

Research Article

**Morphology, geographic distribution, and host preferences are
poor predictors of phylogenetic relatedness in the
mistletoe genus *Viscum* L.**

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Research article containing 6,200 words, 4 color figures, 1 table; 6 additional figures and 7 additional tables are provided as supporting information.

All raw data files and original phylogenetic trees are available from *Mendeley Data*.

1 **Abstract (250 words max.)**

2 Besides their alleged therapeutic effects, mistletoes of the genus *Viscum* L.
3 (Viscaceae) are keystone species in many ecosystems across Europe, Africa, Asia and
4 Australia because of their complex faunal interactions. We here reconstructed the
5 evolutionary history of *Viscum* based on plastid and nuclear DNA sequence data. We obtained
6 a highly resolved phylogenetic tree with ten well-supported clades, which we used to
7 understand the spatio-temporal evolution of these aerial parasites and evaluate the
8 contribution of reproductive switches and shifts in host ranges to their distribution and
9 diversification. The genus *Viscum* originated in the early Eocene in Africa and appeared to
10 have diversified mainly through geographic isolation, in several cases apparently coinciding
11 with shifts in host preferences. During its evolution, switches in the reproductive mode from
12 ancestral dioecy to monoecy imply an important role in the long-distance dispersal of the
13 parasites from Africa to continental Asia and Australia. We also observed multiple cases of
14 photosynthetic surface reduction (evolution of scale leaves) within the genus, probably
15 indicative of increasing specialization associated with the parasitic lifestyle. Even compared
16 with other parasitic angiosperms, where more host generalists than specialists exist, *Viscum*
17 species are characterized by extraordinarily broad host ranges. Specialization on only a few
18 hosts from a single family or order occurs rarely and is restricted mostly to very recently
19 evolved lineages. The latter mostly derive from or are closely related to generalist parasites,
20 implying that niche shifting to a new host represents an at least temporary evolutionary
21 advantage in *Viscum*.

22

23 **Keywords**

24 mistletoes; parasitic plants; host range evolution; geographic range expansion; reproductive
25 switches; *Viscum*

26 **1. Introduction**

27 Mistletoes are keystone resources in forests and woodlands because of their diverse
28 interactions with the ecosystem's fauna (Watson 2001). The mistletoe genus *Viscum* is well-
29 known in Europe, Africa, and Asia since ancient times, where it served as fodder in Neolithic
30 Europe (Heiss 2012). Especially *V. album*, the European or common mistletoe, is still valued
31 as a medicinal plant for its alleged therapeutic activity in cancer and hypertension therapies
32 (Deliorman et al. 2000; Kienle et al. 2009). Species such as *Viscum triflorum*, *V.*
33 *tuberculatum*, and *V. album* represent food resources in different African and Asian countries
34 (Bussmann 2006; Kunwar et al. 2005).

35 The genus *Viscum* (Viscaceae, Santalales) comprises from 70 (Wu et al. 2003) to 120
36 species (Nickrent 1997 onwards) distributed in the tropical and subtropical regions of Africa,
37 Madagascar, Asia, and Australia, the temperate zones of Europe and Asia as well as the
38 temperate southern Africa. One species, *V. album*, has been introduced and persists in the
39 U.S. and Canada. All *Viscum* species are shrubby mistletoes, that is, obtaining water and
40 nutrients via a multi-functional organ (haustorium) that penetrates the shoot of the host to
41 connect with its vascular tissue. These parasites grow endophytically as cortical strands under
42 the hosts' bark (Kuijt 1969), unlike some species of Loranthaceae that form epicortical roots
43 on the surface of the host branch. The genus *Viscum* is characterized by small, unisexual,
44 insect and wind-pollinated flowers (Hatton 1965; Kay 1986) (Figure 1). The axillary or
45 terminal inflorescences consist of petiolate or sessile cymes. Both dioecy, where individuals
46 have either male or female flowers, and monoecy, where one individual carries flowers of
47 both sexes, occur within the genus. Most African and Madagascan species are dioecious,
48 whereas many Asian and all Australasian species exhibit monoecy (Barlow 1983a,b). The
49 fruits are white, yellow, orange, or red typically one-seeded berries that are spread by birds.
50 *Viscum* species retain the ability to photosynthesize to a greater or lesser extent. The leaves
51 are either thick and leathery or scale leaves, often reduced to relic leaves of diminutive size.

52 Furthermore, the genus includes an endoparasitic species, *V. minimum*, a South African
53 endemic, that only emerges from the hosts' tissue for reproduction.

54 The patterns of host specificity within the genus differ widely. For example, *Viscum*
55 *album* is known to parasitize more than 400 host species (Barney et al. 1998), whereas
56 *Viscum minimum* grows only on two closely related species of *Euphorbia*. Besides this,
57 several species such as *V. fischeri* occasionally parasitize other mistletoes (e.g.
58 *Phragmanthera* or *Tapinanthus*), whereas some like *V. loranthicola* are obligate epiparasites
59 on various species of Loranthaceae (Polhill and Wiens, 1998).

60 European mistletoes are well-known to biologists and the public, but the evolutionary
61 history of *Viscum* is still elusive. Several morphological classification systems have been
62 proposed for *Viscum* based on reproductive mode (monoecy or dioecy), the presence or
63 absence of leaves, leaf shape, stem, and fruits, but also structures of the reproductive organs
64 and inflorescences (summarized in Table 1). Although all African *Viscum* species have been
65 monographed (Polhill and Wiens 1998) and their morphology analyzed cladistically (Kirkup
66 et al. 2000), these infraspecific relationships remain to be tested with independent data. To
67 date, the only molecular phylogenetic analysis of the genus was based on the nuclear large-
68 subunit ribosomal DNA (Mathiasen et al. 2008). Although this study sampled only 12
69 *Viscum* species and the phylogram lacked resolution, it did provide preliminary evidence of
70 geographic rather than morphological clades. Expanding this preliminary molecular
71 phylogenetic study to include more species and more genetic markers thus holds the potential
72 to reconstruct the evolutionary history of *Viscum*.

73 Here, we use 33 sequences of the plastid marker *rbcL*, 37 of *trnL-F*, and 19 of *matK* as
74 well as 110 sequences of the nuclear ribosomal internal transcribed spacers 1 and 2 (ITS-1, -
75 2) plus 21 sequences of the 18S ribosomal RNA gene from species and subspecies of *Viscum*
76 and several outgroup taxa of Santalales to elucidate the diversification pattern of these
77 important keystone plants. Our final phylogenetic tree of 110 taxa was subjected to extensive

78 phylogenetic analyses employing maximum likelihood and Bayesian inference. We focused
79 specifically on analyzing whether cladogenesis correlates primarily with morphological
80 differentiation, geographic range distribution, or host preference. To this end, we estimated
81 the age of the genus and the infrageneric divergence times using a relaxed molecular clock.
82 These data provided a solid basis to reconstruct the geographical origin of *Viscum* and trace
83 its subsequent distributional history across four continents. We also reconstructed by
84 maximum likelihood the evolution of morphological characters such as foliage type and
85 reproductive mode, considering phylogenetic uncertainty. We mapped and reconstructed the
86 host preferences and the evolution of host ranges to test if diversification in *Viscum* includes
87 significant host range shifts. This study will aid in understanding diversification of parasitic
88 plants that are important components of flora-faunal interactions in many ecosystems.

