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Running head: Evolutionary history of fig trees.

[Note: Supplementary data to preprint are available upon request to corresponding author].

ABSTRACT

Despite their ecological and evolutionary importance as key components of tropical ecosystems, the phylogeny of fig trees is still unresolved. We use restriction-site-associated DNA (RAD) sequencing (*ca* 420kb) and 102 morphological characters to elucidate the relationships between 70 species of *Ficus* representing all known subgenera and sections and five outgroups. We compare morphological and molecular results to highlight discrepancies and reveal possible inference bias. We analyse marker and taxon properties that may bias molecular inferences, with existing softwares and a new approach based on iterative principal component analysis to reduce variance between clusters of samples. For the first time, with both molecular and morphological data, we recover a monophyletic subgenus *Urostigma* and a clade with all gynodioecious fig trees. However, our analyses show that it is not possible to homogenize evolutionary rates and GC content for all taxa prior to phylogenetic inference and that four competing positions for the root of the molecular tree are possible. The placement of the long-branched section *Pharmacosycea* as sister to all other fig trees is not supported by morphological data and considered as a result of a long branch attraction artefact to the outgroups. Regarding morphological features and indirect evidence from the pollinator tree of life, the topology that divides the genus *Ficus* into monoecious *versus* gynodioecious species appears most likely. Active pollination is inferred as the ancestral state for all topologies, ambiguity remains for ancestral breeding system including for the favored topology, and it appears most likely that the ancestor of fig trees was a freestanding tree. Increasing sampling may improve results and would be at least as relevant as maximizing the number of sequenced regions given the strong heterogeneity in evolutionary rates, and to a lesser extent, base composition among species. Despite morphological plasticity and frequent homoplasy of multiple characters, we advocate giving a central role to morphology in our understanding of the evolution of *Ficus*, especially as it can help detect insidious systematic errors that tend to

become more pronounced with larger molecular data sets.

Keywords: compositional bias, fig trees, long branch attraction, morphology, phylogeny, RAD-seq, traits.

INTRODUCTION

Ficus (Moraceae) is a pantropical and hyperdiverse genus (*ca* 850 species) that includes a broad range of growth forms (trees, hemi-epiphytes, shrubs, climbers) with diverse ecologies (Berg and Corner 2005, Harrison 2005, Harrison and Shanahan 2005). As their inflorescences (figs) are important food source for hundreds of frugivorous species (Shanahan et al. 2001), fig trees are key components of tropical ecosystems. They are also known for their intricate relationships with their pollinating wasps (Agaonidae). Indeed, since *ca* 75 Myr, fig trees and agaonids have been obligate mutualists (Cruaud et al. 2012). The wasp provides pollination services to the fig tree, while the fig tree provides breeding sites for the wasps, and none of the partners are able to reproduce without the other (Galil 1977, Cook and Rasplus 2003). Fifty-two percent of *Ficus* species are monoecious, while 48% are gynodioecious. In monoecious species, figs contain staminate and pistillate flowers and produce pollen, pollinators and seeds. Gynodioecious species are functionally dioecious with male function (pollen and pollinator production), and female function (seed production) segregated on separate individuals. *Ficus* species are pollinated either actively (two-thirds) or passively (one-third) (Kjellberg et al. 2001). In passively pollinated figs, emerging wasps are dusted with pollen before leaving their natal fig. In actively pollinated figs, wasps use their legs to collect pollen they will later deposit on flowers of receptive figs, while laying their eggs.

Despite its ecological importance, the evolutionary history of the genus remains unclear. Several studies have attempted to reconstruct the phylogeny of *Ficus* using Sanger

sequencing of chloroplast markers (Herre et al. 1996), external and/or internal transcribed spacers (ETS, ITS) (Weiblen 2000, Jousselin et al. 2003), a combination of nuclear markers (Rønsted et al. 2005, Rønsted et al. 2008, Xu et al. 2011, Cruaud et al. 2012, Pederneiras et al. 2018, Zhang et al. 2018, Clement et al. 2020) or next-generation sequencing of chloroplast genomes (Bruun-Lund et al. 2017). None of these studies successfully resolved the backbone of the phylogeny and there is no consensus on the relationships between major groups of *Ficus* yet. As a consequence, current classification remains inconsistent with different phylogenetic levels classified under the same taxonomic rank or, to the opposite, identical taxonomic rank appearing at different phylogenetic level (Table 1). In addition, our ability to analyse the evolution of key traits that may have contributed to the evolutionary and ecological success of the genus is limited. Areas of agreement are (i) the position of sect. *Pharmacosycea* as sister to all other fig trees (with poor or high support depending on the study), (ii) a monophyletic and strongly supported *Mixtiflores* (*i.e.* subgenus *Urostigma* excluding sect. *Urostigma, sensu* Clement *et al.* (2020)), and (iii) a monophyletic clade formed by subg. *Synoecia*, sect. *Eriosycea* and subsect. *Frutescentiae,* sometimes recovered sister to subg. *Sycidium*. However, these two last results were challenged by plastome analysis (Bruun-Lund et al. 2017) that provided improved resolution but highlighted a high level of cyto-nuclear discordance with some subgenera undoubtedly monophyletic (e.g. *Sycidium*) recovered as polyphyletic. Non-ambiguous cases of cyto-nuclear discordance were previously detected in African fig trees (Renoult et al. 2009) and are more frequently reported with the use of high throughput sequencing approaches (Huang et al. 2014). Hence, the use of nuclear, genome-wide markers appears more appropriate to resolve the phylogeny of fig trees.

Weiblen (2000) proposed the only phylogenetic hypothesis for the genus (46 species) based on morphological data, with a special focus on gynodioecious fig trees. Representatives of all subgenera of *Ficus* and all sections except one (*Galoglychia*) were scored for 61 characters.

No outgroups were included and trees were rooted in agreement with the only molecular study available at that time that included only three species of *Ficus* and eight outgroups (Herre et al. 1996). The consensus tree was poorly resolved and some groups were recovered as paraphyletic but this study represents a good starting point. As underlined by Clement & Weiblen (2009), botanists have long noticed high levels of homoplasy in Moraceae, which complicates the assembly of morphological matrices. Besides, matrix assembly requires a thorough knowledge of the target group and scoring characters on a representative number of specimens is time consuming. This certainly explains the shift towards molecular approaches, which is the general trend in systematics, especially since the advent of high-throughput sequencing technologies.

However, overconfidence in molecular data is risky. Indeed, while genome-scale data may contribute to better resolve phylogenetic relationships (Philippe et al. 2005), they can also infer incorrect yet highly supported topologies due to the failure of current models /methods to capture the full complexity of evolutionary processes (systematic error; Swofford et al. 1996, Phillips et al. 2004, Kumar et al. 2012). This is why morphological approaches, even though considered old-fashioned or supposed to be difficult to conceptualize and to interpret, are still crucial in a time of big data overflow (Wiens 2004, Giribet 2015, Wipfler et al. 2016). Indeed, they constitute an independent set of characters which can help to detect errors in inferences based on molecular data.

Here we infer the evolutionary history of 70 species of *Ficus* representing all known subgenera and sections and five outgroups from i) 102 morphological characters and ii) Restriction-site-Associated DNA sequencing (RAD-seq). RAD-seq has been used successfully to infer recent and ancient evolutionary histories of groups of plants (e.g. (Eaton and Ree 2013, Hipp et al. 2014, Hipp et al. 2019) including a community of 11 Panamanian strangler figs from the section *Americanae* (Satler et al. 2019). In a preliminary study, we

127 targeted conserved regions with an infrequent 8-cutter restriction enzyme (*SbfI*) and
128 highlighted the power of RAD-seq to infer deep relationships between fig trees (Rasplus et al.
129 2018). However, only forward reads were analysed, taxonomic sampling was reduced and a
130 single outgroup with a high level of missing data was used. Results were encouraging but not
131 supported enough to draw definitive conclusions. Here, we go one step further, by increasing
132 taxon sampling and the length of analysed loci. To mine RAD loci into distant outgroup
133 genomes, we assembled paired reads into long loci. This enabled us to decrease missing data
134 associated with the loss of restriction sites with time, while increasing phylogenetic signal for
135 the ingroup. We critically compare morphological and molecular results and discuss
136 similarities and discrepancies. In addition, we performed a thorough analysis of marker and
137 taxon properties that could bias molecular inferences (heterogeneity in base composition and
138 variable evolutionary rates) with existing software and a new approach based on iterative
139 principal component analysis (PCA) to reduce variance between clusters of samples. We also
140 compared the impact of different rooting strategies on molecular tree topology. Finally, we
141 use our data set to revisit the evolution of key traits (life form, breeding system and
142 pollination mode) that may have contributed to the evolutionary and ecological success of the
143 genus.

144

145 **MATERIALS AND METHODS**

146 ***Sampling and classification.***

147 Here we use the classification by Berg & Corner (2005) with some modifications used in
148 Cruaud *et al.* 2012 (Table 1). Seventy species of *Ficus* representing all known subgenera and
149 sections as well as four outgroups were included in the analysis (Table S1). The same
150 individual was used for molecular and morphological studies. Plants, twigs, leaves and figs

were photographied before sampling of a few leaves that were dried for molecular purposes. Voucher specimens are archived at CBGP, Montpellier.

Morphological Data

Species were scored for 102 morphological characters (Appendix S1). Seventy-eight were extracted from earlier phylogenetic studies (Weiblen 2000, Clement and Weiblen 2009, Chantarasuwan et al. 2015) and sometimes redefined, while 24 characters were used for the first time. Whenever possible we cross-validated our observations and accounted for polymorphism using descriptions available in the literature (Corner 1938, Corner 1967, Corner 1969b, Corner 1969a, Corner 1970, Corner 1978a, Corner 1978b, Berg and Wiebes 1992, Berg and Corner 2005, Berg 2009, Berg et al. 2011) and conspecific specimens from other localities. Data were analysed using Maximum Parsimony (MP) as implemented in PAUP* version 4.0a (Swofford 2003). We used a heuristic search with 5000 random addition sequences (RAS) to obtain an initial tree and "tree bisection and reconnection (TBR)" as branch swapping option, with reconnection limit set to 100. One tree was retained at each step. Characters were equally weighted and treated as unordered and non-additive. Multiple states were interpreted as polymorphism and gaps (characters that were impossible to score because the feature was non-existent) were treated as missing data. Robustness of the topology was assessed by bootstrap procedures (100 replicates; TBR RAS 100; one tree retained at each step). Character transformations were mapped on the majority-rule consensus tree and the four alternative RAD topologies in PAUP* using the ACCTRAN optimization strategy.

