1   Original Article

2   **Incongruent phylogenies and its implications for the study of diversification, taxonomy and**

3   **genome size evolution of *Rhododendron* (Ericaceae)**

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PREMISE: *Rhododendron* L. (Ericaceae Juss.), is the most species-rich genus of woody flowering plants with > 1000 species. Despite the interest in the genus and numerous previous phylogenetic analysis, the infrageneric classification for the genus is still debated, partly due to its huge diversity, partly due to homoplasy in key characters and partly due to incongruence between phylogenetic markers. Here, we provide a broad coverage of representative species of all *Rhododendron* subgenera, sections, and most subsections to resolve its infrageneric phylogeny or highlight areas of incongruence, support previous analyses of diversification patterns and establish a relationship between genome size evolution and its diversification.

METHODS: We generated sequences of two plastid (*trn*K and *trn*L-F) and two nuclear (ITS and *rpb*2-i) markers for a total of 259 *Rhododendron* species, and used likelihood and Bayesian statistics to analyze the data. We analyzed the markers separately to discuss and understand incongruence among the data sets and among previous studies.

RESULTS: We found that the larger a subgenus, the more strongly it is supported as monophyletic. However, the smaller subgenera pose several problems, e.g., *R.* subgen. *Azaleastrum* consists of two sections inferred to be polyphyletic. The main shift to higher diversification in the genus occurred in the Himalayan/SE Asian clade of *R.* subgen. *Hymenanthes*. We found that polyploidy occurs in almost all subgenera but most polyploid species are within *R.* subgen. *Rhododendron* sections *Rhododendron* and *Schistanthe*.

CONCLUSION: Whereas previous reports stated that genome sizes of tropical plants are lower than those of colder and temperate regions in angiosperms in general, our study provides evidence for such a shift to small genome-tropical species within a genus. Taken together, we see the merit in the recognition of the five major clades at the sub generic level but given the amount of incongruence a large amount of species cannot be confidently assigned to one of these five clades. Further, genome-wide data will be necessary to assess whether these currently unassignable taxa are independent taxa, assignable to one of the five major clades or whether they are inter-subgeneric hybrids.

KEYWORDS: genome size evolution; incongruence; phylogeny; polyploidy; *Rhododendron*

Rhododendron L. (Ericaceae Juss.) is the most species-rich genus of woody flowering plants, placed among the twenty largest plant genera >1000 species (Frodin, 2004). The genus has a worldwide distribution (except limited distribution in Central & South America and Africa) with a center of diversity in China (GBIF; Fang and Ming, 1995; Wu and Raven, 2005; Brown et al., 2006). However, many of these species have a limited distribution and are, thus, threatened. According to Gibbs et al. (2011), about 70% of the species of *Rhododendron* are classified as vulnerable, threatened, endangered or critically endangered. Many of the species in the genus are used as ornamentals and/or used as medicinal plants, with abundant literature on their ethno-

56   medicinal use as anti-inflammatory agents, pain killers, in gastro-intestinal disorders, common
57   cold, asthma, skin diseases, and toxic agents (used in the form of insecticide or poison; see
58   Popescu and Kopp, 2013 for a review). Innocenti et al. (2010) suggested that some species of
59   *Rhododendron* have antibacterial activities. Rezk et al. (2015) extended this work and
60   demonstrated that there is a higher susceptibility of Gram-positive bacteria (e.g., *Bacillus subtilis*
61   Ehrenberg) and lesser susceptibility of Gram-negative bacteria (e.g., *Escherichia coli* (Migula)
62   Castellani and Chalmers) towards *Rhododendron* leaf extracts.

63   This interest in the genus as ornamental and medicinal plant has led to a long list of studies
64   investigating its diversification and classification. Linnaeus (1753) classified the species in two
65   genera, *Azalea* (species with five stamens) and *Rhododendron* (species with ten stamens). Over
66   the next century, as the number and diversity of known species increased, additional genera such
67   as *Rhodora* L. (Linnaeus, 1762), *Vireya* Blume (Blume, 1826) and *Anthodendron* Rchb.
68   (Reichenbach, 1827) were proposed. Especially, in the second half of the nineteenth century
69   *Rhododendron* enjoyed an increase of species number based on the botanical exploration of the
70   Himalayas and China (e.g., Hooker, 1849) and starting a rhododendronmania in Europe
71   (Musgrave et al., 1998). Sleumer (1949, 1966) proposed a detailed classification for the genus that
72   proved highly influential until today. He classified all known *Rhododendron* species at that time into
73   five subgenera and 13 sections. Chamberlain et al. (1996), based on a number of more narrowly
74   focused morphological taxonomic studies, refined previous classifications for the genus grouping
75   the species into eight subgenera and 12 sections (Sleumer, 1966; Cullen, 1980; Chamberlain,
76   1982; Philipson and Philipson, 1986; Judd and Kron, 1995; Chamberlain et al., 1996). One notable
77   difference between both classifications is the inclusion of *R.* sect. *Therorhodion* Maxim. In
78   Chamberlain et al. (1996) as a subgenus of *Rhododendron*, whereas Sleumer (1966), following
79   Small (1914) considered it separate from *Rhododendron*. The classification of Chamberlain et al.
80   (1996) is still widely used by specialists and gardeners (Cox and Cox 1997; Goetsch et al., 2005).
81   Phylogenetic analyses started to have an influence on the classification of *Rhododendron* in the
82   last 30 years. The most important changes are the inclusion of three genera, *Ledum* (Kron and
83   Judd, 1990), *Diplarche* Hook.f. & Thomson and *Menziesia* Sm. (Craven, 2011). With respect to the
84   intrageneric classification, Goetsch et al. (2005), based on DNA-based phylogenetic analyses,
85   suggested a reduction to five subgenera, *R.* subgen. *Therorodion*, *R.* subgen. *Rhododendron*, *R.*
86   subgen. *Hymenanthes* (including *R.* subgen. *Pentanthera* sect. *Pentanthera*), *R.* subgen.
87   *Choniastrum* (=*R.* subgen. *Azaleastrum* sect. *Choniastrum*) and *R.* subgen. *Azaleastrum* (incl. *R.*
88   subgen. *Pentanthera* sect. *Sciadorhodion*, *R.* subgen *Mumeazalea*, *R.* subgen. *Candidastrum*, *R.*
89   subgen. *Tsutsusi*; see details in Table S1).

90   Establishing relationships in *Rhododendron* based on morphological characteristics but also
91   molecular markers has been difficult because of frequent convergence and hybridization between
92   species. Hybridization is considered to have played an important role in the evolution and

93   speciation of *Rhododendron* through homoploid or allopolyploid speciation (Milne et al., 1999 and

94   2003; Milne and Abbott, 2008). This is reflected clearly by the large number of horticultural hybrids

95   in *Rhododendron* (over 28,000; Leslie, 2004) as well as the occurrence of natural hybridization

96   indicating weak reproductive barriers (Kron et al., 1993; Milne and Abbott, 2008; Milne et al., 1999

97   and 2003; Zha et al., 2008 and 2010; Zhang et al., 2007). Besides homoploid hybridization,

98   polyploidy, the occurrence of three or more sets of homologous chromosomes in the genome also

99   occurs naturally in *Rhododendron*, ranging from triploids to dodecaploids (Ammal, 1950) and even

100   more extensively exploited in horticulture (Jones et al., 2007). In *Rhododendron* the basic

101   chromosome number is 13 (with exception of *R. camtschaticum* Pall. with n = 12) with more than

102   70% of the species counted (~15% total) being diploid (2n = 26; Ammal et al., 1950; Väinölä, 2000;

103   and Rice et al., 2015), but little is known about the importance of polyploidization in the

104   diversification of the genus. Though hybridization can play diverse roles in promoting speciation

105   (Abbott et al., 2013; and Milne et al., 2010), in *Rhododendron*, hybrid populations have often been

106   found to show higher fitness than their parents only in mosaic habitats created along altitudinal,

107   radiation (Milne et al., 2003) or soil pH (Milne and Abbott, 2008) gradients.

108   The large number of species has stirred interest in investigating the underlying reasons for its

109   diversification. Milne et al. (2010) demonstrated that *R.* subgen. *Hymenanthes* (Blume) K. Koch

110   within South East (SE) Asia has been the clade to diversify fastest. Similarly, Schwery et al. (2015)

111   supported that the greatest diversity within *Rhododendron* occurs in the Himalayas and Malesia,

112   detected a nested Himalayan *Rhododendron* radiation of species of *R.* subgen. *Hymenanthes*, and

113   a separate diversification of *R.* section *Schistanthe* Schltr. (= *Vireya*) accompanied by an

114   eastwards dispersal, as predicted by (Brown et al., 2006) and Goetsch et al. (2011). However,

115   hybridization or polyploidy have not been considered by these studies are, until now, insufficiently

116   considered in the analyses trying to understand the diversification of the genus, in contrast to

117   biogeographic processes (Shreshta et al., 2018). A phylogenetic study analyzing the importance of

118   hybridization and establishing a robust infrageneric classification requires as many species as

119   possible representing all subgenera, sections, and subsections. Till to date about 400 species of

120   the more than 1000 *Rhododendron* species known to data have been studied in different

121   phylogenetic studies. However, these studies mostly focused on some specific subgenera and/or

122   sections. Only few studies considered the phylogeny of the whole genus *Rhododendron* and

123   mostly using a single DNA region, e.g., plastid *mat*K - 51 species (Kurashige et al., 2001); nuclear

124   ITS - 21 species (Gao et al., 2002); nuclear *rpb*2 - 88 species (Goetsch et al., 2005) or *trn*K, *trn*L-F

125   & ITS – 87 species (Grimbs et al., 2017). Shrestha et al. (2018) investigated the global distribution

126   and molecular phylogeny of *Rhododendron* in a biogeographical context, using 423 species with a

127   concatenated dataset of nine plastid genes, nuclear ribosomal ITS data and six introns of one

128   nuclear gene (RPB). However, neither did they report how much missing data was included nor