89

90 **2. Materials and Methods**

91 **2.1. Taxon Sampling**

92 The classification of Santalales families used here follows Nickrent et al. (2010). We
93 used 220 sequences of 5 nuclear and plastid markers from 59 *Viscum* taxa (incl. 2 subspecies)
94 and, in total, 73 outgroup taxa from Viscaceae and three other Santalales families for the final
95 computation of a 110-taxon phylogeny. Our data sets included sequences generated in this
96 study (from one plant individual each), as well as additional data from Genbank.
97 Supplemental Tables S1 and S2 detail the origin and voucher information for all taxa included
98 here. Despite the low availability of useful *Viscum* specimens in herbaria, we successfully
99 sampled the genus representatively in terms of geographical distribution, and morphological
100 trait variability (see 2.3 and 2.4 below).

101

102

103 **2.2. Experimental procedures**

104 We sequenced the nuclear ribosomal ITS region (internal transcribed spacers 1, 2 and
105 5.8S rDNA) for 59 *Viscum* taxa and several outgroups within and outside Viscaceae. In
106 addition, we sequenced the plastid loci *rbcL* and *trnL-F* for nine outgroup and *Viscum*
107 species. DNA was isolated either from herbarium specimens or from fresh or silica-dried
108 tissues using the NucleoSpin Plant II Kit (Macherey-Nagel) according to the manufacturer's
109 protocol. PCR amplification was performed in a 25 µl reaction mix containing 1 µl DNA
110 template (10-30 ng/µl), 2 mM of MgCl₂, 0.2 mM of each dNTP, 0.8 µM of each primer, 1 U
111 GoTaq Flexi DNA polymerase (Promega) and 1× GoTaq Flexi Buffer. The PCR program
112 consisted of 5 min at 94°C, 35 cycles of each 1 min at 94°C, 1 min at 48°C, and 0.45 min at
113 68 °C, plus a final extension step of 10 min at 72 °C.

114 In most cases the amplification of the ITS region was successful with the ITS4 and
115 ITS5 primers (White et al. 1990). We used the following newly designed, *Viscum*-specific
116 primers: SEQITS2_VISCUM (5'-AACGACTCTCGRCAATGG-3') with ITS4 and
117 SEQITS1_VISCUM (5'-TTGCGTTCAAAAACACTCAATGA-3') with ITS5 to amplify the
118 region in two overlapping halves when only low-quality or fragmented template DNA was
119 available. We amplified *rbcL* and *trnL-F* using the *rbcL*-1F primer (Olmstead et al. 1992) in
120 combination with *rbcL*-1368R (Fritsch et al. 2001), and the *trnL-F* universal primers (Noben
121 et al., 2017), respectively. DNA sequencing was carried out by GATC Biotech (Germany) or
122 Macrogen Inc. (Netherlands). We complemented our dataset with several taxa deposited in
123 Genbank (Table S1) to construct two final data sets: data set 1 consisted of the nuclear 18S
124 rRNA gene concatenated with the plastid markers *matK*, *trnL-F*, and *rbcL* (Supplemental
125 Table S1); data set 2 consisted only of the entire ITS region (Supplemental Table S2).

126 **2.3. Phylogenetic analyses**

127 Sequence data editing and manual alignment were performed using PhyDE v0.9971
128 (<http://www.phyde.de>). Because of high variability in the nuclear ribosomal internal tran-

129 scribed spacers across genera and families (51.6% pairwise identity over all taxa; pairwise
130 identity within *Viscum*: 72.4%), we applied a two-step approach, which consisted of the re-
131 construction of a backbone guide tree to aid the resolution of deep nodes in the phylogenetic
132 tree based on a multi-marker data set with the more slowly evolving nuclear 18S rRNA gene
133 and the plastid markers (data set 1) and a subsequent inference of lower-level relationships on
134 a condensed but near-complete matrix of the faster evolving ITS region (data set 2) using
135 constraints resulting from step 1. Rather slowly evolving markers have a higher chance to
136 resolve deep nodes more accurately and suffer less from homoplasy, amongst others (e.g.,
137 Wicke and Schneeweiss 2015).

138 We aligned the data set 1 manually and excluded mutational hotspots in the *trnL-F* spacer
139 where homology assessment was ambiguous (all alignments available from *Mendeley Data*).
140 The backbone guide tree was computed using Bayesian inference (see below), for which we
141 added information from simple gap coding (hereafter: SIC) (Simmons and Ochoterena 2000),
142 obtained with *SeqState* 1.4 (Müller 2005), thereby appending another 266 characters to our
143 data matrix.

144 Additionally, we generated ITS region alignments for the following subgroups within which
145 the variability of this nuclear marker region still allowed confident homology assessment: 1)
146 *Arceuthobium*, 2) *Korthalsella* and *Phoradendron/Dendrophthora*, 3) Loranthaceae, 4) Santa-
147 laceae incl. Amphorogynaceae, 5) *Notothixos*, and 6) *Viscum*, with the *Phoraden-*
148 *dron/Dendrophthora* clade as outgroup. These data subsets were aligned manually (*Viscum*)
149 or using *PRANK* v1.3 (Löytynoja and Goldman 2005; default settings) to generate group-wise
150 sub alignments, to be used as anchors. Phylogenetic relationships within these specific data
151 sets were computed by Bayesian inference, and the reconstruction of a full phylogeny was
152 then constrained with the backbone tree through genera- or cladewise addition. To obtain a
153 complete ITS region alignment of 110 taxa, some of which having served already as sub-
154 alignment anchors, the resulting tree (Supporting Figure S1) was used as a guide tree for
155 *PRANK* under default settings (data set 2; Supporting Table S2).

156 The final ITS region alignment was subsequently analyzed with and without simple
157 indel coding using Bayesian inference (BI) and maximum likelihood (ML), respectively.
158 Bayesian analyses were conducted with *MrBayes* v3.2.5, x64 MPI version (Huelsenbeck and
159 Ronquist 2001) under the GTR+ Γ model. We analyzed six runs with four parallel chains, each
160 with one million generations for the family-specific data sets and ten million generations for
161 the combined analysis, allowing for a burn-in phase of the first 25% of all iterations. ML
162 inferences were computed with RAxML v8.1.2 under the GTR+ Γ model and 10,000 bootstrap
163 replicates (Stamatakis 2014). To evaluate the robustness of our data set 2 alignment and our
164 approach in general, we also employed *Guidance II* (Sela et al. 2015) in combination with
165 *mafft* (Katoh et al. 2002) but in the absence of a guide tree and under three different
166 stringency settings, leading to the elimination of differing proportions of uncertain or
167 homoplasious sites (optimized alignment variants available from *Mendeley Data*). The
168 resulting three data sets were used to re-compute phylogenetic trees with RAxML and
169 MrBayes as described above.