DNA extraction and library construction.

Leaves were either dried with silica gel or sun-dried. Twenty mg of dried leaves were placed in Eppendorf vials and crushed with ceramic beads in liquid nitrogen. DNA was extracted with the Chemagic DNA Plant Kit (Perkin Elmer Chemagen, Baesweller, DE, Part # CMG-194), according to the manufacturer's instructions with a modification of the cell lysis. The protocol was adapted to the use of the KingFisher Flex™ (Thermo Fisher Scientific, Waltham, MA, USA) automated DNA purification workstation. The powder was suspended in 400uL Lysis buffer (200mM Tris pH = 8.0, 50mM EDTA, 500mM NaCl, 1.25 % SDS, 0,5 % CTAB 1% PVP 40000, 1 g/100ml Sodium Bisulfite) and incubated 20 min at 65°C. Then 150 µL of cold precipitation buffer (sodium acetate 3M, pH 5.2) was added. Samples were centrifuged 10 min at 12000 rpm and 350 µL of the supernatant were transferred in a 96 deepwell plate. Binding of DNA on magnetic beads, wash buffer use and elution of purified DNA followed Chemagic kit protocol and KingFisher Flex use.

Library construction followed Baird *et al.* (2008) and Etter *et al.* (2011) with modifications detailed in Cruaud *et al.* (2014) and below. To infer deep phylogenetic relationships, we targeted conserved regions with an infrequent 8-cutter restriction enzyme (*SbfI*). The expected number of cut sites was estimated with the radcounter_v4.xls spread sheet available from the UK RAD Sequencing Wiki (www.wiki.ed.ac.uk/display/RADSequencing/Home). We assumed a 704 Mb approximate genome size (Ohri and Khoshoo 1987), ca 1.44 pg on average for 15 species of *Ficus*) and a 48% GC content (estimated from EST data available on NCBI). Based on those estimates, 9,095 cut sites were expected. 125ng of input DNA was used for each sample. After digestion, 1 uL of P1 adapters (100nM) was added to saturate restriction sites. Samples were then pooled sixteen by sixteen and DNA of each pool was sheared to a mean size of *ca* 400 bp using the Bioruptor® Pico (Diagenode) (15sec ON / 90sec OFF for 8 cycles). After shearing, end repair and 3'-end adenylation, DNA of each pool was tagged with a different barcoded P2 adapter. A PCR enrichment step was performed prior

to KAPA quantification. The 2*125nt paired-end sequencing of the library was performed at MGX-Montpellier GenomiX on one lane of an Illumina HiSeq 2500 flow cell.

Data cleaning and assembly of paired reads into RAD loci.

Data cleaning was performed with RADIS (Cruaud et al. 2016), which relies on Stacks (Catchen et al. 2013) for demultiplexing and removal of PCR duplicates. Individual loci were built using *ustacks* [m=15; M=2, N=4; with removal (r) and deleveraging (d) algorithms enabled]. The parameter n of *cstacks* (number of mismatches allowed between sample loci when building the catalog) was set to 20 to cluster enough loci for the outgroups, while ensuring not to cluster paralogs in the ingroup. To target loci with slow or moderate substitution rate, only loci present in 75% of the samples were analysed. Loci for which samples had three or more sequences were removed from the analysis. Loci were aligned with MAFFT v7.245 (-linsi option) (Katoh and Standley 2013). The 583 loci obtained in this step (mergeR1 dataset) were used as a starting point to assemble paired reads into longer RAD loci. The pipeline, scripts and parameters used for the assembly of paired reads are available from https://github.com/acruaud/radseq_ficus_2020. Briefly, for each sample, forward reads used to build the 583 cstacks loci of the mergeR1 data set as well as corresponding reverse reads were retrieved from original fastq files with custom scripts and assembled with Trinity (Haas et al. 2013). Contigs were aligned to the reference genome of *F. carica* assembly GCA_002002945.1 with Lastz Release 1.02.00 (Harris 2007). Homology between cstacks loci and reference genome and homology between sample contigs within cstacks loci were tested as follows. For each cstacks locus, the genome scaffold with the highest number of alignment hits was considered as likely to contain the RAD locus. When contigs aligned with different parts of the same scaffold, the genome region that showed the highest identity with the sample contigs (as estimated with Geneious 11.1.4: https://www.geneious.com) was

considered as the most likely RAD locus. Cstacks loci for which the sample of *F. carica* JRAS06927_0001 included in the RAD library was not properly aligned with the reference genome (hard or soft clipped unaligned ends > 10 bp) or for which the majority of contigs did not align with the same genome region as *F. carica* JRAS06927_0001 were removed. Finally, loci for which at least one sample contig appeared more than once were discarded. When several contigs were retained per sample (e.g when forward and reverse reads did not overlap; or in case of polyploidy or sequencing mistake) a consensus was built and the IUPAC code was used without considering any threshold, if, for a given position, different nucleotides were present. Of the 583 initial loci, 530 successfully passed quality controls and were retained for phylogenetic analysis.

Retrieval of RAD loci in genome of outgroup species.

As expected for a RAD experiment (Rubin et al. 2012, Gautier et al. 2013), outgroups included in the library had a high level of missing data (*ca* 70%). To decrease missing data, we mined RAD loci in available outgroup genomes. Aside from *F. carica*, only two genomes of Moraceae were available on NCBI when we performed this study: *Morus notabilis* (assembly GCA_000414095.2) and *Artocarpus camansi* (assembly GCA_002024485.1). Pipeline, scripts and parameters used for the retrieval of RAD loci in genome of outgroups are available from https://github.com/acruaud/radseq_ficus_2020. Briefly, the 530 RAD loci extracted from the genome of *F. carica* in the previous step were aligned with the two outgroups genomes using Lastz. Alignment results were parsed with Samtools (Li et al. 2009) to select among the genome regions on which a single RAD locus matched. We considered that the genome region with the highest similarity was the most likely to be homologous with the query RAD locus. Putative RAD loci were extracted from the genome with custom scripts and aligned with the contigs of forward and reverse reads produced in the previous step using

MAFFT v7.245 (-linsi option). The final dataset (mergeR1R2) was composed of 530 loci, 71 ingroup species (70 included in the RAD library plus the genome of *F. carica*) and five outgroup species (*Antiaris toxicaria*, *Artocarpus sp.* and *Morus alba* that were included in the RAD library plus the genomes of *Artocarpus camansi* and *Morus notabilis*). Summary statistics for data sets and samples were calculated using AMAS (Borowiec 2016). Test for phylogenetic signal of sample properties were conducted in R (R Core Team 2018) using the K statistic (Blomberg et al. 2003) implemented in the package Phytools (Revell 2012). The null expectation of K under no phylogenetic signal was generated by randomly shuffling the tips of the phylogeny 1000 times.

Data set cleaning.

To reduce inference bias due to possible misalignment or default of homology, TreeShrink (Mai and Mirarab 2018) was used to detect and remove abnormally long branches in individual gene trees of the mergeR1R2 dataset. As suggested in the manual, the value of b was determined by a preliminary analysis on a subset of loci and set to 20. Four rounds (two with and two without the outgroups) were performed, to ensure a proper cleaning. Following Tan *et al.* (2015) we only performed a light filtering of alignment positions that contained gaps to reduce signal loss. Sites with more than 75% gaps were removed from the locus alignments using the program seqtools implemented in the package PASTA (Mirarab et al. 2014).

Exploration of potential bias.

To explore potential sources of bias, a correlation analysis between locus properties was performed with the R package Performance Analytics (Peterson and Carl 2018).

Then, we explored a possible impact of heterogeneity of evolutionary rates between taxa with three different methods: i) the LS^3 approach (Rivera-Rivera and Montoya-Burgos 2016, Rivera-Rivera and Montoya-Burgos 2019) ii) a custom approach based on iterative principal component analysis (PCA) of long branch (LB) heterogeneity scores of taxa (Struck 2014) in individual gene trees and iii) different rooting approaches : midpoint rooting, minimal ancestor deviation (MAD) approach (Tria et al. 2017) and minimum variance rooting (MinVar) (Mai et al. 2017). We evaluated a possible impact of base composition heterogeneity among taxa and markers using i) incremental removal of the most GC-biased loci and ii) the custom iterative PCA of GC content of taxa in individual gene trees.

For the LS^3 approach we defined four clades of interest which corresponded to the four highly supported clades recovered in the phylogenetic trees inferred from the mergeR1R2 data set: Clade1= sect. *Pharmacosycea*; Clade2=subg. *Urostigma*, Clade3=sect. *Oreosycea*, Clade4= "gynodioecious clade". The LS^3 algorithm was then used to find a subsample of sequences in each locus that evolve at a homogeneous rate across all clades of interests. The minTaxa parameter was set to 1.

We developed a custom iterative PCA approach in R (R Core Team 2018) to analyse LB heterogeneity scores and GC content of the RAD loci. The PCA consisted in the eigenanalysis of the matrix of the correlations between loci and yielded a set of principal axes corresponding to linear combinations of these variables (Manly and Alberto 2017). We used the scores of the taxa along the axes to detect a possible non-random distribution of taxa on the reduced space of the PCA. Depending on the studied properties different groups were highlighted and compared (LB scores: sect. *Pharmacosycea versus* all other fig trees; GC content: *Mixtiflores versus* all other fig trees and then sect. *Pharmacosycea versus* other fig trees, *Mixtiflores* excluded). An initial PCA was performed on all loci and the difference between the groups was statistically assessed by means of a Wilcoxon test applied to the score

of the loci upon the first PCA axis. Then, the locus showing the highest correlation with the axis was removed and another PCA and Wilcoxon test were performed on the thinned dataset. The locus showing the highest correlation with the first axis of this new PCA was removed and so on. Only loci for which no structure could be observed were retained for phylogenetic analysis (*i.e* loci for which Wilcoxon tests were non-significant, indicating no difference between the groups along the first axis of the PCA). This approach was implemented to homogenize taxa properties as much as possible prior to phylogenetic inference. PCA were performed with the R package ade4 (Dray and Dufour 2007). The R script and a tutorial to perform iterative PCAs is available from https://github.com/acruaud/radseq_ficus_2020.

Phylogenetic inference.