129   did they discuss whether and where incongruence between different data sets occurred. In

130   addition, Shrestha et al. (2018) depicted relationships without support values, which does not allow

131 evaluation of the robustness of relationships. Differences between these phylogenies and previous
132 classifications have raised doubts regarding the validity of the morphology-based *Rhododendron*
133 classification and prompted Goetsch et al. (2005) to propose a new classification. However, the
134 differences between analyses based on different DNA regions at the subgeneric and sectional
135 level prevented widespread support for this alternative classification for *Rhododendron*. To this
136 end, here our objective is to reconstruct the phylogeny of *Rhododendron* using representative
137 species of all subgenera, sections, and subsections for discussing support for alternative
138 classifications, especially the DNA-based classification of Goetsch et al. (2005). We used both
139 plastid and nuclear markers for phylogeny reconstruction. Based on this phylogeny, we analyzed
140 the importance of hybridization and polyploidization in diversification of *Rhododendron* in a
141 phylogenetic context. We provide here a critical evaluation of phylogenetic studies and results
142 from different markers in the genus. We, further, use the results to infer patterns of diversification
143 in the genus focusing on the importance of polyploidy and genome size evolution. Our main
144 questions were: (1) Does expansion of the sampling resolve the phylogenetic relationships within
145 *Rhododendron* and support the monophyly of major clades; (2) Do different DNA markers similarly
146 show and support previous diversification patterns? And (3): Are polyploidy and genome size
147 evolution related to the diversification of the genus? To follow these questions, we generated
148 sequences of two plastid (*trn*K and *trn*L-F) and two nuclear (ITS and *rpb*2-i) markers for a total of
149 307 individuals from 259 *Rhododendron* species. We analyzed all four datasets separately and
150 discuss the incongruences among these markers and previous studies. In addition, we used
151 BAMM (Bayesian Analyses of Macroevolutionary Mixtures; Rabosky, 2014) to investigate the
152 pattern of diversification in *Rhododendron*.

MATERIAL AND METHODS

Sampling

155 We based our sampling strategy on two criteria, first to include as many species as possible and
156 second to represent all subgenera, sections, and subsections of *Rhododendron* following
157 Chamberlain et al. 1996 (Table S1), which we managed with the exception of two subsections of
158 *R.* subgen. *Hymenanthes* (subsectt. *Barbata*, *Lanata*), the monotypic *R.* subgen. *Pentanthera*
159 subsect. *Sinensia*, and four monotypic subsections in *R.* subgen. *Rhododendron* (subsect.
160 *Afghanica*, *Campylopogon*, *Camelliflorum*, *Virgata*). We collected fresh leaves of 307 individuals
161 from 259 species at Rhododendron-Park Bremen and downloaded the available sequences of
162 other species from GenBank (Table S2). To root the phylogeny of *Rhododendron*, we used
163 *Calluna vulgaris* (L.) Hull*, Empetrum nigrum* L., *Kalmia angustifolia* L., *Kalmia procumbens* (L.)
164 Desvaux, *Vaccinium* x *intermedium* Ruthe, and *Vaccinium myrtilloides* Michx. as outgroups. The
165 outgroup species belong to the same family, covering the main lineages of family Ericaceae and
166 cultivated in the Botanical Garden of the Carl von Ossietzky-University (Oldenburg, Germany)

167 except *Phyllodoce empetriformis* (Sm.) D.D, collected in Botanical Garden Bochum (Bochum,
168 Germany) with permission. For DNA extraction the leaves were silica gel dried, while for flow
169 cytometry, we used fresh leaves. Vouchers for all species are stored in the herbarium of Carl von
170 Ossietzky-University (OLD).

Genome size estimation, DNA extraction and PCR amplification

172 To estimate nuclear genome sizes of *Rhododendron* species, we used flow cytometry following
173 the basic protocol of Galbraith et al. (1983). We used *Zea mays* L. 'CE-777' (2C = 5.430 pg),
174 *Hedychium gardnerianum* (2C = 4.02 pg) or *Solanum pseudocapsium* (2C = 2.60) as an internal
175 standard (Temsch et al., 2010, Meudt et al., 2015; Table S2). Briefly, we prepared intact nuclei
176 suspensions by chopping 0.5 - 1 cm^2 of fresh leaf tissue of *Rhododendron* and the internal
177 standard together with 1100µl of nuclei extraction buffer (OTTO I buffer: 100mM citric acid;
178 0.5 % (v/v) Tween 20 (pH approx. 2.3) after Otto (1990)). The intact nuclei suspension was filtered
179 through a 30µm CellTrics® nylon mesh filter (Partec). The filtered solution was then incubated at
180 37 °C for 30 min and stained with 2 ml propidium iodide buffer for 1 hour at 4 °C. This staining step
181 also involved a treatment with RNase. Lastly, we ran the suspension on the flow cytometer
182 (CyFlow SL, Partec, Munster, Germany), measuring 5000 particles and at least 1000 nuclei of
183 sample and standard. We repeated this process three times on different days. The genome sizes
184 (2C-value in pg DNA) were determined by comparing the mean relative fluorescence of each
185 sample with the standard. The relationship between ploidy levels and genome sizes (monoploid
186 1Cx- value in pg DNA) was determined with documented chromosome numbers and ploidy levels
187 in the literature.

188 We extracted total genomic DNA from silica gel-dried leaves (Chase and Hills, 1991), with
189 minor modifications (100 µl elution buffer, centrifuge at 6.000 x g for 5 min) using the innuPREP
190 Plant DNA Kit (Analytik Jena, Jena, Germany). Following upon the completion of DNA extraction,
191 we amplified two plastid gene regions, *trn*K (*mat*K and the 3' end *trn*K) & *trn*L-F (*trn*L intron, *trn*L 3´
192 exon, *trn*L-*trn*F spacer); and two nuclear gene regions, the *rpb*2-*i* (segment 2, 3, and 5) and the
193 ribosomal Internal Transcribed Spacer (ITS) through polymerase chain reaction (PCR, Table S3).
194 To amplify *trn*K, we designed two new internal primers based on the existing *trn*K of
195 *Rhododendron setosum* D.Don (OLD00775) using the program PRIMER3 (Rozen and Skaletsky,
196 1999), i.e. MK1538F (TAT GGG TGT TTA AAG AGC) and MK1785R (TCT ATC ATT TGA CTC
197 CGT ACC A). For other regions, we used the available primers (White et al., 1990; Taberlet et al.,
198 1991; Liu et al., 1999). All amplification reactions of target regions were carried out in 25 µl with
199 2.5 µl 10x PCR buffer, 1 µl MgCl$_2$ (50 mM), 0.5 µl dNTPs (10 mM), 1 µl of each primer
200 (10 pmol/µl), 1 µl DMSO (only in case of nuclear regions), 1 µl BSA, 0.2 µl *Taq* polymerase
201 (5 units), and 10-20 ng genomic DNA. The amplification of ITS and *trn*L-F regions were performed
202 on a TProfessional Standard Thermocycler (Biometra GmbH, Goettingen, Germany) and those of

203 the *trn*K and *rpb*2-*i* on a Mastercycler® gradient (Eppendorf AG, Hamburg, Germany; details of

204 PCR reactions profile in Table S3). Sequencing was conducted by GATC Biotech (Konstanz,

205 Germany) on an ABI 3730xl (PE Applied Biosystem) automated sequencers. To check the quality

206 of all sequences, we used GENEIOUS PRO v5.4.6 (Kearse et al., 2012). Lastly, we used MAFFT

207 algorithm (Katoh et al., 2002) to align the sequences and visually inspected the alignment.

Phylogenetic reconstruction and divergence time

209 We analyzed the data considering them as four separate data sets (*trn*K, *trn*L-F, ITS, and *rpb*2-*i*)

210 using the Maximum Likelihood and Bayesian inference approaches. Maximum Likelihood (ML)

211 analyses were conducted in RAxML v.7.9.5 (Stamatakis, 2006) with the substitution model

212 (GTR+Γ with four rate categories for ITS, *rpb*2 and *trn*K; and HKY for *trn*L-F). The selection of best

213 substitution models was implemented in jMODELTEST v.3.7 (Posada, 2008) using the Akaike

214 Information Criterion (AIC). We used non-parametric bootstraps (1000 replicates) to determine

215 support for each node (BS up to 70 was considered as weak, BS 70 – 90 as medium, and BS > 90

216 as strong). Similarly, the Bayesian trees were analyzed in MRBAYES v.3.2.2 (Ronquist and

217 Huelsenbeck, 2003) using the same substitution model with two independent runs, each consisted

218 of four Markov chains. All runs were allowed to proceed for ten million generations, sampled every

219 1000 generations. A consensus tree was generated with a 50 percent majority rule consensus

220 after discarding the first 10 percent generations as burn-in.

221   Similarly, we estimated time-calibrated phylogenetic trees in BEAST v.2.3 (Bouckaert et al.,

222 2014*)* for which, the input file was generated in BEAUTi v.2.3 (Bouckaert et al., 2014) using an

223 uncorrelated lognormal relaxed clock (Drummond et al., 2006) with a birth-death prior and GTR

224 substitution model. Since fossils of *Rhododendron* are scarce and difficult to use for each branch,

225 we used the oldest *Rhododendron* fossil as a first calibration point. According to Schwery et al.,

226 2015, the estimated fossil age of *Rhododendron* (without *R. camtschaticum*) is 58 mya with a

227 standard deviation of 2 my, based on the oldest fossil of *Rhododendron* and 17 other fossils from

228 Ericaceae. As second calibration point, we used 28.10 mya as leaf fossil age of *R.* subgen.

229 *Hymenanthes* from the late Oligocene (Axelrod, 1998) with normally distributed prior and the

230 corresponding standard deviation.. The actual analysis was run for 100 million MCMC each,

231 sampling the results after every 10.000 chains. We used the program Tracer v.1.5 (Rambaut and

232 Drummond, 2009) to check upon the convergence of the chains and estimated sample sizes (ESS

233 > 200). To compute the maximum clade credibility tree, we used TreeAnnotator v.2.3 (Drummond

234 et al., 2012) with node heights being the median of the age estimates deleting the first 10 %

235 generations as burn-in. Finally, we investigated if there were any influence of the priors on the

236 analyses and information content of the data by repeating the analyses with the same settings

237 without data.