170 Results and trees were visualized in *Treegraph* 2.4 beta (Stöver and Müller 2010), or
171 with the R statistical computing framework in combination with the R packages *ape* (Paradis
172 et al. 2004) and *phytools* (Revell 2012).

173 **2.4. Molecular clock dating and estimation of the distribution range evolution**

174 We performed a molecular dating analysis using *PhyloBayes* v3.3 (Lartillot et al. 2009)
175 with the CAT Dirichlet process mixture for among-site substitution heterogeneities (Lartillot
176 2004) and CIR (Lepage et al. 2007) as clock relaxation process in addition to a log-normal
177 autocorrelated relaxed clock (LN) analysis. Using our combined data set 2 and the results
178 from the Bayesian and ML inferences with and without SIC, we applied a primary calibration
179 to constrain our analyses with fossil data with minimal ages according to the records' upper
180 bounds as follows: Loranthaceae – 51 million years (mya; Macphail et al. 2012), Santalaceae
181 – 65 mya (Darrah 1939; Christopher 1979), *Arceuthobium* – 52 mya (Kruttsch 1962), and the

182 most recent common ancestor of *Viscum* to 28 mya (Mai et al. 2001); no root age constraint
183 was set. Per analysis, we ran two parallel chains from which we sampled every 5th generation
184 until 20,000 were collected. MCMC chain convergence was assessed via the discrepancy
185 between the posterior averages obtained from independent runs and the effective size of
186 several summary statistics. Because of the good convergence of both chains, we merged the
187 chains and computed the consensus divergence age estimates per every input tree. Trees were
188 visualized using the *ape* package in R (Paradis et al. 2004).

189 *LaGrange* v2 (Ree and Smith 2008) was run to reconstruct the evolution of geographic
190 ranges in *Viscum* using the four phylogenetic trees (BI, BI-SIC, ML, ML-SIC) and the
191 corresponding *PhyloBayes* inferred root ages (BI: 142.02 mya (CIR), 120.258 mya (LN), BI-
192 SIC: 144.182 mya (CIR), 122.172 mya (LN), ML: 140.969 mya (CIR), 121.558 mya (LN),
193 ML-SIC: 144.195 mya (CIR), 118.287 mya (LN)). For the analysis, we classified seven
194 relevant regions: Europe, Africa north of the Sahara, Sub-Saharan Africa, Madagascar and
195 Comores, continental Asia (north of the Wallace line), Australasia (south of the Wallace line),
196 and the Americas. Distribution ranges of the extant species were coded as a binary text file
197 indicating the presence or absence in these regions (Table S3). We input the species range
198 data matrix, and the phylogenetic tree into the *LaGrange* configurator adding the root node
199 ages from our molecular dating analysis. We excluded the following direct transition
200 combinations from the adjacency matrix: Madagascar and Americas, Europe and Madagascar,
201 and Europe and Australasia, respectively, because we considered such transitions as highly
202 unlikely. All rate parameters were estimated with dispersal constraints consistently set to 1.0,
203 and we calculated with only one-time period from 0 to the respective root age. We visualized
204 the results with *Treegraph* 2.4 beta (Stöver and Müller 2010).

205 **2.5. Ancestral state estimation**

206 Information on the morphological characters regarding foliage was coded with three
207 states as 0 = leafy, 1 = scale leaves, and 2 = leaves and scale leaves; the plant reproductive

208 mode was coded as 0 = monoecious, 1 = dioecious, and 2 = bisexual flowers (Table S4;
209 available from *Mendeley Data*). Ancestral state reconstruction was conducted for both
210 features separately with *BayesTraits* v2.0 under the MultiState option with 1000 maximum
211 likelihood attempts (Pagel et al. 2004). To test all possible topologies of the ingroup, we
212 performed this analysis based on the BI trees with and without SIC, and the ML tree with
213 SIC, respectively (Supporting Figure S2). We did not consider the ML tree without SIC
214 because the topology was identical to the BI tree without SIC.

215 Host range distribution within the genus *Viscum* was primarily assessed on the basis of
216 an extensive literature research (Table S5; also available from *Mendeley Data*). To evaluate
217 whether a broad host range is a derived character, we traced the evolution of host ranges by
218 inferring the ancestral number of potential host species or host genera across the consensus
219 topology via maximum likelihood ancestral state reconstruction, implemented in the *R*
220 packages *ape* (Paradis et al. 2004) and *phytools* (Revell 2012).

221

222 **3. Results**

223 **3.1. Phylogenetic relationships**

224 Maximum likelihood and Bayesian inference based on the concatenated nuclear and
225 plastid gene dataset provided a first comprehensive and statistically well-supported
226 phylogenetic tree of the mistletoe genus *Viscum* (Fig. 2 and Supporting Figures S1, S2, S3;
227 original tree files in *newick* format available from *Mendeley Data*). Our analyses resolve
228 *Notothixos* as sister to all other Viscaceae and Phoradendreae as sister to *Viscum*. Within
229 *Viscum*, we can define ten well-supported clades: Clade A consists of three species from
230 eastern Africa, whereas species of clade B occur in sub-Saharan Africa (*V. congolense*),
231 Northern Africa, Southern Europe, and the Near East (*V. cruciatum*), temperate continental
232 Asia (*V. nudum* and *V. coloratum*), and *Viscum album*, which is widespread from England to
233 Japan. Madagascan species cluster in both clade C, which exclusively comprises Madagascan

234 species, and clade E, that also contains one species occurring also in continental Africa (*V.*
235 *decurrens*) and one species known from continental Africa and the Comoros (*V. triflorum*).
236 Early diverging from clade E, although with low support, is clade D consisting of endemic
237 Australian species (*V. whitei*, *V. bancroftii*). Clades F, G, and H contain species with rather
238 small geographic ranges in Southern Africa, except for *V. tuberculatum*, which expands into
239 eastern Africa. While clade I contains species mostly occurring in both continental Asia and
240 Australasia, Clade J consists of species from sub-Saharan Africa.

241 Our analysis showed that some taxa are not monophyletic: *V. myriophlebium* subsp.
242 *myriophlebium*/*V. myriophlebium* var. *douliotii*, *V. cuneifolium*/*V. cuneifolium* var.
243 *grandifolium*/*V. cuneifolium* var. *demissum*, *Viscum ovalifolium*. The ITS sequences of both
244 specimens of *V. ovalifolium* and *V. orientale* are like one another, forming a clearly definable
245 group.