Gene trees were inferred with a maximum likelihood (ML) approach as implemented in raxmlHPC-PTHREADS-AVX version 8.2.4. A rapid bootstrap search (100 replicates) followed by a thorough ML search (-m GTRGAMMA) was performed. Phylogenetic analyses of the concatenated data set were performed with supermatrix (ML) and coalescent-based summary methods. For the ML approach, we used raxmlHPC-PTHREADS-AVX version 8.2.4 and IQTREE v1.6.7 (Nguyen et al. 2015). Data sets were analysed without partitioning. A rapid bootstrap search (100 replicates) followed by a thorough ML search (-m GTRGAMMA) was implemented for the RAxML approach. IQTREE analysis employed an ML search with the best-fit substitution model automatically selected and branch supports were assessed with ultrafast bootstrap (Minh et al. 2013) and SH-aLRT test (Guindon et al. 2010) (1000 replicates). Trees were annotated with TreeGraph 2.13 (Stöver and Müller 2010) or the R package ape (Paradis et al. 2004).

Evolution of life history traits.

Three key traits (life form, breeding system and pollination mode) were studied. Stochastic mapping (Huelsenbeck et al. 2003) as described in Bollback (2006) and implemented in the R package phytools was utilized to estimate the ancestral state and the number of transitions for each trait. The transition matrix was first sampled from its posterior probability distribution conditioned on the substitution model (10,000 generations of MCMC, sampling every 100 generations). Then, 100 stochastic character histories were simulated conditioned on each sampled value of the transition matrix. Three Markov models were tested: equal rates model (ER) with a single parameter for all transition rates, symmetric model (SYM) in which forward and reverse transition have the same rate and all rates different model (ARD). AIC scores and Akaike weight for each model were computed.

Computational resources.

Analyses were performed on a Dell PowerEdge T630 server with two 10-core Intel(R) Xeon(R) CPUs E5-2687W v3 @ 3.10GHz and on the Genotoul Cluster (INRA, France, Toulouse, http://bioinfo.genotoul.fr/).

RESULTS

Molecular phylogenetic inference with outgroup rooting.

As a reminder, classifications of the genus *Ficus* are provided in Table 1. Data sets are described in Table 2 and features of taxa are reported in Tables S1-S3. An average of 2*416,834 paired reads; 2,520 ustacks loci and 2,308 cstacks loci were obtained for each sample included in the RAD library (Table S1). In an attempt to solve the tree backbone, we kept only the 583 most conserved loci assembled from forward reads (75% complete matrix, mergeR1 data set), 91% of which (530) were retained in the final (mergeR1R2) data set. Mining of loci in outgroup genomes largely reduced the level of missing data (from *ca* 70-

75% in the mergeR1 data set to *ca* 15-30% in the mergeR1R2 data set depending on the outgroup, Table S1). Between 1 and 55 loci were flagged for each sample by Treeshrink (average 15, Table S4). The final data set used for phylogenetic inference (mergeR1R2) was composed of 70 species of *Ficus* and five outgroups. We did not obtain enough reads for *Sparattosyce dioica* to include it in our analysis. Alignment length was 419,945 bp (Table 2). RAxML and IQTREE produced identical topologies with high statistical support (Figure 1, S1; Table 3). Neither gaps (K=0.418, Pvalue=0.342) nor missing data (K=0.384, Pvalue=0.555) were phylogenetically clustered in the ingroup (i.e. taxa with high percentages of missing data /gaps did not cluster together more often than expected by chance). All subgenera except *Ficus* and *Pharmacosycea* were recovered monophyletic with strong support and all non-monospecific sections of the data set except *Ficus* were monophyletic with strong support. Section *Pharmacosycea* was sister to all other *Ficus* species with strong support. The remaining species clustered into two highly supported groups: 1) subg. *Urostigma* and sect. *Oreosycea*; 2) subg. *Ficus*, *Sycomorus, Sycidium* and *Synoecia,* hereafter named the "gynodioecious clade" for brevity as it clusters all gynodioecious species of fig trees (although a few monoecious species are present in subg. *Sycomorus*). Relationships within the "gynodioecious clade" were strongly supported with subg. *Sycidium* + *F. carica* sister to subg. *Sycomorus* + other species of the subg. *Ficus.* Subsection *Frutescentiae* was sister to a clade grouping sect. *Eriosycea* and subg. *Synoecia*, yet with poor support. We must note that ASTRAL recovered sect. *Oreosycea* sister to the "gynodioecious clade" when the whole mergeR1R2 data set was considered (Figure S1C), though with low support (PP=0.2). However, when sequences with less that 50% locus coverage were removed from each RAD locus, ASTRAL inferred sect. *Oreosycea* sister to subg. *Urostigma* (Figure S2, PP=0.8). Therefore, incomplete locus coverage might have misled individual gene tree inference resulting in this observed switch of position for sect. *Oreosycea* in the coalescence tree. Only

374 two unsupported changes were observed between the ASTRAL and ML trees in the
375 shallowest nodes (within sect. *Conosycea* and *Malvanthera*, Figures S1-S2).
376
377 ***Identification of potential bias.***
378 Spearman's rank correlation tests showed a significant negative correlation between the
379 proportion of parsimony informative sites or the average bootstrap support of gene trees and
380 i) GC content of loci and ii) LB score heterogeneity of loci (Figure S3). Furthermore, loci
381 with more homogeneous rates among sites (high alpha) were more informative.
382 With the exception of a single unsupported change within subsect. *Eriosycea* (Figure S6),
383 exclusion of GC-rich loci (Table 3) did not induce topological change, which suggested that
384 inferences were not biased by GC content of loci. However, we highlighted compositional
385 bias among samples. As differences in coverage of RAD loci across samples prevented a
386 proper calculation of GC content in the mergeR1R2 data set (i.e. loci were only partially
387 sequenced in some samples), we focused on the mergeR1 data set to explore GC-content bias
388 among samples with PCA (Figure S4). The first principal component (PC1) discriminated
389 between i) all sections of the subg. *Urostigma* except sect. *Urostigma* [*i.e.* the *Mixtiflores*
390 group *sensu* Clement et al. (2020)] and ii) all other species of *Ficus* (eigenvalues = 9.50% for
391 PC1 and 8.57% for PC2, Figure S4Ab). Within the remaining species of *Ficus*, PC1
392 discriminated between i) sect. *Pharmacosycea* and ii) other species of *Ficus* (eigen-value =
393 12.55% for PC1 and 6.64% for PC2, Figure S4Ac). Wilcoxon tests showed that the distance
394 separating i) *Mixtiflores* and other fig trees on one side and ii) sect. *Pharmacosycea* and other
395 fig trees (*Mixtiflores* excluded) on the other side was significant for all iterations of the PCA
396 (Figures S4B-C). This means that it was not possible to homogenize GC content of taxa prior
397 to phylogenetic inference to fit with model assumptions. GC content of *Mixtiflores* was

398 significantly higher and GC content of sect. *Pharmacosycea* was significantly lower that GC
399 content of other fig trees (Figures S4D-E).
400 In addition to heterogeneity of GC content among *Ficus* lineages, we highlighted
401 heterogeneity in evolutionary rates. Two groups were highlighted on the PCA of LB scores
402 across all RAD loci: sect. *Pharmacosycea* and all other fig trees (Figure S5, eigenvalues =
403 18.76% for PC1 and 4.34% for PC2). Moreover, in average, 29.0% of the loci were flagged
404 for sect. *Pharmacosycea* by LS^3, while only 19.4% were flagged for other fig trees (Table
405 S5). Attempts to reduce heterogeneity in evolutionary rates with LS^3 and the custom PCA
406 approach failed. Whatever the concatenated data set analysed (supposedly cleaned or not from
407 bias), the branch leading to sect. *Pharmacosycea* was the longest (Figures 2, S1E, S6D, S6I,
408 S7D, S8C) and sect. *Pharmacosycea* always had significantly higher LB heterogeneity scores
409 than all other taxa (about 7.5 points more for the mergeR1R2_PCA and _LS3 data sets and
410 about 10 points more for the mergeR1R2, _GCinfmean, _GCsupmean data sets; Table 4).
411
412 ***Impact of rooting strategies on the molecular tree.***
413 Fast-evolving or compositionally biased ingroup taxa can be drawn towards the outgroups,
414 especially when the outgroup is distantly related to the ingroup [Long Branch Attraction
415 (LBA) artefact (Bergsten 2005)], which is the case here (Figure S1). For that reason, we
416 tested alternative rooting methods to outgroup rooting. While outgroup rooting always
417 recovered the long-branched sect. *Pharmacosycea* sister to the remaining fig trees (topology
418 1, Figure 2), other rooting methods suggested three alternative positions for the root: i) on the
419 branch separating the "gynodioecious clade" from other *Ficus* species (topology 2), ii) on the
420 branch separating subg. *Urostigma* from other fig trees (topology 3), iii) on the branch
421 separating sect. *Conosycea*, *Malvanthera*, *Americanae* and *Galoglychia* (*Mixtiflores*) from the
422 remaining fig trees (topology 4). The root ambiguity index calculated by MAD was high

(0.788-0.999; average=0.938) which indicates that root inference was problematic for all data sets.

Morphological study.

The morphological matrix is provided in Appendix S2. The nexus file that contains the majority-rule consensus tree obtained from the morphological data and the four conflicting RAD topologies on which reconstruction of ancestral character states was performed with Mesquite v3.31 (Maddison and Maddison 2018) can be opened with PAUP* or Mesquite. Among the 102 morphological characters used, 100 were parsimony-informative. The heuristic search yielded 213 equally parsimonious trees of 736 steps long (CI = 0.443, RI = 0.710). The majority-rule consensus (MRC) tree and the strict consensus trees are depicted in Figure 3. The consistency index of each character is provided in Appendix S1.

