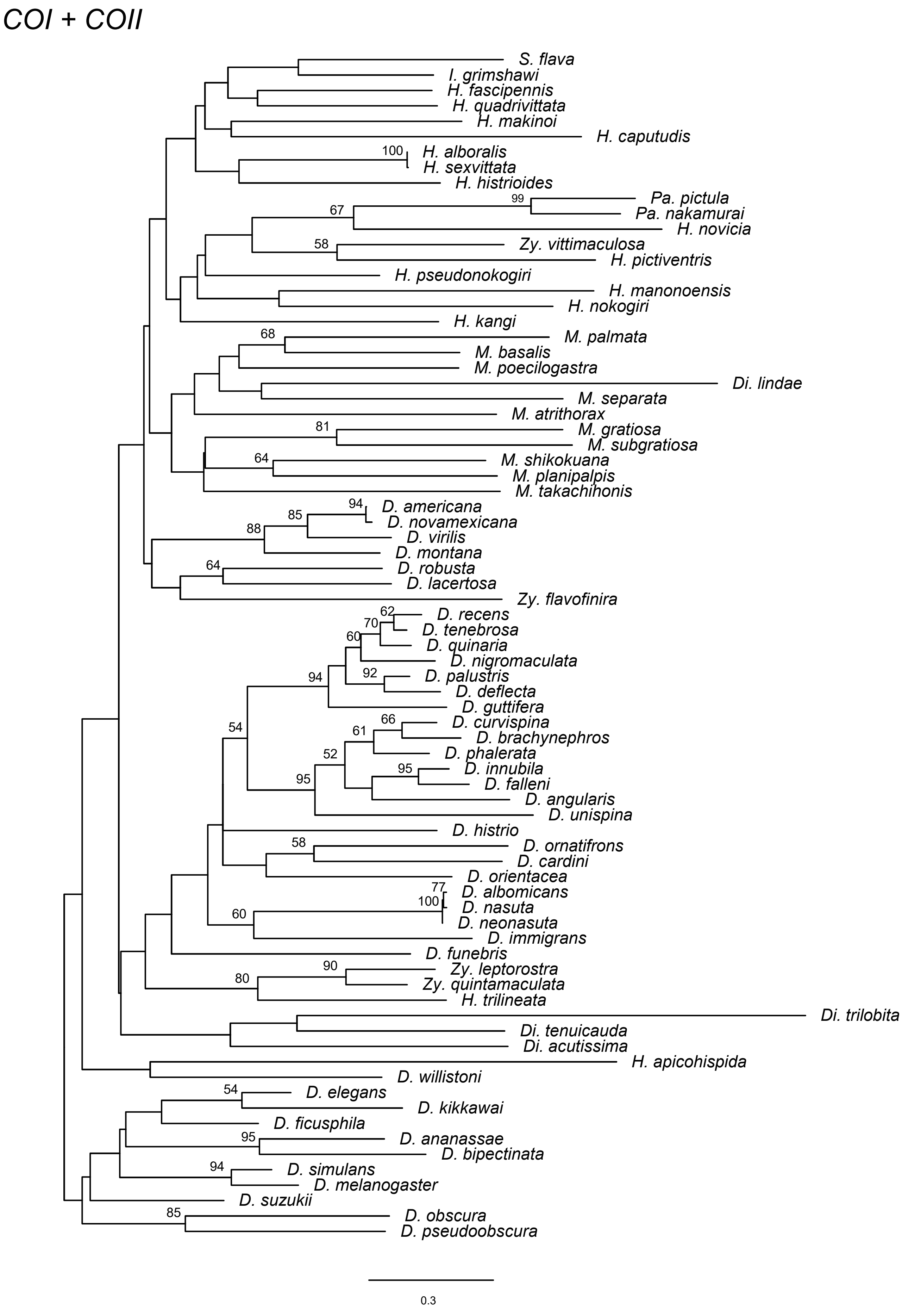
COI + COII

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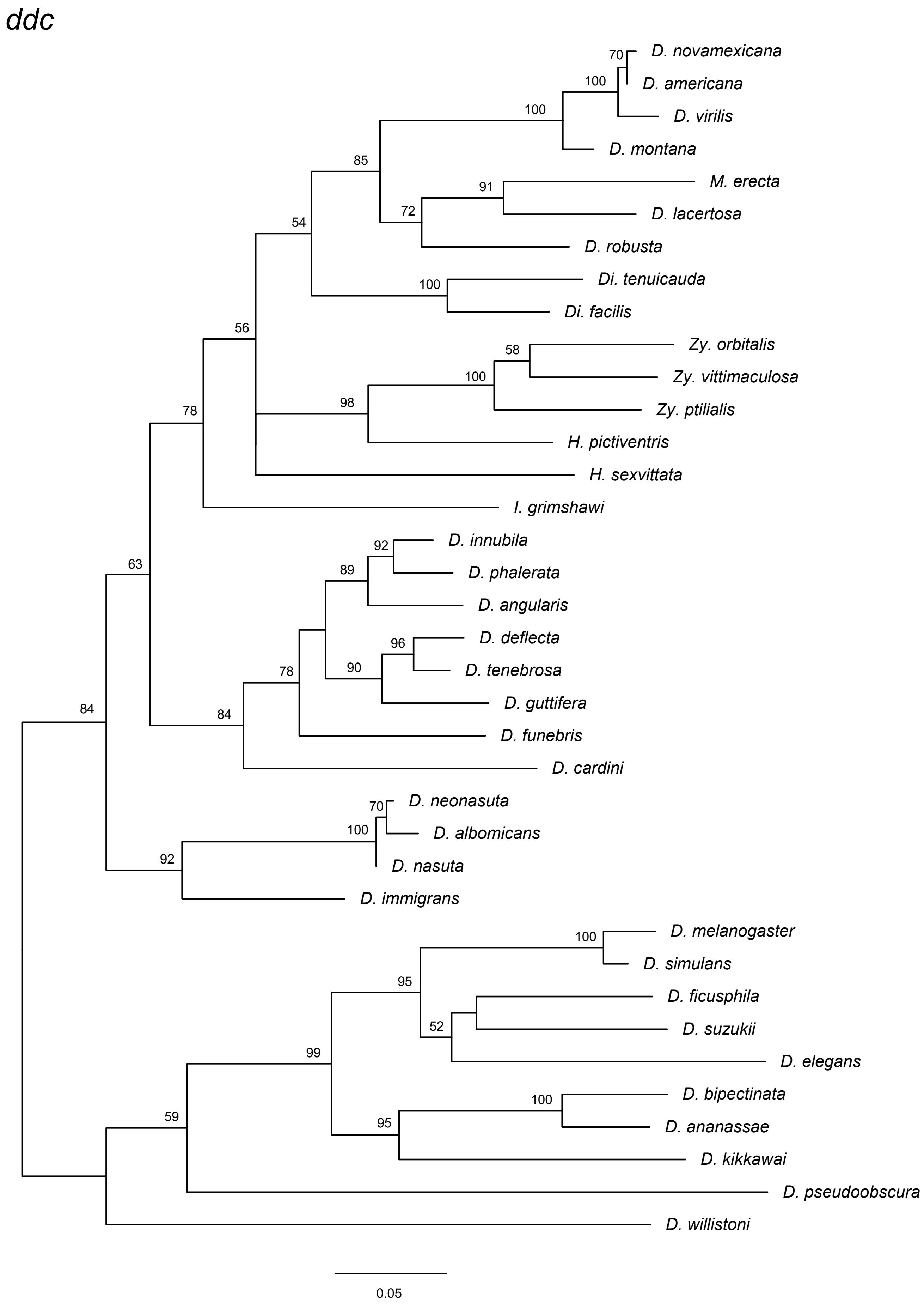

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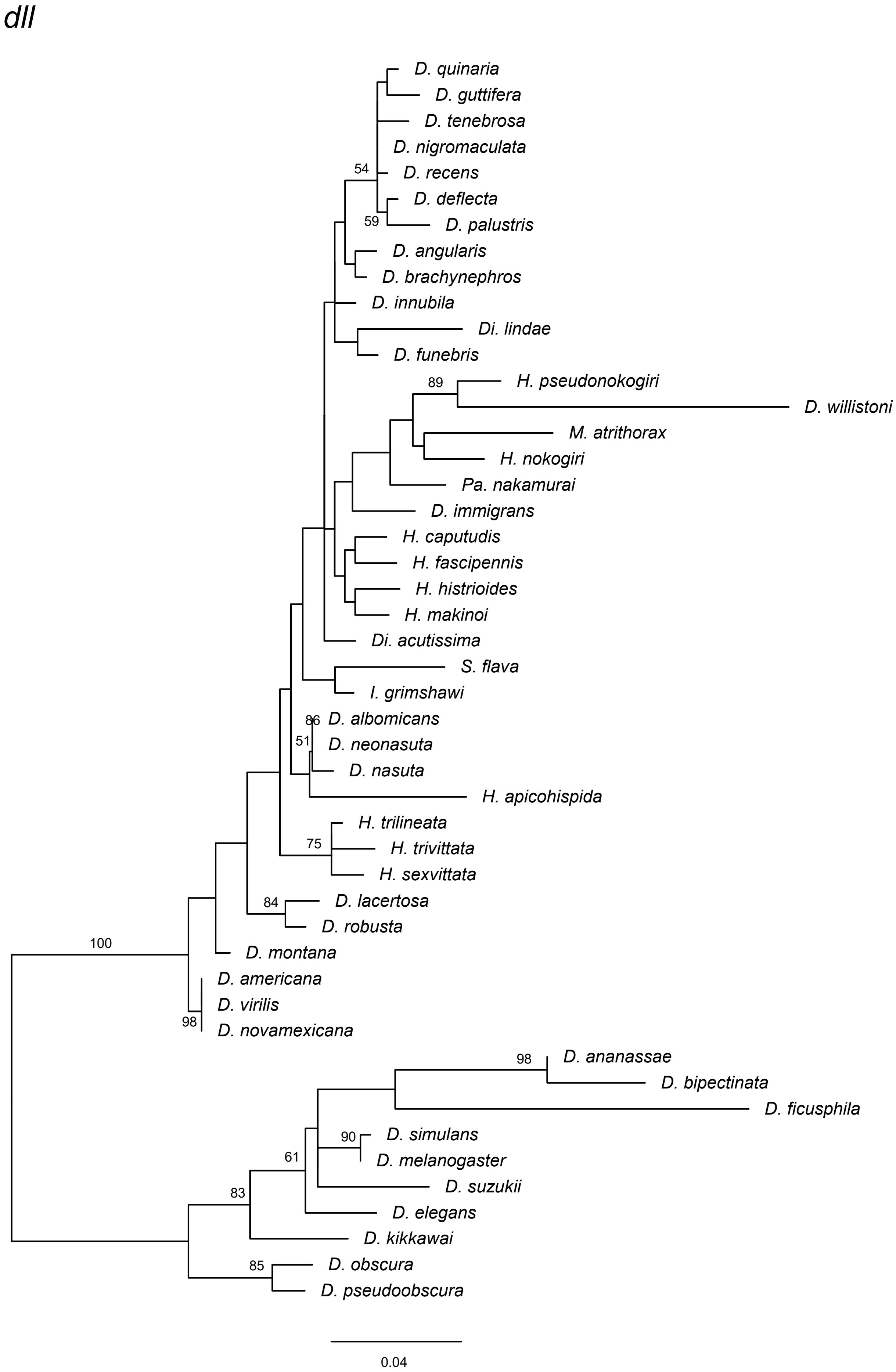

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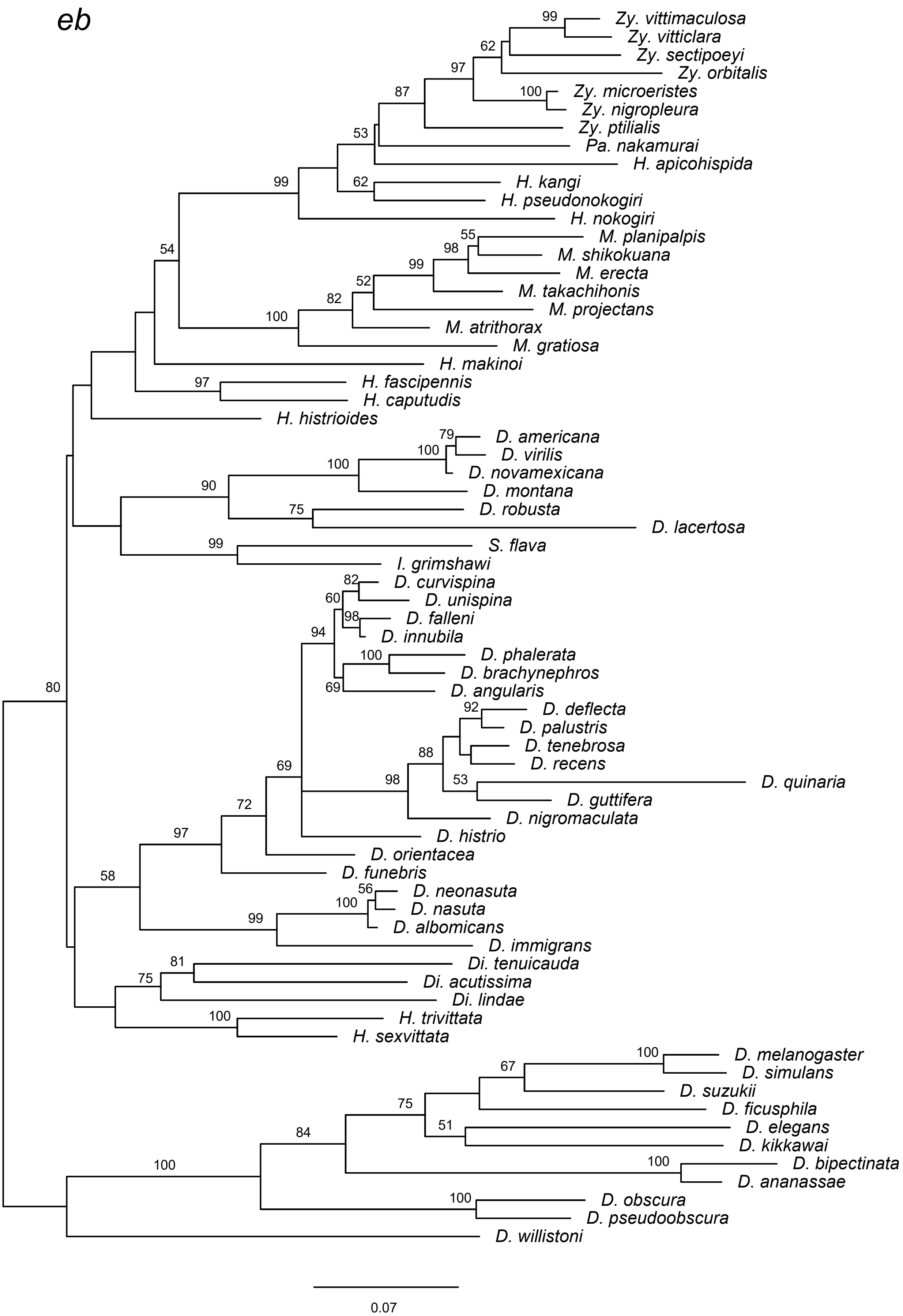

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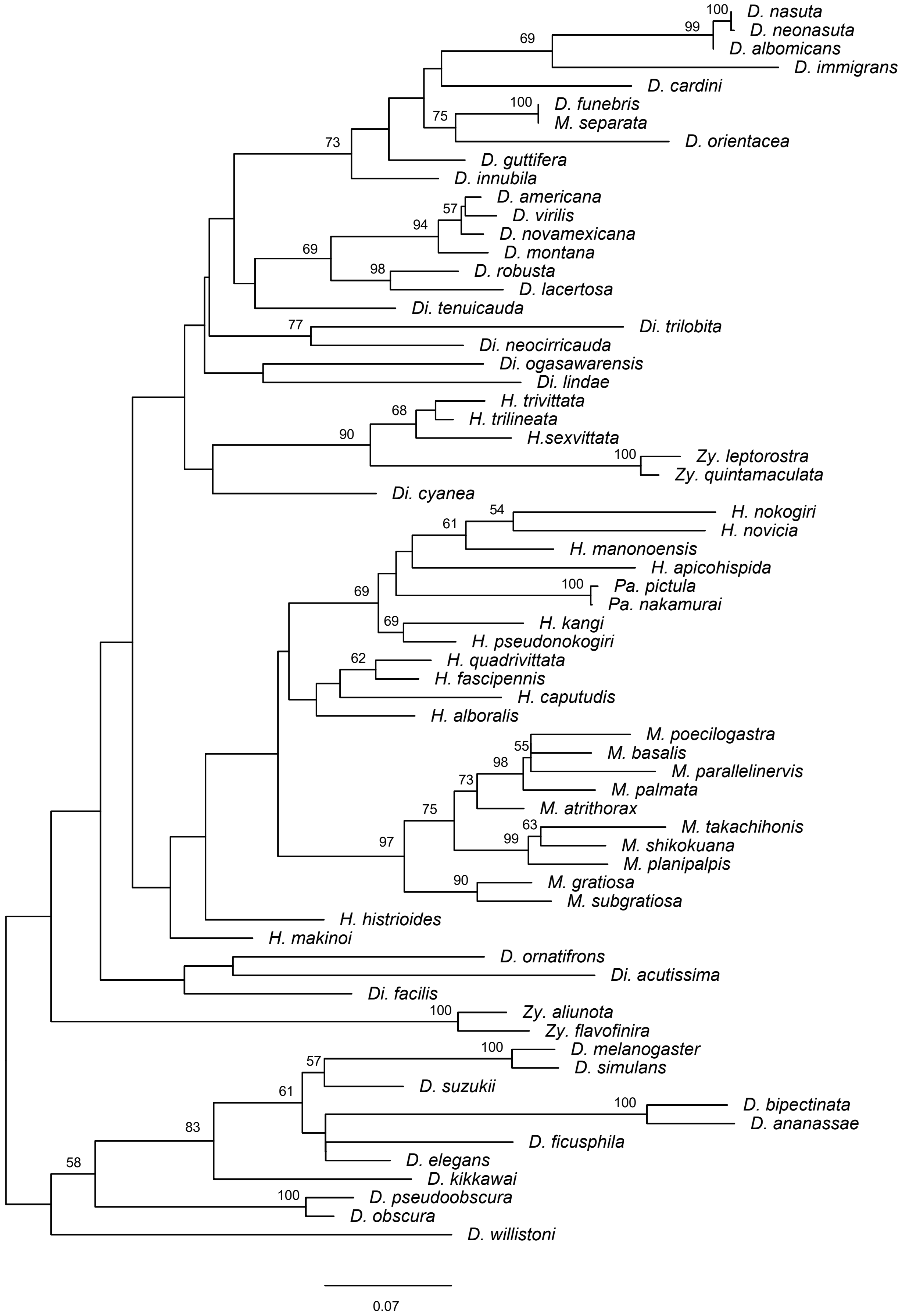
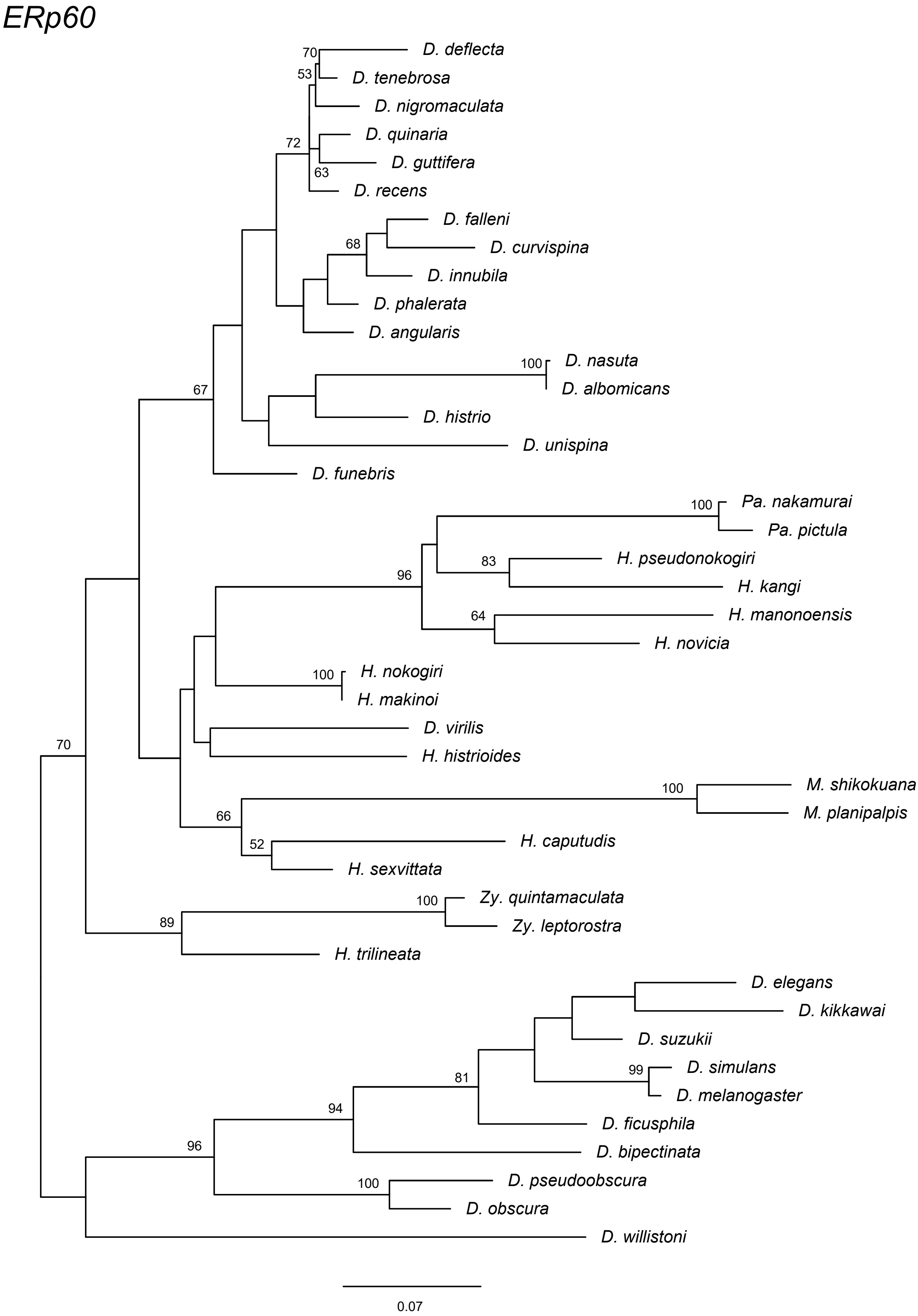
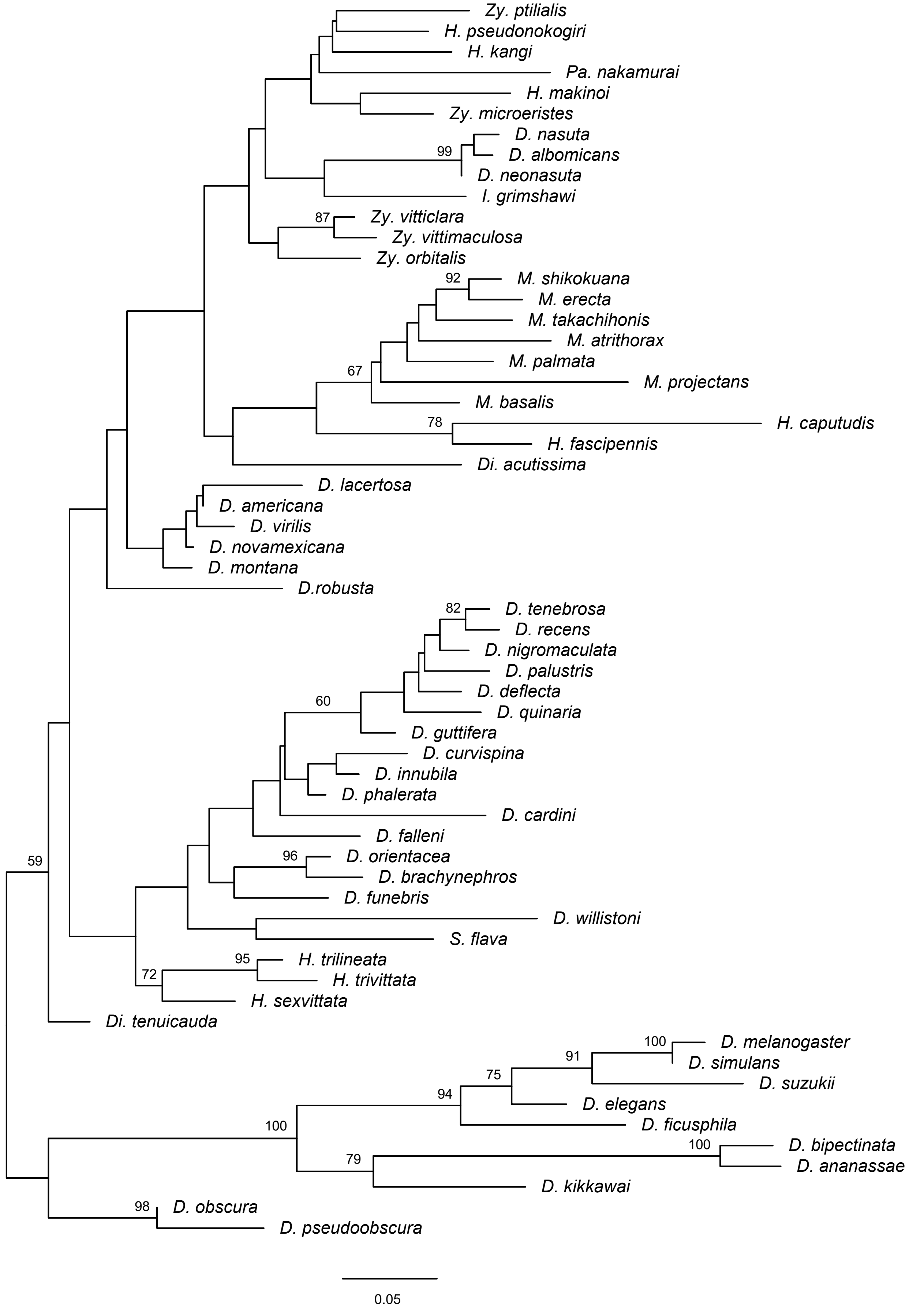