Genome size evolution and diversification

239 To investigate whether the continuous traits related to genome size (2C genome size, 1Cx
240 monoploid genome size, ploidy level) have any significant phylogenetic signal, we estimated the
241 phylogenetic signal of these characters with the function phylosig in the R-package 'phytools'
242 (Revell, 2012) using two different methods: Blomberg's *K* statistic (Blomberg et al., 2003) and
243 Pagel's λ (Pagel, 1999). A bar plot of the trait 'ploidy level' was visualized using 'phytools' by
244 mapping it on the side of the BEAST tree. To calculate and map the ancestral character states for
245 continuous characters 2C- and 1Cx-values on the BEAST tree for the data sets, we used the
246 function contMap in 'phytools' by estimating the maximum likelihood ancestral character states for
247 continuous traits with fastAnc.

248 The net diversification rate and the number and location of monoploid genome size rate shifts
249 in *Rhododendron* were determined using BAMM (Rabosky, 2014) as employed in the R-package
250 'BAMMtools' (Rabosky et al., 2014). Though BAMM has been criticized by Moore et al. (2016),
251 simulation studies suggested robustness of diversification analyses by BAMM (Rabosky et al.,
252 2017; see also in Mitchel et al., 2019). The most important critique of BAMM is an error in the rate
253 of extinction in the absence of fossil records (Rabosky, 2010; Marshall, 2017; and Rabosky, 2018).
254 However, we here used BAMM only for diversification/speciation analysis. Additionally, since
255 *Rhododendron* is monophyletic (details in results section), we only accounted for incomplete
256 sampling to improve the robustness of BAMM based results. In *Rhododendron*, we estimated the
257 globalSamplingFraction as number of species used in the dataset/total number of *Rhododendron*
258 species (for example 0.235 for *trn*K as 259/1100 total *Rhododendron* species) following Igea and
259 Tanentzap (2020; also see in Spriggs et al., 2015). For the actual analysis, we used the BEAST
260 trees as input for running both the trait (1Cx-value) and speciation rate. Three replicates were run
261 in BAMM for 30 million generations and saved after every 5,000th generation. 'BAMMtools' was
262 used to plot the likelihoods of sampled generations after discarding the first 10% of chains. To
263 assess convergence, effective sample size was checked for each prior to be >200. Furthermore,
264 we calculated Bayes factors (BFs), plotted the best shift rate configurations and estimated rates of
265 speciation (diversification analysis) and evolution of monoploid genome size (trait analysis).

266 **RESULTS**

267 **Molecular phylogenetic reconstruction**

268 Details of variables sites, number of informative sites, and species used in each dataset are given
269 in Table 1. The results from phylogenetic analyses of the four DNA regions (Figs. 1-2, S1-6) agree
270 in several aspects. These are the monophyly of *Rhododendron*, the sister-group relationship of *R.*
271 subgen. *Therorhodion* to the rest of the genus, the monophyly of *R.* subgen. *Rhododendron* (the
272 former *Ledum* excluded) and *R.* subgen. *Tsutsusi* (except in *trn*LF), as well as the polyphyly of *R.*
273 subgen. *Azaleastrum* and *R.* subgen. *Pentanthera*, within the latter, *R.* sect. *Pentanthera* is
274 monophyletic if *R. canadense* is included (except in *rpb*2). In *R.* subgen. *Azaleastrum*, the two

275 sections *R*. sect. *Azaleastrum* and *R*. sect. *Choniastrum* are monophyletic. Finally, the analyses

276 agree that in *R*. subgen. *Tsutsusi*, *R*. sect. *Tsutsusi* (excl. *R. tashiroi*) and *R*. sect. *Brachycalyx*

277 (including *R. tashiroi*) are monophyletic. In the following, we will only mention the points, in which

278 the single region analyses differ from the rest.

279 Our results based on the plastid markers, the *trn*K phylogeny (259 species, CI = 0.736, RI =

280 0.917; Table 1; Fig. 1 and S1) and *trn*L-F region (169 species, CI = 0.697, RI = 0.883; Table 1; Fig.

281 S2-3), are highly congruent. Results from the *trn*K dataset suggest that within *R*. subgen.

282 *Hymenanthes*, species of subsections *Argyrophylla* (*R. insigne*, *R. rirei*), *Fortunea* (*R. calophytum*,

283 *R. praevernum*), *Irrorata* (*R. annae*), and all included species of *Pontica* (except *R. degronianum*

284 and *R. smirnowii*) form a group, which includes almost all species from outside SE Asia (South-

285 West Eurasia = Turkey and Caucasus; North-East Asia = Japan, Korea and Manchuria to East

286 Siberia; western North America and eastern North America) and is sister to the remaining

287 subsections of *R*. subgen. *Hymenanthes* (SE Asian clade = mainly southern China, Himalaya

288 Mountains and Taiwan). *Rhododendron* subgen. *Rhododendron* is divided into two clades of which

289 one encompasses the sections *Rhododendron* (excluding subsection *Glauca*) and *Pogonanthum*,

290 and the second contains the vireyas (section *Schistanthe*). The monotypic *R*. subgen.

291 *Candidastrum* is sister to the combined *R*. subgen. *Tsutsusi* and *R*. section *Azaleastrum*, but the

292 clade was weakly supported (BS/PP ≤ 70). The monotypic *R*. subgen. *Mumeazalea* is sister to *R*.

293 sect. *Choniastrum*.

294 Our results based on the *trn*L-F region showed only some, slight differences and was generally

295 less well resolved. The *trn*L-F-based tree recovered *R*. subgen. *Azaleastrum* section *Choniastrum*

296 (with *Mumeazalea*) as sister to *Tsutsusi* section *Tsutsusi*. In addition, *R*. section *Azaleastrum* is

297 sister to the clade of *R*. subgen. *Choniastrum* and *R*. subgen. *Tsutsusi* with strong support (Fig.

298 S2). *Rhododendron vaseyi* (*R*. section *Rhodora*) and *R. pilosum* (*R*. section *Sciadorhodion*, former

299 *Menziesia*) clustered closer to *R*. subgen. *Rhododendron* (Fig. S2). *Rhododendron* subgen.

300 *Hymenanthes* is divided into the same two clades as above and within *R*. subgen. *Rhododendron*

301 section *Schistanthe* (including *R*. section *Rhododendron* subsections *Genestieriana*, *Glauca*, and

302 *Micrantha*) is sister to section *Pogonanthum* and the remaining subsections of section

303 *Rhododendron*.

304 In the phylogeny based on the nuclear ITS region (197 species, CI = 0.662, RI = 0.908; Table

305 1; Fig. 2 and S4) reveals *R*. subgen. *Mumeazalea* clusters with section *Azaleastrum* and with *R*.

306 section *Choniastrum*. In addition, *R*. sections *Rhodora* and *Sciadorhodion* show a sister group

307 relationship to the clade of *R*. section *Azaleastrum*, *Mumeazalea*, and *Tsutsusi*, but this

308 relationship is weakly supported. Within *R*. section *Rhododendron* the subsections *Ledum*,

309 *Micrantha*, and *Rhododendron* form a clade (Fig. 2) which is sister to the clade including *R*. section

310 *Pogonanthum*, the remaining subsections of *R.* section *Rhododendron*, and *R.* section
311 *Schistanthe*.

312   In the phylogeny based on *rpb*2-*i* sequences (170 species, CI = 0.674, RI = 0.864; Table 1;
313 Fig. S5-6) all *Rhododendron* species (except *R. camtschaticum*) fall into three large clades (Fig.
314 S6). The first clade comprises *R.* subgenera *Hymenanthes* and *Pentanthera*, in which *R.* subgen.
315 *Pentanthera* is not monophyletic. The second clade contains *R.* subgenera *Azaleastrum* section
316 *Azaleastrum*, *Candidastrum*, *Mumeazalea*, *Pentanthera* section *Sciadorhodion*, *R. nipponicum*
317 (section *Viscidula*) and *R. vaseyi* (section *Rhodora*), and *R.* subgen. *Tsutsusi*. The remaining clade
318 encompasses *R.* subgen. *Azaleastrum* section *Choniastrum* and *R.* subgen. *Rhododendron*.

Diversification regime shifts

320 All three runs of diversification analysis in BAMM showed similar results (including log likelihoods
321 and number of shifts; data not shown). For the *trn*K species tree the frequent shift configuration of
322 the 95 % credible set of shift configurations (f = 0.15) shows two 'core shifts' to higher
323 diversification rates (red and orange circles and branches) and one 'core shift' to slower
324 diversification rate (light blue circle and branches; Fig. 3). The first shift to diversification rate
325 acceleration (red clade) includes species of *R.* subgen. *Hymenanthes* from the SE Asian clade.
326 The second clade with higher diversification rate (orange clade) is within *R.* subgen.
327 *Rhododendron*. This clade contains species from section *Rhododendron* in part (excluding species
328 from *R.* subsect. *Baileya*, *Boothia*, *Edgeworthia*, *Lapponica*, *Maddenia*, and *Tephropepla*) and the
329 two species included from *R.* section *Pogonanthum*. This diversification shift with rate acceleration
330 is also found in the diversification analysis with only 105 taxa matching those included in the trait
331 analysis (f = 0.35; Supplementary Material, Fig. S7A). The second shift within *R.* subgen.
332 *Rhododendron* shows a diversification rate slowdown (light blue clade). The mean speciation rate
333 in *Rhododendron* as calculated by BAMM was 0.429 speciation events per million years (Myr) and
334 has increased over time (Fig. 3, inset).