As expected, statistical support was generally low. Only a few nodes, all of them also supported in the molecular tree, received bootstrap supports > 80: sections *Eriosycea* (BP=100), *Malvanthera* (BP=96); *Pharmacosycea* (BP=87) and *Urostigma* (BP=92). Three main clades were recovered: 1) a monophyletic subg. *Pharmacosycea*; 2) subg. *Urostigma*; 3) the "gynodioecious clade". Interestingly, the MRC tree differed slightly from the outgroup rooted molecular hypothesis (topology 1, Figure 1). The main differences were: 1) the branching order of the most basal nodes with sect. *Oreosycea* + sect. *Pharmacosycea* (BP=54) sister to all other *Ficus* while only sect. *Pharmacosycea* was recovered sister to the remaining fig trees in the molecular topology; 2) the position of *F. carica* that is sister to section *Eriosycea* in the morphological tree *versus* sister to subg. *Sycidium* in the RAD trees; 3) sect. *Urostigma* sister to section *Conosycea* versus sister to all other sections of *Urostigma*; 4) species of subg. *Synoecia* forming a grade within subg. *Ficus*, while it is monophyletic and nested within subg. *Ficus* in the molecular trees. Character transformations inferred with

PAUP* on the four competing molecular topologies are illustrated in Figure S9 (ACCTRAN optimization). Topologies 1 and 4 were the less compatible with morphological data; while topology 2 was supported by the highest number of unambiguous transformations followed by topology 3.

Reconstruction of traits evolution under stochastic mapping

Because of the low resolution of the morphological tree, reconstructions were performed on the molecular trees only. For all topologies and traits, the ER model had the lowest AIC and highest Akaike weight (Table S6). Therefore, the ER model was subsequently chosen to trace the evolution of breeding system, pollination mode and life form in *Ficus* (Figure S10). Results are given in Table 5, and revealed that the ancestor of all extant *Ficus* was most likely an actively-pollinated, monoecious tree from which hemi-epiphytes /hemi-epilithes and root climbers evolved. Gynodioecy appeared once in the genus and monoecy re-appeared at least twice in the "gynodioecious clade". Active pollination was lost several times independently.

DISCUSSION

A first phylogenomic hypothesis for fig trees and its morphological counterpart.

Here, we propose the first phylogenetic hypothesis for the genus *Ficus* based on pangenomic nuclear markers and a sampling representative of all subgenera and sections (Figure 1). Our analyses highlight heterogeneity in both evolutionary rates and GC content among *Ficus* lineages. We show that sect. *Pharmacosycea* has significantly higher LB heterogeneity scores than all other taxa and, regardless of all the attempts made to reduce this bias (custom PCA approach and LS^3), the branch leading to sect. *Pharmacosycea* is still, by far, the longest (i.e. branch length /evolutionary rates cannot be properly homogenized) (Table 4, Figures S1, S6-8). In addition, the custom PCA approach shows that it was not possible to homogenize GC

content of taxa prior to phylogenetic inference to fit with model assumptions. GC content of *Mixtiflores* (sect. *Americanae, Galoglychia, Conosycea, Malvanthera*) is significantly higher and GC content of sect. *Pharmacosycea* is significantly lower. As heterogeneity in evolutionary rates and base composition are considered important sources of systematic bias (Brinkmann et al. 2005, Philippe et al. 2017), it is crucial to critically interpret results in the light of other line of evidences.

This is why we also built a phylogeny of the same taxa from morphological features. We went one step further than Weiblen (2000) who published the first morphological phylogeny of *Ficus* by increasing the number of characters and taxa analysed. The consistency index values of characters are low (average = 0.375, 32 characters with CI > 0.5), showing a high level of homoplasy as already underlined by Clement & Weiblen (2009) for Moraceae. Nevertheless, the recovered trees are structured enough (Figure 3) to allow comparison with the molecular trees and discuss agreement and discrepancies that could reveal inference bias.

When next generation sequencing corroborates past generation botanists: agreement between morphological and molecular hypotheses.

In agreement with previous molecular studies based on nuclear data, we confirm the monophyly of the subgenera *Sycidium* and *Sycomorus*. The monophyly of *Sycomorus* was not supported in the morphological study by Weiblen (2002) but is confirmed by the morphological analysis presented here. This suggests that the polyphyly of these two subgenera observed by Bruun-Lund *et al.* (2017) in their plastid phylogeny could be due to divergent copies of chloroplastic DNA.

While its monophyly has never been questioned by former botanists (Corner 1958, Berg 1989), the subgenus *Urostigma* had never been recovered as monophyletic in molecular

498 studies so far and one section (*Galoglychia*) was missing from the morphological work by
499 Weiblen (2000) to formally test its monophyly. Here, we highlight a strongly supported
500 subgenus *Urostigma* both with molecular and morphological data. This highly diversified and
501 widespread monoecious subgenus presents a relatively uniform morphology over its range
502 and is well-characterized by non-ambiguous apomorphies (Figure 3): all species have aerial
503 roots and they have only one waxy gland located at the base of the midrib. This subgenus
504 includes, amongst others, sacred banyan trees and giant stranglers.
505
506 For the first time in a molecular framework, we highlight a strongly supported clade that
507 groups all gynodioecious fig trees (Figure 1), which corresponds to a previous
508 circumscription of subgenera within *Ficus* [Table 1, (Corner 1958)]. This monophyly was
509 already highlighted in the morphological work by Weiblen (2000) and is confirmed by our
510 morphological analysis (Figure 3). Aside from the breeding system, this clade is well defined
511 by several non-ambiguous synapomorphies on the morphological tree (species have generally
512 less than 10 lateral veins; figs are frequently stipitate; and bears more than three ostiolar
513 bracts; the stigma in short-styled pistillate flowers is mostly cylindrical; and the fruits are
514 compressed).
515
516 Finally, and as observed in previous molecular work, the subgenus *Ficus* appeared
517 polyphyletic in our molecular and morphological trees, though results differ between the two
518 approaches as discussed in the next section. It is the first time that the non-monophyly of this
519 subgenus is assessed through phylogenetic inference of morphological data as only two
520 representatives of a single section of the subgenus *Ficus* (sect. *Eriosycea*) were included in
521 previous analysis.
522

Systematic bias versus morphological convergences: discrepancies between molecular and morphological evidence.

As mentioned above, subgenus *Ficus* appeared polyphyletic in our molecular and morphological trees. On both trees, subg. *Synoecia* is nested within a clade that comprises subsect. *Frutescentiae* and sect. *Eriosycea* of the subg. *Ficus.* On the morphological tree, *F. carica*, the type species of the subg. *Ficus,* is recovered sister to sect. *Eriosycea.* In the molecular tree, *F. carica* does not cluster with other species of the subg. *Ficus* and is instead recovered sister to subg. *Sycidium*. This sister-taxa relationship has already been observed in the past with molecular data, though with low support (Cruaud *et al*., 2012). This result, which is the most surprising result of our study is nevertheless supported by four homoplastic synapomorphies: deciduousness; asymmetrical lamina; margin of perianth hairy and the presence of pistillodes in male flowers, although several species of subg. *Pharmacosycea* and *Sycomorus* also have pistillodes. More species are needed to confirm this result, especially species of the subseries *Albipilae* Corner. However, if the molecular position of *F. carica* is confirmed, then subsect. *Frutescentiae* and sect. *Eriosycea* should not be considered anymore as belonging to subg. *Ficus*.

The evolutionary history of subg. *Synoecia* seems to be linked to the evolutionary history of subg. *Ficus* in its current circumscription. Indeed, in all molecular studies that were representative enough of the biodiversity of the genus, *Synoecia* always clustered with sect. *Eriosycea* and subsect. *Frutescentiae.* In the morphological tree of Weiblen (2000), subg. *Synoecia* appeared monophyletic and sister to sect. *Eriosycea* (no representative of the subsect. *Frutescentiae* was included). The same sister taxa relationship is observed in our molecular tree, though this is the only part of the tree that received low support. *Synoecia* is not recovered as monophyletic in our morphological tree. However, this may be due to the high level of homoplasy in the analysed characters. Again, further studies are needed but

548 *Synoecia* may simply constitute a lineage that has evolved as root climbers as originally
549 suggested by Corner (1965).
550
551 The second discrepancy between our morphological and molecular results are the
552 relationships between sect. *Malvanthera, Conosycea* and *Urostigma* of subg. *Urostigma*.
553 *Malvanthera* and *Conosycea* are recovered sister in all molecular analyses based on nuclear
554 data including ours. However, all *Conosycea* except *F. elastica* are sister to *Urostigma* in our
555 morphological tree. The morphological results may be due to a lack of signal as all species
556 have aerial roots and show a relatively uniform morphology – at least for a set of characters
557 that are meaningful across the entire genus. These observations lead us to consider that the
558 molecular hypotheses better reflect the history of the subgenus *Urostigma*.
559
560 The last discrepancy between morphological and molecular data concerns the subgenus
561 *Pharmacosycea*. Sections *Pharmacosycea* and *Oreosycea* form one of the few supported
562 clades of our morphological tree (BP > 50, Figure 3), while they form a grade in the
563 molecular tree (Figure 1). The monophyly of the subgenus *Pharmacosycea* has never been
564 questioned by former botanists (Corner 1958, Berg 1989) but has been challenged by all
565 molecular analyses published so far. It is noteworthy that the morphological strict consensus
566 tree presented by Weiblen (2000) was modified to show a monophyletic sect. *Pharmacosycea*
567 that was present in the majority rule consensus of the bootstrap trees but not in the most
568 parsimonious tree in which *F. albipila* (sect. *Oreosycea*) was sister to *F. insipida* (sect.
569 *Pharmacosycea*) [see legend of Figure 5 and text in Weiblen (2000)]. As for other groups of
570 fig trees, apomorphies shared by sections *Pharmacosycea* and *Oreosycea* are difficult to find
571 because there are always a few species that differ from the original ground plan. However, if
572 we consider unambiguous apomorphies that are shared between sect. *Pharmacosycea* and at

573 least one species of sect. *Oreosycea,* six homoplastic characters can be retained (Figure 3):
574 epidermis of petiole flaking off; absence of colored spot on figs; fig stipe present; staminate
575 flowers scattered among pistillate flowers; pistillode present; pistillate perianth partially
576 connate with tepals fused basally.
577 It could be argued that morphological results are due to convergence and this argument cannot
578 be definitely ruled out. However, the morphological tree shows a high level of congruence
579 with the molecular tree for other *Ficus* groups and it is difficult understand why morphology
580 would be only misleading for this subgenus. The ecology of the two sections is close but so is
581 the ecology of all species from subg. *Urostigma* for instance. Denser sampling of subg.
582 *Pharmacosycea* in future molecular works, including species of sect. *Oreosycea* subseries
583 *Albipilae* Corner, may help to better resolve relationships between these two groups. While
584 sect. *Pharmacosycea* may not render sect. *Oreosycea* paraphyletic as observed in the
585 morphological tree, they could be at least closely related. On the opposite, exploration of GC
586 content and evolutionary rates clearly show that sect. *Pharmacosycea* does not exhibit the
587 same properties as all other fig trees, which could mislead molecular inferences.
588
589 ***Where fig trees take root: rooting issues, higher-level relationships and clues from the***
590 ***pollinator tree of life***
591 Indeed, deeper phylogenetic relationships remain the most problematic issue. The unrooted
592 molecular tree highlights the problem we face to resolve the root of fig trees (Figure 2): 1) a
593 long branch leading to a recent diversification of sect. *Pharmacosycea,* and 2) short
594 surrounding branches supporting the "gynodioecious clade", sect. *Oreosycea* and subg.
595 *Urostigma*, suggesting fast diversification of the ancestors of the present lineages. This
596 pattern is predicted to favor artefactual rooting of trees when distant outgroups are used.
597 Further, the recently developed minimal ancestor deviation (MAD) approach that is robust to

