

1 **Colonisation and diversification in the African ‘sky islands’: insights from fossil-**
2 **calibrated molecular dating of *Lychnis* L. (Caryophyllaceae)**

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23 **Summary:**

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- 25 • The flora on the isolated high African mountains or 'sky islands' is remarkable for its
26 peculiar adaptations, local endemism and striking biogeographical connections to
27 remote parts of the world. Ages of the afro-alpine lineages and the timing of their
28 radiations have frequently been debated, but remain contentious as there are no
29 available estimates based on explicit models and fossil-calibrated molecular clocks.
- 30 • We used the plastid region *matK* and a *Caryophylloflora paleogenica* fossil to infer
31 the age of the genus *Lychnis*, and constructed a dataset of three plastid (*matK*, *rps16*,
32 *psbE-petL*) and two nuclear (ITS, *RPB2*) loci for joint estimation of the species tree
33 and divergence time of the afro-alpine representatives.
- 34 • The time of divergence of the afro-alpine lineage was placed in the late Miocene-early
35 Pliocene. Only a single speciation event was inferred in the early Pliocene; subsequent
36 speciation took place sporadically from the late Pliocene to the middle Pleistocene.
- 37 • We provide further support for a Eurasian origin of afro-alpine flora elements, which
38 have been recruited via dispersals at different time periods: some old as in *Lychnis* and
39 others very recent. We also show that dispersal and diversification within Africa plays
40 an important role in shaping the present afro-alpine plant communities.

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43 *Keywords:* African mountains, Afro-alpine, diversification, *Lychnis*, molecular dating

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46 **Introduction**

47

48 Understanding patterns of species diversity, distributions and the processes that govern
49 evolution of distinct regional biota has been a central theme for evolutionary biologists since
50 the times of Darwin and Wallace. Tropical mountain biotas have been recognized as
51 exceptionally diverse (e.g. Mittermeier *et al.*, 2005; Orme *et al.*, 2005; Merckx *et al.*, 2015)
52 and have attracted much attention. Yet the diversity of species assemblies that tropical
53 mountains harbour has been notoriously difficult to explain on the basis of contemporary
54 factors such as climate (e.g. Rahbek & Graves, 2001), indicating the importance of historical
55 processes like lineage diversification and dispersal (e.g. Wiens & Donoghue, 2004; Cadena *et*
56 *al.*, 2011; Dauby *et al.*, 2014; Merckx *et al.*, 2015).

57 The mountains of eastern Africa (i.e. East Africa and Ethiopia) provide an excellent
58 example of a system where the interplay between historical and contemporary processes has
59 produced diverse floras and faunas rich in local endemics (Hedberg, 1969; Mittermeier *et al.*,
60 2005). Based on their geological age and the processes that led to their formation, the eastern
61 African mountains can be divided into two remarkably different groups: the young and
62 volcanic East African Rift System (EARS) and the much older and mostly uplifted mountain
63 blocks of the eastern Afromontane Biodiversity Region (also known as the Eastern Arc or the
64 Eastern Arc Mountains; Mittermeier *et al.*, 2005). In the latter, the basal blocks of the
65 mountains are at least 30 Ma old, but likely much older – perhaps more than 100 Ma
66 (Griffiths, 1993).

67 A recent surge of biogeographical and phylogeographical studies have focused on the
68 origins and evolution of the afro-montane flora and fauna (e.g. Kebede *et al.*, 2007; Couvreur
69 *et al.*, 2008; Tolley *et al.*, 2011; Dimitrov *et al.*, 2012; Ceccarelli *et al.*, 2014; Loader *et al.*,
70 2014) and have underlined the importance of long-term climatic stability for the generation
71 and maintenance of species diversity (Lovett *et al.*, 1988; Fjeldså & Lovett, 1997). As a
72 result, clades that have diversified in this region are often old and have their closest relatives
73 in the African lowland tropical forests (e.g. Couvreur *et al.*, 2008). In sharp contrast, the
74 higher elevations of the EARS mountains are home to a much younger afro-alpine habitat and
75 it has been suggested that several of its local plant lineages have immigrated from Eurasia via
76 the Arabian Peninsula (Koch *et al.*, 2006; Assefa *et al.*, 2007; Ehrich *et al.*, 2007; Popp *et al.*,

77 2008; Gehrke & Linder, 2009). The actual time of colonisation of these mountains is however
78 poorly understood and remains contentious.