elf-s8

Fig. 5. (*continued*)



ERp60

Fig. 5. (*continued*)



eve

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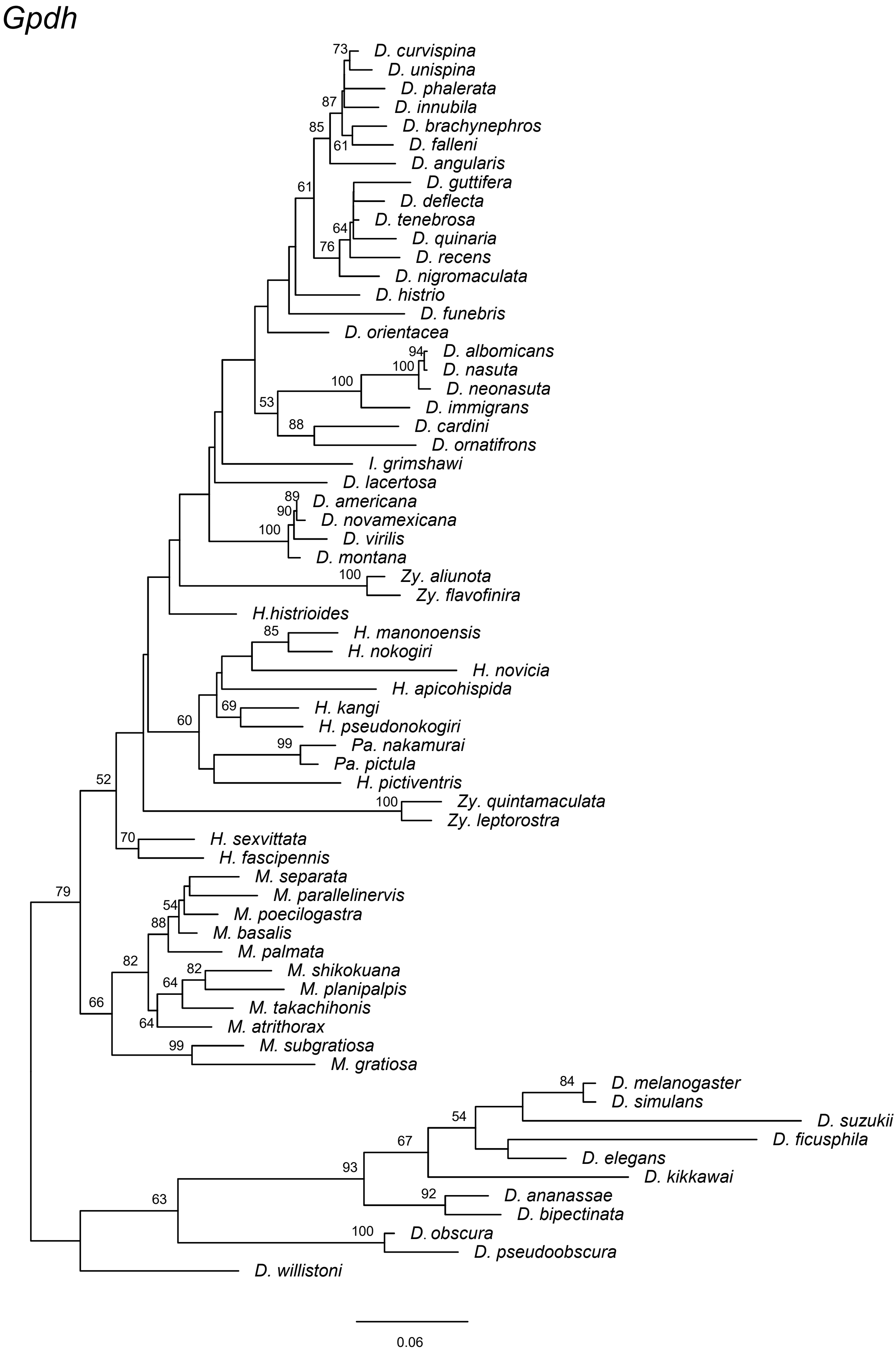

Gpdh

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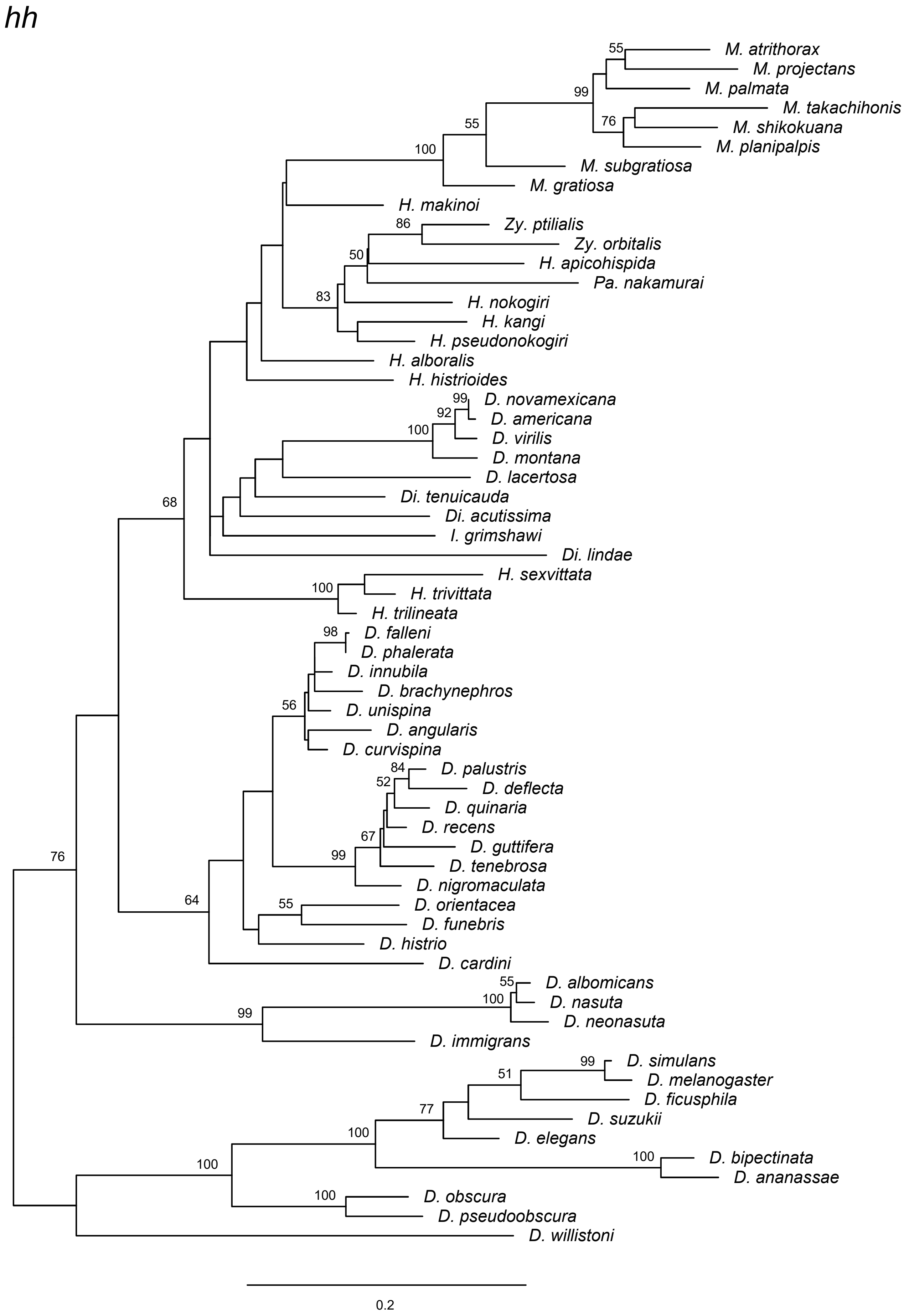

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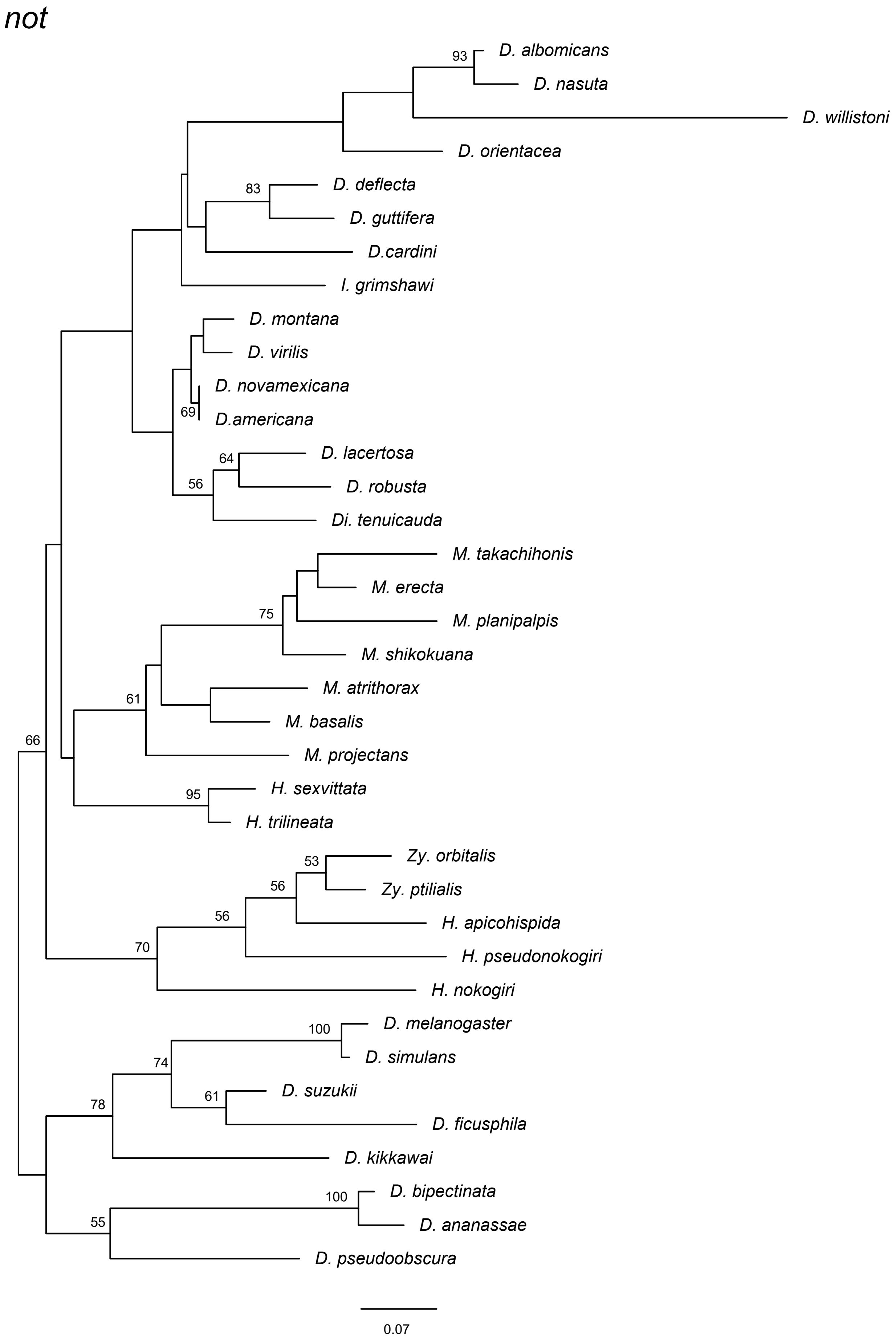

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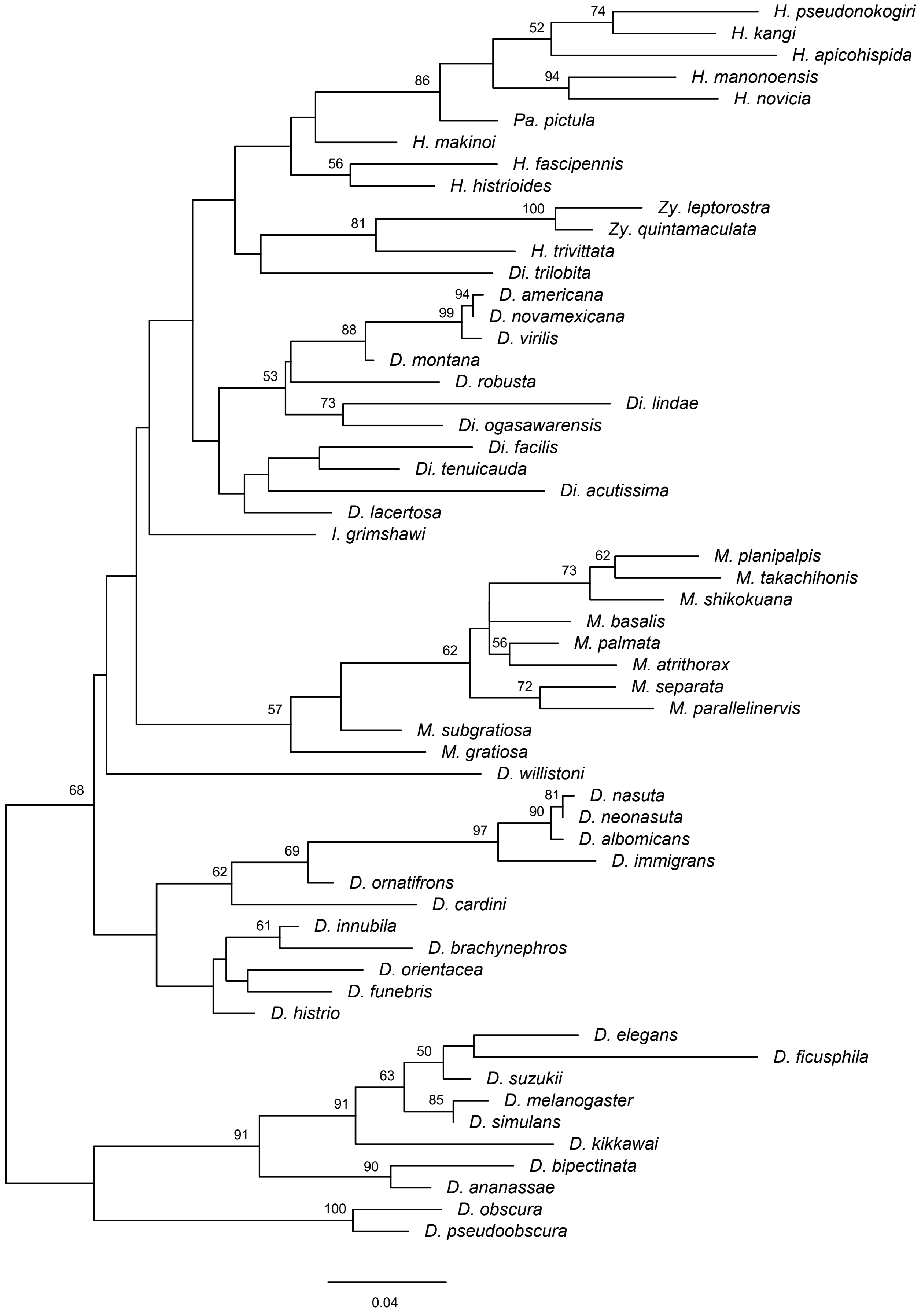
Pdi

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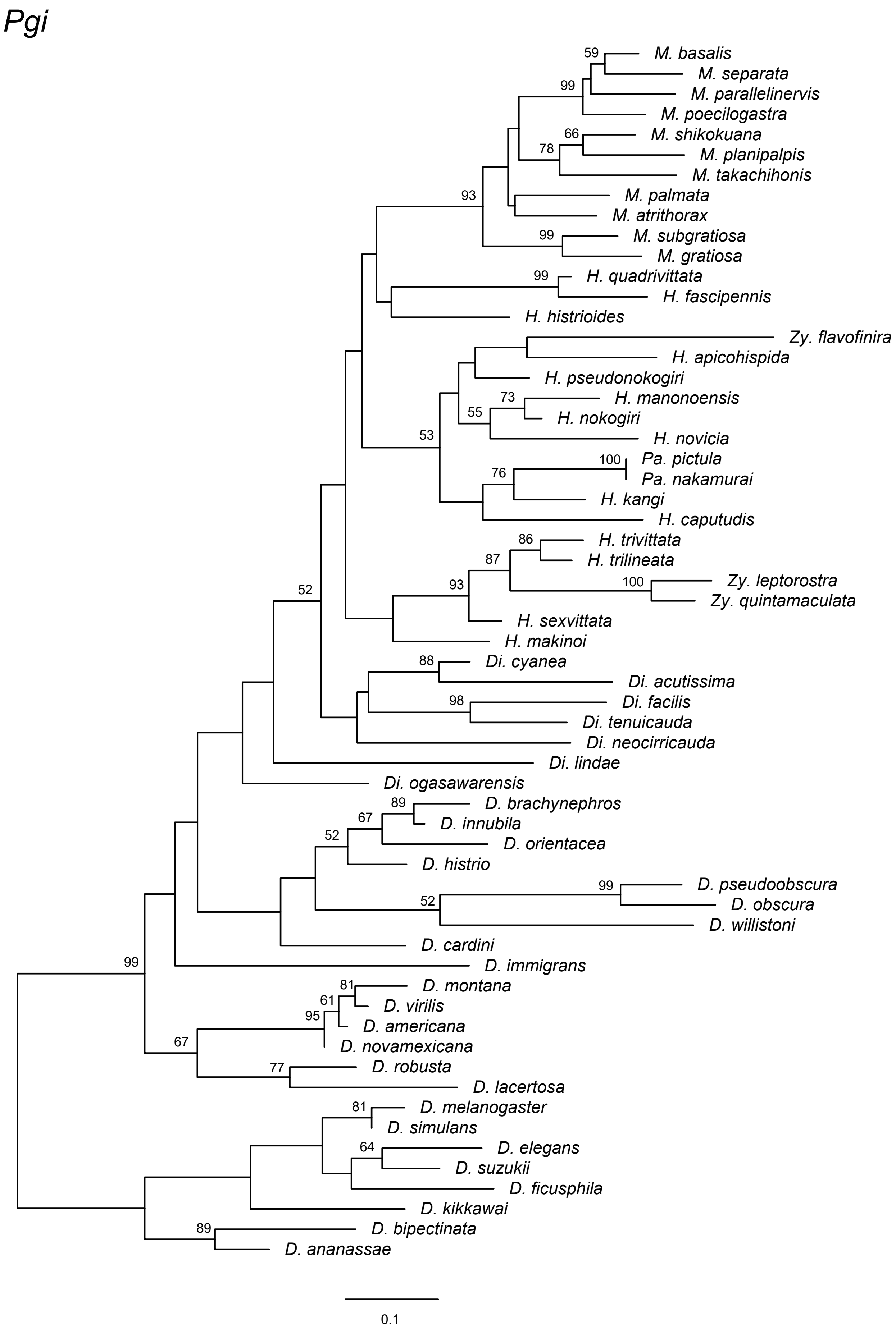