335   However, the diversification analysis of the *rpb*2-*i* species tree (f = 0.14) shows one 'core shift'
336 to a higher diversification rate in *R.* subgen. *Hymenanthes* for the SE Asian clade (Fig. S8A) and a
337 second 'core shift' with diversification rate slowdown for *R.* subgen. *Rhododendron* (excluding
338 subsection *Ledum* (L.) K.A.Kron & W.S.Judd). Both shifts are not seen in the diversification
339 analysis with only 56 taxa matching those for the trait analysis, despite a generally similar pattern
340 (Fig. S8B). The diversification analysis of the ITS species tree shows only one 'core shift' from *R.*
341 *camtschaticum* ssp. *camtschaticum* Pall. to a higher diversification rate for all remaining species of
342 *Rhododendron* (Fig. S9A). In the diversificaton analysis with only 90 taxa a 'core shift' to a slower
343 diversification rate for deciduous species (*R.* subgen. *Azaleastrum* section *Azaleastrum*, *R.*
344 subgen. *Mumeazalea*, *R.* subgen. *Pentanthera* section *Sciadorhodion*, and *R.* subgen. *Tsutsusi*
345 (Fig. S9B) has been revealed.

Genome size evolution

The genome sizes for 125 *Rhododendron* species are listed in Table S2. The 1C-values range from 0.677 pg to 2.182 pg for *R.* subgen. *Hymenanthes*, from 0.543 pg to 1.914 pg for *R.* subgen. *Pentanthera*, from 0.483 pg to 2.777 pg for *R.* subgen. *Rhododendron* and from 0.571 pg to 0.776 pg for *R.* subgen. *Tsutsusi*. For *R.* subgen. *Azaleastrum* the genomes size is 0.583 pg and 1.406 pg, for *R.* subgen. *Mumeazalea* 0.540 pg and for *R.* subgen. *Therorhodion* it is 0.583 pg. Most (87%) species of *R.* subgenera *Hymenanthes*, *Pentanthera*, and *Tsutsusi* are diploid. In contrast, polyploid species (tetra-, hexa- and octoploids) constitute roughly half (53%) of all investigated species of *R.* subgen. *Rhododendron* sections *Rhododendron* and *Schistanthe*.

Among the three continuous traits (2C-value, 1Cx-value, and ploidy level), only the 1Cx-value had a significant phylogenetic signal (Blomberg's K = 0.248, P < 0.005; Pagel's λ = 0.929, P < 0.005). Similarly, the ancestral character state reconstruction analysis using continuous color gradients with 2C-values (Fig. 4A) indicates that the ancestors of *Rhododendron* had small genomes but there have been several increases (yellow to green/blue) of 2C-value along the tree (Fig. 4A). The species with larger genome sizes (green to blue) are mainly species of *R.* subgen. *Rhododendron* sections *Rhododendron* (in part) plus section *Pogonanthum* and section *Schistanthe*. In these two groups almost all polyploid species (87%) are included. The ancestral character state estimation of 1Cx-values indicates genome upsizing for species of *R.* subgen. *Pentanthera* section *Pentanthera*, *R.* subgen. *Hymenanthes*, and *R.* subgen. *Rhododendron* section *Rhododendron* subsect. *Baileya*, *Boothia*, *Edgeworthia*, *Maddenia*, and *Tephropepla* (green to blue). In contrast, species of *R.* subgen. *Rhododendron* section *Schistanthe* show a smaller monoploid genome size (red to yellow) which indicates genome downsizing for this group.

In the BAMM trait analysis of monoploid genome size (1Cx-value) the mean rate of monoploid genome size evolution in *Rhododendron* is 0.0005 and has not varied much over time (Fig. S10B, inset). Based on the *trn*K BEAST phylogeny three 'core shifts' are indicated in the trait analysis (Fig. S7B). Two of them are shifts to increased rates, i.e. in *R.* subgen. *Tsutsusi* section *Tsutsusi* and *R.* subgen. *Rhododendron* section *Malayovireya*, whereas a third shift to a decreased rate is shown for *R.* subgen. *Rhododendron* sections *Rhododendron* and *Pogonanthum*. Species of section *Rhododendron* subsect. *Baileya*, *Boothia*, *Edgeworthia*, *Maddenia*, and *Tephropepla* are excluded from this decrease.

The trait analysis based on the phylogeny of *rpb*2-*i* shows only one shift to an increased rate for *R.* subgen. *Tsutsusi* section *Tsutsusi* (Supplementary Material, Fig. S8C) and based on ITS only one 'core shift' to an increased rate for species of *R.* subgen. *Rhododendron* sections *Rhododendron* and *Pogonanthum* (except species of subsections *Micrantha* and *Rhododendron*; Fig. S9C) are indicated.

DISCUSSION

Our results agree in a well-supported monophyly of *Rhododendron* (Figs. 1-2), which is in line with previous studies e.g., Shrestha et al., 2018; and Schwery et al., 2015), and when species of *Ledum* and *Menziesia* are included (Craven (2011), Goetsch et al. (2005), Kron and Judd (1990) and Kurashige et al. (2001). *Rhododendron camtschaticum*, representing *R.* subgen. *Therorhodion* is sister to the rest of the genus in all analyses. This agrees with other analyses and allows recognition of *R. camtschaticum* and *R. redowskianum* as separate genus (e.g., Judd and Kron, 2009). However, we continue recognizing them within *Rhododendron*.

In contrast, we found a marked difference concerning the internal relationships beyond the sectional level. Overall, the results revealed the larger a subgenus, the stronger is the support for its monophyly. The smaller subgenera pose several problems, for example *R.* subgen. *Azaleastrum* is inferred to be polyphyletic, as previously indicated (Goetsch et al., 2005; Kurashige et al., 2001; Schwery et al., 2015; and Yan et al., 2015). However, both sections are monophyletic in all analyses (inclusion of *R. charitopes* in the ITS analysis is dubious). However, several incongruent placements of species among analyses complicate a new classification of the genus. Shrestha et al. (2018) provided the most comprehensive phylogenetic analysis to date with multiple plastid and nuclear markers, but did not reflect incongruent placements among datasets (Table 2).

Phylogeny of *Rhododendron*

Goetsch et al. (2005) grouped the species in five subgenera, *R.* subgen. *Therorhodion* with two species and *R.* subgen. *Rhododendron*, *Hymenanthes*, *Azaleastrum* and *Choniastrum* (Table 2). The largest of these subgenera is *R.* subgen. *Rhododendron* with more than 500 species (Table S1). There have been debates on whether to recognize the vireyas as separate subgenus or section (Craven et al., 2008; Argent and Twyford, 2012). Recognition of a separate subgenus in the traditional sense would lead to recognition of a diphyletic clade since *R.* subsection *Discovireya* is separate from the rest in most analyses as found earlier by Goetsch et al. (2011). The phylogeny presented by Shrestha et al. (2018) would allow recognition of three subclades, one containing most of the members of *R.* sect. *Schistanthe*. However, these clades are inconsistent among studies and markers and we refrain from suggesting a new sectional classification. The only question regarding the circumscription of this subgenus is the inclusion of the former genus *Ledum*, which appears as sister to *R.* subgen. *Rhododendron* in all nuclear DNA-based analyses (Fig. 2 and S1; Gao et al., 2002; and Goetsch et al., 2005) but as sister to *R. albrechtii* (Fig. 1 and S5; Kurashige et al., 2001) or sister to *R.* subgen. *Hymenanthes* and *R.* sect. *Pentanthera* (Schwery et al., 2015) and distant to *R.* subgen. *Rhododendron* in the plastid DNA-based analyses. Shrestha et al. (2018) has some species as sister to *R.* subgen. *Rhododendron* and some as sister to *R. albrechtii* in his combined analysis of plastid and nuclear DNA, which suggests that missing data in their dataset causes different accessions of species from this group

418   to end up in different positions. In Grimbs et al. (2017) the plastid DNA signal apparently overruled
419   the nuclear signal.

420   The second largest group in *Rhododendron* is *R.* subgen. *Hymenanthes*. Whereas there is
421   mostly strong support for the morphologically well-circumscribed subgenus in the traditional sense
422   (except in *rpb*2; Fig. S5-6; Goetsch et al., 2005), Shrestha et al. (2018) enlarged the subgenus to
423   include *R.* sect. *Pentanthera*. This relationship is strongly supported by *rpb*2 (Fig. S5-6; Goetsch et
424   al., 2005) and shown without support by *trn*K (Fig. 1; Kurashige et al., 2001; Schwery et al., 2015).
425   The relationship is not shown but also not strongly refuted by analyses of ITS (Fig. 2 and S4; Gao
426   et al., 2002). The two groups differ in leaves being either deciduous or evergreen, a character
427   traditionally in taxonomy and horticulture important in the distinction between rhododendrons and
428   azaleas. We, therefore, prefer to keep *R.* sect. *Pentanthera* separate from *R.* subg. *Hymenanthes*.

429   The second-smallest subgenus in the classification of Goetsch et al. (2005) is *R.* subg.
430   *Choniastrum* with 15 species. The signal in Shrestha et al. (2018) depicting it as sister to *R.*
431   subgen. *Rhododendron* is largely derived from the *rpb*2-dataset (Fig. S5-6; Goetsch et al. 2005).
432   However, ITS puts the group in a position as sister to *R.* subg. *Azaleastrum* sensu Goetsch et al.
433   (2005; Fig. 2) or in unresolved position in one clade with this subgenus (Gao et al., 2002).
434   Schwery et al. (2015) resolves the section including former genus *Diplarche* based on *mat*K and
435   *rbc*L as sister to this clade, as well. Other studies based on plastid DNA markers even group the
436   section among the members of *R.* subgen. *Azaleastrum* sensu Goetsch et al. (2005). We,
437   therefore, consider it premature to recognize this clade at the subgeneric level.