79 The EARS is a prominent geological feature, extending from Syria in the north
80 through East Africa to Mozambique in the south. Its formation began around 45 Ma and
81 divided the region into the western and eastern Rift branches (Fig. 1; Ebinger *et al.*, 2000;
82 Trauth *et al.*, 2005). Associated volcanism resulted in the formation of extensive mountain
83 systems in the Ethiopian highlands and scattered high mountains in East Africa, whose origins
84 range from the Miocene to the late Pliocene (Griffiths, 1993). The mountains are famous for
85 their unique ecosystem with distinct altitudinal zonation of the vegetation: the uppermost
86 afro-alpine zone proper, the ericaceous transition zone, and the subalpine montane forest zone
87 (Hedberg, 1951). Some 77% of the vascular plant species of the afro-alpine zone are
88 recognized as local or regional endemics (Hedberg, 1961; Hedberg, 1969; Gehrke & Linder,
89 2014), with a large proportion having their closest relatives in other temperate areas rather
90 than in the adjacent tropical lowlands (Hedberg, 1965; Hedberg, 1970). This led Hedberg
91 (1961) to state that “[the afro-alpine flora] seems to have existed in tropical East Africa before
92 the formation of the mountains [Miocene – late Pleistocene] now harbouring it, and to have
93 been strongly isolated from other high-mountain (or temperate) floras for a long time”. On the
94 contrary, others have suggested that at least parts of the flora are rather young (Koch *et al.*,
95 2006; Assefa *et al.*, 2007; Ehrich *et al.*, 2007; Gehrke & Linder, 2014; Linder, 2014) and that
96 the colonisation and current distribution of the mountain flora were mainly shaped by the
97 climatic oscillations of the Plio-Pleistocene period (e.g. Livingstone, 1962; Hamilton, 1982;
98 Mohammed & Bonnefille, 1998; Trauth *et al.*, 2005). This view was also adopted in a recent
99 study of the evolution of African plant diversity, where the afro-alpine flora was suggested to
100 represent the youngest of all the African floras (Linder, 2014). However, only one study has
101 so far addressed the time of colonisation of the alpine zones of the EARS by plants. Koch *et*
102 *al.* (2006) used synonymous mutation rates and sequence distances calculated from the
103 chloroplast gene *matK* and the nuclear *adh* and *chs* genes to infer a Pleistocene origin of the
104 afro-alpine populations of the arctic-alpine plant *Arabis alpina* (Brassicaceae). The use of
105 fixed rates and sequence distances is, however, not optimal for molecular dating as it assumes
106 a strict clock with no rate heterogeneity.

107 Here we provide the first results based on explicit fossil-calibrated molecular clocks to
108 estimate the time of the origin and colonisation of the afro-alpine habitat by plants. We
109 selected the afro-alpine representatives of *Lychnis* L. (Caryophyllaceae), which are distributed

110 in the Ethiopian highlands and in the high mountains of East/Central Africa (Kenya,
111 Tanzania, Uganda, Rwanda, Burundi, the Democratic Republic of Congo) and West Africa
112 (Cameroon, Nigeria). The genus *Lychnis* comprises some 30 species occurring mostly in
113 temperate areas of the northern hemisphere (Oxelman *et al.*, 2000). In previous works the six
114 afro-alpine species of the genus (= *Uebelinia* Hochst.) have been referred to as afro-montane
115 rather than afro-alpine (e.g. Popp *et al.*, 2008) because they are sometimes found at lower
116 altitudes (occasionally down to 1500 m; Ousted, 1985). Traditionally, the afro-montane
117 concept has been used as a loose framework applied to most African mountains south of
118 Sahara (Carbutt & Edwards, 2015). Occurrence restricted to areas above the tree line or above
119 specific elevations provides an objective criterion to classify plants into montane or alpine
120 species (e.g. Linder, 2014; Carbutt & Edwards, 2015). However, its strict application may
121 lead to biased assessments as, for variety of reasons, tropical alpine plants commonly occur at
122 lower elevations (Hedberg, 1957; Rundel *et al.*, 1994; Leuschner, 1996 and references
123 therein). Here we apply a less restrictive definition of afro-alpine plants to include species that
124 may occasionally occur at lower elevations (and thus, often referred to as afro-montane) but
125 have their main occurrences at elevations above 2700 m and are adapted to night frost. In
126 *Lychnis*, all afro-alpine species occur above 2700 m and only few are also found at lower
127 altitudes (Table 1). These lower-altitude plants are typically found as weeds in fields, along
128 roads, and in other disturbed habitats. With the exception of the Moroccan endemic *L.*
129 *lagrangei*, we therefore consider the rest of African *Lychnis* to fit best in the afro-alpine
130 category.