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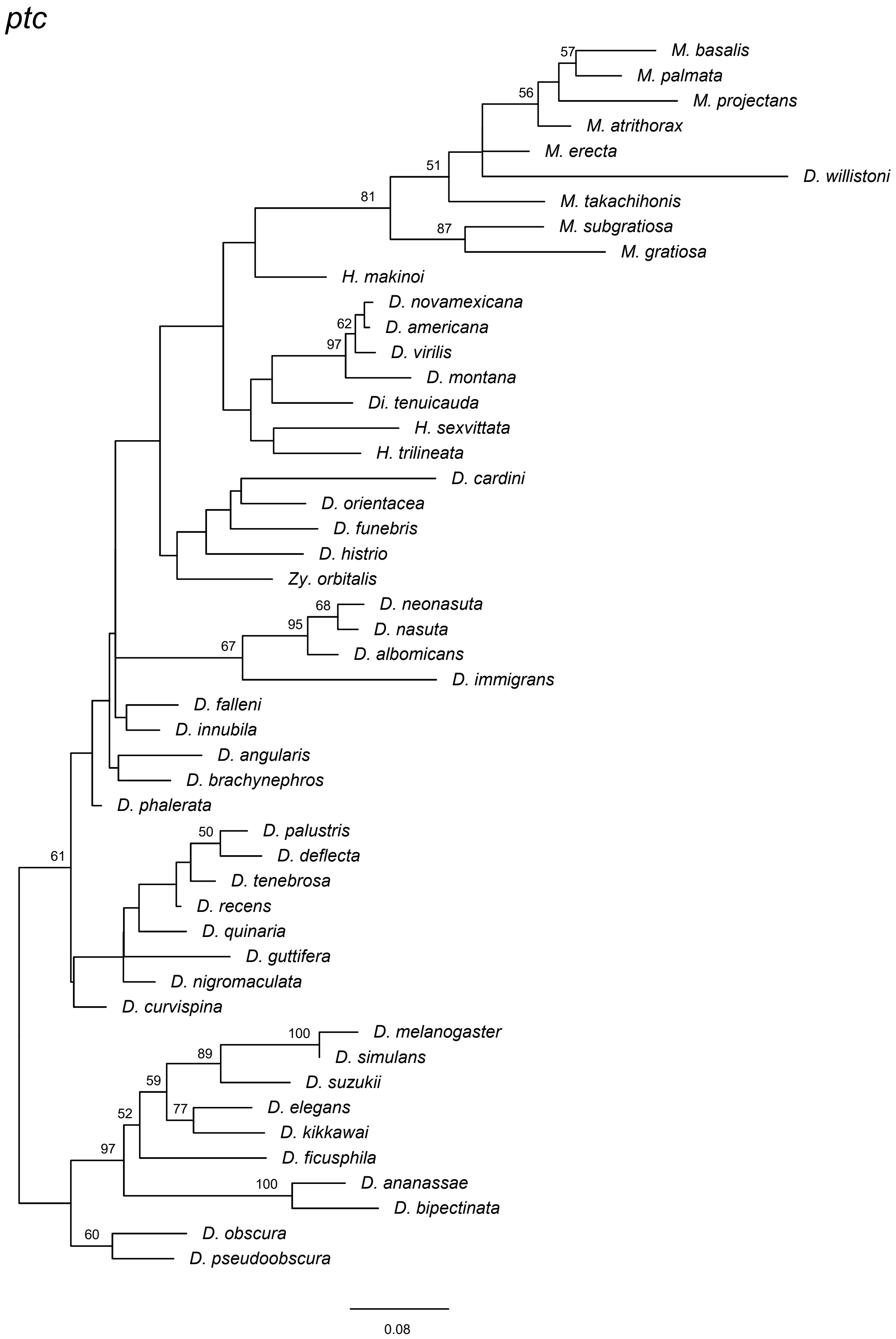
ptc

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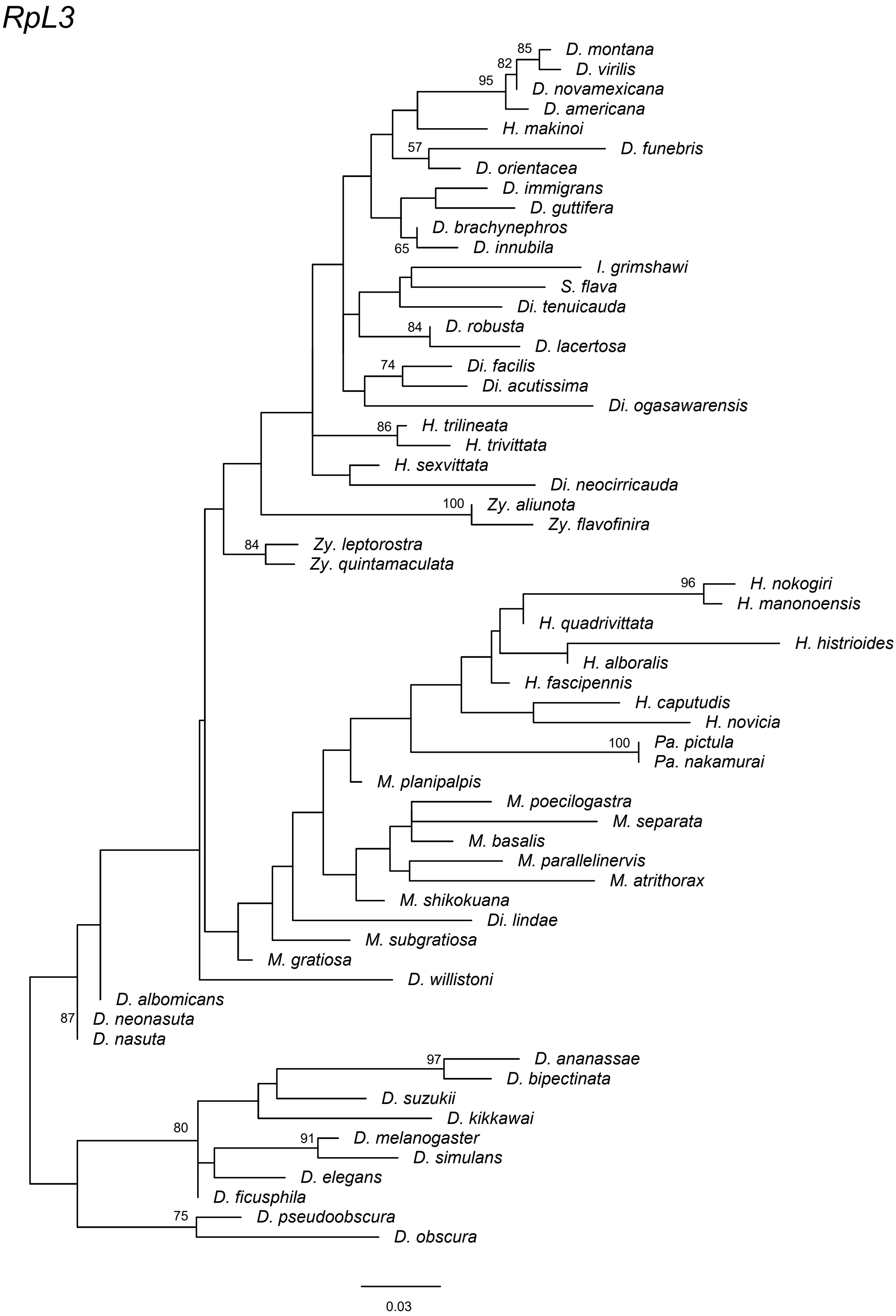

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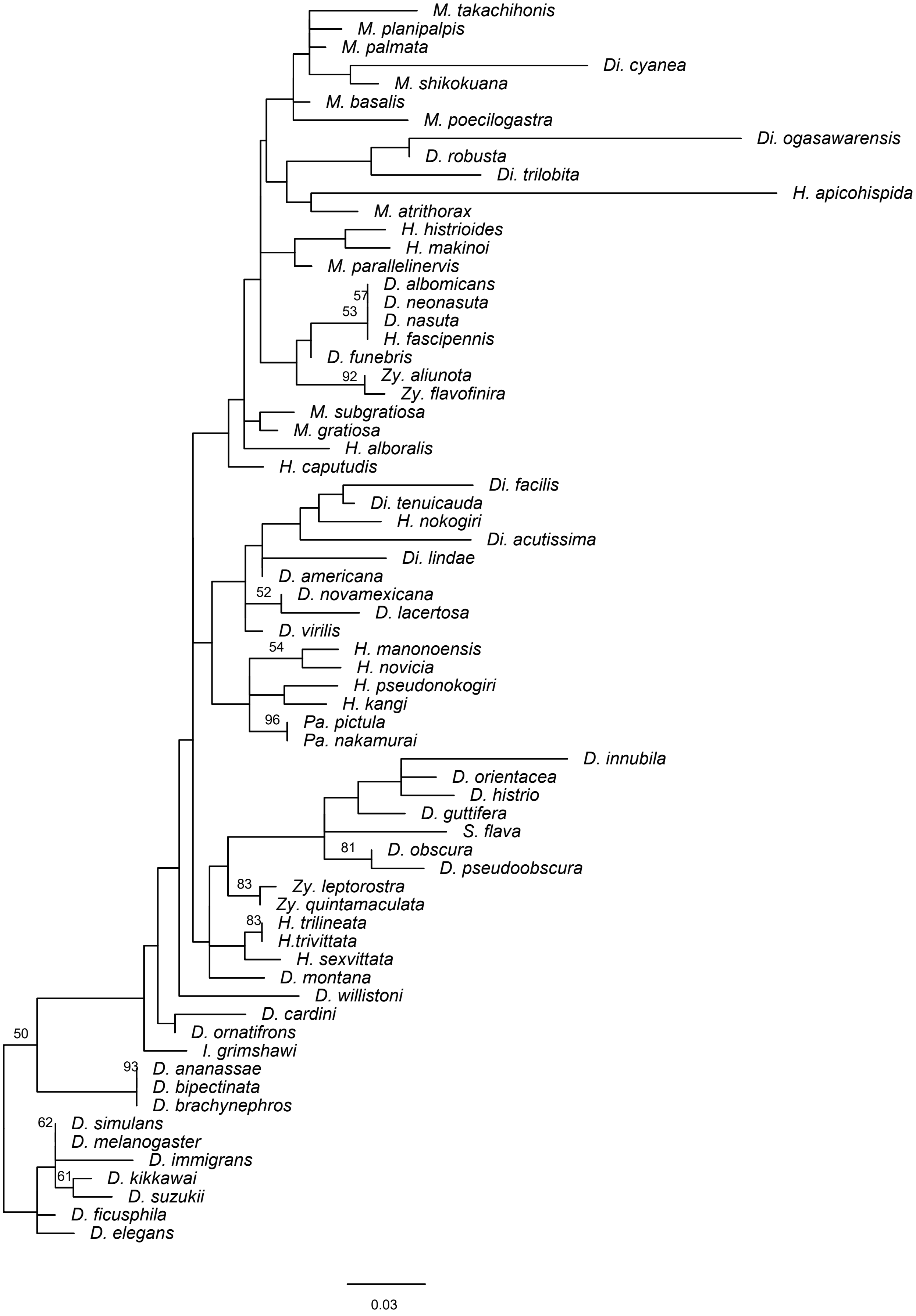

RpS17

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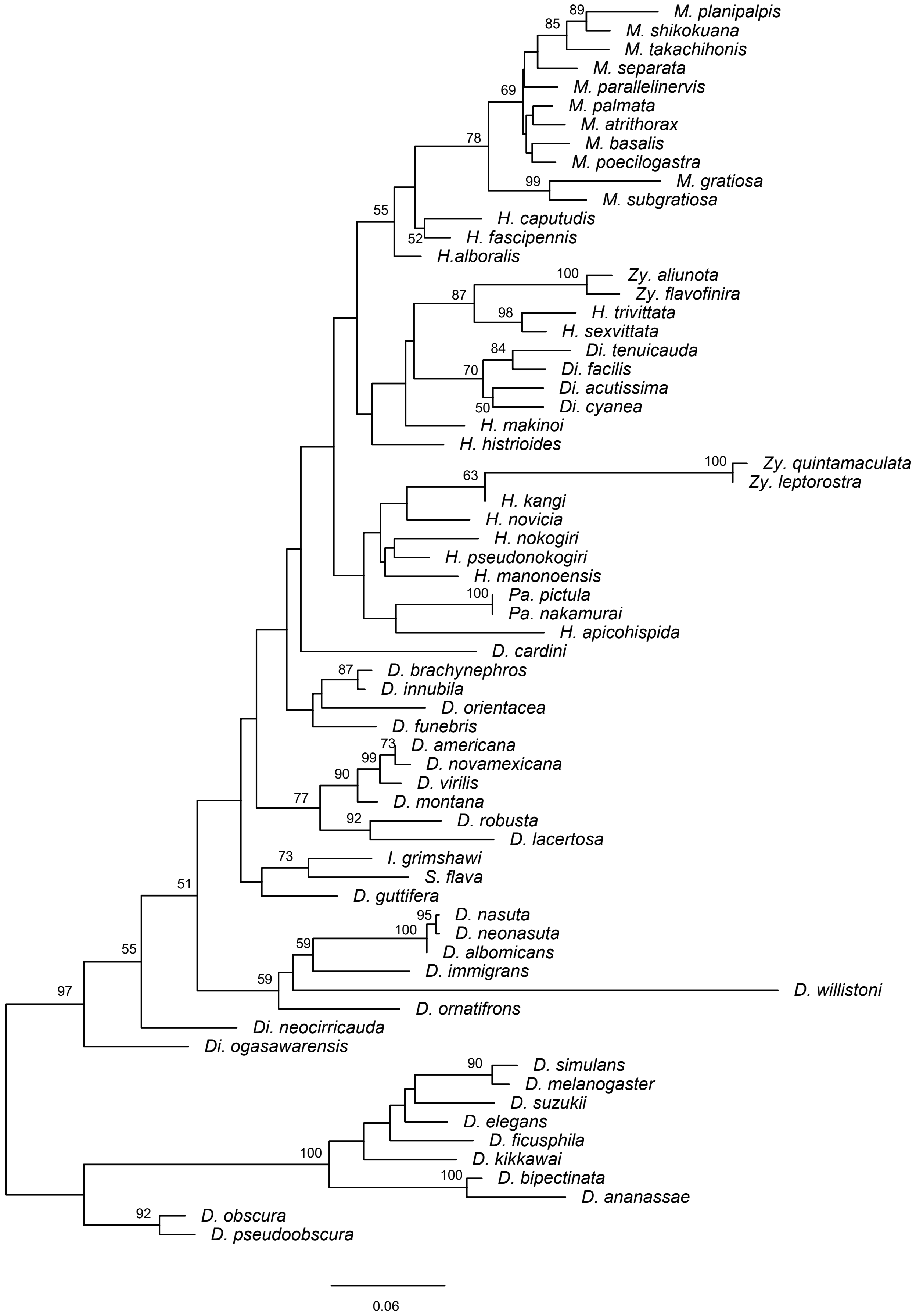
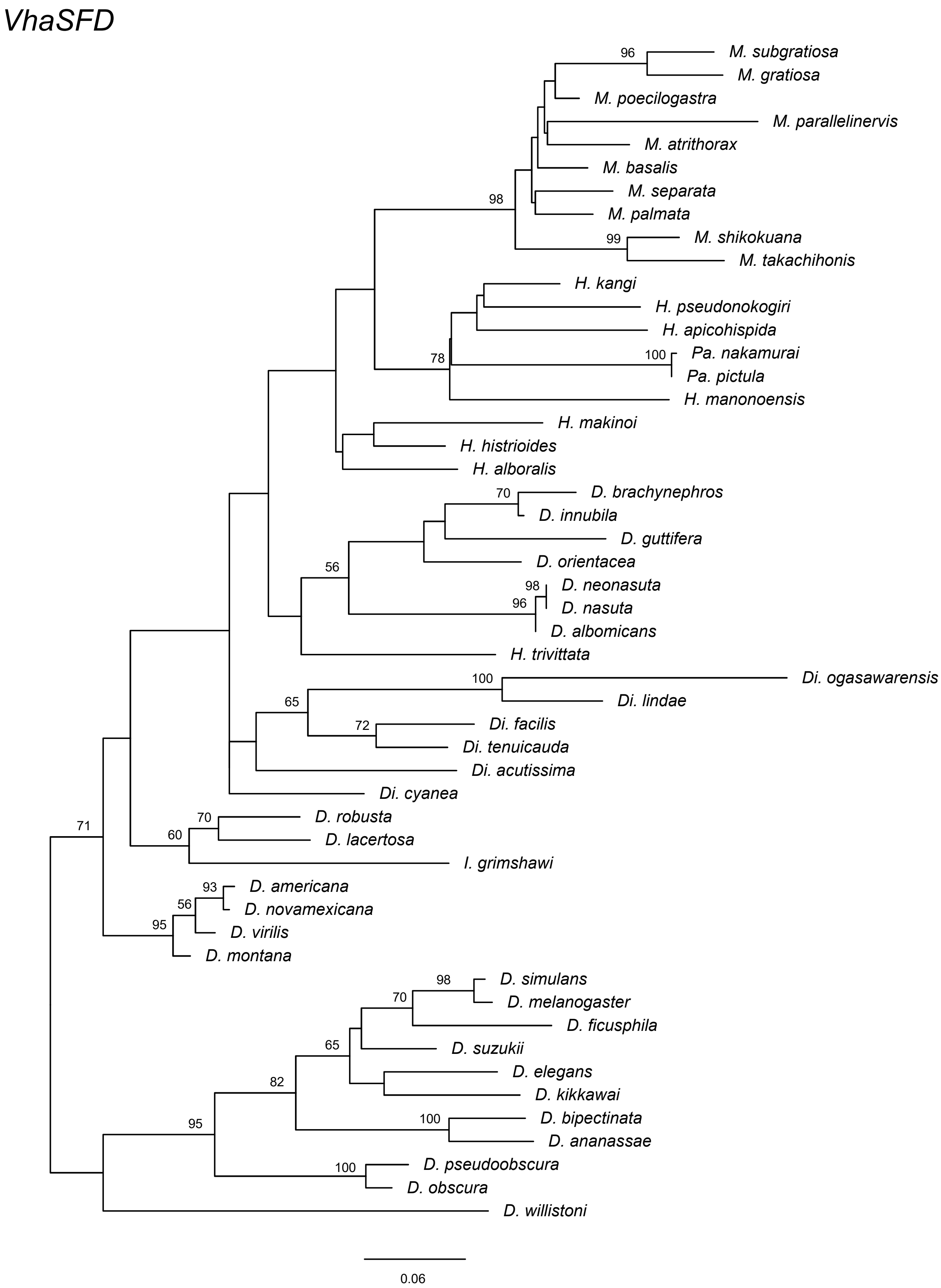

sina

Fig. 5. (*continued*)