246 Minor topological differences between our various phylogenetic reconstruction methods
247 (BI with and without SIC, and ML with and without SIC, respectively) only occur within two
248 outgroup clades (Loranthaceae and *Notothixos*), and on three positions inside *Viscum*. Both
249 trees obtained with SIC (BI and ML) are congruent within *Viscum*, except for the topology of
250 clade I: In the ML-SIC tree, the *V. ovalifolium*/*V. orientale* subclade is sister to the remaining
251 clade, whereas the *V. articulatum*/*V. stenocarpum* subclade is sister to the remaining clade I
252 in all other trees. No incongruency is seen between inferences without SIC (Fig. S3). Within
253 *Viscum* they differ slightly from the SIC trees in the position of *V. menyhartii* in clade J and in
254 the position of *V. minimum* and *V. pauciflorum*, which is sister to clade H with low support in
255 the trees without SIC but forms a well-supported discrete clade being sister to clades H-J in
256 the SIC analyses (Fig. S3). Except for the branch leading to extant *Arceuthobium* species,
257 phylograms (Supporting Figures S2 and S5) from any of the conducted analyses show no
258 conspicuously long branches within or between clades that could hint to a long-branch
259 attraction phenomenon or elevated nucleotide substitution rates. Also, optimization of the

260 alignment of data set 2 has no influence on the recovered topology (Supporting Fig. S4;
261 results also available in *newick* format from *Mendeley Data*), only affecting node support
262 values. These results suggest that the primary nucleotide data already exhibits a rather
263 powerful phylogenetic signal for a robust phylogenetic inference even without SIC.

264 **3.2. Molecular dating and ancestral range distribution**

265 Our *PhyloBayes*-based divergence time estimations indicate that Viscaceae diverged in
266 the late Lower Cretaceous, with an origin between 124.72 ± 31.55 mya (CIR) and $102.15 \pm$
267 33.67 mya (LN) for the stem group and 108.06 ± 27.35 mya (CIR) and 86.01 ± 27.94 mya
268 (LN) for the crown group (Fig. 2 and Table S5). The root node age estimates based on our BI-
269 SIC tree vary between 144.18 ± 36.96 mya (CIR model) and 122.17 ± 41.86 mya (LN model).
270 The reconstruction of the ancestral distribution suggests an Australasian origin of Viscaceae
271 (more than two log-likelihood units in all analyses) (Fig. 3; Table S6). The genus *Viscum*
272 separated from Phoradendreae 73.78 ± 18.99 (CIR), or 61.76 ± 19.62 (LN) mya. The crown
273 group of *Viscum* evolved in the early Eocene (CIR: 51.31 ± 13.49 mya, LN: 45.61 ± 14.48
274 mya), when the ancestor of clade A/B diverged from that of the remaining *Viscum*.

275 The Lagrange results do not clarify the geographical origin of *Viscum*, although Africa
276 as the ancestral area of the *Viscum* stem group has slightly better support than alternatives.
277 The African range is retained throughout the whole backbone of *Viscum*. Continental Asia
278 was most likely colonized 29.72 ± 10.19 (LN), or 26.38 ± 7.85 (CIR) mya (clade B). The
279 Madagascan clades C and E, together with the Australasian clade D separated from the
280 ancestor of remaining *Viscum* taxa 42.86 ± 11.24 (CIR), or 40.9 ± 12.95 (LN) mya. Given the
281 tree topology is correct, Africa seems to have been colonized twice from Madagascar (CIR:
282 16.36 ± 4.82 , LN: 16.6 ± 5.65 mya; CIR: 4.06 ± 1.55 , LN: 3.27 ± 1.43 my). *Viscum*
283 recolonized Australasia most probably 24.27 ± 7.78 (LN), 19.47 ± 5.45 (CIR) mya from Africa
284 at the base of clade I (*V. articulatum*). Slightly higher support values suggest that *Viscum*
285 spread to continental Asia three times independently from Australasia within Clade I (LN:

286 9.76 ± 3.7 , CIR: 5.36 ± 1.88 mya; LN: 7.04 ± 2.89 , CIR: 5.11 ± 1.99 mya; LN: 3.35 ± 1.84 ,
287 CIR: 1.76 ± 0.97 mya; results from alternative tree topologies: Supporting Table S6).

288 **3.3. Ancestral state estimation**

289 The reconstruction of reproductive modes suggests that dioecy likely was the ancestral
290 sexual condition in *Viscum* (posterior probability [pp]: 0.81 for dioecy versus 0.19 for
291 monoecy) (Fig. 2; Supporting Fig. S6 and Table S7 for alternative tree topologies). Clades A,
292 B, and C contain only dioecious species. Within the remaining clades reproductive mode
293 varies. Monoecy evolved at least eight times independently, being replaced again by dioecy in
294 five cases. Our analysis suggests that the most-recent common ancestors of the (Austral)
295 Asian clades D and I likely both were monoecious (pp: 1.00 (D), 1.00 (I); Fig. 2; Fig. S6 and
296 Table S7).

297 Our ancestral state reconstruction of foliage evolution suggests that the *Viscum* ancestor
298 was leafy (pp: 0.72). The reduction of leaves occurred independently ten times during the
299 evolution of *Viscum*, nine times from an ancestrally leafy condition and once from an ancestor
300 with both leaves and scale leaves (Fig. 2). The extreme reduction to minute scale leaves
301 evolved rather late during the diversification of *Viscum*, with one exception: within the
302 African clade J, scale leaves are present in all species, thus it is likely that this feature was
303 already present in the ancestor of this clade. Our analyses suggest that full foliage was
304 maintained over ca. 19.61 mya (LN) or 25.71 mya (CIR), after the reconstructed origin of the
305 genus (see below), and reject the possibility of a recurrence of the leafy habit in a clade that
306 had previously already evolved scale leaves. Conversely, our reconstruction suggests that
307 within clade C, leafiness has evolved again from the intermediate habit of both leaves and
308 scales, once from the clade's most recent common ancestor and a second time within the
309 clade.

310 Visual inspection of host range distribution within the genus suggests that a shift in host
311 specificity may have contributed to speciation and diversification in *Viscum* (Fig. 4). Based

312 on an extensive literature search (summarized in Supporting Table S5), we observe that
313 several sister taxa show distinct host specificities (e.g. *Viscum album* subsp. *album* and *V.*
314 *album* subsp. *abies*; *V. pauciflorum* and *V. minimum*; the *V. schimperi/V. loranthicola/V.*
315 *shirense* clade). Many species grow on core rosids. A clear ancestral order cannot be
316 identified, partly because of the lack of host information for some taxa. The number of
317 potential hosts is highest in *V. album* subsp. *album* and covers the broadest taxonomic
318 diversity, ranging from basal angiosperms to lamiids and asterids (Fig. 4; Supporting Table
319 S5). A few other Asian taxa such as *V. articulatum* are known to parasitize many different
320 host plants, too, but the diversity of their hosts is narrower than that of *V. album* subsp.
321 *album*.