438   The morphologically most heterogeneous subgenus of Goetsch et al. (2005) is *R.* subgen.
439   *Azaleastrum*, which includes *R.* sect. *Azaleastrum*, three sections formerly assigned to *R.* subgen.
440   *Pentanthera* (*R.* sect. *Rhodora*, *R.* sect. *Sciadorhodion* (incl. *Menziesia*), *R.* sect. *Viscidula*), the
441   monotypic *R.* subgen. *Mumeazalea* (*R. semibarbatum*) and *R.* subgen. *Candidastrum* (*R.*
442   *albiflorum*) and the large (66 species) *R.* subgen. *Tsutsusi*. The signal for the monophyly of this
443   clade is derived mostly from *rpb*2 (Fig. S5-6; Goetsch et al., 2005) with ITS supporting it with
444   inclusion of *R.* sect. *Choniastrum* (Fig. 2 and S4; Gao et al., 2002). Plastid DNA does not support
445   the group as monophyletic. The core of the group is constituted by *R.* subgen. *Azaleastrum* and *R.*
446   subgen. *Tsutsusi*. The monotypic *R.* subgen. *Candidastrum* (*R. albiflorum*) clusters with these two
447   based on *trn*K (Fig. 1 and S1; Kurashige et al., 2001), is unresolved together with these based on
448   ITS (Gao et al., 2002) and strongly supported sister to *R. albrechtii* based on *rpb*2 (Goetsch et al.,
449   2005) and in Shrestha et al. (2018). The case is the other way around for the monotypic *R.*
450   subgen. *Mumeazalea* (*R. semibarbatum*), which is sister to *R.* subgen. *Tsutsusi* in Shrestha et al.
451   (2018) or *R.* subgen. *Azaleastrum* with ITS (Fig. 2 and S4; Gao et al., 2002) or in one clade with
452   these two and *R.* sect. *Viscidula* in *rpb*2 (Fig. S2-3; Goetsch et al., 2005) but distantly to those and
453   strongly supported sister to *R.* sect. *Choniastrum* in the plastid DNA-based phylogenies (Fig. 1 and

454 S2-3; Kurashige et al., 2001). A third species (group) related to this core group is mono- or ditypic
455 *R.* sect. *Viscidula* (*R. nipponicum*), which clusters with this core in *rpb*2 (Fig. S5-6; Goetsch et al.,
456 2005) and in Shrestha et al. (2018) but not using *trn*K (Fig. 1; Kurashige et al., 2001) with weak
457 support. A fourth species changing positions in different analyses is *R. vaseyi*, the second species
458 of *R.* sect. *Rhodora* apart from *R. canadense*, which clusters with *R.* sect *Pentanthera.*
459 *Rhododendron vaseyi* is related to *R. schlippenbachii* of *R.* sect. *Sciadorhodion* and the species of
460 former genus *Menziesia* with medium to strong support based on *rpb*2 (Fig. S5-6; Goetsch et al.,
461 2005) and is found there in Shrestha et al. (2018), too. However, ITS and the plastid DNA markers
462 retrieve it in different positions (Fig. 1-2 and S1-6; Kurashige et al., 2001; Grimbs et al., 2017). The
463 former genus *Menziesia* has been found to be nested in *Rhododendron* since the studies of Kron
464 (1997) and Kurashige et al. (2001). Goetsch et al. (2005) found strong support for a clade
465 consisting of *Menziesia* with *R. schlippenbachii* and *R. vaseyi* and a relationship with either holds
466 in all analyses but not always with both but almost always with *R. schlippenbachii*. In turn, *R.*
467 *schlippenbachii* (and *Menziesia*) have different positions, but always close to the Azaleastrum-
468 Tsutsusi-group. It is, however, noteworthy that other members of *R.* sect. *Sciadorhodion* rarely
469 form a monophyletic group with these. For example, *R. albrechtii*, which takes only a distant
470 relationship with *R. schlippenbachii* in the plastid DNA-based analyses (Fig. 1 and S1-3; Kurashige
471 et al., 2001; Grimbs et al., 2017).

472 Taken together, we consider it premature to group all species of *Rhododendron* in five
473 subgenera. We see the merit in the recognition of the five major clades at the subgeneric level but
474 given the amount of incongruence a large number of species cannot be confidently assigned to
475 one of these five clades. Further, genome-wide data will be necessary to assess whether these
476 currently unassignable taxa are independent taxa, assignable to one of the five major clades or
477 whether they are inter-subgeneric hybrids.

Diversification regime shifts

479 Based on this incongruence, we considered it necessary to conduct diversification analyses
480 separate for each DNA marker, although the *trn*LF-region did not have enough variation to allow a
481 reliable analysis. Diversification analyses demonstrated nearly the same speciation shifts for the
482 plastid *trn*K and nuclear *rpb*2-*i* regions (Fig. 3 and Fig. S8). The main shift to higher diversification
483 was found for species of *R.* subgen. *Hymenanthes* from the Himalayan/SE Asian clade, although
484 the exact species included is not consistent. Most species of subsection *Pontica* with a distribution
485 outside SE Asia (e.g., SW Eurasia, NE Asia, and N America) show no shift in diversification rate.
486 This pattern is consistent with the findings of other analyses. For example, Milne et al. (2010)
487 hypothesized, based on divergence time estimations, a slow diversification outside SE Asia
488 followed by more rapid diversification of one lineage within SE Asia, followed by immigration of at
489 least one additional lineage to the region. Additional support for a nested Himalayan radiation is

490 given by Schwery et al. (2015) using a BAMM analysis similar to ours but with much smaller taxon
491 sampling. The Himalayan-Southwest China region is known as a species-rich area of
492 *Rhododendron* since Joseph D. Hooker famous travels to India and the Himalayas (1847 – 1851)
493 but the region is also well known for other highly diverse groups of plants (Qiu et al., 2011). Most
494 intriguing is the parallel increase in diversification in shrubby *Viburnum* of the region, which has
495 been dated to the Eocene (Spriggs et al., 2015), similar to *Rhododendron*. Suggested reasons for
496 the high diversity that may apply to *Rhododendron* are the climatic and physiographic
497 heterogeneity, a complex geological history and the absence of major Quaternary glaciations (Qiu
498 et al., 2011) coupled with an increase in precipitation (Wang et al., 2012). These factors may have
499 spurned a diversification by causing barriers to plant migration (Zhao et al., 2013) and providing
500 opportunities for frequent niche shifts between temperate and tropical biomes with limited vertical
501 migration (Spriggs et al., 2015).

502 Similar to *R.* subgen. *Hymenanthes*, a significant rate increase was found in most
503 diversification analyses in *R.* subgen. *Rhododendron* (Figs. 3 and Fig. S7) and comprises most
504 species of sections *Pogonanthum* and *Rhododendron*. In contrast, a clade within section
505 *Rhododendron* containing species of section *Rhododendron* subsections *Baileya*, *Boothia*,
506 *Edgeworthia*, *Maddenia*, and *Tephropepla* shows no change in diversification rate. All members of
507 this clade without rate shift occur in the Himalayan Mountains above 1500 meters with many at
508 least facultative epiphytic species and in an ecological diverse array of habitats such as conifer
509 forests, grassy hillsides, among rocks, steep slopes, or cliffs. In contrast to our expectations, no
510 shift in diversification rate was found within the species-rich group of tropical *Rhododendron*
511 (vireyas, section *Schistanthe*) for subsection *Euvireya* (Fig. 3; Figs. S7-8) which includes almost all
512 species endemic to the Philippines, Borneo, New Guinea, Sulawesi, and the Solomon Islands.
513 Nevertheless, here we only included 22% species of *Rhododendron* out of their total 587 species
514 (Table S1). *Euvireya* species exhibit considerable variation in their ecology, being epiphytic or
515 terrestrial and occurring from sea level to over 4000 m. Within *Euvireya*, the molecular-
516 phylogenetic clustering follows geography more closely than traditional taxonomy based upon
517 morphology, which is similar to the results from (Goetsch et al., 2011). The geographical pattern of
518 the group was intensively discussed by Brown et al. (2006b). It is considered a classic example of
519 Malayan radiation (Brown et al., 2006b; and Goetsch et al., 2011). An adaptive radiation can be
520 accompanied by a diversification rate slowdown, which may sound counter-intuitive at first.
521 However, since speciation rates usually decrease after an initial rapid diversification either due to
522 increased competition for resources or niche filling, diversity- or time-dependent factors must be
523 considered as potential drivers for observed decreases in the rates of diversification (Soulebeau et
524 al., 2015). Our results are in contradiction with Shrestha et al. (2018), who suggested that tropical
525 and subtropical mountains are not only the biodiversity and endemism hotspots for the genus
526 *Rhododendron*, but also function as cradles of *Rhododendron* diversification.

527   While support for a diversification increase in *R.* subgen. *Hymenanthes* is more or less

528   unambiguous, we have found here a discordance of results from different DNA regions but cannot

529   really distinguish between diversification rate shifts depending on species sampling, evolutionary

530   rate or topology (Fig. 3; and Figs. S7 and S9). Based on the similarity of our analysis using *trnK*

531   with 260 taxa and that of Schwery et al. (2015) with 60 taxa but also using *trn*K (and *rbc*L),

532   topology seems to be the most important. Therefore, more complete taxon sampling and,

533   especially, resolving incongruences between markers in the future seems to be required for more

534   conclusive diversification analyses.

535   **Genome size evolution**

536   Our analysis is the first to analyze genome sizes in a larger number of species of *Rhododendron*

537   (Table S2). Previous genome size estimations had been generated using Feulgen densitometry

538   (Ammal, 1950; Ammal et al., 1950) or flow cytometry using DAPI staining (Jones et al., 2007).