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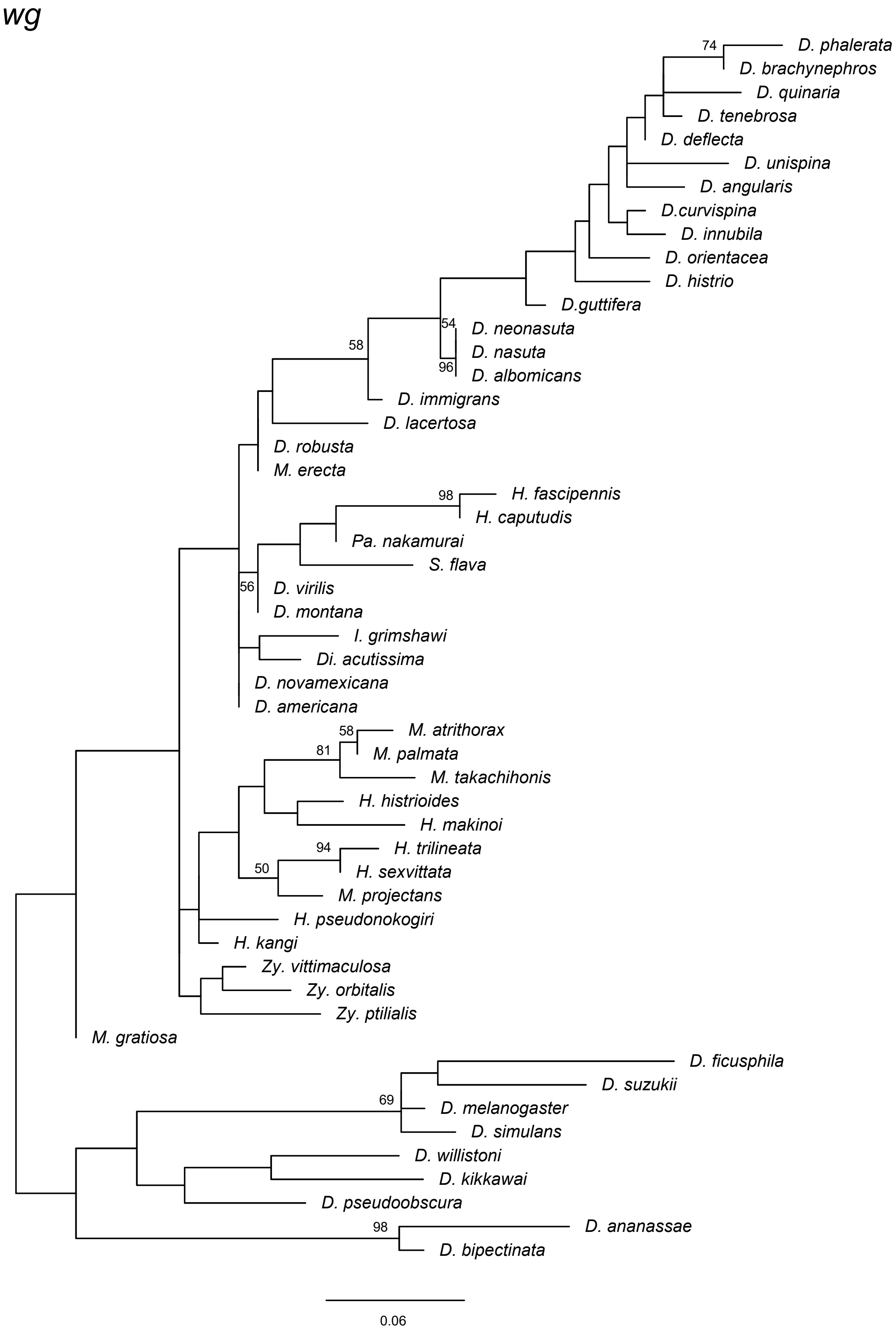

wg

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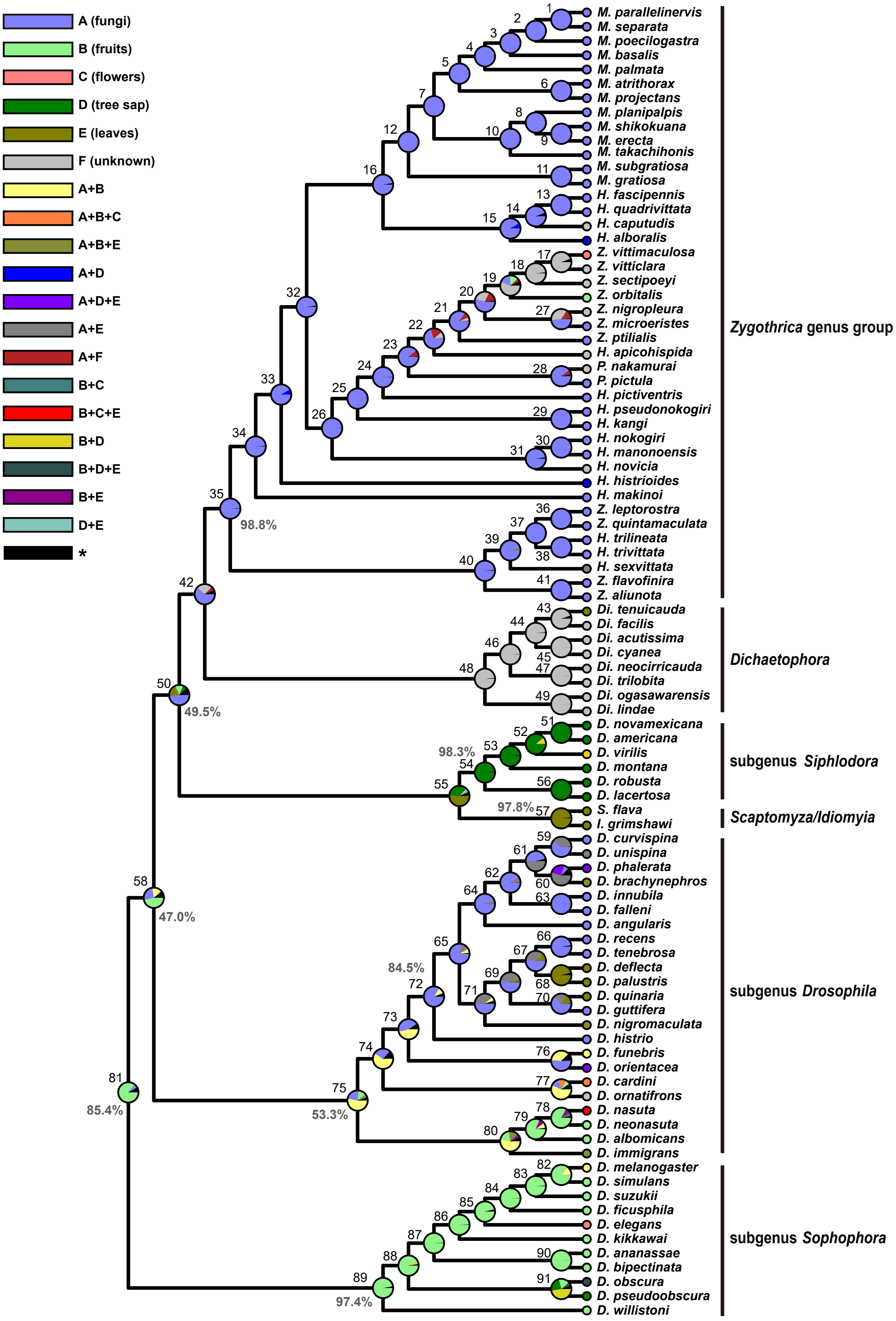
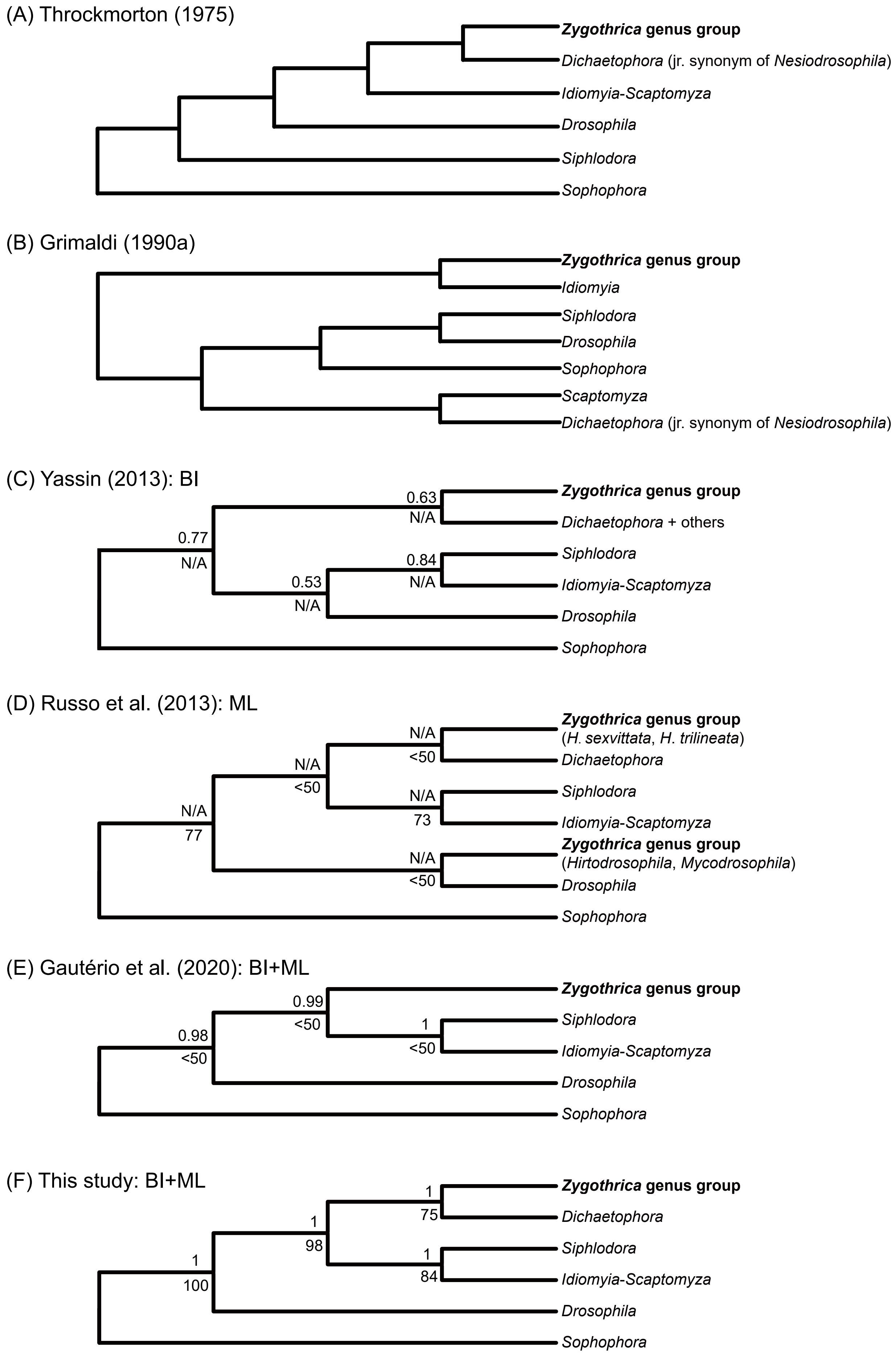


Fig. 6. Ancestral reconstruction of food habits by the BBM analysis. Nodes are numbered. Pie charts on nodes show the probabilities of possible ancestral state. *, ancestral state with relative probability <10%. The determined probability of the food habit of the MRCA at each node discussed in the text is indicated.



(A) Throckmorton (1975)

(B) Grimaldi (1990a)

(C) Yassin (2013): BI

(D) Russo et al. (2013): ML

(E) Gautério et al. (2020): BI+ML

(F) This study: BI+ML

Fig. 7. Hypotheses for the phylogenetic position of the *Zygothrica* genus group. A, Throckmorton (1975); B, Grimaldi (1990a); C, Yassin (2013); D, Russo et al. (2013); E, Gautério et al. (2020); F, the present study. Posterior probabilities for Bayesian tree and bootstrap supports for ML tree are shown above and below internal branches, respectively.

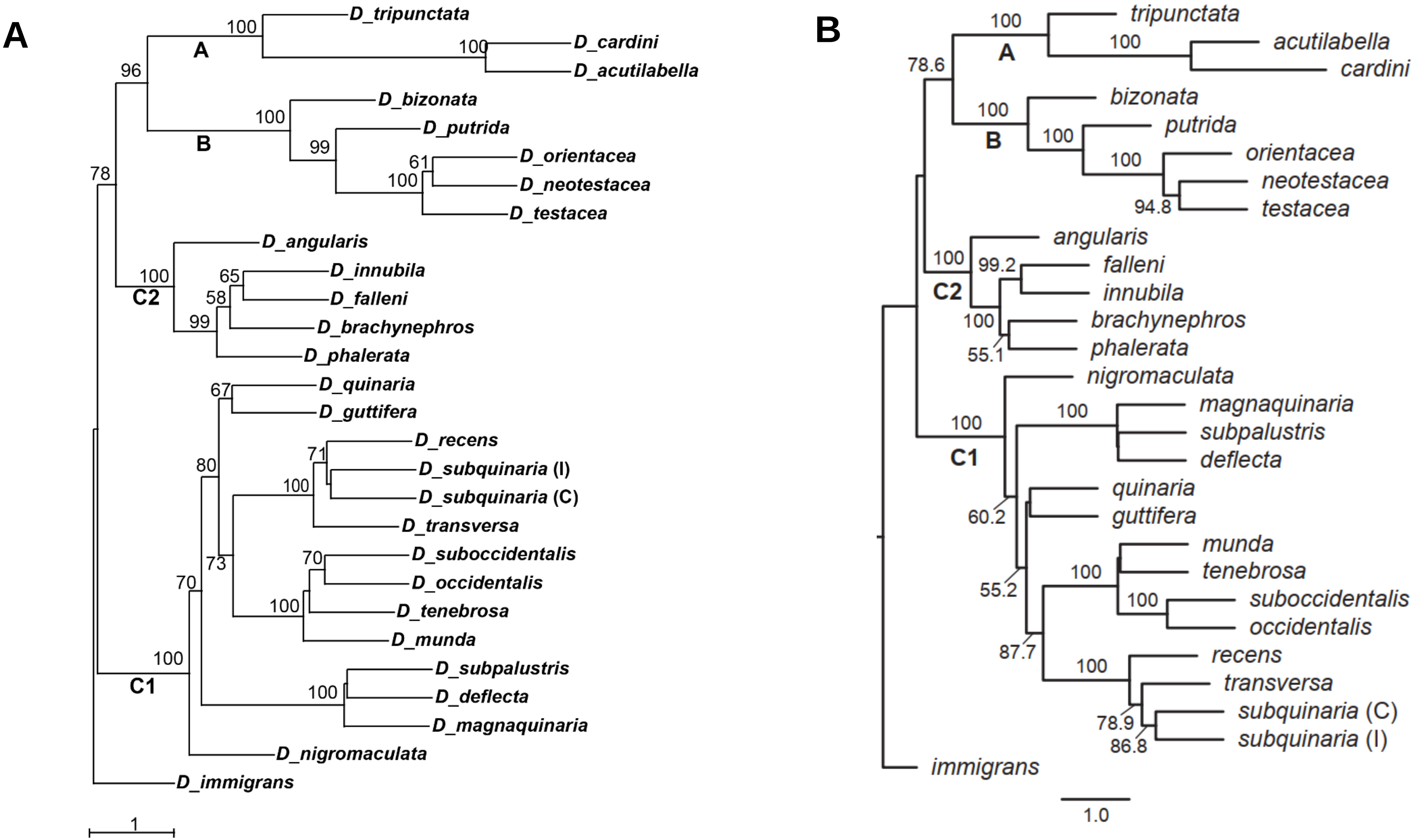


Fig. 8. (A) Species tree resulted from the ASTRAL analysis of 22 nuclear and one concatenated mitochondrial gene trees. Branch supports are indicated by bootstrap values (≥50%). (B) “Fig. 1C” of Scott Chialvo et al. (2019) that the species tree resulting from the ASTRAL analysis of all 43 gene trees.

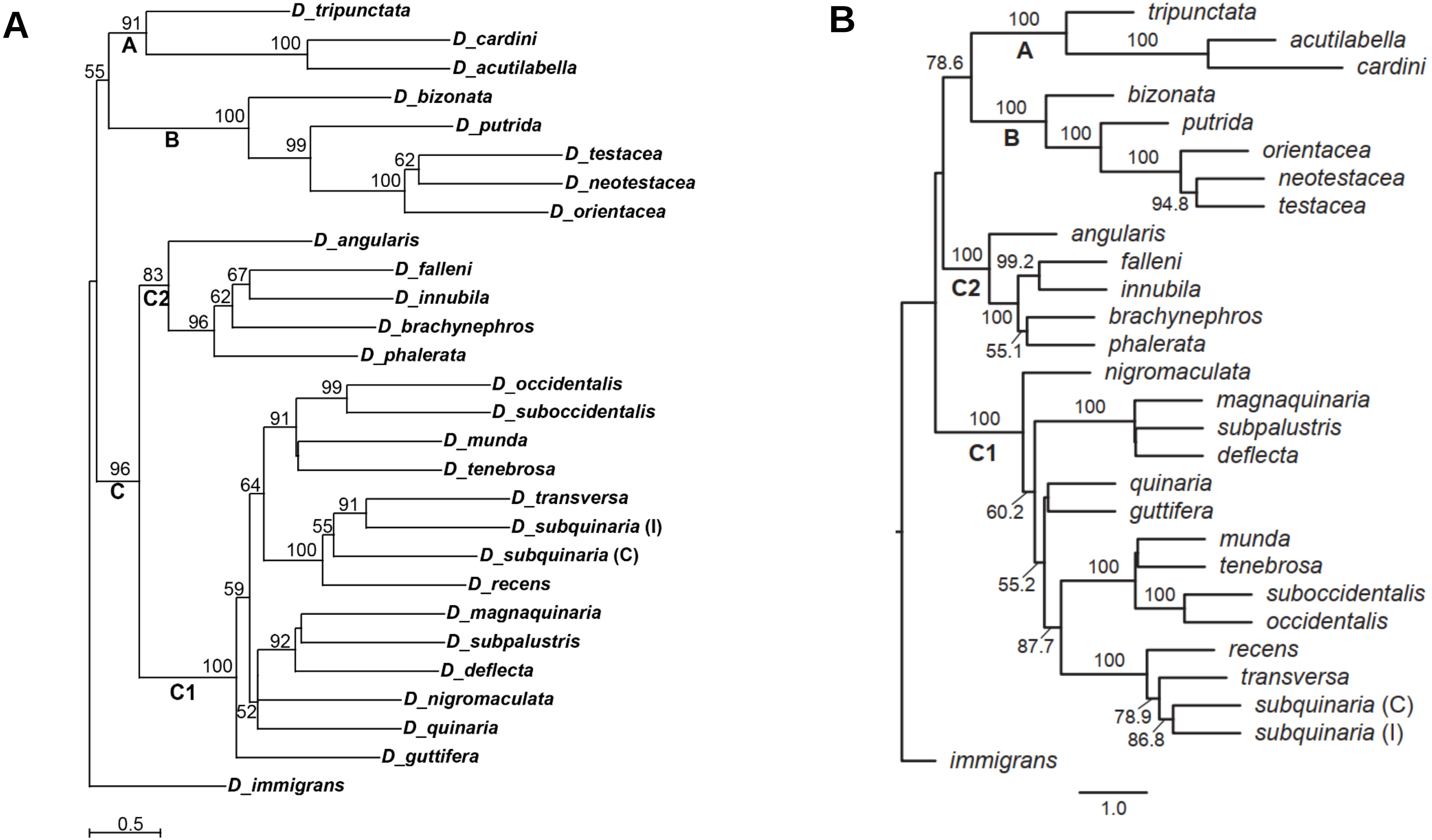


Fig. 9. (A) Species tree resulted from the ASTRAL analysis of the gene trees using 39 nuclear loci of without 3rd codons and one concatenated mitochondrial gene tree, Branch supports are indicated by bootstrap values (≥50%). (B) "Fig. 1C" of Scott Chialvo et al. (2019) that the species tree resulting from the ASTRAL analysis of all 43 gene trees.

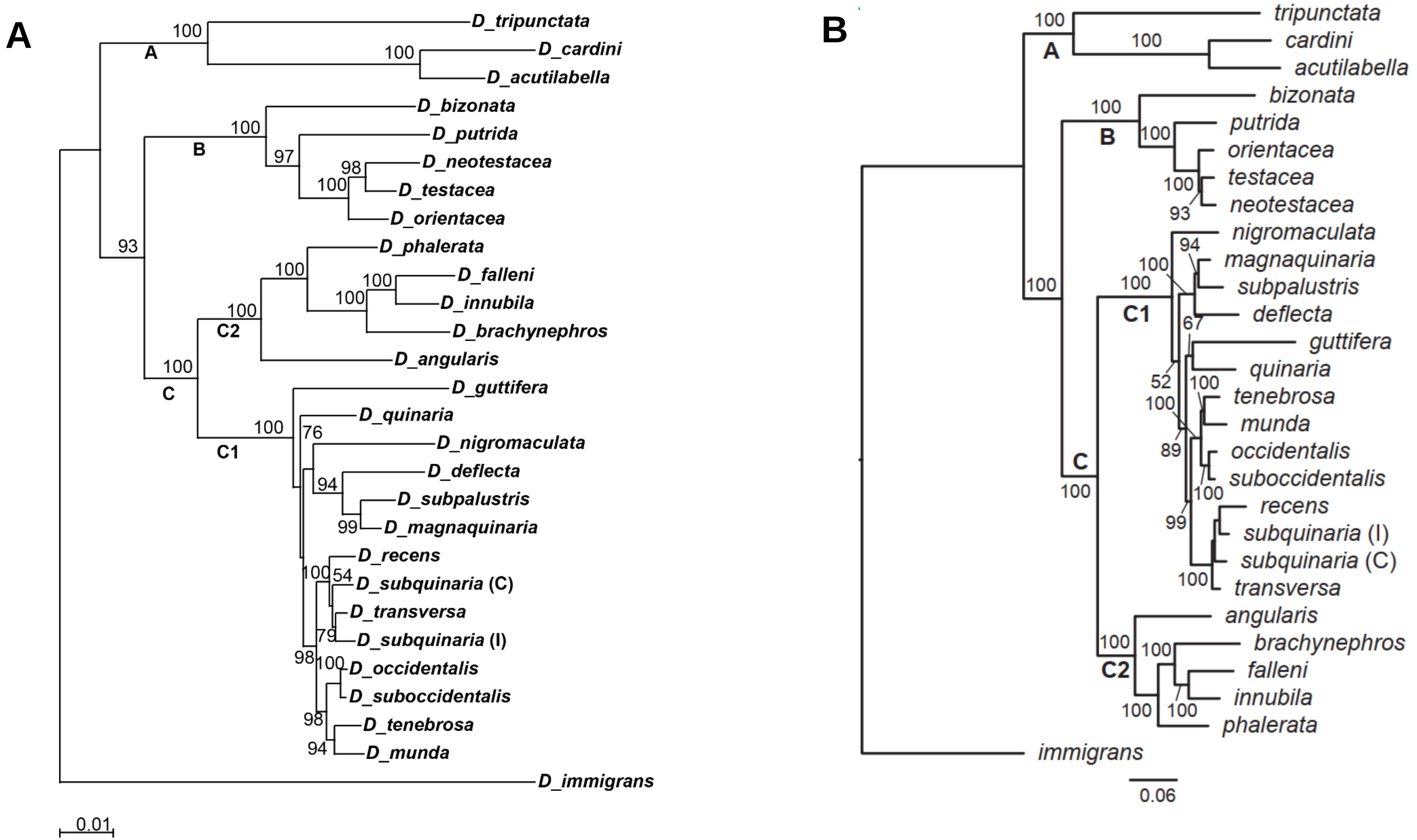


Fig. 10. (A) Phylogenetic tree constructed by the maximum likelihood (ML) analysis of the concatenated sequence dataset of 39 nuclear genes without 3rd codons and 3 mitochondrial genes. Branch supports are indicated by bootstrap values (≥50%). (B) “Fig. 1A” of Scott Chialvo et al. (2019) that the phylogenetic tree constructed by the ML analysis of the concatenated sequence dataset of 43 genes.