131 In our recent biogeographical study, we found support for a single Eurasian origin of
132 the afro-alpine *Lychnis*, which formed a monophyletic group and inferred to be sister to the
133 Eurasian *L. flos-cuculi* (Popp *et al.*, 2008). The only other African representative of the genus,
134 the Moroccan *L. lagrangei*, thus seems to have immigrated independently to Africa. The afro-
135 alpine *Lychnis* comprised two distinct lineages: one diploid lineage consisting of *L.*
136 *rotundifolia*, *L. scottii*, *L. abyssinica* and *L. kigesiensis*, and one tetraploid lineage consisting
137 of *L. crassifolia* and *L. kiwuensis* (Popp *et al.*, 2008). Because of its single origin and wide
138 distribution in the East African mountains, the afro-alpine *Lychnis* is an excellent system to
139 study the tempo and mode of afro-alpine habitat colonisation. In addition, the occurrence of
140 *Lychnis* in Eurasia and Africa provides a broader context that allows us to investigate afro-
141 alpine links to other regional floras. In the current study, we first used a fossil-calibrated
142 relaxed molecular clock for the plastid *matK* gene to infer the age of the genus *Lychnis*. Then

143 we used this estimated age for joint estimation of the species tree and divergence time of the
144 afro-alpine *Lychnis* based on sequences from three plastid (*matK*, *rps16*, and *psbE-petL*) and
145 two nuclear (ITS and *RPB2*) DNA regions. We used our dated phylogeny to address the
146 following questions: (1) When did *Lychnis* colonise the afro-alpine habitat? (2) When did
147 subsequent speciation events within the afro-alpine region occur? (3) Which factors may have
148 contributed to speciation and diversification within afro-alpine *Lychnis*? (4) Can the
149 colonisation and diversification events be correlated to past climatic and/or geological events?
150 Answering these questions is central to our quest for better understanding of the origins and
151 evolution of afro-alpine diversity in general and of *Lychnis* in particular.

152

153

154 **Materials and Methods**

155

156 **Plant materials and DNA extraction**

157 Leaf tissue of afro-alpine *Lychnis* was collected in Ethiopia, Kenya, Uganda, Tanzania, and
158 Cameroon in 2004-2009 (Table 1) and dried in silica gel. Voucher specimens were deposited
159 in the National Herbarium of Ethiopia, Addis Ababa University, Ethiopia (ETH); with
160 duplicate sets in the Natural History Museum, University of Oslo, Norway (O; full duplicate
161 set of the 2007-2009 collections); the East African Herbarium, National Museum of Kenya,
162 Kenya (EA; Kenyan collections); Sokoine University of Agriculture, Tanzania (SUA;
163 Tanzanian collections); and Makerere University Herbarium, Uganda (MU; Ugandan
164 collections). In addition, herbarium material of the Moroccan *L. lagrangei* and the Eurasian *L.*
165 *subintegra* and silica-dried leaf samples of the Eurasian *L. flos-cuculi* were used. *Lychnis*
166 *abyssinica* consists of two morphologically indistinguishable lineages with significantly
167 different genome sizes (Popp *et al.*, 2008). We sampled large genome size (LGS) *L.*
168 *abyssinica*, which is a putative hybrid (Popp *et al.*, 2008), from its currently known
169 geographical range in the Ethiopian highlands (Fig.1b) and small genome size (SGS) *L.*
170 *abyssinica* from across its Ethiopian range as well as from its strikingly disjunct area in West
171 Africa (Bamenda, North-west Cameroon). Although we included samples of *L. abyssinica* for
172 both genome size variants from most of its range, we were not able to obtain collections from
173 its small East African range (Fig. 1). Samples of the narrow East African endemics were
174 collected in Mt Kilimanjaro (*L. rotundifolia*), Mt. Aberdare/Cherangani Hills (*L. crassifolia*),
175 and the Echuya Forest, Kanaba Swamp in Uganda (*L. kigesiensis* subsp. *kigesiensis*). We also
176 obtained representative samples of the narrow Ethiopian endemics *L. kiwuensis* subsp.
177 *erlangeriana*, *L. kigesiensis* subsp. *ragazziana*, and *L. scottii*. The widespread *L. kiwuensis*
178 subsp. *kiwuensis* was sampled from its disjunct areas in the Ethiopian highlands and from Mts
179 Muhavura and Mgahinga in Uganda (Fig. 1).