598 variation in evolutionary rates among lineages (Tria et al. 2017) shows that root inference is
599 problematic in the original data set (mergeR1R2) and in all other data sets built to test for
600 potential bias (Figure 2).
601
602 Four competing topologies are suggested. Given the long branch leading to sect.
603 *Pharmacosycea* and the impossibility to homogenize its evolutionary rate (and GC content)
604 with those of other fig trees, it seems reasonable to suspect that Topology 1 results from an
605 LBA artefact (Bergsten 2005). Indeed, long-branched taxa can cluster with outgroups with
606 high statistical support irrespective of their true phylogenetic relationships (convergent
607 changes along the two long branches are interpreted as false synapomorphies because current
608 models do not reflect evolutionary reality) (Phillips et al. 2004). It is known that LBA tend to
609 be reinforced as more and more data are considered (e.g. (Boussau et al. 2014), which is
610 probably the case here, even though we used an outgroup belonging to Castillae, *i.e.* the
611 closest relative of *Ficus* (Clement and Weiblen 2009). So far, in all molecular studies, sect.
612 *Pharmacosycea* was recovered sister to all other fig trees [with low or high support, but see
613 Figure 1 of Clement *et al.* (2019), which depicts the phylogeny of Involucraoideae, where
614 section *Albipilae* was sister to all other fig trees with low support. A result that is not
615 recovered from the data set centered on *Ficus* spp. on their Figure 2]. Topology 1 contradicts
616 morphological evidences (Figure 3) and previous classification (Table 1). We must note that
617 the unbalance between overall short internal branches and long branches leading to sect.
618 *Pharmacosycea* on one side and outgroups on the other is recurrently observed in all
619 molecular-based analyses of the *Ficus* phylogeny. Hence, all molecular analyses might have
620 been trapped by an LBA artefact with a misplacement of the root on the long branch leading
621 to sect. *Pharmacosycea*. There is a last line of evidence that should be considered to critically
622 interpret the phylogenies presented here: the evolutionary history of the pollinators.

623 Interestingly, the position of the pollinators of sect. *Pharmacosycea* (genus *Tetrapus*) as sister
624 to all other species of Agaonidae recovered in early molecular studies has been shown to
625 result from an LBA artefact (Cruaud et al. 2012). In addition, pollinators of sect. *Oreosycea*
626 (*Dolichoris*) and sect. *Pharmacosycea* (*Tetrapus*) are also grouped by morphology (see
627 supplementary data of Cruaud *et al.* 2012). Notably *Dolichoris* and *Tetrapus* share a unique
628 metanotal structure in males of agaonids (Figures S14A & B in Cruaud et al., 2012). This
629 structural character advocates close relationships instead of convergence between their host
630 figs.
631 Although they cannot be used as direct evidence, these results are converging evidence
632 suggesting that topology 1 does not accurately reflect early divergence events within fig trees.
633 There are three known techniques to reduce LBA (Bergsten 2005). The first possibility is
634 reducing the branch length of the ingroup taxa that are drawn towards the outgroups. Here, we
635 show that the branch leading to sect. *Pharmacosycea* was still significantly longest regardless
636 of the attempt made to reduce this bias. The second possibility is outgroup removal (Bergsten
637 2005). However, as sect. *Pharmacosycea* is among the first lineages to diverge this method is
638 not helpful. The third possibility is increasing sampling. This should definitely be attempted
639 in the future but without guarantee as LBA may be too strong to be broken (Boussau et al.
640 2014). More generally, given strong bias highlighted here, increasing species sampling
641 appears at least as relevant (if not more) as increasing the number of sequenced regions for
642 each species analysed.
643
644 Topology 4 is the least supported by morphological data (Figure S9) and appears unlikely
645 given the highly supported monophyly of subg. *Urostigma* in morphological analyses (Figure
646 3). A possible explanation for its recovery would be the high GC content of *Mixtiflores* that
647 could be linked to a high number of SNPs supporting the group and an increased branch

length, which distorts the calculation of ancestral deviation. In addition, given life forms observed in Moraceae if appears most likely that the ancestor of fig trees was a freestanding tree, which contradicts the scenario of trait evolution based on topology 4 (Table 5, Figure S10).

Therefore, two topologies still remain likely (topologies 2 & 3, Figure 1). They only differ by the position of the grade composed by sections *Oreosycea* and *Pharmacosycea*. In topology 2, these sections cluster with subg. *Urostigma* and the genus *Ficus* is divided into two groups: monoecious and gynodioecious species. In topology 3, the sections cluster with the "gynodioecious clade" and the genus is split into species with aerial roots on one side and other fig trees on the other side. Topology 2 is the most supported by morphological data on fig trees (Figure S9). Importantly, unambiguous transformations that support the monoecious /gynodioecious split are not only linked to breeding system (or pollination mode). Indeed, characters supporting the split are related to tree height; number of lateral veins; fig stipitate or not; pistillate flowers sessile or not; shape of stigma in short-styled pistillate flowers; lamina thickness; lamina margin; furcation of lateral veins; tertiary venation; presence of interfloral bracts. On the opposite, topology 3 and, more specifically, the sister taxa relationships between sect. *Oreosycea*, sect. *Pharmacosycea* and the "gynodioecious clade" is not supported by any unambiguous morphological transformation (Figure S9).

Finally, the current molecular phylogeny of pollinators of fig trees provide more support to topology 2. Indeed, and again even though this argument should be used only as an indirect evidence, reconciliation of the agaonid tree of life with topology 3 would require a partial reversal of the pollinator tree that contradicts transformation series for the structure of male mesosoma and female basal flagellomeres (anelli) (see Cruaud *et al.* 2012, Figure S14). The

673 current pollinator tree supports a progressive fusion of male mesosoma and female anelli,
674 while topology 3 would favor a subdivision of mesosoma and basal flagellomere as the most
675 derived character state, a trend that has never been observed in Hymenoptera.
676 Therefore, after considering all the evidence (bias, morphology, pollinators) we consider that
677 the most likely topology for the *Ficus* tree of life is topology 2. Interestingly, this topology
678 agrees with one of the first statement of Corner (1958): "the first division of *Ficus* is into the
679 monoecious and dioecious species, but it is more convenient to recognize three subgenera,
680 namely the monoecious banyans (*Urostigma*), the monoecious trees (*Pharmacosycea*) and the
681 dioecious *Ficus*".
682 Nevertheless, increasing sampling is required to resolve the root of the *Ficus* tree of life. Until
683 now, no investigation was undertaken to identify another root for *Ficus* than the one identified
684 by outgroup rooting. However, resolving this issue is important not only to understand the
685 evolution of *Ficus*, but is also crucial to our understanding of key questions such as how
686 species' traits have changed over time and how fig trees co-diversified with their pollinating
687 wasps. Here we go one step further in our understanding of the evolution of key traits as
688 previous trees were not enough resolved to allow inference. While there is a consensus
689 concerning the ancestral pollination mode that is inferred as active for all topologies,
690 ambiguity remains for ancestral breeding system (including for our favored topology, Figure
691 4). Finally, it appears most likely that the ancestor of fig trees was a freestanding tree.
692

693 **CONCLUSION**

694 We present the first phylogenetic hypothesis for the genus *Ficus* based on pangenomic
695 nuclear markers and a sampling effort representative of all subgenera and sections. For the
696 first time, we recover a monophyletic and strongly supported subgenus *Urostigma* and a
697 strongly supported clade grouping all gynodioecious fig trees. Our in-depth analysis of biases,

general pattern of rooting preferences and morphological data completed with indirect evidence from the pollinator tree of life, highlights that previous molecular studies might have been trapped by LBA, which resulted in an artefactual placement of sect. *Pharmacosycea* as sister to all other fig trees. The next key step will be to increase sample size. Indeed, confidence in phylogenetic inference may increase with increasing data sets encompassing as much as possible the overall diversity in the studied group. Increasing species sampling appears at least as relevant as increasing the number of sequenced regions for each species analysed. This work is a first step towards a clarification of the classification and evolutionary history of fig trees. Taxonomic changes foreseen by previous molecular works and new ones will need to be undertaken. But taxonomists will need to be cautious and humble as invalidating current and widely used names will generate more confusion than clarity.

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AUTHOR CONTRIBUTIONS

J.Y.R. and A.C. conceived the study, analyzed data and drafted the manuscript. J.Y.R, L.J.R, Y.Q.P, D.R.Y, F.K., A.B, R.D.H., R.U., R.A.S.P, S.F, A.C. collected samples. J.Y.R identified or verified identification of all samples. L.J.R., L.S. C.T.C. and A.C. performed lab work. M.G and J.P.R contributed scripts. All authors gave final approval for publication.

DATA ACCESSIBILITY

Raw paired reads are available as an NCBI Sequence Read Archive (#SRPXXXX).

Pipeline and scripts are available from: https://github.com/acruaud/radseq_ficus_2020.