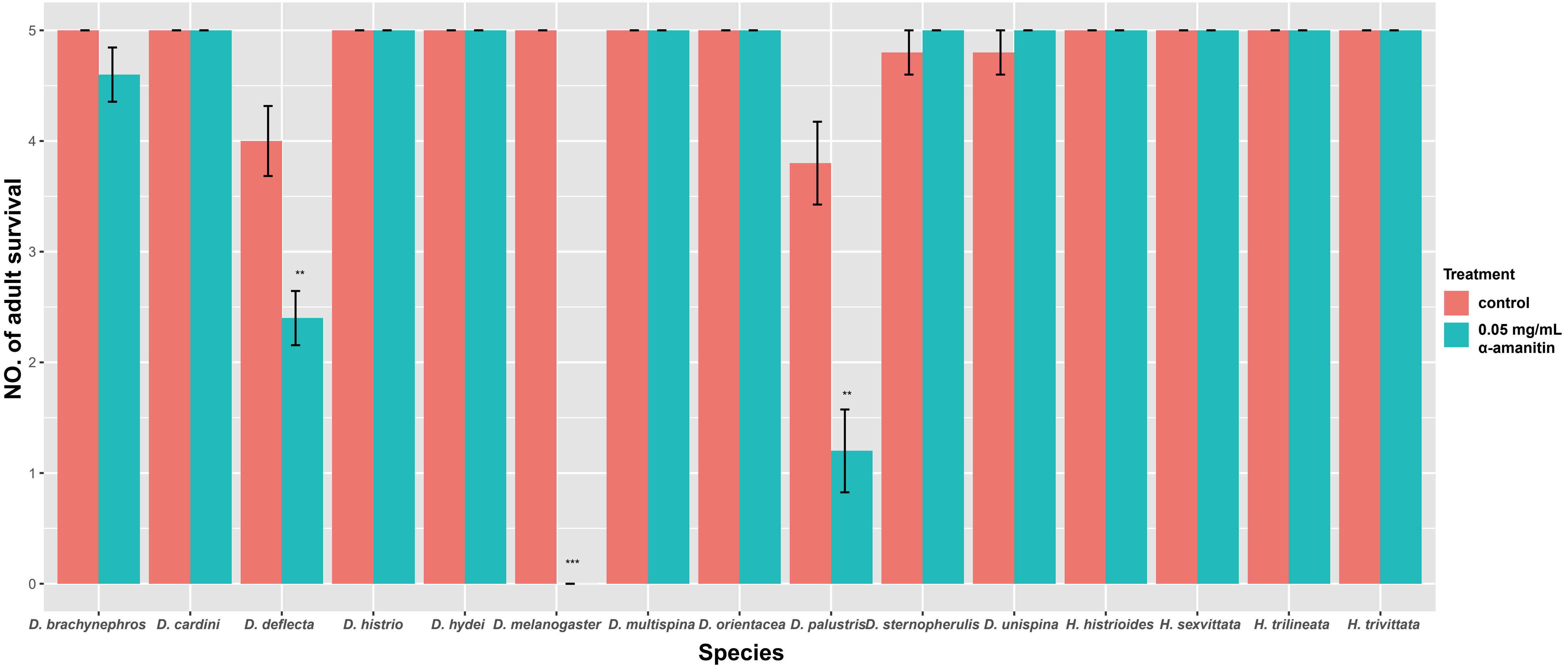


Fig. 11. The effects of deionized water and α-amanitin on survival of adult flies.

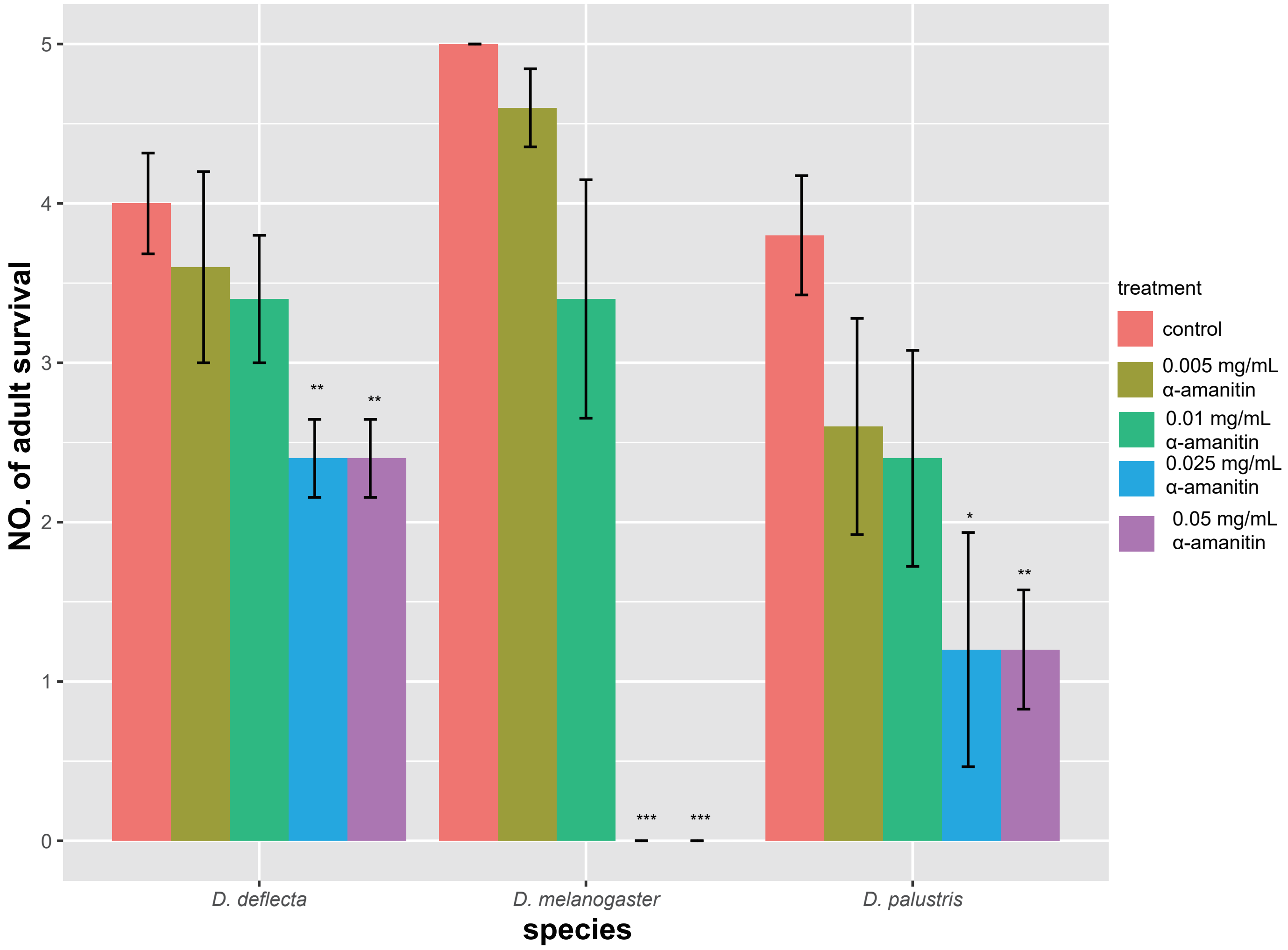


Fig. 12. The effects of deionized water and different α-amanitin concentration on survival of adult flies.

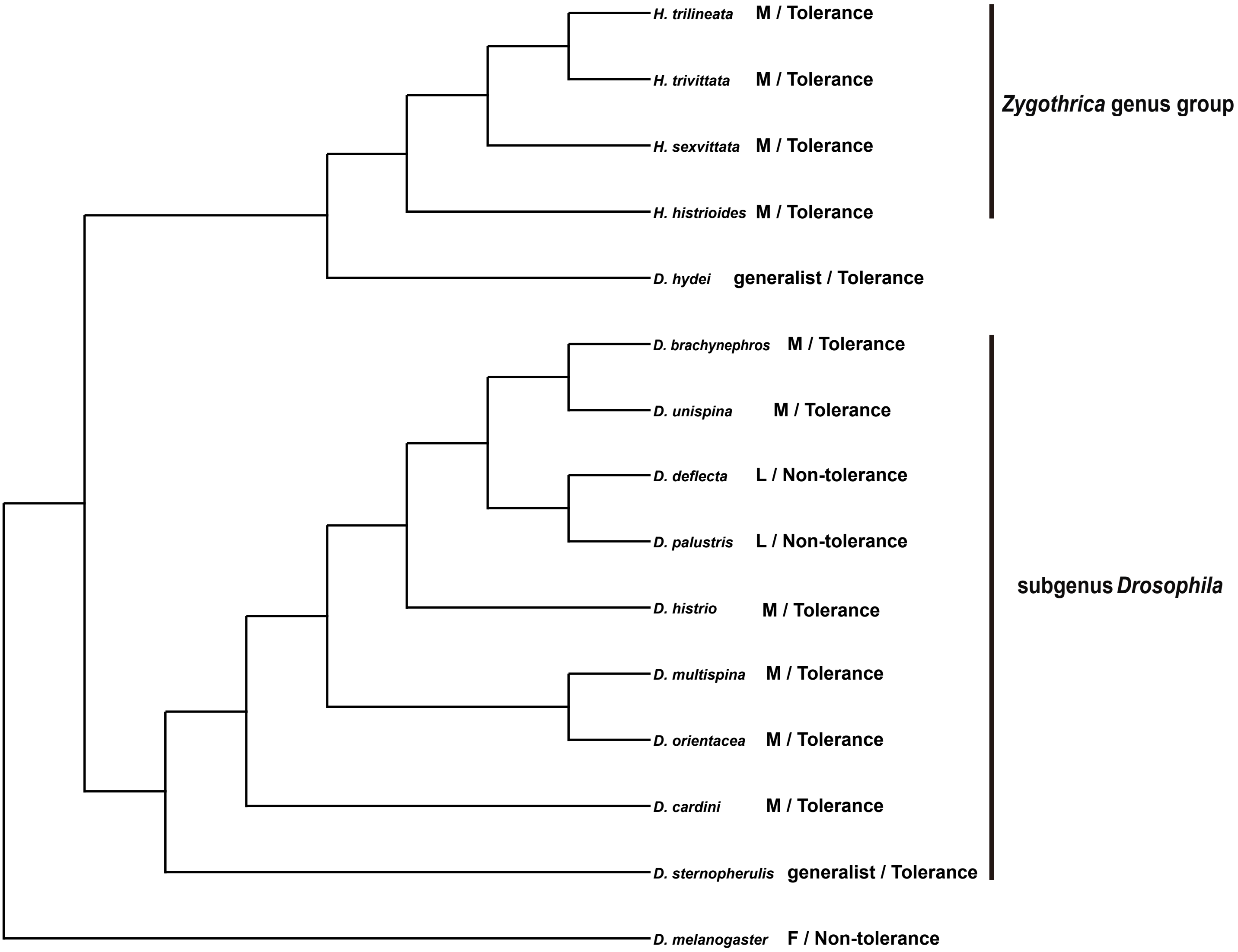


Fig. 13. A phylogeny of species tested in this study for which the α-amanitin tolerance and main food habits are known.

Table 1. Species used in this study with their Genbank accession numbers for gene *Adh*, *Adss*, *ATPsynB*, *bur*, *ced-6*, *COI*, *COII*, *Ddc*, *Dll*, *eb*, *elf3-s8* and *ERp60* (underlined for newly determined sequences; see Table 4 for the specimens used).

Species	***Adh***	***Adss***	***ATPsynB***	***bur***	***ced-6***	***COI***	***COII***	***Ddc***	***Dll***	***eb***	***elf3-s8***	***ERp60***
Ingroup												
Dichaetophora acutissima	-	MT662334	MT662435	MT662508	MT662603	-	KC192948	-	MT668855	MT648815	MT662699	-
Dichaetophora cyanea	-	MT662288	MT662389	MT662474	MT662557	-	-	-	-	-	MT662653	-
Dichaetophora facilis	-	MT662333	MT662434	MT662507	MT662602	-	-	MT668723	-	-	MT662698	-
Dichaetophora lindae	-	MT662322	MT662423	-	MT662590	-	KC192941	-	MT668856	-	MT662685	-
Dichaetophora neocirricauda	-	MT662309	MT662412	MT662489	MT662578	-	-	-	-	-	MT662673	-
Dichaetophora ogasawarensis	-	MT662277	MT662379	MT662468	MT662548	-	-	-	-	-	MT662644	-
Dichaetophora tenuicauda	-	MT662317	MT662418	MT662496	MT662586	-	KC192949	MT668724	-	MT648816	MT662680	-
Dichaetophora trilobita	-	MT662312	MT662414	MT662491	MT662581	-	KY809848	-	-	-	MT662675	-
Drosophila (*Drosophila*)	AB033642	JXOX01005004	WJDZ01000008	WJDZ01000009	JXOX01000917	AB932717	AB932762	LC058354	WJDZ01000008	JXOX01002498	ACVV01140528	LC058477
Drosophila (*Drosophila*) *angularis*	AB932646	-	-	-	-	AB932718	AB932763	MT668728	MT668859	MT668735	-	LC566650
Drosophila (*Drosophila*)	AB932649	LC567699	LC569962	-	LC570084	AB932721	AB932766	-	MT668858	MT668733	-	-
Drosophila (*Drosophila*) *cardini*	AB932650	LC567698	LC569963	LC570057	LC570085	AB932722	AB932767	MT668725	-	-	LC570119	-
Drosophila (*Drosophila*) *curvispina*	AB932652	-	-	-	-	AB932724	AB932769	-	-	MT668736	-	LC566651
Drosophila (*Drosophila*) *deflecta*	AB932653	-	-	-	-	AB932725	AB932770	LS992509	LS992508	LS992462	-	LC566652
Drosophila (*Drosophila*) *falleni*	AB932655	-	-	-	-	AB932727	AB932772	-	-	MT668737	-	LC566653
Drosophila (*Drosophila*) *funebris*	AB932656	LC567697	LC569964	-	LC570086	AB932729	AB932774	MT668726	LS992505	LS992463	LC570120	LC566654
Drosophila (*Drosophila*) *guttifera*	AB932658	LC567695	LC569966	-	LC570088	AB932731	AB932776	LS992513	LS992504	LS992464	LC570122	LC566655
Drosophila (*Drosophila*) *histrio*	AB932659	LC567694	LC569967	LC570059	LC570089	AB932732	AB932777	-	-	MT668731	-	LC566656
Drosophila (*Drosophila*) *immigrans*	M97638	LC567693	LC569968	LC570060	LC570090	AB932734	AB932779	MT668727	MT668857	MT668732	LC570123	-
Drosophila (*Drosophila*) *innubila*	AB932660	SKCT01000003	SKCT01000001	-	SKCT01000002	AB932735	AB932780	SKCT01000005	SKCT01000002	SKCT01000003	SKCT01000002	LC566657
Drosophila (*Drosophila*) *nasuta*	AB261132	LYTC01000876	LYTC01006881	LYTC01006520	LYTC01001599	AB932738	AB932783	LC058370	LYTC01004245	LYTC01002918	LYTC01010136	LC058493
Drosophila (*Drosophila*) *neonasuta*	AB261138	-	MRUE01031461	MRUE01005559	MRUE01001073	AB932740	AB932785	MRUE01025707	MRUE01001955	MRUE01012166	MRUE01008991	MRUE01011285
Drosophila (*Drosophila*)	AB932664	-	-	-	-	AB932742	AB932787	-	MT668860	MT668738	-	LC566658
Drosophila (*Drosophila*) *orientacea*	AB932665	LC567692	LC569969	-	LC570091	AB932743	AB932788	-	-	MT668734	LC570124	-
Drosophila (*Drosophila*)	AB932657	LC567696	LC569965	LC570058	LC570087	AB932730	AB932775	-	-	-	LC570121	-
Drosophila (*Drosophila*) *palustris*	LC578831	-	-	-	-	LC578832	AF147122	-	MT668864	MT668744	-	-
Drosophila (*Drosophila*) *phalerata*	AB932667	-	-	-	-	AB932746	AB932791	MT668729	-	MT668739	-	LC566659
Drosophila (*Drosophila*) *quinaria*	AB932670	-	-	-	-	AB932749	AB932794	-	MT668861	MT668740	-	LC566660
Drosophila (*Drosophila*) *recens*	AB932671	-	-	-	-	AB932750	AB932795	-	MT668862	MT668741	-	LC566661
Drosophila (*Drosophila*) *tenebrosa*	AB932676	-	-	-	-	AB932756	AB932801	MT668730	MT668863	MT668742	-	LC566662
Drosophila (*Drosophila*) *unispina*	AB932680	-	-	-	-	AB932760	AB932805	-	-	MT668743	-	LC566663
Drosophila (*Siphlodora*) *americana*	DQ471655	UEJX01006296	UEJX01006426	UEJX01001086	UEJX01001007	DQ471597	AY646730	AY165520	UEJX01001189	EU545905	CWKB01009300	UEJX01000286
Drosophila (*Siphlodora*) *lacertosa*	AY750122	SCEA01000007	SCEA01000861	SCEA01000177	SCEA01000145	DQ471590	EU493740	SCEA01000280	SCEA01000059	SCEA01000164	SCEA01000072	SCEA01000160
Drosophila (*Siphlodora*) *montana*	DQ471665	LUVX01028740	LUVX0106099	LUVX01039516	LUVX01007120	DQ471608	DQ426799	LUVX01012356	LUVX01052633	LUVX01059892	LUVX01056581	LUVX01063591
Drosophila (*Siphlodora*)	AY165542	UBJT01000306	UBJT01000104	UBJT01000678	UBJT01007204	JF735929	JF735934	AY165545	XM_030704300	UBJT01000898	UBJT01000274	XM_030704596
Drosophila (*Siphlodora*) *robusta*	AY750138	SCDW01001187	SCDW01009640	SCDW01002713	SCDW01018689	AY750112	GQ244457	AF293747	SCDV01002006	SCDW01003055	SCDV01000054	SCDW01001141
Drosophila (*Siphlodora*) *virilis*	AB033640	XM_002054109	XM_002047036	XM_002052867	XM_015168418	JQ679111	EU493791	AF293749	XM_002048839	GQ457354	XM_002049824	XM_002059295
Hirtodrosophila alboralis	LC566617	LC567700	LC569970	-	LC570092	-	LC566593	-	-	-	LC570125	-
Hirtodrosophila apicohispida	-	LC567727	-	-	LC570093	LC566147	-	-	MT668850	MT648796	LC570126	-

Table 1. (*Continued*)

Hirtodrosophila caputudis	-	LC567726	LC569971	LC570061	LC570094	LC566148	LC566594	-	MT668852	MT648799	LC570127	LC566664
Hirtodrosophila fascipennis	-	-	LC569972	LC570062	LC570095	-	LC566595	-	MT668851	MT648798	LC570128	-
Hirtodrosophila histrioides	-	LC567708	LC569973	LC570063	LC570096	LC566149	LC566596	-	MT668845	MT648792	LC570129	LC566665
Hirtodrosophila kangi	-	LC567724	-	LC570064	LC570097	LC566150	-	-	-	MT648797	LC570130	LC566666
Hirtodrosophila makinoi	-	LC567723	LC569974	LC570065	LC570098	LC566151	LC566597	-	MT675052	MT648791	LC570131	LC566667
Hirtodrosophila manonoensis	-	LC567722	LC569975	LC570066	LC570099	LC566152	LC566598	-	-	-	LC570132	LC566668
Hirtodrosophila nokogiri	-	LC567721	LC569976	LC570067	LC570100	LC566153	LC566599	-	MT668844	MT648790	LC570133	LC566669
Hirtodrosophila novicia	-	LC567720	LC569977	LC570068	LC570101	-	LC566600	-	-	-	LC570134	LC566670
Hirtodrosophila pictiventris	AF021832	-	-	-	-	-	AF478434	MT668718	-	-	-	-
Hirtodrosophila pseudonokogiri	-	LC567719	LC569978	LC570069	LC570102	LC566154	LC566601	-	MT668846	MT648793	LC570135	LC566671
Hirtodrosophila quadrivittata	LC566618	-	LC569979	LC570070	LC570103	-	LC566602	-	-	-	LC570136	-
Hirtodrosophila sexvittata	LC566619	LC567718	-	LC570071	-	LC566155	LC566603	MT668717	MT668847	MT648794	LC570137	LC566672
Hirtodrosophila trilineata	LC566620	LC567717	-	LC570072	LC570104	-	LC566604	-	MT668848	-	LC570138	LC566673
Hirtodrosophila trivittata	-	LC567716	-	LC570073	LC570105	-	-	-	MT668849	MT648795	LC570139	-
Idiomyia grimshawi	-	-	-	-	AAPT01021596	AAPT01003399	AAPT01003399	AAPT01020916	AAPT01021521	AAPT01020159	-	AAPT01021521
Mycodrosophila atrithorax	-	-	-	-	LC570106	LC566156	LC566605	-	MT668853	MT648802	LC570140	-
Mycodrosophila basalis	-	LC567715	LC569980	LC570074	-	LC566157	LC566606	-	-	-	LC570141	-
Mycodrosophila erecta	-	-	-	-	-	-	-	MT668719	-	MT648805	-	-
Mycodrosophila gratiosa	-	LC567714	LC569982	LC570075	LC570107	LC566158	LC566607	-	-	MT648804	LC570142	-
Mycodrosophila palmata	-	LC567713	LC569983	LC570076	LC570108	LC566159	-	-	-	-	LC570143	-
Mycodrosophila parallelinervis	-	LC567712	LC569984	LC570077	-	-	-	-	-	-	LC570144	-
Mycodrosophila planipalpis	-	LC567711	LC569985	-	-	-	LC566608	-	-	MT648803	LC570145	LC566674
Mycodrosophila poecilogastra	LC566621	LC567710	LC569986	-	-	LC566160	-	-	-	-	LC570146	-
Mycodrosophila projectans	-	-	-	-	-	-	-	-	-	MT648806	-	-
Mycodrosophila separata	-	LC567709	LC569987	-	LC570109	LC566161	LC566609	-	-	-	LC570147	-
Mycodrosophila shikokuana	LC566622	LC567725	LC569988	-	LC570110	LC566162	LC566610	-	-	MT648801	LC570148	LC566675
Mycodrosophila subgratiosa	-	LC567707	LC569989	-	LC570111	LC566163	LC566611	-	-	-	LC570149	-
Mycodrosophila takachihonis	LC566623	LC567706	LC569990	-	LC570112	LC566164	LC566612	-	-	MT648800	LC570150	-
Paramycodrosophila nakamurai	-	LC567705	LC569991	LC570078	LC570113	-	LC566613	-	MT668854	MT648814	LC570151	LC566677
Paramycodrosophila pictula	-	LC567704	LC569992	LC570079	LC570114	LC566165	LC566614	-	-	-	LC570152	LC566676
Scaptomyza flava	-	-	RKRM01000033	-	RKRM01000154	-	HQ170738	-	RKRM01000037	RKRM01000195	-	RKRM01000027
Zygothrica aliunota	-	LC567703	LC569993	LC570080	LC570115	-	-	-	-	-	LC570153	-
Zygothrica flavofinira	-	-	LC569994	LC570081	LC570116	LC566166	LC566615	-	-	-	LC570154	-
Zygothrica leptorostra	-	LC567702	LC569995	LC570082	LC570117	LC566166	LC566616	-	-	-	LC570155	LC566678
Zygothrica microeristes	-	-	-	-	-	-	-	-	-	MT648809	-	-
Zygothrica nigropleura	-	-	-	-	-	-	-	-	-	MT648808	-	-
Zygothrica orbitalis	-	-	-	-	-	-	-	MT668722	-	MT648813	-	-
Zygothrica ptilialis	-	-	-	-	-	-	-	MT668721	-	MT648812	-	-
Zygothrica quintamaculata	-	LC567701	LC569996	LC570083	LC570118	LC566168	-	-	-	-	LC570156	LC566679
Zygothrica sectipoeyi	-	-	-	-	-	-	-	-	-	MT648807	-	-
Zygothrica vitticlara	-	-	-	-	-	-	-	-	-	MT648810	-	-
Zygothrica vittimaculosa	-	-	-	-	-	-	KP859241	MT668720	-	MT648811	-	-