180 Total genomic DNA was extracted from leaf material using an automated GeneMole[®]
181 robot (Qiagen Nordic, Oslo, Norway) or DNeasy[™] Plant Mini Kit (Qiagen, Valencia, CA).
182 Leaf tissue was ground with two tungsten carbide beads for 2 min at 15 Hz in a mixer mill
183 (MM301, Retsch GmbH & Co., Haan, Germany).

184

185 **Polymerase Chain Reaction (PCR) and DNA sequencing**

186 Amplification and DNA sequencing protocols as described in Popp *et al.* (2008) were used to
187 generate sequences for two non-coding plastid DNA regions, the *rps16* intron and the *psbE*-
188 *petL* interspacer. Approximately 1.5 kb of the plastid *matK* region was amplified and
189 sequenced in three overlapping fragments using primer pairs *matK*-F2b/*matK*-547.rc, *matK*-
190 526/*matK*-R2, and *matK*-1066/*matK*-1463.rc (Mower *et al.*, 2007; Sloan *et al.*, 2009) using
191 Ready-To-GoTM PCR Beads (Amersham Pharmacia Biotech, UK), 0.5 μ L of each 10 μ M
192 forward and reverse primers, and 1 μ L genomic DNA of 100 ng/ μ L concentration. The PCR
193 products were purified using 2 μ L Exosap-IT (USB Corp., Cleveland, OH, USA), diluted 1:10
194 for 5 μ L PCR products, and incubated for 45 min at 37 °C and for 15 min at 80 °C.

195 Sequencing was performed using the PCR primers and BigDye Terminator v 1.1
196 Cycle Sequencing Kit (Applied BiosystemsTM, Foster City, CA, USA) according to the
197 manufacturer's manual (except for using 10 μ L reaction volumes) and visualized with an ABI
198 3100 capillary sequencer (Applied Biosystems). The sequencing program consisted of
199 denaturing at 96°C for 1 min followed by 25-35 cycles of 96°C 10 sec, 50°C 5 sec, and 60°C
200 4 min, ending with extension at 60°C for 10 min.

201 The sequences were edited using CodonCode aligner v3.5.7 (CodonCode Corporation,
202 Dedham, MA, USA), translated into amino acids for visual inspection, and manually aligned
203 using MEGA v. 4 (Tamura *et al.*, 2007). Substitution model selection was done in MrAIC v.
204 1.4.4 (Nylander, 2004) in conjunction with PhyML v. 3.0 (Guindon & Gascuel, 2003) using
205 the Akaike Information Criterion. The phylogenetic analyses and dating were performed in
206 two steps. The first analysis was carried out to obtain an estimate of the age for the entire
207 genus *Lychnis* using *matK* DNA sequences representing the subfamilies Alsinoideae and
208 Caryophylloideae and a single fossil to calibrate the inferred phylogeny. The inferred age of
209 the genus *Lychnis* from the *matK* phylogeny was then used as a secondary calibration point in
210 a subsequent analysis including all available afro-alpine *Lychnis* sequences.

211

212 **Phylogenetic analyses and dating of the *matK* phylogeny**

213 A total of 33 *matK* sequences representing Alsinoideae and Caryophylloideae as well as one
214 sequence of *Polycarpon tetraphyllum* from the subfamily Paronychioideae (for outgroup
215 rooting) were downloaded from GenBank (Table 2). The resulting dataset consisted of 46

216 sequences, including 13 additional *matK* sequences of afro-alpine *Lychnis*, the Moroccan *L.*
217 *lagrangei*, and the Eurasian *L. flos-cuculi* and *L. subintegra* generated in this study. The input
218 files for Bayesian Evolutionary Analysis Sampling Trees (BEAST) were prepared using
219 BEAUti v. 1.6.1 (Drummond & Rambaut, 2007; Heled & Drummond, 2010).