539   Ammal et al. (1950) completed an extensive survey of chromosome numbers and ploidy levels in

540   *Rhododendron* and found the elepidote rhododendrons (*R.* subgen. *Hymenanthes*), evergreen

541   azaleas (*R.* subgen. *Tsutsusi*), and the deciduous azaleas (*R.* subgen. *Pentanthera*) to be

542   predominantly diploid. However, they also demonstrated the occurrence of triploids, hexaploids,

543   octoploids, and dodecaploids (2n = 12x = 156) within *R.* subgen. *Rhododendron* and natural

544   tetraploids in other subgenera as well, e.g. in *R.* subgen. *Pentanthera* (*R. canadense* and *R.*

545   *calendulaceum;* Ammal, 1950; Ammal et al., 1950; and Jones et al., 2007). Our results confirm

546   and expand the pattern that polyploidy occurs in almost all subgenera but most polyploid species

547   are within *R.* subgen. *Rhododendron* sections *Rhododendron* and *Schistanthe* (Fig. 4A). This is

548   rather surprising given the high frequency of polyploids among garden cultivars belonging to *R.*

549   subgen. *Hymenanthes* (Perkins et al., 2012). Within *R.* section *Schistanthe*, the polyploid species

550   are restricted to *R.* subsections *Euvireya* and *Malayovireya*, which occur in Indonesia and New

551   Guinea and most of them are endemic to these islands while the species of the Asian mainland

552   are diploid based on our data. There seems to be no correlation of genome size with habit

553   (epiphytic, terrestrial or both) in this group of vireyas but the monoploid genome sizes are smaller

554   (1Cx-values = 0.483 up to 0.618 pg; light green/yellow to red; Fig. 4B). This genome downsizing

555   has been inferred to be accompanied by a slow-down in the evolution of genome size. Thus, the

556   group seems to have stabilized on a lower level of genome size. Such a pattern has also been

557   shown in the New Zealand radiation of *Veronica*, in which genome downsizing and a slow-down of

558   rate in genome size evolution are associated with a radiation on the polyploid level (Meudt et al.,

559   2015). While previous studies have shown that genome sizes of tropical angiosperms in general

560   are lower than those of colder, temperate regions (Levin and Funderburg, 1979; Ohri, 2005), our

561   study seems to be the first to indicate such a shift to small genome-tropical clade within a specific

562   genus. It remains to be studied whether genome downsizing in this clade is functionally related

563   with the higher frequency of polyploidy and/or its species richness.

Within section *Rhododendron,* almost all polyploid species form a monophyletic clade and seem to be restricted to subsections *Heliolepida* and *Triflora.* In contrast to the former group, here polyploidy is not associated with genome downsizing. Diploid species from the latter subsection do not cluster with the monophyletic clade of polyploids but with other diploid species from different subsections based on plastid DNA markers (Fig. 4A; *trn*K gene region). In the same analysis using ITS (Fig. S10) diploids are nested among polyploids suggesting an origin of at least four polyploid clades involving the same unknown diploid species. Hybridization and polyploidization, often referred to as whole genome duplication, are both potential speciation mechanism. Genome doubling creates instantaneously lineages reproductively isolated from its diploid progenitors and must overcome competition with their parents for abiotic resources (Soltis et al., 2010). Diversification analyses have demonstrated that polyploids have higher extinction rate and lower diversification rate than diploid lineages (Mayrose et al., 2011) but nevertheless have given rise to major radiations in angiosperms (Tank et al., 2015). In line with this pattern, we did not detect a change in diversification in polyploids. Thus, polyploidy did not increase speciation rate in *Rhododendron* but may however be associated with evolutionary novelties. Our hypothesis is that in this Heliolepida/Triflora-group species have a higher chance to produce unreduced gametes, thus the repeated origins of polyploids from similar ancestors, but the lack of genome downsizing prevents diversification on the polyploid level.

Hybridization facilitates the transfer of traits between species, which has been shown to promote adaptive evolutionary change in *Rhododendron* (Milne and Abbott, 2000) and other species. Such introgressed traits may affect various stages of life history including resistance to herbivores and pathogens (Whitney et al., 2015). Recent reviews suggest that resistance is an important component of hybrid survival (Orians, 2000) and that hybridization and polyploidy may be important evolutionary mechanisms for generating novel secondary chemicals important in the diversification of plant-animal interactions (Soltis et al., 2014). Oswald and Nuismer (2007) explored the possibility that new polyploids are initially more resistant to pathogens than their diploid progenitors by using mathematical models and confirmed that polyploids are significantly more resistant. *Rhododendron* may prove to be a living example for the evolution of novel chemicals in polyploids since four of the ten species shown to exhibit the highest antibacterial effects against Gram-positive bacteria by Rezk et al. (2015) are shown here to be polyploid (*R. ambiguum, R. cinnabarinum, R. concinnum* and *R. rubiginosum*). It remains to be shown that the high antimicrobial activity is due to the origin of novel gene combinations in polyploids.

CONCLUSIONS

Given the large numbers of species, including many rare species with restricted range sizes, there are inherent difficulties in the analyses of phylogenetic relationships, diversification, and trait evolution. Here, we included only 25-30% of the total species from the genus in the study and analyzed all data sets separately. However, by using state of the art analytical tools, relying on

601 strong support in terms of bootstraps and posterior probabilities, and discussing thoroughly and

602 critically the results, we provide the most up-to-date knowledge of *Rhododendron* phylogeny,

603 diversification and genome size evolution. We compared our results with the DNA-based

604 classification by Goetsch et al. (2005) and warn of adopting this classification uncritically (Table 2).

605 Our results agree in a well-supported monophyly of *Rhododendron*, with *R.* subgen. *Therorhodion*

606 sister to all other taxa of *Rhododendron.* The results recovered marked incongruences between

607 markers in retrieval of internal relationships but also finding many results common across DNA

608 markers and morphology-based classifications. Our results demonstrate that the definition of

609 enlarged *R.* subgen. *Azaleastrum* and *R.* section *Sciadorhodion* by Goetsch et al. (2005) including

610 *R.* subgen. *Candidastrum*, *R.* subgen. *Mumeazalea*, *R. vaseyi* and other species of *R.* sect.

611 *Sciadorhodion* may be premature. Similarly, our results do not consistently support the placement

612 of *R.* subsect. *Ledum* in *R.* subgen. *Rhododendron* and the inclusion of *R.* sect. *Pentanthera* in *R.*

613 subgen. *Hymenanthes*. The diversification analysis revealed that a major rate shift in

614 *Rhododendron* occurred in the Himalayan Mountains above 1500 meters with many at least

615 facultative epiphytic species and in an ecologically diverse array of habitats such as conifer

616 forests, grassy hillsides, among rocks, steep slopes, or cliffs. These results are in contradiction to

617 Shrestha et al. (2018), who suggested tropical and subtropical mountains to be not just the

618 biodiversity and endemism hotspot for the genus but also a cradle of its diversification. However,

619 the topology of the phylogeny is indicated to influence largely the results. Therefore, more

620 complete taxon sampling, especially, resolving incongruences between markers in the future

621 seems to be required for more conclusive diversification analyses. Lastly, we confirm and expand

622 that polyploidy occurs in almost all subgenera but most polyploid species are within *R.* subgen.

623 *Rhododendron* sections *Rhododendron* and *Schistanthe*. The two groups differ, however, in their

624 pattern with the polyploids in *R.* sect. *Rhododendron* originating frequently but not diversifying on

625 the polyploid level and not exhibiting genome downsizing. In contrast, *R.* sect. *Schistanthe* seems

626 to be another example for a polyploid radiating after genome downsizing. It further can serve as an

627 example for the frequently cited reduction in genome size of tropical plants versus their temperate

628 relatives.

633 **DATA ACCESSIBILITY**

634 Postprocess data will be available on Dryad.

635

636 **AUTHOR CONTRIBUTIONS**

637 Conceived the idea: DCA, JN, HS and KG; Performed experiments: JN; Contributed

638 reagents/materials/analysis tools: DCA, and HS; Analyzed data and authored the drafts of the

639 paper: KG, DCA. All authors contributed, made multiple revisions, and approved the final draft.

640 **LITERATURE CITED**

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844

845   **TABLE 1.** Numerical attributes of the four likelihood phylogenetic trees.

Data set	*trn*K	*trn*L-F	ITS	*rpb2-i*
No. individuals (Species)	307(259)	199(169)	237(197)	170(148)
Newly generated sequences	204	119	162	82
Aligned sequence length (bps)	1750	983	660	3306
No. of variable sites	285 (16.30%)	130 (13.20%)	173 (26.20%)	574 (17.30%)
No. of informative sites	399 (22.80%)	277 (28.10%)	252 (38.10%)	572 17.30%)
Consistency Index (CI)	0.736	0.697	0.662	0.674
Retention Index (RI)	0.917	0.883	0.908	0.864

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TABLE 2. Comparison of different molecular phylogenetic analyses with the results of this particular study. The abbreviated letters showed in the table represents, R − R. subgen. Rhododendron; A − *R.* sect. Azaleastrum; T − R. subgen. Tsutsusi; M − R. subgen. Mumazalea; C − R. sect. Choniastrum; S − R. sect. Sciadorhodion and P − R. sect. Pentanthera; H − R. subgen. Hymenanthes; L − R. sect. Ledum; V − R. sect. Viscidula.