Data sets and trees are available from the Dryad Digital Repository:

http://dx.doi.org/10.5061/dryad.[NNNN]

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952
953
954 **FIGURE LEGENDS**
955 **Figure 1. Phylogenetic trees obtained with the molecular data set.**
956 RAxML and IQTREE trees were identical. Genomes are indicated with a (G). Subgenera
957 (larger font size) and (sub) sections (smaller font size) are illustrated with pictograms and
958 highlighted with different colors. Classification follows Cruaud *et al.* (2012) as reported in
959 Table 1. All nodes were supported with RAxML Bootstrap (BP) ≧ 90 and IQTREE SH-aLRT
960 ≧ 80 / UFBoot ≧ 95 unless specified with a grey square: BP < 90 or SH-aLRT < 80 / UFBoot
961 < 95 or a white square: BP < 90 and SH-aLRT < 80 / UFBoot < 95. Although a few
962 *Sycomorus* are monoecious, we refer to the clade that groups subgenera *Ficus, Sycidium,*
963 *Sycomorus* and *Synoecia* as the "gynodioecious clade" for brevity. *Topology 1*: tree obtained
964 from the mergeR1R2 data set with outgroup rooting. This topology is not supported by
965 morphological data (Figures 3 & S9) and supposed to result from an LBA artefact of sect.
966 *Pharmacosycea* to the outgroups. *Topology 2* is obtained for a few data sets when alternative
967 rooting methods are used (Figure 2). It is the most supported by morphological data and

968 evolutionary history of pollinators. *Topology 3* is obtained for a few data sets when alternative
969 rooting methods are used. It is less supported by morphological data and evolutionary history
970 of pollinators than topology 2. *Topology 4* is obtained for a few data sets when alternative
971 rooting methods are used. It is considered unlikely as the position of sect. *Urostigma* is not
972 supported by morphological data. The position of the root could be driven by the GC-content
973 bias exhibited by *Mixtiflores.*
974
975 **Figure 2. Impact of rooting methods on tree estimation.**
976 Three alternative methods to outgroup rooting were tested: midpoint rooting, minimal
977 ancestor deviation (MAD) approach (Tria et al. 2017) and minimum variance rooting
978 (MinVar) (Mai et al. 2017). Outgroups were removed from the data sets described in Table 2
979 before analysis with IQTREE (best-fit substitution model automatically selected). Full trees
980 are available as supplementary data. *Top figure*: Unrooted tree obtained from the mergeR1R2
981 data set with branch colors corresponding to their ancestor relative deviation value AD;
982 alternative positions for the root are indicated with arrows. *Middle figure*: Illustration of the
983 four topologies obtained with different rooting strategies. *Bottom figure*: Summary table of
984 the topologies obtained from different data sets and rooting strategies. In all cases, the MAD
985 approach resulted in very high ambiguity scores for the root (0.788-0.999; average=0.938).
986 When alternative positions for the root were identified by MAD the preferred topology is
987 listed first in the table, then, topologies within 0.01 units of ancestral deviation scores are
988 listed by decreasing order of ancestral deviation. Statistical support of nodes varied with
989 analysed data sets. Black squares indicate strong support: IQTREE SH-aLRT ≧ 80 / UFBoot
990 ≧ 95; white squares indicate low support: IQTREE SH-aLRT < 80 / UFBoot < 95. *Although
991 a few *Sycomorus* are monoecious, we refer to the clade that groups subgenera *Ficus,*
992 *Sycidium, Sycomorus* and *Synoecia* as the "gynodioecious clade" for brevity.

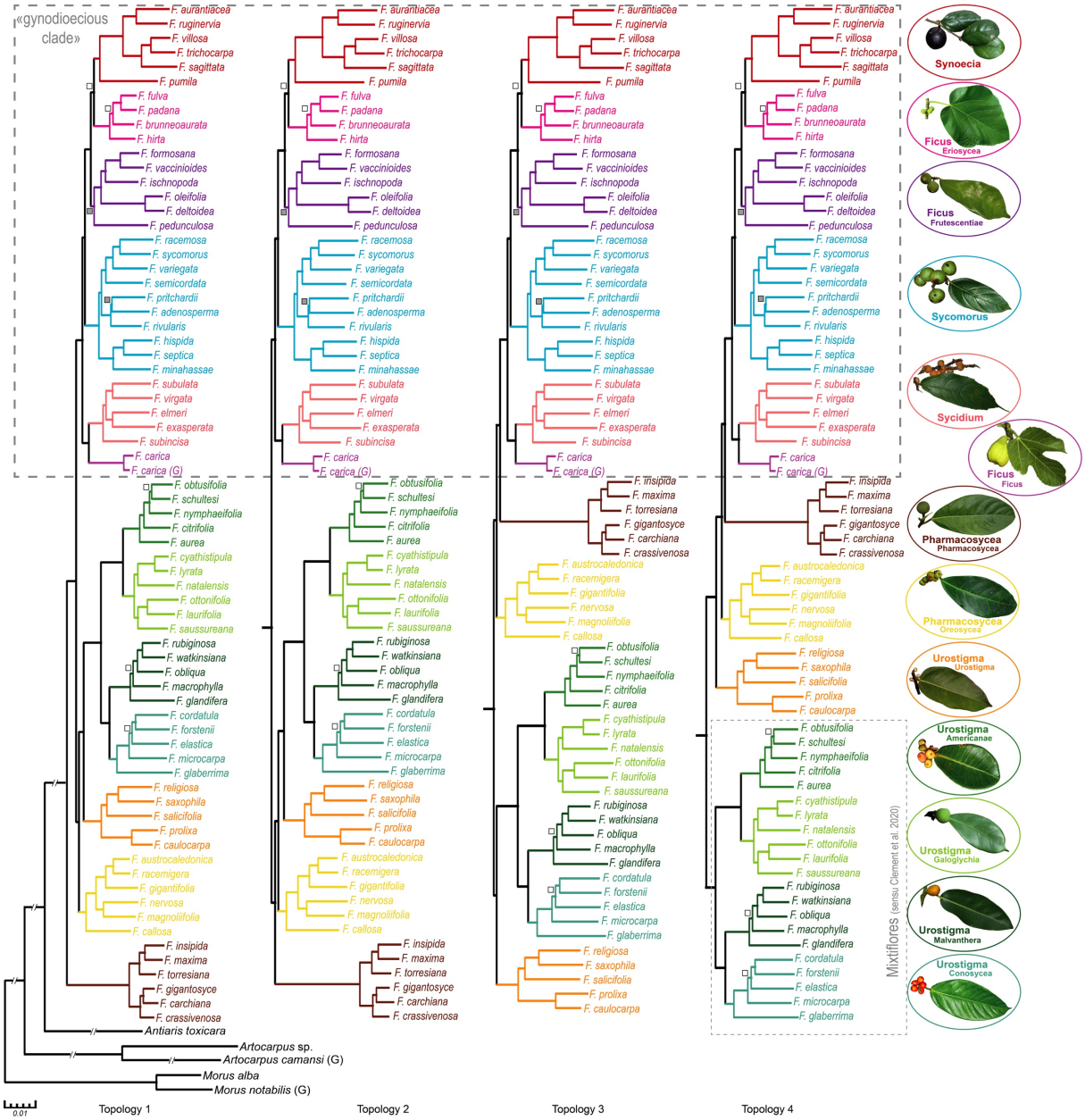
Figure 3. **Phylogenetic trees obtained with the morphological data set.**

Left: majority rule consensus tree; *Right*: strict consensus tree. Bootstrap (100 replicates) at nodes. Ambiguous (single arrow) and non-ambiguous (double arrow) transformations inferred by PAUP* (ACCTRAN optimization) are listed for key nodes as follows: character: ancestral state -->/=> derived state (see list of character /states in Appendix S1). Subgenera (larger font size) and (sub)sections (smaller font size) are illustrated with pictograms and highlighted with different colors as for Figure 1. Classification follows Cruaud *et al.* (2012) as reported in Table 1. Although a few *Sycomorus* are monoecious, we refer to the clade that groups subgenera *Ficus, Sycidium, Sycomorus* and *Synoecia* as the "gynodioecious clade" for brevity.

Figure 4. Reconstruction of traits evolution on the favored topology (topology 2).

All reconstructions are available in Figure S10. *Nota:* the word hemi-epiphytes is used for all species with aerial roots (i.e. hemi-epiphytes *s.s.* and the few hemi-epilithes).

1007 **Fig.1**



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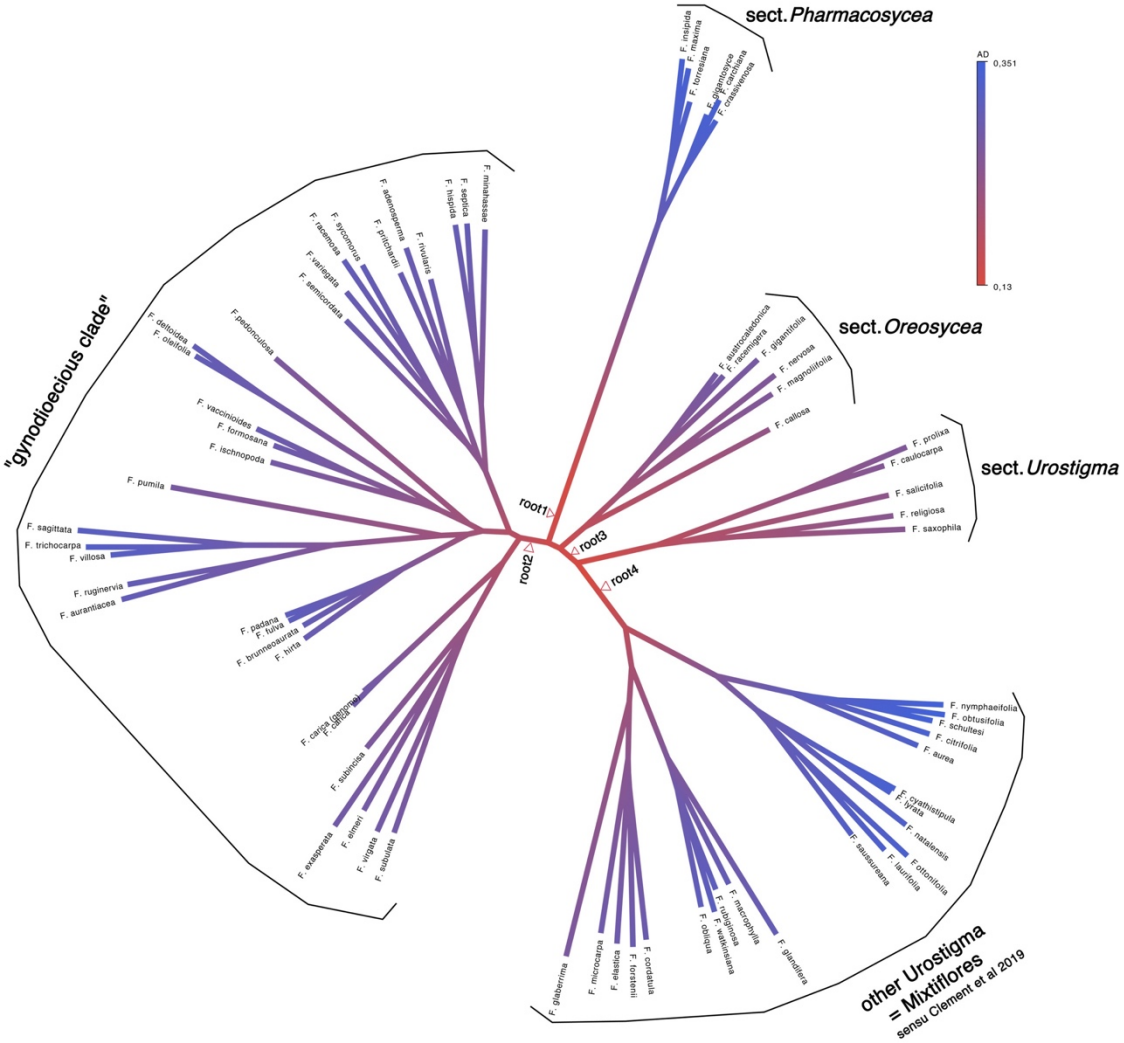
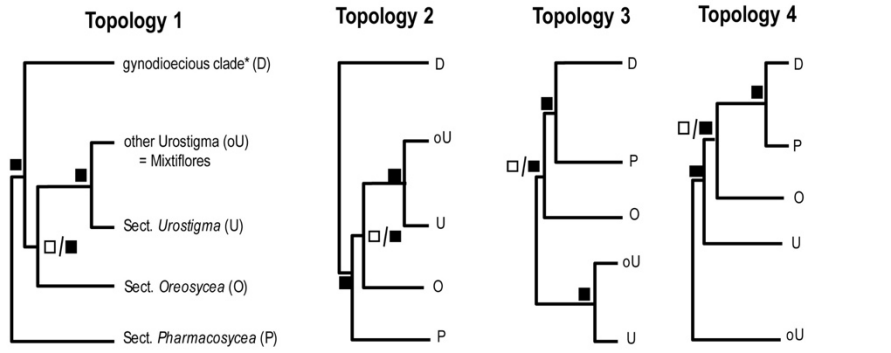
1009