220 Monophyly was enforced for the Caryophylloideae and Alsinoideae sequences to
221 ensure the tree was rooted with *Polycarpon tetraphyllum* from the subfamily Paronychioideae
222 (Bittrich, 1993; Fior *et al.*, 2006). A fossil inflorescence of *Caryophylloflora paleogenica*
223 (Caryophyllaceae), inferred as sister to either one or both of the subfamilies Alsinoideae and
224 Caryophylloideae (Jordan & Macphail, 2003), was used to calibrate the tree. The inferred
225 fossil age is Middle to Late Eocene, which corresponds to an age of 48.6-33.9 Ma
226 (<http://www.paleodb.org>). A lognormal prior distribution with log mean = 0.0, standard
227 deviation = 1.37 and offset of 33.9 Myr was assigned to the Alsinoideae/Caryophylloideae
228 crown node (Fig. 2). This approximates a 95% probability distribution spanning the inferred
229 minimum and maximum age of the fossil with a fixed minimum age as suggested by (Ho &
230 Phillips, 2009).

231 Initial analyses using a relaxed molecular clock in BEAST v. 1.6.1 with uncorrelated
232 lognormal distributed substitution rates for each branch showed that the posterior distribution
233 of the standard deviation of the clock rate variation did not include 0, and thus a strict
234 molecular clock could be rejected (Drummond *et al.*, 2007). All subsequent *matK* analyses
235 were consequently modelled using a relaxed molecular clock. All other prior distributions
236 used the defaults in BEAUti v. 1.6.1. The final analysis included four independent Markov
237 Chains Monte Carlo (MCMC) simulations run for 10 million generations with tree and
238 parameter values sampled every 1000 generations. To test the influence of the priors on the
239 posterior estimates, one additional chain was run for 10 million generations without data,
240 sampling only the priors. Chain convergence was confirmed by inspection of the MCMC
241 samples from each run using Tracer v.1.5, and joint estimates were produced using
242 LogCombiner v. 1.6.1 (Drummond & Rambaut, 2007).

243

244 **Phylogenetic dating and inference of the species tree**

245 A species tree and divergence times for the afro-alpine *Lychnis* and the closely related *L.*
246 *lagrangei*, *L. flos-cuculi*, *L. flos-jovis* and *L. coronaria* were co-estimated using the *matK*,
247 *rps16*, and *psbE-petL* sequences from this study and the nuclear ITS and *RPB2* sequences

248 generated using the same samples by Popp *et al.* (2008) in *BEAST (Heled & Drummond,
249 2010). A total of 224 sequences were analysed (Table 3). Substitution models, clock models
250 and tree models were unlinked among datasets, except for the *rps16* and *psbE-petL* tree
251 models, which were linked as these regions are inherited as a single linkage block. Although
252 this is also true for *matK*, the tree model for this region was unlinked from the *rps16* and
253 *psbE-petL* models, because we were unable to produce *matK* sequences for all taxa (Tables 1,
254 2).

255 The posterior age estimate for the *Lychnis* crown group obtained from the fossil-
256 calibrated *matK* BEAST analysis was used as prior age in the *BEAST species tree analysis.
257 The XML input file was manually edited to include a prior for the species tree root age
258 following a normal distribution approximating the posterior estimate of the age of *Lychnis*
259 from the *matK* analysis. Preliminary *BEAST analyses were run to test how well the datasets
260 fit a strict molecular clock as described for the *matK* analysis above. Strict clocks could not be
261 rejected for ITS and *rps16*; the remaining datasets were analysed using uncorrelated
262 lognormal clocks.

263 The final *BEAST analysis was performed using constant population size coalescent
264 models for each species with an autosomal nuclear ploidy type set for ITS and *RPB2* and a
265 mitochondrial type for the linked *rps16* and *psbE-petL* as well as for *matK*. The species were
266 linked with a Yule tree prior. Seven independent analyses were run for 150 million
267 generations and trees and parameter values were sampled every 15,000th generations. To test
268 the influences of the prior on the posterior estimate, two additional chains were run for 300
269 million generations without data, sampling only the prior. Convergence of the chain to
270 stationary distributions was confirmed by inspection of the MCMC samples in each analysis
271 using the program Tracer 1.5 (Rambaut & Drummond, 2007) and joint estimates were
272 produced using LogCombiner v. 1.6.1 (Rambaut & Drummond, 2007). To avoid violating the
273 assumption of non-reticulate evolution in *BEAST, we included plastid data only for the LGS
274 *L. abyssinica*, which together with *L. scottii* was inferred as a sister group to *L. kigesiensis*
275 and the SGS *L. abyssinica* (cf. Fig. 3).