322

323 **4. Discussion**

324 **4.1. Diversification of *Viscum* is geography-driven – to some extent**

325 Using nuclear and plastid markers, we here showed that cladogenesis within the
326 mistletoe genus *Viscum* is more consistent with geographical ranges of the species they
327 contain than with classifications based on morphology. Although some geographic patterning
328 exists, thus corroborating to some degree an earlier hypothesis of a geography-driven
329 diversification pattern (Mathiasen et al. 2008), the geographic distribution pattern of the
330 extant *Viscum* species is complex and cannot alone be used to conclude interspecific
331 relationships within the genus. The independent colonizations of Europe, continental Asia, as
332 well as the recolonizations of the African continent and Australasia does not allow the
333 discrimination of clades based solely on their distribution. We found that African *Viscum*
334 species are polyphyletic (Figs. 2 and 3), present in six different clades. We also present
335 evidence that the Australasian-continental Asian clade I most likely split from the African
336 species possibly by stepping stone dispersal, although we cannot exclude that Australasia has
337 been colonized via continental Asia (see below). In clade B, the species of temperate Asia

338 appear to be more closely related to those occurring in temperate to subtropical regions of
339 Europe, the Middle East, the Mediterranean and North-Saharan Africa. All other
340 Australasian/Continental Asian species are phylogenetically distinct (clade I). For two
341 species, where subspecific taxa were sampled (*V. cuneifolium* and *V. myriophlebium*), the
342 accessions were not monophyletic at the species level. Future research using a greater
343 sampling density with multiple accessions per species will be necessary to address the
344 potential paraphyly of those taxa and to clarify the taxonomy within the genus *Viscum*. The
345 estimation of species numbers in the genus has a broad range (see 1; Nickrent 1997, and
346 onwards; Wu et al. 2003;), and our study thus covers between 50 % and 80 % of the
347 recognized *Viscum* diversity. Although we consider our inference a valuable and rather robust
348 first hypothesis of the evolutionary history of *Viscum*, analyses with a more exhaustive
349 species representation might yield higher statistical support of the ancestral area and trait
350 estimations, and could contribute towards the clarification of evolutionary processes of
351 *Viscum*. Including multiple specimens identified as the same species should also be included
352 in future work, especially to address the herein reported paraphyly of some taxa.

353 Besides our detailed analysis of the genus *Viscum*, the topology of the phylogenetic tree
354 based on our concatenated four-marker “backbone” data set (Fig. S1) is congruent with a
355 previously published phylogeny (Der and Nickrent 2008) regarding all deeper splits of the
356 sandalwood order, Santalales. Our analyses of nuclear and plastid markers strongly support
357 the monophyly of Viscaceae, in line with earlier studies (Soltis et al. 2000; Der and Nickrent
358 2008; Vidal-Russell and Nickrent 2008). Our multigene data set resolves *Notothixos* as sister
359 to the remaining Viscaceae genera, and *Dendrophthora/Phoradendron* as sister to *Viscum*,
360 which contradicts earlier data (Wiens and Barlow 1971; Der and Nickrent 2008; Mathiasen et
361 al. 2008) and prompting for further studies, ideally involving phylogenomic approaches.

362

363

364 **4.2. Eocene origin of *Viscum* results in the subsequent dispersal of a widely distributed**
365 **ancestor**

366 Our analyses based on Bayesian relaxed clock approaches date the crown group
367 estimates of Viscaceae to 108.06–84.47 mya (Fig. 2; Supporting Fig. S6 and Table S7) and
368 the reconstruction of the ancestral geographic range indicates an Australasian distribution. A
369 Gondwanan origin of the family agrees with Barlow (1990), rejecting the hypothesized
370 Laurasian origin suggested by Wiens and Barlow (1971). After the split of *Notothixos*, the
371 ancestor of the remaining genera dispersed to the Americas during the Upper Cretaceous.
372 Antarctica might have served here as a connection as it was still within close reach of both
373 continents after the Gondwana breakup (McLoughlin 2001 and references therein). During
374 that time, the Antarctic landmass had a warm and humid climate (Dingle and Lavelle 1998).
375 Further evidence of the evolution of Viscaceae in the Upper Cretaceous comes from the
376 Cretaceous Gondwana origin and subsequent diversification of modern frugivorous birds (the
377 most important seed dispersers of *Viscum*), for which the Antarctic also represents an
378 important factor for the distribution pattern of extant birds (Cracraft 2001). The
379 *Dendrophthora/Phoradendron* clade may have evolved by vicariance as a consequence of a
380 general temperature drop around the Cretaceous/Tertiary boundary. Critical in this respect
381 will be the genus *Arceuthobium* because its species occur in both the Old and New World.
382 However, the selection of *Arceuthobium* species for our study is not adequate to shed any
383 further light onto this issue. We would also like to point out that our Likelihood and Bayesian
384 inference place *Ginallia* (South-East Asia) as sister to *Dendrophthora/Phoradendron* (New
385 World), which differs from earlier, maximum parsimony and ML analysis, where it is sister to
386 *Korthalsella* (South East Asia, Australia, Indian and Pacific Islands).

387 Puzzling is the reconstructed Late Jurassic/Early Cretaceous origin of many of the
388 included Santalales families, even though some of the fossils used herein were also taken for
389 primary calibrations earlier (e.g., Vidal-Russell and Nickrent 2008). The stem (root) age is in
390 congruence with angiosperm-wide studies of Bell et al. (2005), Naumann et al. 2013, or

391 Magallón et al. (2015), whose representations of all flowering plant lineages came at the
392 expense of a meager sampling of the Santalales diversity. However, our family-based age
393 estimates are partly divergent from earlier reports. For example, with 81–68 mya, the origin
394 of Viscaceae was inferred as much younger by Vidal-Russell and Nickrent (2008), who
395 included only two species of *Viscum* and one of *Arceuthobium*. In contrast, our estimation of
396 the crown group age of Loranthaceae (68.24–42.31 my) are close to or even congruent with
397 earlier studies (e.g., Grímsson et al. 2017; Liu et al. 2018). The focus and, thus, sampling
398 strategies and tree topologies differ largely between earlier works and ours, and so did the
399 preferred molecular dating approaches regarding the employed methods, software programs,
400 models, type and number of genetic markers, fossil information, calibration and constraint
401 strategies. The main difference between ours and comparable existing work is that we
402 refrained from applying any sort of root age constraint or other maximum age constraints. We
403 believe that analysis restrictions based on earlier inferences – no matter how meticulously
404 these were carried out – bear the risk of amplifying error, and that fossil records can not
405 provide evidence of a maximum age. Hence, we present here an alternative age hypothesis for
406 Viscaceae and prefer to avoid judging the correctness of our results versus those of others. To
407 test existing (and newly emerging) hypotheses of divergence times in groups of the
408 sandalwood order, a comprehensive evaluation of an order-wide approach that also assesses
409 the effect of topological uncertainties and methodological robustness is desirable.