Table 1. (*Continued*)

Outgroup												
Drosophila (*Sophophora*)	EU877944	XM_001953846	XM_001956245	XM_032451841	XM_032450591	AB032132	BK006336	XM_014906446	XM_001960364	XM_032456386	XM_014907337	-
Drosophila (*Sophophora*)	DQ363233	XM_017248041	XM_017232806	XM_017251682	XM_017250479	KM486996	GQ376042	XM_017242132	XM_017241360	XM_017243153	XM_017238628	LYTD01001424
Drosophila (*Sophophora*) *elegans*	DQ363230	XM_017263664	XM_017278248	XM_017277088	XM_017264715	AB032130	AF461307	XM_017261133	XM_017269819	XM_017264390	XM_017269798	WVIB01005222
Drosophila (*Sophophora*) *ficusphila*	DQ363232	XM_017193311	XM_017184878	XM_017188364	XM_017189702	AY757285	AY757273	XM_017205407	XM_017189860	XM_017201583	XM_017194241	AFFG02007896
Drosophila (*Sophophora*) *kikkawai*	AB669864	XM_017162025	XM_017172768	XM_017176563	XM_017171783	AF050746	AB669790	XM_017178245	XM_017163475	XM_017167821	XM_017164475	AFFH02007704
Drosophila (*Sophophora*)	NM_001032099	NM_142661	NM_079287	NM_001299191	NM_001259297	EF531192	KT174472	NM_078876	NM_079133	NM_079707	NM_001299616	NM_165849
Drosophila (*Sophophora*) *obscura*	JF735883	XM_022353744	XM_022358255	XM_022368740	XM_022376162	JF735919	AF081356	-	XM_022377586	XM_022376083	XM_022371298	BDQP01000094
Drosophila (*Sophophora*)	X64470	XM_001358592	XM_001353846	XM_001357455	XM_001361512	FJ899745	AF519348	XM_015188929	XM_001361171	XM_001358017	XM_015184611	XM_002138877
Drosophila (*Sophophora*) *simulans*	GQ376038	XM_016176336	XM_002084282	XM_016177987	XM_016167172	AF200837	GQ376045	XM_016178103	XM_002083004	XM_016176314	XM_016168347	XM_002081055
Drosophila (*Sophophora*) *suzukii*	XM_017082035	XM_017087248	XM_017077551	XM_017084079	XM_017074633	KU588141	LN867083	XM_017079235	XM_017075148	XM_017080525	XM_017074593	AWUT01000945
Drosophila (*Sophophora*) *willistoni*	L08648	XM_002072120	XM_002067952	XM_002069238	XM_023175003	JQ679116	HQ110560	XM_023176532	XM_002062924	XM_002074112	XM_023174953	XM_002066199
Total (species)	48	64	64	54	66	63	72	37	48	67	67	51

Table 2. Species used in this study with their Genbank accession numbers for gene *eve*, *Gpdh*, *hh*, *not*, *Pdi*, *Pgi*, *ptc*, *RpL3*, *RpS17*, *sina*, *VhasFD*, *wg* (underlined for newly determined sequences; see Table 4 for the specimens used).

Species	***eve***	***Gpdh***	***hh***	***not***	***Pdi***	***Pgi***	***ptc***	***RpL3***	***RpS17***	***sina***	***VhasFD***	***wg***	**Total**
Ingroup													
Dichaetophora acutissima	MT668804	-	-	-	MT662754	MT662824	-	MT662908	MT662992	MT663061	MT663138	MT668900	16
Dichaetophora cyanea	-	-	-	-	-	MT662787	-	-	MT662956	MT663027	MT663106	-	9
Dichaetophora facilis	-	-	-	-	MT662753	MT662823	-	MT662907	MT662991	MT663060	MT663137	-	12
Dichaetophora lindae	-	-	MT668767	-	MT662748	MT662815	-	MT662897	MT662982	-	MT663127	-	12
Dichaetophora neocirricauda	-	-	-	-	-	MT662804	-	MT662887	-	MT663042	-	-	8
Dichaetophora ogasawarensis	-	-	-	-	MT662725	MT662783	-	MT662860	MT662947	MT663021	MT663096	-	11
Dichaetophora tenuicauda	MT668805	-	MT668766	MT668879	MT662746	MT662810	MT668829	MT662893	MT662978	MT663048	MT663125	-	18
Dichaetophora trilobita	-	-	-	-	MT662743	-	-	-	MT662973	-	-	-	8
Drosophila (*Drosophila*) *albomicans*	WJDZ01000008	AB261148	WJDZ01000006	WJDZ01000008	WJDZ01000008	-	WJDZ01000008	WJDZ01000006	WJDZ01000008	WJDZ01000008	JXOX01036341	WJDZ01000009	23
Drosophila (*Drosophila*) *angularis*	-	AB932682	MT668773	-	-	-	MT668835	-	-	-	-	MT668906	11
Drosophila (*Drosophila*) *brachynephros*	MT668807	AB932685	MT668771	-	LC570157	LC570275	MT668833	LC570310	LC570342	LC570380	LC570417	MT668904	19
Drosophila (*Drosophila*) *cardini*	MT668806	AB932686	MT668768	MT668880	LC570158	LC570276	MT668830	-	LC570343	LC570381	-	-	18
Drosophila (*Drosophila*) *curvispina*	MT668809	AB932688	MT668774	-	-	-	MT668836	-	-	-	-	MT668907	10
Drosophila (*Drosophila*) *deflecta*	LS992280	AB932689	LS992521	LS992527	-	-	LS992530	-	-	-	-	MT668908	13
Drosophila (*Drosophila*) *falleni*	MT668810	AB932691	MT668775	-	-	-	MT668837	-	-	-	-	-	9
Drosophila (*Drosophila*) *funebris*	LS992281	AB932692	LS992522	-	LC570159	-	LS992534	LC570311	LC570344	LC570382	-	-	19
Drosophila (*Drosophila*) *guttifera*	LS992282	AB261155	LS992526	LS992528	-	-	LS992531	LC570312	LC570346	LC570384	LC570419	MT668901	21
Drosophila (*Drosophila*) *histrio*	-	AB932694	MT668769	-	LC570161	LC570277	MT668831	-	LC570347	-	-	MT668902	16
Drosophila (*Drosophila*) *immigrans*	-	AB261142	MT668770	-	LC570162	LC570278	MT668832	LC570313	LC570348	LC570385	-	MT668903	20
Drosophila (*Drosophila*) *innubila*	SKCT01000002	AB932695	SKCT01000003	-	SKCT01000001	SKCT01000002	SKCT01000002	SKCT01000003	SKCT01000001	SKCT01000001	SKCT01000005	SKCT01000005	22
Drosophila (*Drosophila*) *nasuta*	LYTC01015811	AB261144	LYTC01030748	LYTC01007632	LYTC01000985	-	LYTC01006470	LYTC01010173	LYTC01000499	LYTC01013528	LYTC01017694	LYTC01039268	23
Drosophila (*Drosophila*) *neonasuta*	MRUE01007099	AB261151	MRUE01000218	MRUE01030378	MRUE01002523	-	MRUE01011047	MRUE01002577	MRUE01003861	MRUE01017655	MRUE01017952	MRUE01000502	22
Drosophila (*Drosophila*) *nigromaculata*	MT668811	AB932699	MT668776	-	-	-	MT668838	-	-	-	-	-	10
Drosophila (*Drosophila*) *orientacea*	MT668808	AB932700	MT668772	MT668881	LC570163	LC570279	MT668834	LC570314	LC570349	LC570386	LC570419	MT668905	20
Drosophila (*Drosophila*) *ornatifrons*	-	AB932693	-	-	LC570160	-	-	-	LC570345	LC570383	-	-	12
Drosophila (*Drosophila*) *palustris*	MT668816	-	MT668782	-	-	-	MT668843	-	-	-	-	-	8
Drosophila (*Drosophila*) *phalerata*	MT668812	AB932702	MT668777	-	-	-	MT668839	-	-	-	-	MT668909	11
Drosophila (*Drosophila*) *quinaria*	MT668813	AB932705	MT668778	-	-	-	MT668840	-	-	-	-	MT668910	11
Drosophila (*Drosophila*) *recens*	MT668814	AB932706	MT668779	-	-	-	MT668841	-	-	-	-	-	10
Drosophila (*Drosophila*) *tenebrosa*	MT668815	AB932711	MT668780	-	-	-	MT668842	-	-	-	-	MT668911	12
Drosophila (*Drosophila*) *unispina*	-	AB932715	MT668781	-	-	-	-	-	-	-	-	MT668912	8
Drosophila (*Siphlodora*) *americana*	UEJX01003354	D50090	CWKC01026616	CWKB01013232	CWKC01021563	UEJX01000181	UEJX01000412	UEJX01004833	CWKC01020879	CWKB01013066	UEJX01000173	UEJX01005536	24
Drosophila (*Siphlodora*) *lacertosa*	SCEA01000059	SCEA01000012	SCEA01000090	SCEA01002385	SCEA01000636	SCEA01000067	SCEA01000072	SCEA01000600	SCEA01000587	SCEA01000783	SCEA01000063	SCEA01000379	24
Drosophila (*Siphlodora*) *montana*	LUVX01033013	AB019546	LUVX01001536	LUVX01031507	LUVX01045422	LUVX01001693	LUVX01005664	LUVX01059791	LUVX01020304	LUVX01015468	LUVX01007897	LUVX01022437	24
Drosophila (*Siphlodora*) *novamexicana*	XM_030704645	D50088	XM_030713346	XM_030714313	UBJT01000066	UBJT01000231	XM_030711724	UBJT01000166	UBJT01000735	UBJT01006387	UBJT01000601	UBJT01003193	24
Drosophila (*Siphlodora*) *robusta*	SCDW01004306	-	-	SCDV01004749	SCDW01014522	SCDW01000546	-	SCDW01002708	SCDW01017556	SCDW01023573	SCDW01000114	SCDW01000460	21
Drosophila (*Siphlodora*) *virilis*	XM_002048819	D10697	XM_002054702	XM_032434859	XM_002048180	XM_002048878	XM_002049158	XM_002053569	XM_002047228	XM_002046652	XM_015172508	XM_002052538	24
Hirtodrosophila alboralis	-	-	MT668754	-	-	-	-	LC570315	LC570350	LC570387	LC570420	-	11
Hirtodrosophila apicohispida	-	LC566624	MT668752	MT668869	LC570164	LC570280	-	-	LC570351	LC570388	LC570421	-	14

Table 2. (*Continued*)

Hirtodrosophila caputudis	MT668790	-	-	-	-	LC570281	-	LC570316	LC570352	LC570389	-	MT668889	16
Hirtodrosophila fascipennis	MT668789	LC566625	-	-	LC570165	LC570282	-	LC570317	LC570353	LC570390	-	MT668888	15
Hirtodrosophila histrioides	-	LC566626	MT668747	-	LC570166	LC570283	-	LC570318	LC570354	LC570391	LC570422	MT668883	19
Hirtodrosophila kangi	MT668788	LC566627	MT668753	-	LC570167	LC570284	-	-	LC570355	LC570392	LC570423	MT668887	16
Hirtodrosophila makinoi	MT668783	-	MT668746	-	LC570168	LC570285	MT668817	LC570319	LC570356	LC570393	LC570424	MT668882	20
Hirtodrosophila manonoensis	-	LC566628	-	-	LC570169	LC570286	-	LC570320	LC570357	LC570394	LC570425	-	15
Hirtodrosophila nokogiri	-	LC566629	MT668745	MT668865	-	LC570287	-	LC570321	LC570358	LC570395	-	-	17
Hirtodrosophila novicia	-	LC566630	-	-	LC570170	LC570288	-	LC570322	LC570359	LC570396	-	-	13
Hirtodrosophila pictiventris	-	L41649	-	-	-	-	-	-	-	-	-	-	4
Hirtodrosophila pseudonokogiri	MT668784	LC566631	MT668748	MT668866	LC570171	LC570289	-	-	LC570360	LC570397	LC570426	MT668884	20
Hirtodrosophila quadrivittata	-	-	-	-	-	LC570290	-	LC570323	-	-	-	-	8
Hirtodrosophila sexvittata	MT668785	LC566632	MT668749	MT668867	-	LC570291	MT668818	LC570324	LC570361	LC570398	-	MT668885	20
Hirtodrosophila trilineata	MT668786	-	MT668750	MT668868	-	LC570292	MT668819	LC570325	LC570362	-	-	MT668886	16
Hirtodrosophila trivittata	MT668787	-	MT668751	-	LC570172	LC570293	-	LC570326	LC570363	LC570399	LC570427	-	14
Idiomyia grimshawi	AAPT01021521	AAPT01021686	AAPT01020215	AAPT01020541	AAPT01020645	-	-	AAPT01018771	AAPT01020574	AAPT01020608	AAPT01021727	AAPT01020969	17
Mycodrosophila atrithorax	MT668793	LC566633	MT668757	MT668872	LC570173	LC570294	MT668821	LC570327	LC570364	LC570400	LC570428	MT668891	18
Mycodrosophila basalis	MT668794	LC566634	-	MT668874	LC570174	LC570295	MT668822	LC570328	LC570365	LC570401	LC570429	-	16
Mycodrosophila erecta	MT668796	-	-	MT668875	-	-	MT668826	-	-	-	-	MT668894	6
Mycodrosophila gratiosa	-	LC566635	MT668760	-	LC570175	LC570296	MT668824	LC570329	LC570366	LC570402	LC570430	MT668893	18
Mycodrosophila palmata	MT668795	LC566636	MT668759	-	LC570176	LC570297	MT668823	-	LC570367	LC570403	LC570431	MT668892	16
Mycodrosophila parallelinervis	-	LC566637	-	-	LC570177	LC570298	-	LC570330	LC570368	LC570404	LC570432	-	11
Mycodrosophila planipalpis	-	LC566638	MT668758	MT668873	LC570178	LC570299	-	LC570331	LC570369	LC570405	-	-	14
Mycodrosophila poecilogastra	-	LC566639	-	-	-	LC570300	-	LC570332	LC570370	LC570406	LC570433	-	11
Mycodrosophila projectans	MT668797	-	MT668762	MT668876	-	-	MT668827	-	-	-	-	MT668895	6
Mycodrosophila separata	-	LC566640	-	-	LC570179	LC570301	-	LC570333	-	LC570407	LC570434	-	12
Mycodrosophila shikokuana	MT668792	LC566641	MT668756	MT668871	LC570180	LC570302	-	LC570334	LC570371	LC570408	LC570435	-	19
Mycodrosophila subgratiosa	-	LC566642	MT668761	-	LC570181	LC570303	MT668825	LC570335	LC570372	LC570409	LC570436	-	15
Mycodrosophila takachihonis	MT668791	LC566643	MT668755	MT668870	LC570182	LC570304	MT668820	-	LC570373	LC570410	LC570437	MT668890	19
Paramycodrosophila nakamurai	MT668803	LC566644	MT668765	-	-	LC570305	-	LC570336	LC570374	LC570411	LC570438	MT668899	18
Paramycodrosophila pictula	-	LC566645	-	-	LC570183	LC570306	-	LC570337	LC570375	LC570412	LC570439	-	15
Scaptomyza flava	RKRM01000037	-	-	-	-	-	-	RKRM01000038	RKRM01000007	RKRM01000211	-	RKRM01000004	11
Zygothrica aliunota	-	LC566646	-	-	-	-	-	LC570338	LC570376	LC570413	-	-	9
Zygothrica flavofinira	-	LC566647	-	-	-	LC570307	-	LC570339	LC570377	LC570414	-	-	11
Zygothrica leptorostra	-	LC566648	-	-	LC570184	LC570308	-	LC570340	LC570378	LC570415	-	-	14
Zygothrica microeristes	MT668798	-	-	-	-	-	-	-	-	-	-	-	2
Zygothrica nigropleura	-	-	-	-	-	-	-	-	-	-	-	-	1
Zygothrica orbitalis	MT668802	-	MT668764	MT668878	-	-	MT668828	-	-	-	-	MT668898	7
Zygothrica ptilialis	MT668801	-	MT668763	MT668877	-	-	-	-	-	-	-	MT668897	6
Zygothrica quintamaculata	-	LC566649	-	-	LC570185	LC570309	-	LC570341	LC570379	LC570416	-	-	13
Zygothrica sectipoeyi	-	-	-	-	-	-	-	-	-	-	-	-	1
Zygothrica vitticlara	MT668799	-	-	-	-	-	-	-	-	-	-	-	2
Zygothrica vittimaculosa	MT668800	-	-	-	-	-	-	-	-	-	-	MT668896	5

Table 2. (*Continued*)

Outgroup												
Drosophila (*Sophophora*) *ananassae*	XM_001960811	XM_014906626	XM_014911255	XM_001957909	XM_001958056	XM_014907813	HQ631896	XM_032449792	XM_001956299	XM_001957583	XM_014905103	XM_001963224	23
Drosophila (*Sophophora*) *bipectinata*	XM_017241031	XM_017243708	XM_017248274	XM_017246017	XM_017252234	XM_017250312	XM_017250526	XM_022354273	XM_017254053	XM_017232129	XM_017233435	XM_017243470	24
Drosophila (*Sophophora*) *elegans*	XM_017264555	XM_017265037	XM_017276166	-	XM_017257102	XM_017277501	HQ631907	XM_017258430	XM_017266930	XM_017272838	XM_017278706	XM_017260177	23
Drosophila (*Sophophora*) *ficusphila*	XM_017189430	XM_017185792	XM_017190981	XM_017187931	XM_017197935	XM_017189040	HQ631909	XM_017184640	XM_017185010	XM_017187627	XM_017200930	XM_017193337	24
Drosophila (*Sophophora*) *kikkawai*	XM_017171561	XM_017166662	-	XM_017173024	XM_017164046	XM_017177821	HQ631912	XM_017181267	XM_017173016	XM_017172646	XM_017176591	XM_017164159	23
Drosophila (*Sophophora*) *melanogaster*	NM_078946	NM_001273184	NM_001038976	NM_168643	NM_001300141	NM_165636	AH001024	NM_169378	NM_079278	NM_001300160	NM_001273581	NM_078778	24
Drosophila (*Sophophora*) *obscura*	XM_022373075	XM_022373127	XM_022359851	-	XM_022355574	XM_022370200	XM_022364580	XM_022354273	XM_022365742	XM_022359840	XM_022372373	-	21
Drosophila (*Sophophora*) *pseudoobscura*	XM_001361190	XM_033379246	XM_001358266	XM_015188357	XM_001353135	XM_015184900	XM_001361482	XM_001359900	XM_001354266	XM_001352776	XM_015180465	XM_001356360	24
Drosophila (*Sophophora*) *simulans*	XM_016183263	XM_016179628	XM_016174893	XM_002085009	XM_002084896	DSU20560	HQ631935	XM_002103859	XM_002084225	XM_016171608	XM_016178194	XM_016184008	24
Drosophila (*Sophophora*) *suzukii*	XM_017071991	XM_017083167	XM_017076039	CAKG01042255	XM_017080289	XM_017085223	HQ631937	XM_017069449	XM_017078548	XM_017088090	XM_017080790	XM_017083763	24
Drosophila (*Sophophora*) *willistoni*	XM_002062900	XM_023175947	XM_002073983	XM_023178055	XM_002068439	XM_023182072	XM_002063044	XM_023179974	XM_002062454	XM_002065898	XM_015178369	XM_002066877	24
Total (species)	60	66	61	38	57	60	50	61	68	66	51	53	

Table 3. Species used in this study with their food habits.