276

277 **Results**

278

279 Detailed information on each DNA region including the number of characters and terminals is
280 presented in Table 3.

281

282 **Dating and phylogenetic inference based on *matK***

283 GTR+G was selected as the best-fitting model for the *matK* dataset including the additional
284 Alsinoideae, Caryophylloideae and Paronychioideae representatives. Inspection of the
285 MCMCs upon completion of the BEAST analyses showed that all individual runs had
286 converged and the effective sample size (ESS) was >200 for all parameters (>>1000 in most
287 cases). The maximum clade credibility tree with estimated node ages based on the combined
288 result from all runs is presented in Figure 2. The time to the most common ancestor (tMRCA)
289 of the genus *Lychnis* was estimated to 8.0 Ma (95% HPD 5.5-10.7; node B in Fig. 2). The
290 model analysed without data returned the prior distributions and we therefore conclude that
291 the model is dominated by the data.

292

293 **Multigene *Lychnis* species tree and molecular dating**

294 GTR+G was again the best fitting model for *matK* (including only *Lychnis* taxa), aK3Puf+G
295 for *rps16*, aK3Puf+I for *psbE-petL*, SYM+G for ITS, and HKY+G for *RPB2*. The results from
296 preliminary *BEAST analyses suggested strict clock models for the ITS, *rps16* and *matK*
297 regions. Uncorrelated relaxed lognormal clocks were used for the *RPB2* and *psbE-petL*
298 regions. Inspection of the *BEAST MCMCs showed convergence of model parameters in all
299 individual analyses with ESS's >200 for all parameters (>>1000 for most of them). Likewise,
300 the model analysed without data resulted the prior distributions indicating the model being
301 dominated by the data.

302 The species tree supported the monophyly of the afro-alpine *Lychnis* including two
303 well-supported clades: one diploid (*L. abyssinica*, *L. kigesiensis*, *L. scottii*, and *L.*
304 *rotundifolia*) and one polyploid (*L. crassifolia* and *L. kiwuensis*) as in Popp *et al.* (2008). The
305 analysis recovered two distantly related plastid lineages in *L. abyssinica* (Fig. 3, Suppl. Fig.
306 1a-d). The Moroccan *L. lagrangei* and the Eurasian *L. flos-cuculi* formed a sister group to the
307 afro-alpine clade. The tMRCA of the afro-alpine *Lychnis* and its sister lineage was estimated
308 to 5.1 Ma (95% HPD 3.0–7.4; node A in Fig. 3 and Table 4). The tMRCA for the afro-alpine

309 *Lychnis* was estimated to 4.1 Ma (95% HPD 2.3–5.9; node B in Fig. 3 and Table 4). The
310 tMRCAs for the diploid and polyploid afro-alpine lineages were estimated to 2.5 Ma (95%
311 HPD 1.5–3.7; node C) and 1.4 Ma (95% HPD 0.6–2.2; node D), respectively.

312

313

314 **Discussion**

315

316 **Origin and diversification of the afro-alpine *Lychnis***

317 The multilocus species tree presented here (Fig. 3) largely corroborates the single and
318 concatenated gene tree analyses of Popp *et al.* (2008), including the rejection of a direct sister-
319 group relationship between the afro-alpine *Lychnis* and the only other African species of
320 *Lychnis*, the Moroccan endemic *L. lagrangei*. We have thus corroborated the hypothesis of
321 two independent colonisations of Africa by *Lychnis* (Popp *et al.*, 2008). However, the inferred
322 position of *L. lagrangei* differs between the present study and that of Popp *et al.* (2008). The
323 concatenated analysis of Popp *et al.* (2008) resolved the Eurasian *L. flos-cuculi* as immediate
324 sister to the afro-alpine *Lychnis*, with *L. lagrangei* as sister to this group, while in our results
325 *L. lagrangei* and *L. flos-cuculi* form a clade that is the sister to the afro-alpine lineage. We
326 consider the latter result to be more reliable because it is based on a larger dataset, both in
327 number of specimens and loci. In the current study, we increased the sample size with the
328 inclusion of two additional samples of *L. lagrangei* from herbarium specimens collected in
329 Morocco (Table 1). In addition, phylogenetic analysis of concatenated sequence data may be
330 misleading, in particular if the internal branches are short (Kubatko & Degnan, 2007). Popp *et*
331 *al.* (2008) used maximum parsimony to analyse a concatenated dataset of both plastid and
332 nuclear DNA regions, whereas the present study uses a coalescent-based approach that may
333 be more robust to incomplete lineage sorting. Both hypotheses, however, reject a single
334 colonisation of Africa.