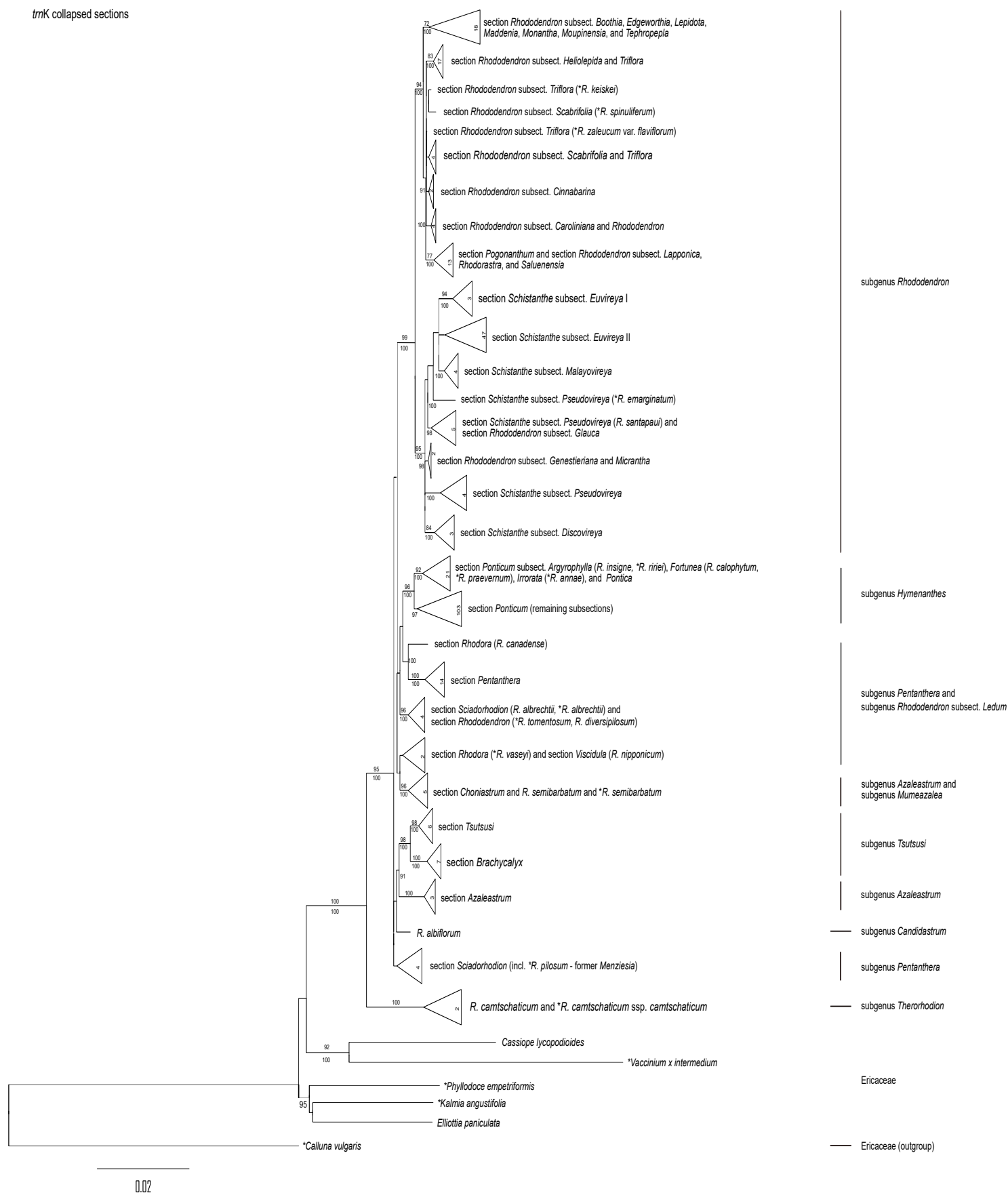|	Kurashige et al. (2001) – *mat*K	This study – *trn*K	This study – *trn*LF	Grimbs et al. (2017) – *trn*K, *trn*LF, ITS	Gao et al. (2002) – ITS	This study – ITS	Goetsch et al. (2005) – rpB2	This study – rbB2	Shrestha et al. (2018) – multiple regions
Sect. Schistanthe	Triphyletic (subsect. Discovireya/R. santapaui/rest) nested in R	unresolved within R	unresolved in R	-	single species nested in R	monophyletic nested in R	monophyletic Discovireya not sampled	diphyletic (Subsect. Discovireya/rest) nested in R	Diphyletic (subsect. Discovireya/rest) nested in R
Ledum	R. hypoleucum sister to R. albrechtii	R. tomentosum, R. diversipilosum sister to R. albrechtii	R. tomentosum close to R. albrechtii but sister to P	R. tomentosum sister to R. albrechtii	R. tomentosum sister to R	R. tomentosum sister to R	R. hypoleucum, R. tomentosum sister to R	R. hypoleucum, R. tomentosum sister to R	R. hypoleucum, R. tomentosum sister to R except R. diversipilosum sister to R. albrechtii
Sect. Pentanthera	sister to R. canadense, together sister to H	Sister to R. canadense, together sister to V, M, C, R. vaseyi	sister to R. canadense, together sister to L	sister to H (R. canadense not sampled)	sister to R. canadense in unresolved position	basally branching (R. canadense not sampled)	incl. R. canadense sister to H	together with R. canadense in H	incl. R. canadense; sister to H
Sect. Choniastrum	sister to M	sister to M	sister to M	sister to T, A	unresolved with A, T, R. albiflorum, R. schlippenbachii	sister to A, T, S, M, R. vaseyi	sister to R, L	sister to R, L	sister to R, L
R. subgen. Tsutsusi	sister to A	sister to A	paraphyletic sister to A+M	sister to A	sister to A+M	sister to A+M	unresolved with A, M, V	sister to V, together sister to M, together sister to A	sister to A+M
R. sect. Azaleastrum	sister to T	sister to T	sister to T, C, M	sister to T	Sister to M	sister to M	unresolved with T, M, V	unresolved with T, M, V	sister to T, M
R. albiflorum (R. subgen. Candidastrum)	unresolved with T, A, R. sect. Sciadorhodion	sister to T, A	-	-	Unresolved with T, A, C, R. sect. Sciadorhodion	-	sister to R. albrechtii	-	sister to R. albrechtii + R. diversipilosum
R. semibarbatum (R. subgen. Mumeazalea)	sister to C	sister to C	sister to C	-	sister to A	sister to A	sister to V	sister to V, T, together sister to A	sister to T
R. nipponicum (R. sect. Viscidula)	sister to M, C	sister to R. vaseyi	-	-	-	-	sister to M	sister to T	Sister to R. quinquefolium (sect. Sciadorhodion), together sister to A, T, M
R. vaseyi (R. sect. Rhodora)	-	sister to V	sister to R	sister to R, H	-	sister to R. albrechtii	sister to Menziesia, together sister to R. schlippenbachii	sister to R. schlippenbachii, together sister to Menziesia	with R. pentaphyllum sister to Menziesia, together sister to R. schlippenbachii
Menziesia	Unresolved with A, T, C, R. sect. Sciadorhodion	R. pilosum incl. in R. sect. Sciadorhodion	R. pilosum with R. vaseyi sister to R	-	-	5 spp. sister to R. schlippenbachii	2 spp. sister to R. vaseyi, together sister to R. schlippenbachii	-	9 spp. sister to R. vaseyi and R. pentaphyllum, together sister to R. schlippenbachii
R. schlippenbachii (R. sect. Sciadorhodion)	unresolved with Menziesia, A, T, R. pentaphyllum, R. quinquefolium	together with Menziesia and R. pentaphyllum, R. quinquefolium, close to A, T, V, albiflorum	unresolved	basally branching	unresolved with T, A, R. albiflorum	sister to Menziesia, together sister to T, A, M	sister to R. vaseyi + Menziesia, then to R. albiflorum, then to A, T, M, V	sister to R. vaseyi, together sister to Menziesia, sister to A, T, M, V	sister to Menziesia + R. vaseyi
R. albrechtii (R. sect. Sciadorhodion)	sister to R. hypoleucum (L), together unresolved	R. albrechtii sister to L, together sister to H	unresolved	sister to L, together sister to P, H	-	sister to R. vaseyi, together sister to C	R. albrechtii sister to R. albiflorum	sister to R. vaseyi, R. schlippenbachii + Menziesia	sister to R. diversipilosum (L), together sister to R. albiflorum

Figure 1. Phylogenetic tree of *Rhododendron* inferred from the plastid *trn*K gene region, showing species arranged by subgenus and section. Numbers above branches are bootstrap values, numbers below branches are posterior probabilities (in percent). Numbers in triangles are the number of the included individuals (species list in Table S2). Newly generated sequences are marked with a star (*).
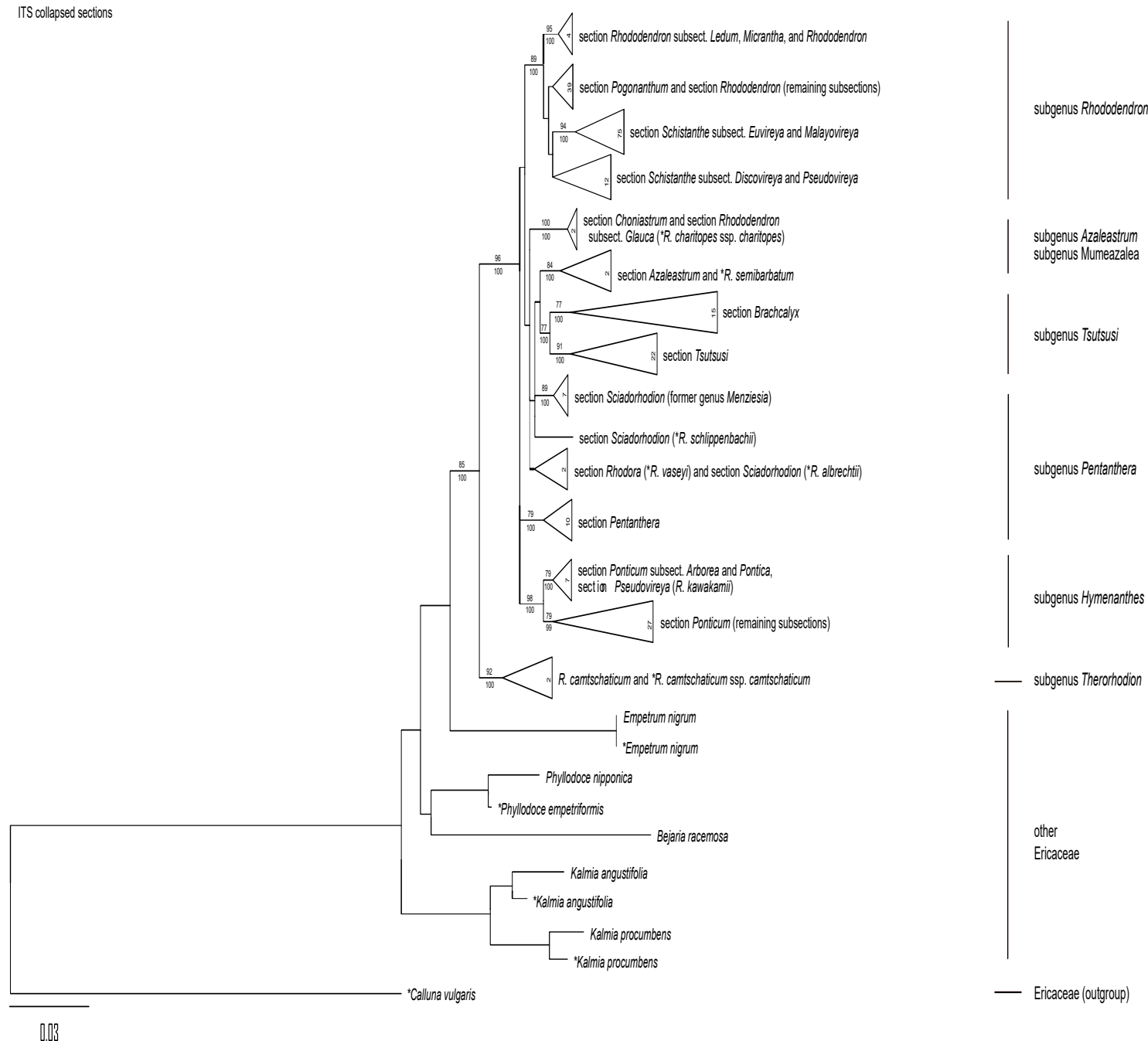
ITS collapsed sections