Species	Food†	Species	Food†
Ingroup		*Hirtodrosophila novicia*	?
Dichaetophora acutissima	?	*Hirtodrosophila pictiventris*	M
Dichaetophora cyanea	?	*Hirtodrosophila pseudonokogiri*	M
Dichaetophora facilis	?	*Hirtodrosophila quadrivittata*	M
Dichaetophora lindae	?	*Hirtodrosophila sexvittata*	Ml
Dichaetophora neocirricauda	?	*Hirtodrosophila trilineata*	M
Dichaetophora ogasawarensis	?	*Hirtodrosophila trivittata*	M
Dichaetophora tenuicauda	L	*Idiomyia grimshawi*	L
Dichaetophora trilobita	?	*Mycodrosophila atrithorax*	M
Drosophila (*Drosophila*) *albomicans*	F	*Mycodrosophila basalis*	M
Drosophila (*Drosophila*) *angularis*	M	*Mycodrosophila erecta*	M
Drosophila (*Drosophila*) *brachynephros*	Mlf	*Mycodrosophila gratiosa*	M
Drosophila (*Drosophila*) *cardini*	FMB	*Mycodrosophila palmata*	M
Drosophila (*Drosophila*) *curvispina*	M	*Mycodrosophila parallelinervis*	M
Drosophila (*Drosophila*) *deflecta*	L	*Mycodrosophila planipalpis*	M
Drosophila (*Drosophila*) *falleni*	M	*Mycodrosophila poecilogastra*	M
Drosophila (*Drosophila*) *funebris*	Mf	*Mycodrosophila projectans*	M
Drosophila (*Drosophila*) *guttifera*	M	*Mycodrosophila separata*	M
Drosophila (*Drosophila*) *histrio*	M	*Mycodrosophila shikokuana*	M
Drosophila (*Drosophila*) *immigrans*	Fml	*Mycodrosophila subgratiosa*	M
Drosophila (*Drosophila*) *innubila*	M	*Mycodrosophila takachihonis*	M
Drosophila (*Drosophila*) *nasuta*	Fbl	*Paramycodrosophila nakamurai*	?
Drosophila (*Drosophila*) *neonasuta*	F	*Paramycodrosophila pictula*	M
Drosophila (*Drosophila*) *nigromaculata*	Lfm	*Scaptomyza flava*	L
Drosophila (*Drosophila*) *orientacea*	Mlt	*Zygothrica aliunota*	M
Drosophila (*Drosophila*) *ornatifrons*	?	*Zygothrica flavofinira*	M
Drosophila (*Drosophila*) *palustris*	?	*Zygothrica leptorostra*	M
Drosophila (*Drosophila*) *phalerata*	Mlt	*Zygothrica microeristes*	M
Drosophila (*Drosophila*) *quinaria*	L	*Zygothrica nigropleura*	?
Drosophila (*Drosophila*) *recens*	M	*Zygothrica orbitalis*	F
Drosophila (*Drosophila*) *tenebrosa*	M	*Zygothrica ptilialis*	M
Drosophila (*Drosophila*) *unispina*	Ml	*Zygothrica quintamaculata*	M
Drosophila (*Siphlodora*) *americana*	T	*Zygothrica sectipoeyi*	?
Drosophila (*Siphlodora*) *lacertosa*	T	*Zygothrica vitticlara*	?
Drosophila (*Siphlodora*) *montana*	T	*Zygothrica vittimaculosa*	B
Drosophila (*Siphlodora*) *novamexicana*	T	Outgroup	
Drosophila (*Siphlodora*) *robusta*	T	*Drosophila* (*Sophophora*) *ananassae*	F
Drosophila (*Siphlodora*) *virilis*	Tf	*Drosophila* (*Sophophora*) *bipectinata*	F
Hirtodrosophila alboralis	Mt	*Drosophila* (*Sophophora*) *elegans*	B
Hirtodrosophila apicohispida	?	*Drosophila* (*Sophophora*) *ficusphila*	F
Hirtodrosophila caputudis	?	*Drosophila* (*Sophophora*) *kikkawai*	F
Hirtodrosophila fascipennis	M	*Drosophila* (*Sophophora*) *melanogaster*	Fm
Hirtodrosophila histrioides	Mt	*Drosophila* (*Sophophora*) *obscura*	Tfl
Hirtodrosophila kangi	M	*Drosophila* (*Sophophora*) *pseudoobscura*	T
Hirtodrosophila makinoi	M	*Drosophila* (*Sophophora*) *simulans*	F
Hirtodrosophila manonoensis	M	*Drosophila* (*Sophophora*) *suzukii*	F
Hirtodrosophila nokogiri	M	*Drosophila* (*Sophophora*) *willistoni*	F

† M: fungi, F: fruits, B: flowers, T: tree sap, L: leaves, ?: unknow; capital: primary food, lowercase: secondary one.

Table 4. Sources of specimens used for newly sequencing some of the marker genes in this study.

Genus	Subgenus	Species group	Species subgroup	Species	Distribution[†]	Collection locality/Culture strain[‡]
Drosophila	*Drosophila*	*cardini*		*cardini*	NE, NT	from DSSC (15181–2181.03)
Drosophila	*Drosophila*	*funebris*		*funebris*	PA	from TMU
Drosophila	*Drosophila*	*guarani*	*guarani*	*ornatifrons*	NT	from DSSC (15172–2151.00)
Drosophila	*Drosophila*	*guttifera*		*guttifera*	NE	from TMU
Drosophila	*Drosophila*	*histrio*		*histrio*	PA, OR	Sapporo, Hokkaido, JAPAN
Drosophila	*Drosophila*	*immigrans*	*immigrans*	*immigrans*	Cosmopolitan	from TMU
Drosophila	*Drosophila*	*quinaria*		*angularis*	PA, OR	from TMU
Drosophila	*Drosophila*	*quinaria*		*brachynephros*	PA, OR	Sapporo, Hokkaido, JAPAN
Drosophila	*Drosophila*	*quinaria*		*curvispina*	PA	Sapporo, Hokkaido, JAPAN
Drosophila	*Drosophila*	*quinaria*		*deflecta*	NE	from Dr. Shigeyuki Koshikawa
Drosophila	*Drosophila*	*quinaria*		*falleni*	NE	from TMU
Drosophila	*Drosophila*	*quinaria*		*innubila*	NE	University of Rochester
Drosophila	*Drosophila*	*quinaria*		*nigromaculata*	PA	Sapporo, Hokkaido, JAPAN
Drosophila	*Drosophila*	*quinaria*		*palustris*	NE	from Dr. Shigeyuki Koshikawa
Drosophila	*Drosophila*	*quinaria*		*phalerata*	PA	from TMU
Drosophila	*Drosophila*	*quinaria*		*quinaria*	NE	from DSSC (15130–2011.00)
Drosophila	*Drosophila*	*quinaria*		*recens*	NE	from Dr. Shigeyuki Koshikawa
Drosophila	*Drosophila*	*quinaria*		*tenebrosa*	NE	from Dr. Shigeyuki Koshikawa
Drosophila	*Drosophila*	*quinaria*		*unispina*	PA, OR	Sapporo, Hokkaido, JAPAN
Drosophila	*Drosophila*	*testacea*		*orientacea*	PA	Sapporo, Hokkaido, JAPAN
Hirtodrosophila		*hirticornis*	*hirticornis*	*kangi*	PA, OR	Kamou-cho, Kagoshima Pref., JAPAN
Hirtodrosophila		*hirticornis*	*hirticornis*	*manonoensis*	OR, AU	Hamamoto T.F.W., GUAM
Hirtodrosophila		*hirticornis*	*hirticornis*	*nokogiri*	PA, OR	Tomakomai, Hokkaido, JAPAN
Hirtodrosophila		*hirticornis*	*hirticornis*	*novicia*	AU	Hamamoto T.F.W., GUAM
Hirtodrosophila		*hirticornis*	*hirticornis*	*pseudonokogiri*	PA	Tomakomai, Hokkaido, JAPAN
Hirtodrosophila		*melanderi*		*makinoi*	PA	Sapporo, Hokkaido, JAPAN
Hirtodrosophila		*quadrivittata*	*confusa*	*histrioides*	PA, OR	Tomakomai, Hokkaido, JAPAN
Hirtodrosophila		*quadrivittata*	*quadrivittata*	*caputudis*	OR	Poring, Sabah, MALAYSIA
Hirtodrosophila		*quadrivittata*	*quadrivittata*	*fascipennis*	PA, OR	Hirai, Wakayama Pref., JAPAN
Hirtodrosophila		*quadrivittata*	*quadrivittata*	*quadrivittata*	PA, OR	Metaki R, Kazamaura, Shimokita, Aomori Pref.,
Hirtodrosophila		*quadrivittata*	*trivittata*	*alboralis*	PA	Ikokuma R, Kazamaura, Shimokita, Aomori Pref.,
Hirtodrosophila		*quadrivittata*	*trivittata*	*sexvittata*	PA, OR	Tomakomai, Hokkaido, JAPAN
Hirtodrosophila		*quadrivittata*	*trivittata*	*trilineata*	PA	Hirai, Wakayama Pref., JAPAN
Hirtodrosophila		*quadrivittata*	*trivittata*	*trivittata*	PA, OR	Tomakomai, Hokkaido, JAPAN
Hirtodrosophila				*apicohispida*	PA, AU	Nametoko-keikoku, Ehime Pref., JAPAN
Mycodrosophila	*Mycodrosophila*			*gratiosa*	PA, OR, AU, AF	Hirai, Wakayama Pref., JAPAN
Mycodrosophila	*Mycodrosophila*			*parallelinervis*	OR, AU	Hamamoto T.F.W., GUAM
Mycodrosophila	*Mycodrosophila*			*poecilogastra*	PA, OR	Tomakomai, Hokkaido, JAPAN
Mycodrosophila	*Mycodrosophila*			*subgratiosa*	PA, OR	Matsuno-cho, Ehime Pref., JAPAN
Mycodrosophila	*Mycodrosophila*			*takachihonis*	PA, OR	Tomakomai, Hokkaido, JAPAN
Mycodrosophila	*Promycodrosophila*			*separata*	OR, AU	Bogor, West Java, INDONESIA
Mycodrosophila				*atrithorax*	PA	Tomakomai, Hokkaido, JAPAN
Mycodrosophila				*basalis*	PA, OR	Uwajima, Ehime Pref., JAPAN
Mycodrosophila				*palmata*	PA, OR	Hirai, Wakayama Pref., JAPAN
Mycodrosophila				*planipalpis*	PA, OR	Matsuyama, Ehime Pref., JAPAN
Mycodrosophila				*shikokuana*	PA	Tomakomai, Hokkaido, JAPAN
Paramycodrosophila				*nakamurai*	OR	Yaseo Water Fall, Kagoshima Pref., JAPAN
Paramycodrosophila				*pictula*	PA, OR, AU	Poring, Sabah, MALAYSIA
Zygothrica		*samoaensis*	*samoaensis*	*aliunota*	OR	Poring, Sabah, MALAYSIA
Zygothrica		*samoaensis*	*samoaensis*	*flavofinira*	OR	Cikaniki, Gn. Halimun, West Java, INDONESIA
Zygothrica		*samoaensis*	*leptorostra*	*leptorostra*	OR, AU	Cikaniki, Gn. Halimun, West Java, INDONESIA
Zygothrica		*samoaensis*	*leptorostra*	*quintamaculata*	OR	PHQ, Mt. Kinabalu, Sabah, MALAYSIA

[†] AF: Afrotropical, AU: Australasian, NE: Nearctic, NT: Neotropical, OR: Oriental, PA: Palearctic.

[‡] DSSC: UC San Diego Drosophila Stock Center, TMU: Tokyo Metropolitan University.

Table 5. Genes used in this study.

Gene loci		Primer name	Sequence (5'-3')	Annealing Temp	Length
Symbol	**Name**			**(°C)**	**(bp)**
Adh	Alcohol dehydrogenase	Adh_a2[α]	ATGGCAATCGCTAAGAA	49	~711
		Adh_b1[α]	TTAGATGCCAGAGTCCCAGT		
AdSS	Adenylosuccinate Synthetase	AdSS_F[β]	TGGGYACCACCAAAAAGGG	50	~400
		AdSS_R[β]	GGATACGTGCCAAARTCAATG		
ATPsynB	ATP synthase, subunit B	ATPsynB_F[β]	GAGGARTGGTTCCAGTTYTT	50	~450
		ATPsynB_R[β]	GCAATRTTYTCCTTCTTGGC		
bur	burgundy	bur_F[β]	GGCATYGATTTRATAGTGCG	48	~280
		bur_R[β]	GTTTCCGCRTTCKTGCTGAC		
ced-6	cell death protein 6	ced-6_F[β]	GARACGGGCACACAGGAGAA	53	~390
		ced-6_R[β]	CCTGTAGGCCAAATCAAARG		
COI	Cytochrome c oxidase subunit I	COI_a1[α]	GAAGGAGTTTACCAAYAAGG	46	~699
		COI_b1[α]	CCWACCCAATCCCARAAACC		
COII	Cytochrome c oxidase subunit II	COII_a1[α]	AGTGCTGGGAACACATCMTC	55	~666
		COII_b1[α]	AACGCCCTCGTGTATGGAAC		
Ddc	Dopa decarboxylase	527[γ]	TTCCASGAGTACTCCATGTCCTCG	58	~1200
		528[γ]	GGCAGGATGTKATGAAGGACATTGAG		
Dll	Distal-less	677[γ]	TGATACCAATACTGSGGCACATA	56	~600
		678[γ]	ATGATGAARGCMGCTCAGGG		
eb	ebony	525[γ]	CCCATSACCTCKGTGGAGCCGTA	59	~900
		526[γ]	CTGCATCGCATCTTYGAGGAGCA		
eIF3-S8	Eukaryotic translation initiation factor 3, subunit C	eIF3-S8_F[β]	GYCAAATGCCATTCCAYATG	52	~650
		eIF3-S8_R[β]	AAGTTGCCCTGCTTCATGTC		
ERp60	Endoplasmic reticulum p60	ERp60F[δ]	AAGGGCACCAACTACTGGCG	64	~621
		ERp60R[δ]	AGCTCGGTCTTCTTGGGCTTG		
eve	even skipped	689[γ]	TGCCTVTCCAGTCCRGAYAACTC	55	~1000
		690[γ]	TACGCCTCAGTCTTGTAGGG		
Gpdh	Glycerol-3-phosphate dehydrogenase	Gpdh_a1[α]	GTTTCTAGATCTGGTTGAGGCTGCCAAGAA	60	~699
		Gpdh_b1[α]	ACATATGCTCTAGATGATTGCGTATGCA		
hh	hedgehog	681[γ]	ACCTTGTABARGGCATTGGCATACCA	56	~600
		682[γ]	ATCGGWGATCGDGTGCTRAGCATG		
not	notum	464[γ]	TGGAACTAYATHCAYGADATGGGCGG	56	~800
		465[γ]	GAGCAGYTCVAGRAADCGCATCTC		
Pdi	Protein disulfide isomerase	Pdi_F[β]	GATTGGGACAARCARCCCGTC	50	~400
		Pdi_R[β]	TTACAACTCRTCCTTCTTRGGC		
Pgi	Phosphoglucose isomerase	Pgi_F[β]	GAAGGAGTTTACCAAYAAGG	46	~780
		Pgi_R[β]	CCWACCCAATCCCARAAACC		
ptc	patched	652/655[γ]	ACCCAGCTGCGCATSAGRAAGG/ACCCAGCTGCGCATSAGRAACG	54	~600
		656[γ]	GCTGACGGCSGCSTATGCGG		
RpL3	Ribosomal protein L3	RpL3_F[β]	AAAAGAAGGCGCACATCATG	50	~340
		RpL3_R[β]	GATCTTCTTGTTGATCTCGG		
RpS17	Ribosomal protein S17	RpS17_F[β]	TCGCGTCAGAACCAAGACWG	53	~290
		RpS17_R[β]	CCTCYTCCTGCAGCTTAATGG		
sina	seven in absentia	sina_F[β]	AGTGCTGGGAACACATCMTC	55	~740
		sina_R[β]	AACGCCCTCGTGTATGGAAC		
VhaSFD	Vacuolar H[+]-ATPase SFD subunit	VhaSFD_F[β]	TAYATGCARTCGCAAATGAT	44	~390
		VhaSFD_R[β]	TRAGGAACTGCAAGTAGAAG		
wg	wingless	447[γ]	AGCACGTyCArGCrGAGATGCG	58	~420
		449[γ]	CACTGTTkGGCGAyGGCATrTTGGG		

[α] Izumitani et al. (2016); [β] Katoh et al. (2021); [γ] Finet et al. (2021); δ Satomura and Tamura (2016).

Table 6. Best-fit partitioning scheme for 24 genes used in the phylogenetic analyses of the concatenated dataset.

Subset	Partition names	Optimal model	Length (bp)
1	*Erp60*_codon1, *ATPsynB*_codon1	GTR+I+G	308
2	*ATPsynB*_codon2, *ddc*_codon2	TVM+I+G	494
3	*ATPsynB*_codon3, *AdSS*_codon3, *VhaSFD*_codon3	GTR+I+G	308
4	*wg*_codon1, *ddc*_codon1, *AdSS*_codon1	GTR+I+G	535
5	*Pdi*_codon2, *VhaSFD*_codon2, *AdSS*_codon2, *ced-6*_codon2, *Erp60*_codon2	GTR+I+G	606
6	*Pgi*_codon1, *Pdi*_codon1, *ced-6*_codon1	GTR+I+G	316
7	*Pdi*_codon3, *RpS17*_codon3, *wg*_codon3	GTR+G	216
8	*Pgi*_codon2	GTR+I+G	126
9	*Pgi*_codon3	GTR+I+G	126
10	*RpL3*_codon1	TRN+I+G	97
11	*sina*_codon1, *RpL3*_codon2	SYM+I+G	327
12	*RpL3*_codon3	GTR+I+G	96
13	*eve*_codon1, *RpS17*_codon1	SYM+I+G	203
14	*RpS17*_codon2, *sina*_codon2	JC+I	290
15	*eb*_codon1, *VhaSFD*_codon1	GTR+G	405
16	*bur*_codon1	SYM+G	80
17	*bur*_codon2	F81+G	80
18	*bur*_codon3	TVMEF+I+G	80
19	*hh*_codon1, *Adh*_codon1	GTR+I+G	387
20	*hh*_codon2, *Adh*_codon2	GTR+I+G	387
21	*Adh*_codon3	GTR+I+G	237
22	*COII*_codon1, *COI*_codon1	GTR+I+G	489
23	*COI*_codon2, *ptc*_codon2	HKY+I+G	356
24	*COI*_codon3	K81UF+G	258
25	*COII*_codon2	GTR+I+G	229
26	*COII*_codon3	TIM+G	229
27	*Gpdh*_codon1	GTR+G	234
28	*elF3-S8*_codon2, *Gpdh*_codon2	TVM+I	433
29	*Gpdh*_codon3, *ced-6*_codon3	GTR+I+G	315
30	*eb*_codon3, *ddc*_codon3	GTR+I+G	682
31	*dll*_codon1	TVM+I+G	116
32	*dll*_codon2	F81+I	116
33	*dll*_codon3	SYM+G	116
34	*eb*_codon2, *wg*_codon2	GTR+I+G	337
35	*elF3-S8*_codon1	TIMEF+I+G	199
36	*elF3-S8*_codon3	GTR+I+G	199
37	*eve*_codon2	SYM+I+G	142
38	*eve*_codon3	GTR+I+G	142
39	*Erp60*_codon3	GTR+G	207
40	*hh*_codon3	TVM+I+G	150
41	*not*_codon1	TIMEF+G	101
42	*not*_codon2	GTR+G	101
43	*not*_codon3	TVM+I+G	101
44	*ptc*_codon1	SYM+G	98
45	*ptc*_codon3	TVM+I+G	97
46	*sina*_codon3	TVM+I+G	230

Table 7. Percentage of GC content at third codon positions of gene *abdA*, *abdB*, *adhr*, *amy*, *bab2*, *black*, bt, *burs*, *cp36*, *desatI*, *desatII*, *dll*, *dsx*, *ebony*, *elav*, *en*, *esc*, *fru*, *gawky*, *gl* and *hb* in 28 *Drosophila* species studied by Scott Chialvo et al. (2019).

Clade	**species**	**group**	***abdA***	***abdB***	***adhr***	***amy***	***bab2***	***black***	***bt***	***burs***	***cp36***	***desatI***	***desatII***	***dll***	***dsx***	***ebony***	***elav***	***en***	***esc***	***fru***	***gawky***	***gl***	***hb***
A	*acutilabella*	*cardini*	-	58.73	44.94	65.91	61.82	65.96	42.00	57.14	61.93	62.22	-	40.23	74.58	64.76	-	60.29	56.91	67.06	33.70	59.63	54.65
	cardini	*cardini*	-	53.97	40.61	65.71	59.09	65.96	45.56	66.07	62.26	65.00	63.20	41.38	84.75	62.39	-	58.82	56.10	60.00	-	60.25	54.65
	tripunctata	*tripunctata*	-	48.41	43.95	56.82	59.09	-	-	74.55	-	59.44	-	41.86	72.41	68.81	63.45	63.24	63.72	72.94	-	60.00	46.51
B	*bizonata*	*bizonata*	-	47.24	-	-	48.62	56.74	32.79	60.71	-	63.89	57.60	40.48	-	60.89	53.64	54.41	47.97	69.41	31.91	56.67	51.76
	orientacea	*testacea*	-	-	-	60.00	50.91		26.52	60.71	-	58.79	58.87	41.38	-	57.33	50.89	52.94	49.59	70.59	34.04	57.69	-
	neotestacea	*testacea*	-	-	48.17	54.93	49.09	60.43	26.89	57.41	-	61.11	61.60	41.38	-	58.64	-	52.94	51.64	70.59	35.11	57.96	47.06
	putrida	*testacea*	-	-	43.75	-	52.73	59.57	28.41	57.14	63.30	62.78	63.20	42.53	-	60.79	53.64	55.88	49.59	69.41	34.04	57.96	-
	testacea	*testacea*	-	-	47.88	60.00	49.09	61.70	27.27	53.57	-	60.56	60.00	40.23	-	59.03	-	52.94	51.67	69.41	35.11	58.55	-
C2	*angularis*	*quinaria*	66.39	55.12	49.33	80.25	68.81	61.43	-	64.29	-	70.00	-	44.83	77.97	67.61	-	64.71	69.92	67.06	-	68.15	57.65
	brachynephros	*quinaria*	-	52.76	49.70	65.88	68.18	72.34	50.00	64.29	66.06	-	-	42.86	74.58	66.67	66.18	55.74	68.29	67.06	53.76	63.29	48.24
	falleni	*quinaria*	-	53.54	48.48	60.49	57.27	65.96	39.77	62.50	63.94	65.00	-	42.37	74.58	66.22	-	-	63.41	-	39.78	58.60	56.47
	innubila	*quinaria*	-	55.91	49.69	65.91	64.55	-	43.15	58.93	64.68	65.56	-	42.65	74.58	65.64	66.47	64.71	65.04	65.88	40.86	61.78	56.47
	phalerata	*quinaria*	60.87	56.69	48.48	66.67	62.73	63.83	41.37	67.86	-	63.89	60.42	42.53	66.10	63.77	60.35	55.88	69.11	69.41	-	62.18	-
C1	*deflecta*	*quinaria*	70.09	58.27	-	69.32	65.14	70.37	47.73	69.64	70.18	63.69	65.60	48.28	84.75	75.60	58.02	67.65	66.67	78.82	-	66.67	69.41
	magnaquinaria	*quinaria*	69.83	57.14	51.55	71.11	64.55	69.50	-	73.21	70.18	62.22	66.40	49.43	77.97	74.45	59.77	64.71	74.36	76.47	-	69.81	61.90
	munda	*quinaria*	71.96	59.52	-	75.00	68.18	75.54	43.56	73.21	71.56	66.86	71.90	43.68	81.36	77.38	61.81	67.65	64.23	72.94	-	69.38	64.56
	nigromaculata	*quinaria*	69.83	65.79	52.73	68.18	67.27	73.57	45.83	67.86	-	64.44	66.94	45.98	69.49	71.30	60.35	67.65	71.54	74.12	-	68.42	61.18
	occidentalis	*quinaria*	71.79	58.27	54.55	75.56	69.09	73.88	-	73.21	71.56	63.33	71.20	44.83	79.66	77.09	63.27	64.71	64.23	75.29	-	69.38	67.06
	quinaria	*quinaria*	69.23	58.40	56.36	73.33	66.36	74.47	49.62	80.36	69.72	-	71.20	44.83	77.97	86.34	64.14	65.67	70.25	75.29	32.91	71.88	68.24
	recens	*quinaria*	73.39	-	55.15	77.78	65.42	76.52	49.24	80.77	74.31	-	72.88	45.98	86.21	77.17	77.84	67.65	77.24	74.12	-	72.08	62.20
	suboccidentalis	*quinaria*	71.56	64.29	53.66	76.67	69.09	73.05	-	76.36	71.56	64.44	71.20	44.83	79.66	75.33	63.56	64.71	72.36	75.00	-	70.59	63.53
	subpalustris	*quinaria*	70.09	56.69	52.73	72.22	66.36	72.26	50.38	73.21	70.18	62.78	66.40	48.28	81.36	76.21	59.18	65.57	69.92	76.47	-	71.07	67.06
	subquinaria coast	*quinaria*	70.09	57.26	55.76	73.86	65.45	74.26	46.97	69.64	72.48	-	71.20	45.98	81.36	76.21	72.59	67.65	72.36	74.12	32.18	71.25	61.18
	subquinaria inland	*quinaria*	70.94	58.06	53.94	74.44	66.06	76.64	48.86	76.79	73.85	-	71.20	45.98	81.36	74.21	73.18	63.24	77.87	72.94	-	73.13	60.00
	tenebrosa	*quinaria*	71.79	56.44	53.94	76.67	67.27	75.18	46.21	71.43	71.70	63.89	68.80	44.83	79.66	77.09	64.14	66.18	73.17	76.54	47.31	69.81	63.53
	transversa	*quinaria*	70.09	66.67	53.80	74.16	66.36	76.60	47.35	77.78	74.77	66.11	70.40	44.83	81.36	75.80	71.43	68.18	76.72	74.12	-	73.38	60.24
	guttifera	*guttifera*	75.21	59.84	58.79	75.56	73.64	73.05	44.70	71.43	68.35	67.22	62.60	46.51	86.44	71.63	59.48	60.29	67.48	-	-	76.88	68.24
	immigrans	*immigrans*	-	62.20	61.39	-	87.27	74.47	-	66.67	78.50	72.22	70.40	47.13	88.14	82.67	72.01	79.41	77.39	81.18	-	72.15	70.59
	Avg.		70.17	56.98	51.24	69.63	63.55	69.35	42.00	68.06	69.57	64.17	66.56	44.19	78.97	70.41	63.44	62.73	65.62	72.15	37.64	66.05	60.07
	NO. of sequences:		16	23	24	25	28	26	22	28	20	23	22	28	23	28	22	27	28	26	12	28	25