410 Our ancestral area reconstruction does not assign the *Viscum* ancestor unequivocally to
411 one area, but assumes a widespread range comprising Africa, and most probably, Australasia,
412 and Madagascar. Starting from this widely-distributed ancestor, the crown group of *Viscum*
413 seems to have evolved during the early Eocene. The colonization of Australasia and
414 Madagascar by *Viscum*, therefore, must have occurred via long-distance dispersal, assuming
415 the most likely scenario of an African origin of the stem group is inferred correctly. Within
416 clade A, *Viscum* reached continental Asia in the Oligocene from Africa, potentially via India
417 (Fig. 2 and Fig. 3), which is in line with *Viscum* fossils from the Miocene flora of Eastern

418 Georgia (Dzhaparidze 1979). From continental Asia, *Viscum* dispersed to North-Saharan
419 Africa and Europe. The Madagascan clades C and E, together with the Australasian clade D
420 separated in the Eocene from the remaining African clades and the second Asian clade I.
421 Within clade E, the African continent was re-colonized from Madagascar twice, probably
422 through intra-African bird migration. A vicariant event about 30 million years ago separated
423 the two lineages of the Madagascan clade E and the Australasian clade D, which contains two
424 species endemic to Australia. *Viscum* most likely spread to Australasia again between 24 (LN)
425 and 19 (CIR) mya from Africa (clade I) via long distance dispersal. At ca. 25–20 mya
426 (Oligocene), when said split seems to have occurred (Fig. 2), Africa was well separated from
427 Madagascar and Australia, not contiguous with India (and then Australia) since the
428 Cretaceous. Thus, vicariance from direct land mass connections seems unlikely. Stepping
429 stone dispersal via the Kerguelen Plateau, Crozet Plateau, and Ninetyeast ridge represents an
430 alternative explanation, one that has also been proposed for some palms and birds, the latter
431 of which important seed dispersers of *Viscum* (Carpenter et al. 2010; Liu et al. 2018). With
432 some certainty, continental Asia was colonized by *Viscum* at least three times independently
433 from Australasia. In the light of our data, we cannot exclude the possibility of colonizing
434 Australasia outward from Africa via continental Asia. There are well known flyways of
435 migrating birds connecting Africa and continental Asia, while there are no such connections
436 known between Africa and Australia. However, flotsam carrying or infected with fruiting
437 mistletoes, whose berries might get picked up and placed on a new suitable tree, represents
438 another alternative to the lack of flyway routes. Dispersal within the Asian and Australasian
439 regions could be caused by birds migrating along the East-Asian-Australasian flyway, using
440 again islands as stepping stones. Within the African continent, the present-day diversity
441 hotspot of *Viscum*, different clades evolved during the Oligocene with a hotspot in the South-
442 African Cape region (Clades F, G, H). The most recent African clade evolved in the Miocene
443 with a migration towards the Southeast and east of Africa, maybe aided by the establishment
444 of more open woodlands and savannahs during the early Miocene (Jacobs 2004).

445 **4.3. Multiple independent reductions of leaves and switches of the reproductive system**

446 The reduction of leaves evolved at least ten times independently in eight of the here
447 defined ten clades (Fig. 2), thus confirming the observations of Danser (1941). At least some
448 species in most geographical regions (Africa, continental Asia, Australasia, and Madagascar)
449 possess scale leaves. Moreover, there is a trend towards this habit prevalent in Africa, and all
450 the species contained in the youngest African clade J possess only vestigial leaves. This
451 drastic reduction in photosynthetic leaf tissue might be a contributor to an advanced parasitic
452 habit for viscaceous mistletoes in general.

453 Many *Viscum* species are dioecious, reflecting the general trend of a slight
454 overrepresentation of dioecy in parasitic plants compared to nonparasitic angiosperms (Bellot
455 and Renner 2013). Lacking knowledge of the phylogenetic relationships within the genus, the
456 predominance of monoecy within the remaining genera of Viscaceae led Wiens and Barlow
457 (1979) to assume that *Viscum* had evolved from a monoecious ancestor. Our analyses that
458 also considered alternative tree topologies (Fig. 2; Fig. S6 and Table S7) clearly indicate that
459 dioecy is the ancestral reproductive mode in *Viscum*. Despite the higher risk of pollination
460 failure that unisexuality brings, particularly where population densities are small, dioecy
461 guarantees the exchange of genetic material derived from genetically distinct individuals
462 (assuming there is no sex switching), among others by eliminating the opportunity for selfing
463 (as present in bisexuals). Efficient gene flow within and between populations could thus be a
464 beneficial driver in the parasite-host arms race, allowing the parasite to rapidly overcome
465 evolving resistances in host populations or to evolve new mechanisms to exploit novel host
466 systems. However, unisexual plants cannot establish new populations successfully in the
467 absence of the opposite sex. Successful mating in dioecious species inevitably requires two
468 individuals of the opposite sex, plus pollination agents, to overcome the distance between
469 both. The same may apply for shifts in the parasite's host range. In addition, a seed-shadow
470 handicap (i.e., male plants do not contribute to seed dispersal, through which fewer seeds of
471 dioecious populations may reach new local patches than hermaphrodite ones; Heilbuth et al.

472 2007) may contribute to the trend towards monoecy, which we find to have evolved at least
473 eight times independently. Therefore, the evolution of this form of hermaphroditism as seen
474 multiple times within *Viscum* also represents an evolutionary advantage for (long-distance)
475 dispersal events – despite the risk of inbreeding-depression. Thus, the ability to self-fertilize
476 apparently may have aided the dispersal and subsequent successful establishment of
477 monoecious *Viscum* species. It is noteworthy to mention that also the dioecious *Viscum album*
478 occurs over great geographic distances. However, the evolutionary success and dispersal
479 range of the European mistletoe may be linked primarily to its broad host range, unparalleled
480 within the genus *Viscum* (Fig. 4; Table S5). The ability to parasitize a great diversity of plants
481 appears to be a rather derived character, which has evolved independently. There is little
482 evidence from our analysis that a direct link exists between the independent reduction of
483 leaves or the shift in the reproductive mode with shifts in host ranges or increasing host
484 specificity in *Viscum* (Figs. 2 and 4). While no causal relationship between reproductive traits
485 and host selection is evident, the reduction of leaves to minute scales, which *de facto* means
486 the loss of photosynthetic surface, may indicate an increasing parasitic specialization and
487 dependence upon host-derived photosynthates. The extreme reduction of surface area for
488 photosynthesis, which in *Viscum* can be reduced to photosynthetic stems only, and thus
489 autotrophic carbon supply, in consequence to this near-loss of leaves requires that the uptake
490 of organic carbon is secured, perhaps through a more efficient connection to its host's
491 vascular tissue. Leafy mistletoes such as *V. album* obtain a large though host-specific fraction
492 of carbon from their host although they establish only xylem connections (Richter and Popp
493 1992).