Figure 2. Phylogenetic tree of *Rhododendron* inferred from the nuclear Internal Transcribed Spacer (ITS) gene region, showing species arranged by subgenus and section *Calluna vulgaris* (Ericaceae) was used as root. Numbers above branches are bootstrap values, numbers below branches are posterior probabilities (in percent). Numbers in triangles are the number of the represented individuals (species list in Table S2). Newly generated sequences are marked with a star (*).
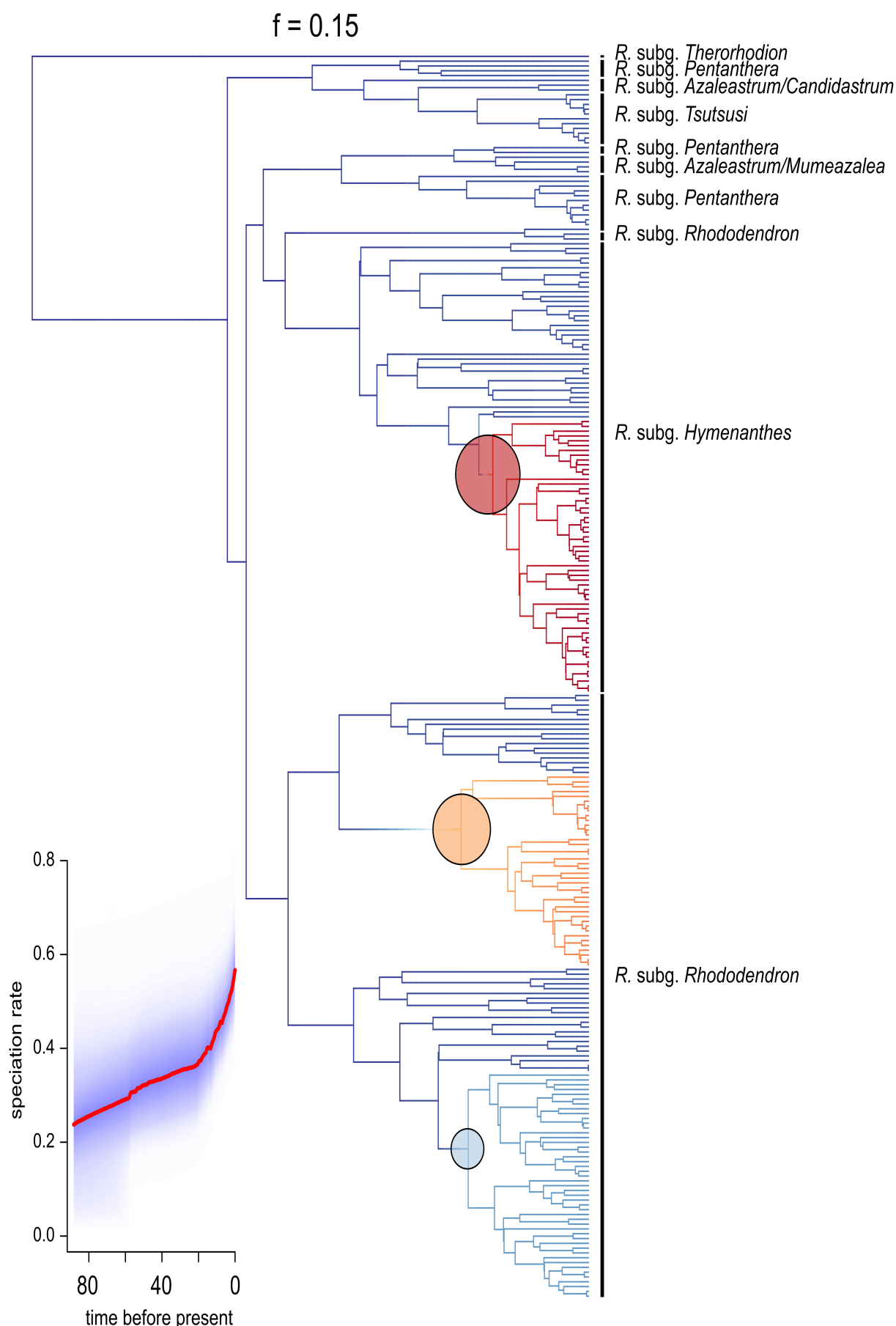
Figure 3. Result from the Bayesian Analysis of Macroevolutionary Mixtures (BAMM) diversification analysis of *Rhododendron trn*K. Colors of phylogeny refer to decrease (blue) or increase (red) in diversification rates. The size of the circles shows rate of diversification shift i.e., larger circle higher rate shift and smaller circle lowers rate shift. Inset shows the rate vs. time plot from the diversification analysis, where the 'speciation rate' (speciation events) is given per million years, and 'time before present' is in millions of years.
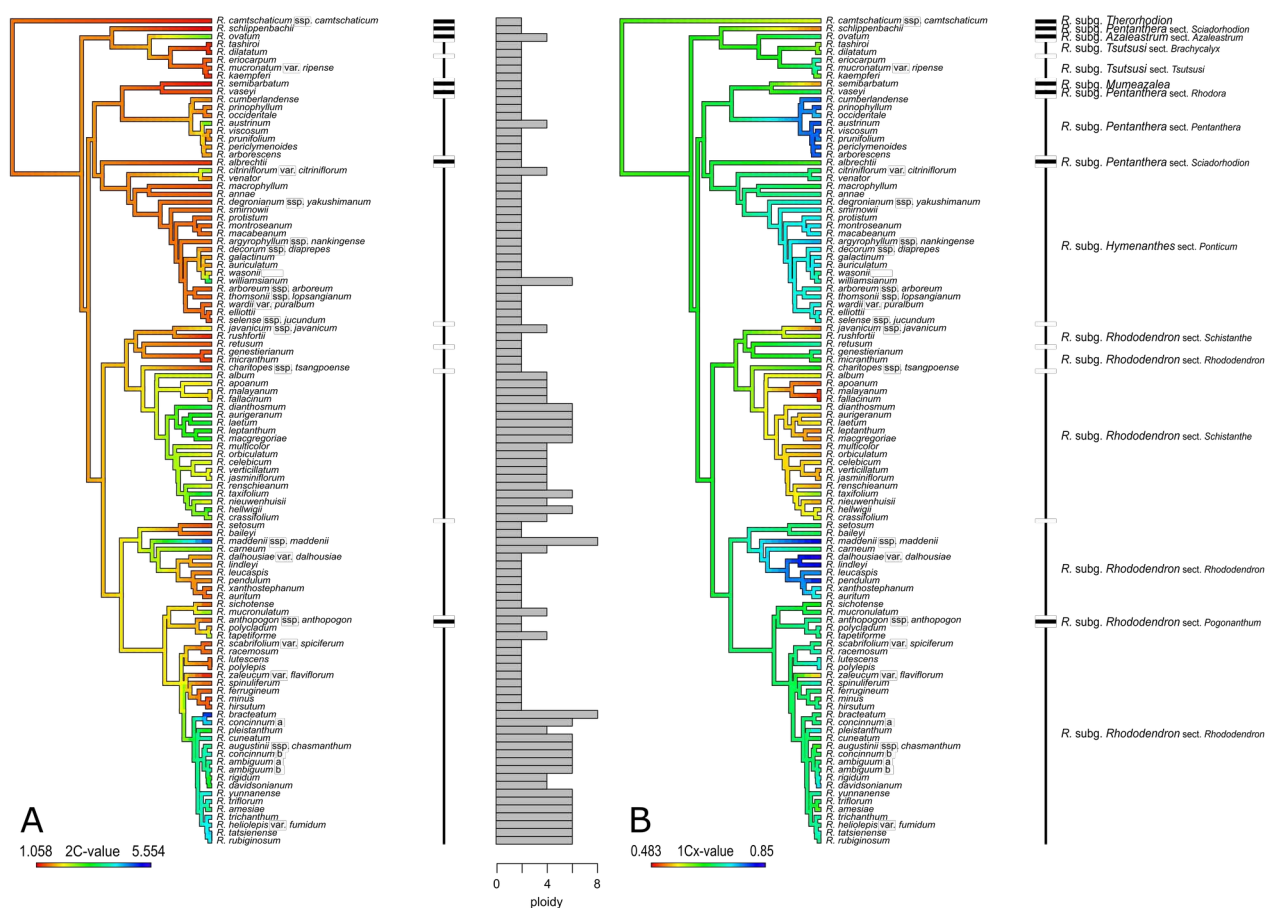
Figure 4. Ancestral genome size values mapped using a continuous color gradient on a BEAST tree of 105 Rhododendron species based on the trnK gene region. A, 2C-values and ploidy levels indicated as bar plot; B, 1Cx-values.