1010 **Fig.2**



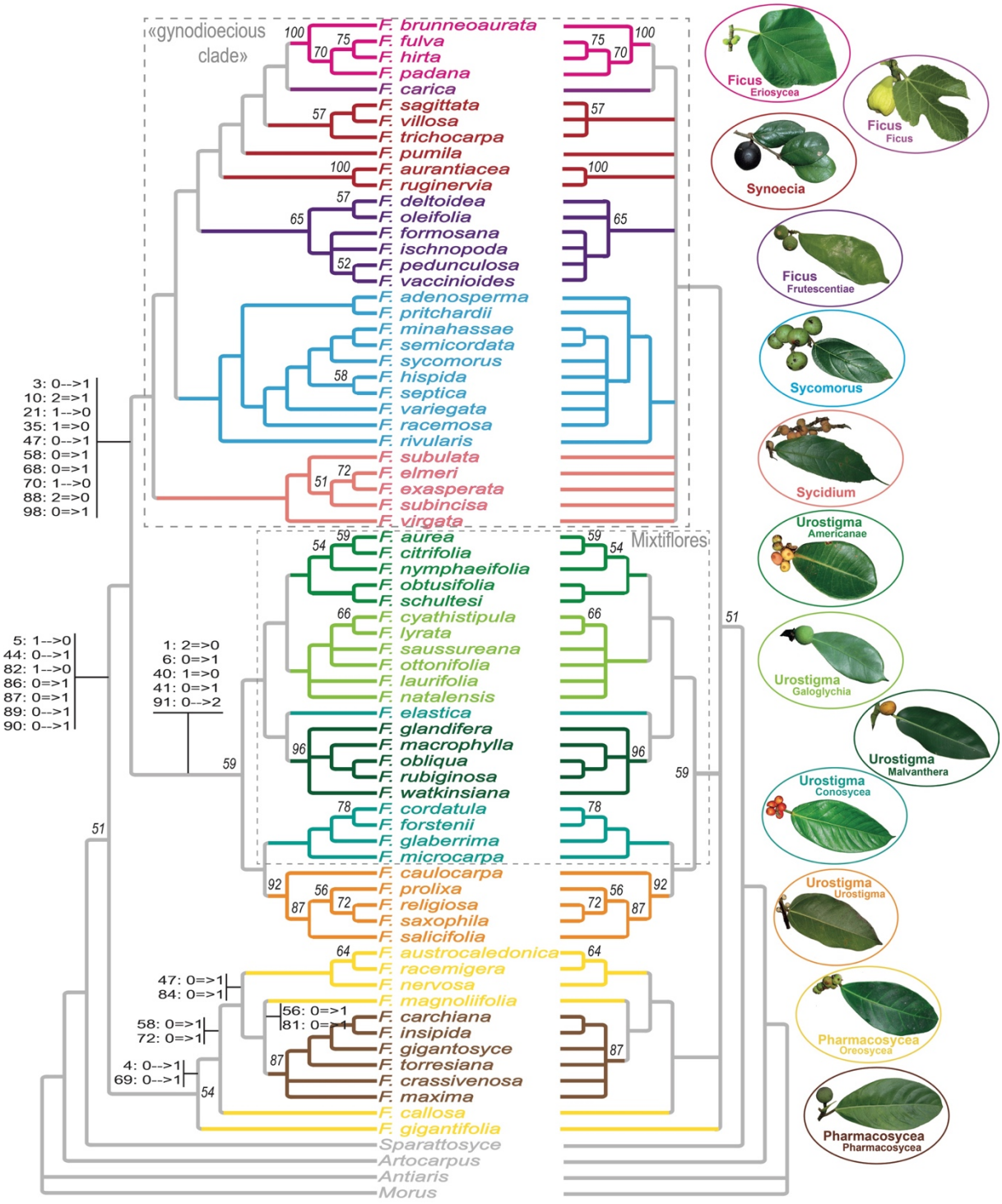


	Rooting method			
Data sets	outgroup	midpoint	MAD	MinVar
mergeR1R2	1	1	4,3,1	1
mergeR1R2_PCA	1	2	1,4,3,2	3
mergeR1R2_LS3	1	1	4,3,1,2	4
mergeR1R2_GCinfmean	1	1	4,3	4
mergeR1R2_GCsupmean	1	1	1,4,3	1

1011

1012

1013 **Fig.3**

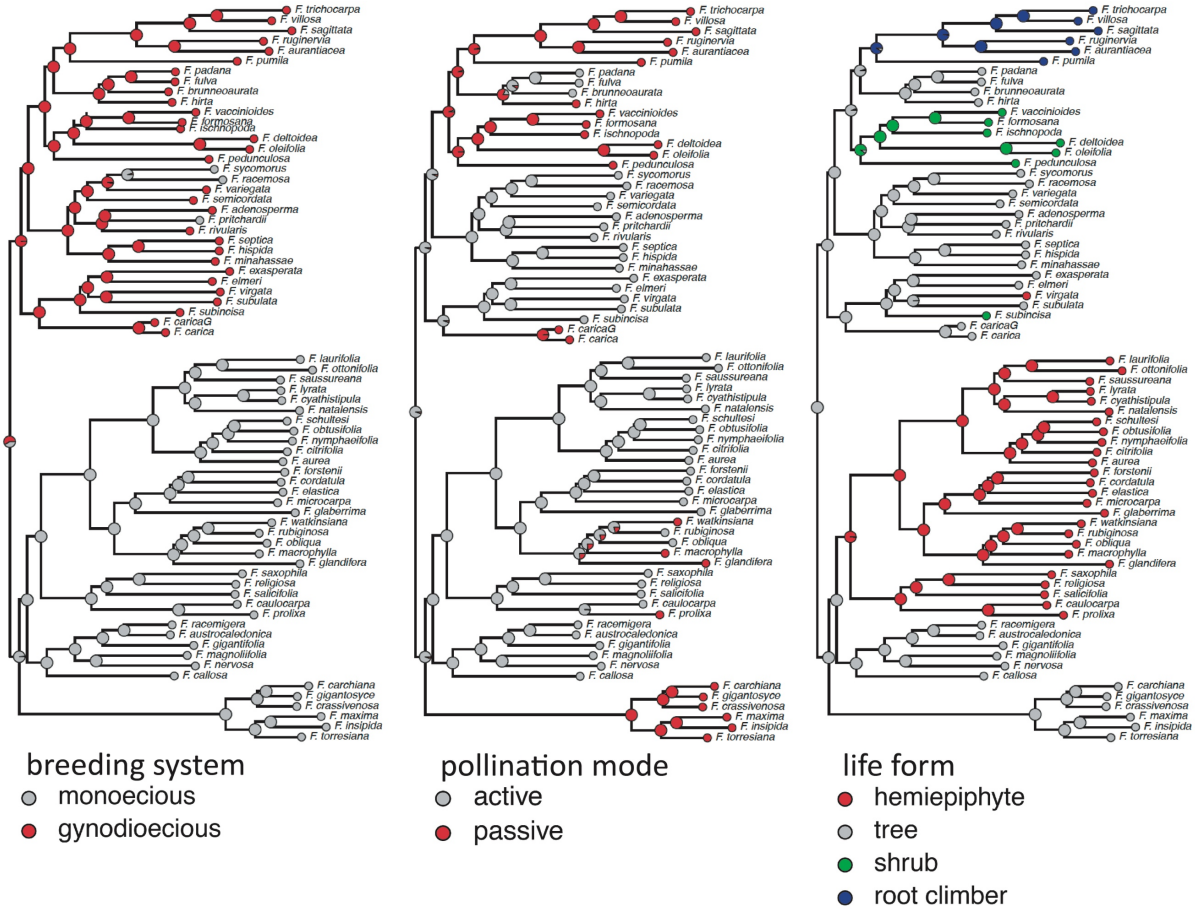


1014

1015

1016

1017 **Fig.4**



1018

TABLES

Table 1. Classification of the genus *Ficus.*
In this paper, we use the simplified classification adapted from Cruaud *et al.* 2012.
*the proper spelling of this section should be *Americanae*, which is the original spelling proposed by Miquel. Indeed, it is a plural adjective.
** *Conosycea, Malvanthera* and *Urostigma* are considered as sections instead of subsections and *F. elastica* (the only member of the subsect. *Stilnophyllum sensu* Berg & Corner (2005) is included in *Conosycea* as recovered in previous molecular studies.
Nota: Subgenus *Urostigma* excluding section *Urostigma* is referred to as *Mixtiflores* by Clement *et al.* (2020).