494 *Viscum*, as well as other obligate parasites, depend on the availability of a compatible
495 host plant, which adds an additional isolating barrier during the speciation process that is
496 absent from nonparasites. Beyond that, geographic barriers are important, too, not only for the
497 mostly bird-driven distribution of the parasites but also for the host. The sedentary nature of
498 plants, thus, may contribute a plausible explanation why many parasitic plants tend to have

499 broader host ranges compared to most animal parasites, whereas the specialization on only a
500 few closely related host species (as in *V. minimum*) is observed rather rarely. Thus, the
501 interaction of factors like the degree of host specificity, the abundance of and distances
502 between different host individuals, plus the abundance of dispersing birds and the distance
503 these dispersers cover may also have played a critical role during the evolution of the genus
504 *Viscum* and mistletoes in general.

505 To summarize, we have shown here that morphology, geographic range, and the
506 similarity in host preferences are inconclusive indicators of phylogenetic relatedness in
507 *Viscum* mistletoes. Despite the probable beneficial switch of mating systems within this genus
508 aiding their establishment in new geographic areas, the biotic-abiotic factors and their
509 interactions driving the speciation and diversification within *Viscum* remain to be clarified,
510 thus leaving us with the pressing question whether mistletoes might have diversified in the
511 shadow of birds.

512

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525

526 **6. Authors' contributions**

527 K.M. and S.W. designed this study, generated and analyzed the data, and wrote the
528 manuscript. D.L.N. and K.F.M. contributed to the study design, to performing this research,
529 and to critically revising the manuscript. M.K. and D.Q. contributed to the phylogenetic
530 analyses or data generation. All authors have read and approved the final manuscript.

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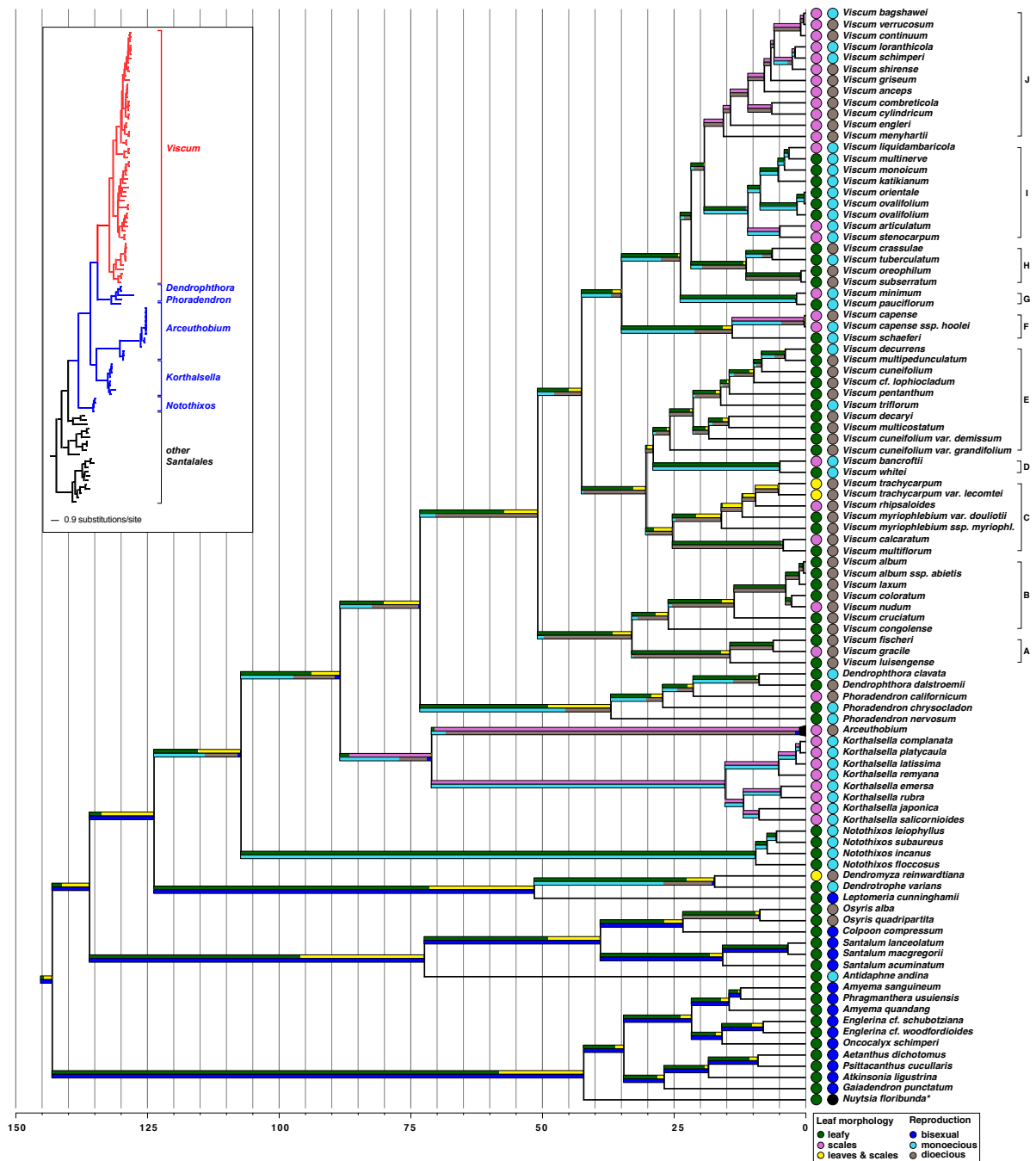
688 **8. Figures**