Table 8. Percentage of GC content at third codon positions of gene *ix*, *kl3*, *kl5*, *mago*, *mof*, *ntid*, *pale*, *pgd*, *rpl11*, *rpl36*, *runt*, *sina*, *skp*, *svr*, *sxl*, *tim*, *tpi, wee*, *COI*, *COII* and *COIII* in 28 *Drosophila* species studied by Scott Chialvo et al. (2019).

Clade	**species**	**group**	***ix***	***kl3***	***kl5***	***mago***	***mof***	***ntid***	***pale***	***pgd***	***rpl11***	***rpl36***	***runt***	***sina***	***skp***	***svr***	***sxl***	***tim***	***tpi***	***wee***	***COI***	***COII***	***COIII***	Total
A	*acutilabella*	*cardini*	45.60	-	28.86	55.00	62.26	74.65	61.70	58.52	60.76	74.71	59.62	55.30	58.90	52.63	58.73	-	-	54.84	5.84	3.79	5.07	37
	cardini	*cardini*	-	-	28.75	60.00	61.79	64.08	57.45	52.40	54.43	74.71	59.62	54.55	58.22	55.98	57.14	-	74.80	55.91	5.11	7.59	5.84	34
	tripunctata	*tripunctata*	50.41	-	30.13	56.00	53.73	70.07	76.60	58.95	53.16	74.71	63.16	60.77	60.27	54.40	-	76.47	70.49	54.12	8.03	10.53	7.81	41
B	*bizonata*	*bizonata*	-	30.72	38.93	57.00	60.56	64.79	56.84	53.28	45.57	66.67	-	59.85	54.11	47.85	61.67	-	66.14	48.31	12.98	10.53	8.70	35
	orientacea	*testacea*	-	32.67	40.25	55.00	53.99	66.90	73.40	52.40	50.63	70.73	-	59.23	48.63	50.72	59.68	61.40	64.29	45.35	10.53	7.46	6.52	39
	neotestacea	*testacea*	45.60	31.13	39.49	57.00	55.40	66.90	70.21	51.97	49.37	70.73	-	61.36	50.68	-	59.68	60.12	63.20	45.98	18.32	10.96	9.49	37
	putrida	*testacea*	51.20	30.91	37.47	52.00	57.28	66.90	75.53	51.53	50.63	70.27	59.62	61.36	50.74	53.50	59.68	-	64.29	49.41	12.21	7.46	4.35	40
	testacea	*testacea*	50.40	32.74	39.49	56.00	55.50	67.63	67.02	52.84	49.37	69.51	-	60.61	49.32	50.83	59.68	59.65	64.00	47.19	10.69	7.46	6.52	38
C2	*angularis*	*quinaria*	47.20	-	-	63.00	69.48	67.86	75.53	69.00	62.03	-	-	68.18	59.59	59.81	62.50	75.44	79.53	52.75	7.30	4.39	6.52	36
	brachynephros	*quinaria*	50.43	32.66	-	61.00	67.61	68.46	71.28	73.80	62.03	65.52	61.54	66.67	67.12	62.20	64.06	63.53	77.17	56.99	10.95	10.09	6.52	40
	falleni	*quinaria*	45.45	34.00	36.71	62.11	67.01	67.18	75.53	62.45	62.03	67.82	53.85	65.91	62.33	58.37	64.06	60.23	75.59	49.46	12.31	7.66	7.25	38
	innubila	*quinaria*	48.00	32.67	35.44	60.00	69.48	67.61	76.60	68.12	56.96	67.82	61.54	67.69	64.38	61.72	60.94	59.43	75.59	47.31	8.76	10.09	7.25	41
	phalerata	*quinaria*	50.40	32.45	34.43	60.42	61.50	64.29	62.11	69.00	60.76	66.22	54.29	66.67	63.70	61.24	60.94	62.01	74.02	50.54	11.68	8.33	7.97	36
C1	*deflecta*	*quinaria*	53.60	32.89	35.95	61.00	-	66.90	75.53	76.60	55.70	72.41	63.46	69.23	61.64	66.03	66.67	68.16	77.95	54.44	9.49	10.05	10.32	40
	magnaquinaria	*quinaria*	52.00	-	-	63.00	61.50	69.01	78.72	74.24	58.23	72.41	61.54	67.42	-	64.59	61.90	68.36	79.53	53.76	8.76	6.14	7.25	39
	munda	*quinaria*	48.80	31.13	35.44	66.00	61.97	73.24	60.00	73.80	60.76	73.56	61.54	66.67	60.27	66.51	65.08	69.94	83.06	57.30	8.40	8.37	12.32	35
	nigromaculata	*quinaria*	48.00	31.54	35.95	57.00	59.62	69.72	65.96	74.24	58.23	73.56	-	68.94	60.27	64.59	63.49	66.48	79.17	53.76	10.22	9.65	12.78	41
	occidentalis	*quinaria*	48.80	32.44	-	63.00	64.62	71.83	62.11	-	59.49	74.71	61.54	67.94	60.27	67.46	66.67	69.27	82.68	58.06	8.87	7.46	10.14	36
	quinaria	*quinaria*	50.40	31.79	35.19	61.00	62.44	71.83	79.79	74.24	56.96	82.76	71.15	68.18	58.22	65.55	63.49	67.60	81.10	54.84	11.45	9.65	10.14	42
	recens	*quinaria*	58.40	31.46	36.20	65.00	72.77	74.65	77.66	89.78	63.29	74.71	71.15	72.09	63.19	78.47	66.67	72.07	87.80	58.06	11.68	11.40	10.14	40
	suboccidentalis	*quinaria*	51.20	32.23	36.20	63.00	64.32	74.65	77.66	74.24	62.03	75.86	63.46	67.42	59.59	66.51	65.08	70.39	83.06	58.89	8.03	7.89	10.22	41
	subpalustris	*quinaria*	-	32.01	33.42	61.00	62.44	69.01	75.53	75.11	58.23	68.92	65.38	67.69	-	63.16	65.08	67.60	77.95	55.91	9.49	10.53	4.35	41
	subquinaria coast	*quinaria*	53.60	31.69	36.04	64.00	64.79	70.42	77.66	86.46	60.26	80.46	69.23	67.18	60.27	70.81	65.08	71.51	85.04	56.99	8.76	8.33	9.42	41
	subquinaria inland	*quinaria*	58.40	31.46	36.20	63.16	70.42	70.42	79.79	78.17	59.49	75.86	71.15	66.67	61.64	73.68	65.08	73.74	84.25	53.93	9.49	7.02	10.14	41
	tenebrosa	*quinaria*	52.00	33.33	35.88	62.00	63.85	73.94	78.72	75.11	60.76	74.71	63.46	66.67	59.59	66.51	64.52	73.18	81.89	59.14	8.13	8.33	-	43
	transversa	*quinaria*	53.60	31.79	36.20	62.89	66.19	72.66	61.05	78.60	60.76	75.86	71.15	68.18	60.27	70.81	65.08	72.00	85.04	54.84	10.69	7.46	10.87	36
	guttifera	*guttifera*	54.40	31.35	34.07	58.00	70.42	76.26	80.85	70.59	60.76	73.56	67.31	74.24	58.22	60.77	65.00	75.58	83.46	53.76	9.92	9.65	7.25	42
	immigrans	*immigrans*	72.00	-	28.75	62.00	72.04	75.35	64.89	82.97	74.68	82.76	80.77	77.10	73.97	70.33	-	-	92.13	57.14	12.21	8.77	14.49	34
	Avg.		51.68	32.05	35.25	60.22	63.46	69.91	71.26	68.76	58.12	73.12	64.53	65.84	59.46	62.94	62.99	68.03	77.54	53.59	9.99	8.48	8.50	
	NO. of ssequences:		24	22	24	28	27	28	26	27	28	27	22	28	26	27	26	23	27	28	28	28	27	

Table 9. Base compositional homogeneity (X^2 P-value and relative composition variability, RCV), calculated on each codon of the datasets from Scott Chialvo et al. (2019).

Locus ID	P-value	RCV	Locus ID	P-value	RCV	Locus ID	P-value	RCV
abdA-1st	1.00	0.01	*elav*-1st	1.00	0.01	*pgd*-1st	1.00	0.03
abdA-2nd	1.00	0.03	*elav*-2nd	1.00	0.00	*pgd*-2nd	1.00	0.02
abdA-3rd	1.00	**0.06**	*elav*-3rd	**0.00**	**0.11**	*pgd*-3rd	**0.00**	**0.19**
abdB-1st	1.00	0.04	*en*-1st	1.00	0.03	*rpl11*-1st	1.00	0.01
abdB-2nd	1.00	0.03	*en*-2nd	1.00	0.02	*rpl11*-2nd	1.00	0.00
abdB-3rd	1.00	**0.08**	*en*-3rd	1.00	**0.11**	*rpl11*-3rd	1.00	**0.09**
adhr-1st	1.00	0.03	*esc*-1st	1.00	0.03	*rpl36*-1st	1.00	0.03
adhr-2nd	1.00	0.02	*esc*-2nd	1.00	0.01	*rpl36*-2nd	1.00	0.02
adhr-3rd	0.83	**0.09**	*esc*-3rd	**0.00**	**0.16**	*rpl36*-3rd	1.00	**0.09**
amy-1st	0.93	**0.11**	*fru*-1st	1.00	0.02	*runt*-1st	1.00	0.03
amy-2nd	1.00	**0.08**	*fru*-2nd	1.00	0.00	*runt*-2nd	0.95	0.02
amy-3rd	0.14	**0.15**	*fru*-3rd	1.00	**0.09**	*runt*-3rd	1.00	**0.13**
bab2-1st	1.00	0.02	*gawky*-1st	1.00	0.04	*sina*-1st	1.00	0.02
bab2-2nd	1.00	0.00	*gawky*-2nd	1.00	0.03	*sina*-2nd	1.00	0.00
bab2-3rd	**0.01**	**0.13**	*gawky*-3rd	0.94	**0.11**	*sina*-3rd	1.00	**0.08**
black-1st	1.00	0.03	*gl*-1st	1.00	0.03	*skp*-1st	1.00	0.01
black-2nd	1.00	0.02	*gl*-2nd	1.00	0.02	*skp*-2nd	1.00	0.00
black-3rd	0.21	**0.12**	*gl*-3rd	0.27	**0.12**	*skp*-3rd	0.61	**0.10**
bt-1st	1.00	0.03	*hb*-1st	1.00	0.03	*svr*-1st	1.00	0.02
bt-2nd	1.00	0.01	*hb*-2nd	1.00	0.01	*svr*-2nd	1.00	0.02
bt-3rd	**0.00**	**0.13**	*hb*-3rd	0.76	**0.13**	*svr*-3rd	**0.00**	**0.12**
burs-1st	1.00	0.02	*ix*-1st	1.00	0.02	*sxl*-1st	1.00	0.02
burs-2nd	1.00	0.00	*ix*-2nd	1.00	0.00	*sxl*-2nd	1.00	0.03
burs-3rd	1.00	**0.14**	*ix*-3rd	0.98	**0.09**	*sxl*-3rd	1.00	**0.06**
cp36-1st	1.00	0.02	*kl3*-1st	1.00	0.01	*tim*-1st	1.00	0.04
cp36-2nd	1.00	0.01	*kl3*-2nd	1.00	0.01	*tim*-2nd	1.00	0.02
cp36-3rd	0.65	**0.08**	*kl3*-3rd	1.00	0.02	*tim*-3rd	0.54	**0.10**
desatI-1st	1.00	0.03	*kl5*-1st	1.00	0.02	*tpi*-1st	1.00	0.03
desatI-2nd	1.00	0.01	*kl5*-2nd	1.00	0.01	*tpi*-2nd	1.00	0.01
desatI-3rd	1.00	**0.05**	*kl5*-3rd	0.93	**0.06**	*tpi*-3rd	**0.00**	0.13
desatII-1st	1.00	**0.09**	*mago*-1st	1.00	0.02	*wee*-1st	1.00	0.03
desatII-2nd	1.00	0.02	*mago*-2nd	1.00	0.00	*wee*-2nd	1.00	0.03
desatII-3rd	1.00	**0.09**	*mago*-3rd	1.00	**0.08**	*wee*-3rd	1.00	**0.08**
dll-1st	1.00	0.01	*mof*-1st	1.00	0.02	*COI*-1st	1.00	0.00
dll-2nd	1.00	0.02	*mof*-2nd	1.00	0.01	*COI*-2nd	1.00	0.02
dll-3rd	1.00	**0.07**	*mof*-3rd	0.21	**0.10**	*COI*-3rd	1.00	**0.06**
dsx-1st	1.00	0.03	*ntid*-1st	1.00	0.03	*COII*-1st	1.00	0.02
dsx-2nd	1.00	0.00	*ntid*-2nd	1.00	0.03	*COII*-2nd	1.00	0.01
dsx-3rd	0.98	**0.10**	*ntid*-3rd	1.00	**0.09**	*COII*-3rd	1.00	0.04
ebony-1st	1.00	0.03	*per*-1st	1.00	0.01	*COIII*-1st	1.00	0.03
ebony-2nd	1.00	0.01	*per*-2nd	1.00	0.01	*COIII*-2nd	1.00	0.02
ebony-3rd	**0.00**	**0.13**	*per*-3rd	0.11	**0.15**	*COIII*-3rd	1.00	**0.06**

X^2 (p) < 0.05, RCV > 0.05 are in bold.

Table 10. Best-fit partitioning scheme for 39 nuclear genes without 3rd codons and 3 mitochondrial genes used in the phylogenetic analyses of the concatenated dataset.

Subset	**Partition names**	**Optimal model**	**Length (bp)**
1	*bab2*_codon1, *Abd-B*_codon1, *gl*_codon1	TVM+I+G	400
2	*Abd-B*_codon2	F81+G	127
3	*AbdA*_codon1, *dsx*_codon2, *Dll*_codon2, *Dll*_codon1	F81	352
4	*rpl11*_codon2, *AbdA*_codon2, *rpl36*_codon2	F81	285
5	*Adhr*_codon1, *wee*_codon1, *ebony*_codon1, *pgd*_codon1	GTR+I+G	715
6	*mof*_codon1, *Adhr*_codon2, *fru*_codon1	SYM+I+G	463
7	*desatI*_codon1, *desatII*_codon1, *tim*_codon1	SYM+I+G	486
8	*desatI*_codon2, *mof*_codon2, *kl5*_codon2	K81UF+I	789
9	*amy*_codon1	TVMEF+G	90
10	*amy*_codon2	TVMEF+I	90
11	*b*_codon1, *kl5*_codon1, *sxl*_codon1, *kl3*_codon1	GTR+I+G	1056
12	*tpi*_codon2, *svr*_codon2, *b*_codon2, *cp36*_codon2	TVM+I+G	696
13	*runt*_codon2, *burs*_codon2, *bab2*_codon2, *sina*_codon2	JC+I	350
14	*bt*_codon1	GTR+I	264
15	*bt*_codon2, *gawky*_codon2	HKY+I	358
16	*burs*_codon1, *sina*_codon1	SYM+I	188
17	*COI*_codon1	TIM+G	137
18	*COI*_codon2	F81	137
19	*COIII*_codon3, *COI*_codon3	TIM+I+G	275
20	*COII*_codon1, *COIII*_codon1	TRN+I+G	367
21	*esc*_codon2, *tim*_codon2, *COII*_codon2	GTR+I	531
22	*COII*_codon3	HKY+I+G	228
23	*COIII*_codon2	F81+I	138
24	*cp36*_codon1, *tpi*_codon1	GTR+G	345
25	*kl3*_codon2, *desatII*_codon2	K81UF+I+G	580
26	*dsx*_codon1	TRNEF+I	59
27	*ebony*_codon2	GTR	228
28	*skp*_codon1, *mago*_codon1, *rpl36*_codon1, *elav*_codon1	GTR+I+G	677
29	*elav*_codon2, *pale*_codon2, *ix*_codon2	F81	563
30	*en*_codon1	SYM+I	68
31	*sxl*_codon2, *en*_codon2	TVM+I	132
32	*esc*_codon1, *ix*_codon1	TRNEF+I+G	249
33	*fru*_codon2	K81	85
34	*runt*_codon1, *gawky*_codon1	K81UF+I	146
35	*gl*_codon2	TVM+G	163
36	*hb*_codon1	TIM+G	86
37	*hb*_codon2	TVM	86
38	*skp*_codon2, *mago*_codon2	F81	247
39	*ntid*_codon1	GTR+G	142
40	*wee*_codon2, *ntid*_codon2	TVM+I+G	235
41	*pale*_codon1, *rpl11*_codon1	TRN+I	175
42	*pgd*_codon2	TRNEF+I+G	229
43	*svr*_codon1	TRN+G	210

Table 11. Survivals on control food and food containing α-amanitin.

Species	Treatment	Mean(±SE) survival	Statistical significance
D. brachynephros	D.W.	5	-
	0.05mg/ml α-amanitin	4.6±0.24	-
D. cardini	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
D. deflecta	D.W.	4±0.32	-
	0.005mg/ml α-amanitin	3.6±0.6	-
	0.01mg/ml α-amanitin	3.4±0.4	-
	0.025mg/ml α-amanitin	2.4±0.24	0.00395 **
	0.05mg/ml α-amanitin	2.4±0.24	0.00395**
D. histro	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
D. hydei	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
D. melanogaster	D.W.	5	-
	0.005mg/ml α-amanitin	4.6±0.24	-
	0.01mg/ml α-amanitin	3.4±0.75	0.065
	0.025mg/ml α-amanitin	0	<2e-16 ***
	0.05mg/ml α-amanitin	0	<2e-16***
D. multispina	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
D. orientacea	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
D. palustris	D.W.	3.8±0.37	-
	0.005mg/ml α-amanitin	2.6±0.68	-
	0.01mg/ml α-amanitin	2.4±0.68	-
	0.025mg/ml α-amanitin	1.2±0.73	0.0135 *
	0.05mg/ml α-amanitin	1.2±0.37	0.00117**
D. sternopherulis	D.W.	4.8±0.2	-
	0.05mg/ml α-amanitin	5	-
D. unispina	D.W.	4.8±0.2	-
	0.05mg/ml α-amanitin	5	-
H. histrioides	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
H. sexvittata	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
H. trilineata	D.W.	5	-
	0.05mg/ml α-amanitin	5	-
H. trivittata	D.W.	5	-
	0.05mg/ml α-amanitin	5	-

Table 12. Food habits and α-amanitin tolerance for each species.

species	food habits (main)	previous record (for eggs)	tolerance (for adults)
D. brachynephros	M	yes[β]	yes
D. cardini	M	yes[β]	yes
D. deflecta	L	no[β]	no
D. histrio	M	no record	yes
D. hydei	**generalist**	**no record**	**yes**
D. melanogaster	F	no[α]	no
D. multispina	M	no record	yes
D. orientacea	M	no record	yes
D. palustris	L	no[γ]	no
D. sternopherulis	**generalist**	**no record**	**yes**
D. unispina	M	no record	yes
H. histrioides	M	no record	yes
H. sexvittata	M	no record	yes
H. trilineata	M	no record	yes
H. trivittata	M	no record	yes

M:Mushroom; F :Fruits; T:Tree; L: leaves

[α]Jaenike, 1983; [β]Stump et al., 2011; [γ]Spicer and Jaenike, 1996