Corner (1958)		**Corner (1960a, 1960c, 1960b, 1960d, 1960e, 1965)**		**Berg & Corner (2005), Rønsted *et al.* (2008)**		**Traditional simplified classification adapted from Cruaud *et al.* (2012)**	
Subgenera	**Sections**	**Subgenera**	**Sections / Subsections**	**Subgenera**	**Sections / Subsections**	**Subgenera**	**Sections / Subsections**
Pharmacosycea Miq.		*Pharmacosycea*	*Pharmacosycea* (Miq.) Benth. & Hook	*Pharmacosycea*	*Pharmacosycea / Bergianae* & *Petenses*	*Pharmacosycea*	*Pharmacosycea*
			Oreosycea (Miq.) Corner		*Oreosycea / Glandulosae* & *Pedunculatae*		*Oreosycea*
				Ficus	*Ficus / Ficus* & *Frutescentiae*	*Ficus*	*Ficus / Ficus* & *Frutescentiae*
Ficus	*Ficus* (Eusyce)	*Ficus*	*Ficus / Ficus* & *Eriosycea*		*Eriosycea* Miq. */ Eriosycea* & *Auratae*		*Eriosycea / Eriosycea* & *Auratae*
	Synoecia (Miq.) Benth. & Hook		*Kalosyce* (Miq.) Corner = Synoecia (Miq)	*Synoecia*	*Kissosycea*	*Synoecia*	*Kissosycea*
			Rhizocladus Endl.		*Rhizocladus / Plagiostigma, Pogonotrophe, Punctulifoliae, Trichocarpeae*		*Rhizocladus*
	Sycidium Miq.		*Sycidium* Miq. / *Sycidium*, *Varinga, Palaeomorphe*	*Sycidium*	*Sycidium*	*Sycidium*	*Sycidium*
			Sinosycidium Corner		*Palaeomorphe* King		*Palaeomorphe*
	Adenosperma Corner		*Adenosperma* Corner	*Sycomorus*	*Sycomorus / Sycomorus* & *Neomorphe*	*Sycomorus*	*Sycomorus / Sycomorus* & *Neomorphe*
			Neomorphe King		*Sycocarpus / Sycocarpus* & *Macrostyla*		*Sycocarpus / Sycocarpus* & *Macrostyla*
	Sycocarpus Miq. (Covellia auct.)		*Sycocarpus* Miq. / *Auriculisperma, Dammaropsis, Papuasyce, Lepidotus, Macrostyla, Sycocarpus*		*Adenosperma*		*Adenosperma*
					Hemicardia C.C. Berg		*Hemicardia*
	Sycomorus (Gasp.) Miq.	*Sycomorus*			*Papuasyce* (Corner) C.C. Berg		*Papuasyce*
					Bosscheria (Teijsm. & de Vriese) C.C. Berg		*Bosscheria*
					Dammaropsis (Warb.) C.C. Berg		*Dammaropsis*

Urostigma (Gasp.) Miq.	*Americana* Miq.	*Urostigma*	*Americana* (Miq.) Corner	*Urostigma*	*Americana*	*Urostigma*	*Americanae**
	Bibracteata Milxdbr. & Burr.		*Conosycea* (Miq.) Corner / *Conosycea, Dictyoneuron, Benjamina*		*Galoglychia*		*Conosycea***
	Urostigma		*Galoglychia* (Gasp.) Endl.		*Stilnophyllum / Stilnophyllum, Malvanthera*		*Galoglychia*
			Leucogyne Corner		*Urostigma / Conosycea* & *Urostigma*		*Malvanthera***
			Malvanthera Corner				*Urostigma***
			Stilpnophyllum Endl.				
			Urostigma				

Table 2. Description of the concatenated data sets analysed in the study.
**gap content refers to indels inserted during alignment or missing parts of RAD loci following assembly of forward and reverse reads*
***missing data refers to missing position in the matrix due to the absence of RAD loci*

a-description of the data sets

Data set name	Description
mergeR1	Original data set, clustering of forward reads (R1) into RAD loci with RADIS and stacks, no filtering of loci (m ustacks=15, M ustacks = 2, N ustacks = 4; n cstacks = 20; matrix completeness = 75%, Npbloci radis = 3 (i.e. RAD loci were removed from the analysis if at least one sample had more than 3 sequences for this locus in the cstacks catalog), alignment of individual loci = mafft-linsi, no alignment cleaning)
mergeR1R2	Final data set obtained with the assembly of paired reads into RAD loci using the mergeR1 data set as a starting point, filtering of sequences with treeshrink (4 rounds), alignment of individual loci with mafft-linsi, alignment cleaning = seqtools 25 (i.e. for each locus, alignment positions with more than 75% of gaps were removed)
mergeR1R2_50%locuscoverage	Starting from the mergeR1R2 data set, sequences with more than 50% gaps in individual loci were removed
mergeR1R2_PCA	Starting from the mergeR1R2 data set, loci filtered out while Wilcoxon test significant between scattered plots of LB scores for sect. *Pharmacosycea* and other ingroups
mergeR1R2_LS3	Starting from the mergeR1R2 data set, filtering based on LS^3 (loci with less than one sample per group (N=93) filtered out before analysis as required by the program)
mergeR1R2_GCsupmean	Starting from the mergeR1R2 data set, loci filtered out when GC content inferior or equal to mean GC content (0.406)
mergeR1R2_GCinfmean	Starting from the mergeR1R2 data set, loci filtered out when GC content strictly superior to mean GC content (0.406)

b-properties of the data sets

Data set name	mergeR1	mergeR1R2	mergeR1R2_50%locuscoverage	mergeR1R2_PCA	mergeR1R2_LS3	mergeR1R2_GCinfmean	mergeR1R2_GCsupmean
Nb taxa	73	76	76	76	76	76	76
Nb loci	583	530	530	134	377 (rate homogeneity could not be reached for 60 loci)	306	224
Alignment length (bp)	66,197	419,945	419,945	107,499	299,524	239,709	180,236
Variable sites content	0.314	0.453	0.441	0.438	0.432	0.482	0.415
Parsimony informative sites content	0.156	0.203	0.195	0.192	0.186	0.215	0.188
GC content	0.444	0.405	0.404	0.408	0.408	0.373	0.449
Gap content*	0.004	0.187	0.099	0.183	0.168	0.185	0.189
Missing data content**	0.157	0.193	0.311	0.195	0.267	0.198	0.185
Supplementary figure	NA	S1	S2	S7	S8	S6 (B-F)	S6 (G-K)

Table 3. Summary of the phylogenetic relationships inferred from the different data sets when topologies were rooted with outgroups. Data sets are described in Table 2 and topologies are available as supplementary data (Figures S1, S6-8). *Although a few *Sycomorus* are monoecious, we refer to the clade that groups subgenera *Ficus, Sycidium, Sycomorus* and *Synoecia* as the "gynodioecious clade" for brevity. N=Not recovered; Y=recovered. Color coding is as follows: white = not recovered; light grey = RAxML Bootstrap < 90 and IQTREE SH-aLRT < 80 / UFBoot < 95; dark grey = RAxML Bootstrap ≧ 90 and IQTREE SH-aLRT ≧ 80 / UFBoot ≧ 95.

	mergeR1R2		mergeR1R2_PCA		mergeR1R2_LS3		mergeR1R2_GCinfmean		mergeR1R2_GCsupmean	
Relationships	RAxML	IQTREE	RAxML	IQTREE	RAxML	IQTREE	RAxML	IQTREE	RAxML	IQTREE
subg. *Pharmacosycea* monophyletic	N	N	N	N	N	N	N	N	N	N
sect. *Pharmacosycea* sister to other *Ficus*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
subg. *Urostigma* monophyletic	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
(sect. *Urostigma* (*Americanae* + *Galoglychia*) + (*Conosycea* + *Malvanthera*))	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
gynodioecious clade* monophyletic	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
sect. *Oreosycea* + sg. *Urostigma*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
subg. *Ficus* monophyletic	N	N	N	N	N	N	N	N	N	N
F. carica + sg. *Sycidium*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
subg. *Sycidium* sister to other gynodioecious *Ficus*	Y	Y	Y	Y	Y	Y	Y	Y	N	N
subg. *Sycomorus* sister to other gynodioecious *Ficus*	N	N	N	N	N	N	N	N	Y	Y
ssect. *Frutescenciae* incl. *F. pedunculosa*	Y	Y	N	N	Y	Y	Y	Y	Y	Y
subg. *Synoecia* + *F. pedunculosa*	N	N	Y	Y	N	N	N	N	N	N
sect. *Eriosycea* + ssect. *Frutescentiae*	N	N	N	N	N	N	N	N	N	N
sect. *Eriosycea* + subg. *Synoecia*	Y	Y	N	N	Y	Y	Y	Y	N	N
ssect. *Frutescentiae* + subg. *Synoecia*	N	N	Y	Y	N	N	N	N	Y	Y

Table 4. Comparison of average Long Branch heterogeneity scores (LB scores) between sect. *Pharmacosycea* and all other fig trees in concatenated data sets.
Data sets are described in Table 2. Taxa properties are in Table S1. Topologies are available as supplementary data (Figures S1, S6-8)

	mergeR1R2	mergeR1R2_PCA	mergeR1R2_LS3	mergeR1R2_GCinfmean	mergeR1R2_GCsupmean
Sect. *Pharmacosycea*	-8.39	-10.58	-11.40	-9.58	-17.75
Other fig trees	-18.44	-18.00	-18.93	-19.17	-6.97
Wilcoxon test (P-value)	6.9e-05	0.00018	0.00012	6.3e-05	7.5e-05

Table 5. Summary of the reconstruction of traits evolution on the molecular trees.
The character state of the most recent common ancestor to all fig trees is mentioned for the three analyzed traits.
The full reconstructions can be found in Figure S10. *Nota:* the word hemi-epiphytes is used for all species with aerial roots (i.e. hemi-epiphytes *s.s.* and the few hemi-epilithes).

	Topology 1	**Topology 2**	**Topology 3**	**Topology 4**
Breeding system	monoecious	ambiguous (gynodioecious /monoecious)	monoecious	monoecious
Pollination mode	active	active	active	active
Life form	tree	tree	ambiguous (tree /hemi-epiphyte)	hemi-epiphyte