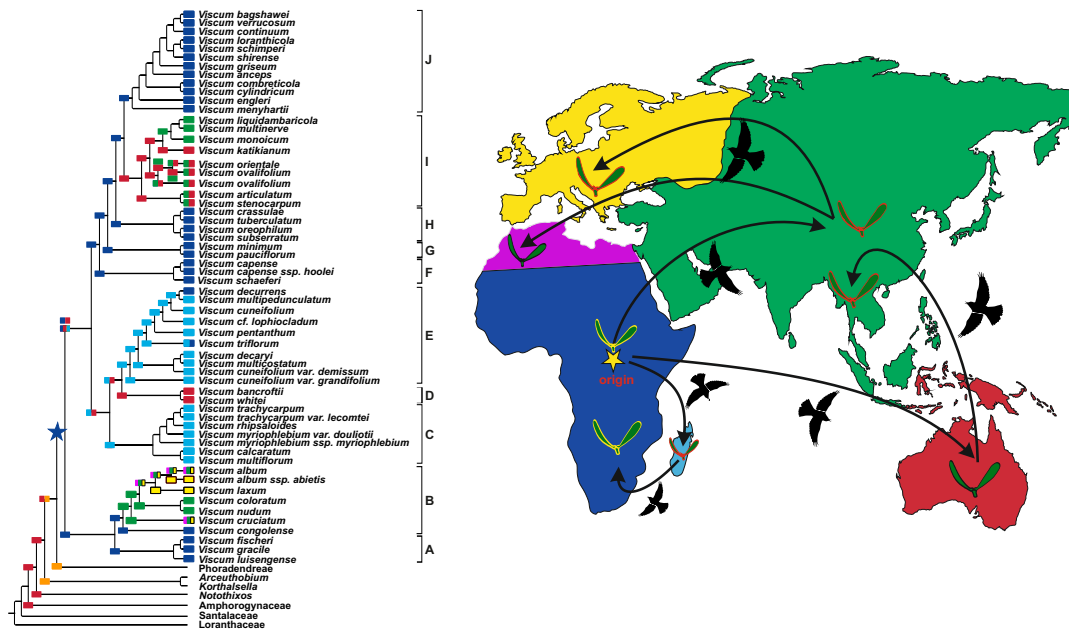
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690 **Figure 1 Growth forms of *Viscum*.** **A.** *Viscum album* (clade B), a dioecious species from
691 Europe. Plant in fruit. Insets: top – female flowers, bottom – male flower. Photo credits:
692 Karola Maul, Gerhard Glatzel. **B.** *Viscum trachycarpum* (clade C), a dioecious, leafless
693 species from Madagascar. Inset: male flowers. Photo credits: Peter Phillipson. **C.** Pendant
694 shoots with young inflorescences of *Viscum whitei* (clade D), a monoecious species from
695 Australia. Inset: closer view of inflorescences. Photo credits: Roger Fryer and Jill Newland.
696 **D.** Flowering female plant of *Viscum cuneifolium* (clade E), a dioecious species from
697 Madagascar. Inset: young fruits clustered in bibracteal cup. Photo credits: Peter Phillipson,
698 Christopher Davidson (Flora of the World). **E.** Female plant with mature fruits of *Viscum*
699 *capense* (clade F), a dioecious species from South Africa. Inset: male flowers. Photo credits:
700 Marinda Koekemoer. **F.** Inflorescences of the monoecious, South African *Viscum minimum*
701 (clade G) arising from the stem of a *Euphorbia* host plant. Insets: top – close-up view of male
702 and female flowers, bottom – mature fruits. Photo credits: Karola Maul and Daniel Nickrent.
703 **G.** *Viscum articulatum* (clade I), a monoecious, leafless species from Asia and Australia (here
704 Philippines). Habit of mistletoe showing flattened branches. Insets: top – male flowers,
705 bottom – fruit. Photo credits: Pieter Pelser. **H.** Branches bearing mature fruits of *Viscum*
706 *combreticola* (clade J), a dioecious, leafless species from tropical east Africa. Insets: top –
707 female flowers and developing fruits that are tuberculate when young, bottom – male flowers.
708 Photo credits: Karola Maul, Bart Wursten. Clade names B–J (in parentheses) refer to our
709 circumscriptions from phylogenetic inferences presented herein.

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 712 **Figure 2 Evolution of leaves and reproductive systems in *Viscum*.** The ancestral states of
 713 foliage and reproductive systems are illustrated by colored stacked bars below or above
 714 branches of the BI-SIC tree, obtained from the analysis of the ITS region alignment (data set
 715 2), respectively. The lengths of the individual stacks represent, proportionally, the probability
 716 of the ancestor adopting a certain state (color-coded as detailed in the bottom right corner).
 717 The lengths of the stacks (corresponding to 100 % cumulated probability per branch)
 718 are scaled according to evolutionary time in millions of years. Colored circles at the tips of the
 719 tree show the extant foliage or reproductive morphology per species, which was used for
 720 ancestral state estimation. The inset depicts the simplified phylogram of the same MB-SIC
 721 tree, which is provided in full in Supporting Figure S2-A. *Note that the category
 722 „polygamous“ (more specifically, polygamonoecious) was not included here, which is the
 723 actual reproductive condition in *Nuytsia*.
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Figure 3 Biogeographical history of *Viscum*. The most probable ancestral geographic ranges as well as the extant ranges are illustrated by colored squares at all nodes on the dated phylogenetic BI-SIC tree of *Viscum* and outgroups, based on data set 2 (ITS region). Two different squares on top of each other indicate an ambiguity in the reconstructed geographic ranges, where two or more ancestral areas were equally likely (see Table S6 for details). Distributions exceeding one geographic region are shown by multi-color squares. The colonization history of Australia, continental Asia and Europe from Africa is graphically summarized. An arrow indicates the independent colonization event, and the star suggests the likely origin of *Viscum* in Africa.

744 **9. Tables**

745 Table 1. Summary of major classification systems for *Viscum*.

Author(s) [#]	Basis for classification system	Number of species included (geographical distribution)
Korthals (1839)	Presence vs. absence of leaves, monoecy vs. dioecy	8 (Java, Sumatra, Borneo)
van Tieghem (1896)	Modification of Korthals' system, prioritizing the composition and position of inflorescences	27 (Madagascar, continental Africa, Asia, Europe)
Engler and Krause (1935)	Inflorescence characters: position, number of flowers; phyllotaxy	42 (continental Africa, Madagascar, Europe, Asia)
Danser (1941)	Structure of inflorescences	24 (India, SE-Asia, Malaysia, Australia)
Balle (1960)	Structure and composition of inflorescences	99 (Madagascar, continental Africa, Europe, Asia, Oceania)
Polhill and Wiens (1998)	Macroscopic morphological characteristics of the whole plants	45 (continental Africa)

746 [#] Several other regional treatments exist (e.g., Rao 1957, Sanjai and Balakrishnan 2006, or
747 Barlow 1997), which we here omitted for simplicity.

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750 **10. Supporting Information**

- 751 Figure S1 Topological constraints inferred with the four-marker data set 1 used as
752 topological constraints for ingroup inferences ¹
- 753 Figure S2 Phylogenetic trees obtained from BI and ML analysis of the 110-taxa data set 2
754 used as input data for ancestral state estimations ¹
- 755 Figure S3 Phylogenetic tree and summary of topological conflicts within the genus *Viscum*
756 and outgroups ¹
- 757 Figure S4 Cladograms with support values from alignment optimizations of dataset 2 ¹
- 758 Figure S5 Phylograms obtained from alignment optimizations of data set 2 ¹
- 759 Figure S6 Node delimitation for PhyloBayes and BayesTraits results, as detailed in Table S7
- 760 Table S1 Voucher information and NCBI Genbank accession numbers of all taxa used for
761 the reconstruction of a backbone guide tree with data set 1
- 762 Table S2 Voucher information and NCBI Genbank accession numbers for all taxa used for
763 the reconstruction of the final 110-taxa phylogeny with data set 2
- 764 Table S3 Geographic ranges for ancestral area estimation ²
- 765 Table S4 Morphological features for ancestral state estimation ²
- 766 Table S5 Host information for *Viscum* and outgroup taxa ²
- 767 Table S6 Results of the *LaGrange* analyses
- 768 Table S7 Results of the *PhyloBayes* and *BayesTraits* analyses

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771 ¹ Phylogenetic trees also available in *newick* format from *Mendeley Data*.

772 ² Trait data also available in excel table format and as easy-to-parse plain text files (tab-delimited with
773 comment lines) from *Mendeley Data*.