335 Our molecular dating analysis resulted in an estimated mean age for the stem of the
336 afro-alpine *Lychnis* of 5.1 Ma (95% HPD 3.05-7.41 Ma; node A/B, Fig. 3 and Table 4),
337 suggesting a late Miocene – early Pliocene origin, coinciding with a period of increased
338 aridity in East Africa 6-7 Myr (Cerling *et al.*, 1993; Cerling *et al.*, 1997). Aridification
339 happened again in the late Pliocene around 3 Ma (Bobe, 2006). With minor variations, warm
340 climate has been predominant during the last 5 Myr (Wara, 2005) and several studies have
341 shown that trees and shrubs dominated East African plant communities during this period
342 (e.g. Corlett, 2014). These climatic conditions also had an effect on the EARS, where warm
343 and humid climate towards the end of the Miocene has been inferred based on the paucity of

344 grass pollen in the Northwestern Ethiopian highlands, Gonder (Yemane *et al.*, 1985).
345 Evidence from the Turkana Basin of Kenya indicates that there was a humid closed rainforest
346 environment at about 4 Ma (Bonnefille, 1995; Bobe, 2006). The rainforest expanded in
347 eastern Africa (Williamson, 1985; Pickford *et al.*, 2004) and lasted until the late Pliocene
348 (about 3.4 Ma) in the Ethiopian highlands (Woldegabriel *et al.*, 1994; Bonnefille, 1995). Such
349 a warm and moist climate might have promoted the expansion of forest in the region and
350 provided more favourable conditions for dispersal over the north and central African plains by
351 reducing the extent of deserts acting as biogeographical barriers for many plants. During this
352 period, most of the eastern African mountains that today are high enough to sustain alpine
353 habitats started to rise (Wichura *et al.*, 2010). Most of the 95% HPD of the estimated time of
354 origin and initial diversification of the afro-alpine *Lychnis* corresponds to this warm and moist
355 period. It is, therefore, possible that the ancestor of the afro-alpine *Lychnis* dispersed from
356 Eurasia to eastern Africa and diversified into two lineages at the time when the first high
357 mountains started to rise. The capability of some *Lychnis* species (Table 1) to establish at
358 lower elevation (as low as 1500 m under current conditions) shows that they can tolerate a
359 wide range of climatic conditions. Such environmental flexibility may have also played an
360 important role for the early establishment of these plants in the EARS mountains.

361 Interspecific divergence within each of the two main afro-alpine lineages of *Lychnis*
362 commenced around 2.5 Ma (95% HPD 1.5-3.7 Ma, diploids) and 1.4 Ma (95% HPD 0.6-2.2
363 Ma, polyploids). These time estimates roughly fit with renowned periods of aridification in
364 East Africa peaking at 2.8, 1.7, and 1.0 Ma (Cane & Molnar, 2001; deMenocal, 2004; Bobe,
365 2006; Sepulchre *et al.*, 2006). Thus, habitat fragmentation triggered by aridification and
366 subsequent allopatric speciation in widely distributed ancestors may explain the
367 diversification pattern in both diploids and tetraploids. However, the 95% HPD divergence
368 time estimates of both lineages span beyond these arid intervals and overlaps with periods
369 known to have been more humid. Such more humid interglacial periods in Africa occurred
370 about 2.7-2.5, 1.9-1.7 and 1.1-0.9 Ma (Trauth *et al.*, 2005). The uncertainty in time estimates
371 does not allow us to distinguish between the two alternatives hypotheses of diversification
372 during arid periods or diversification under humid periods. The distributions of divergence
373 times inferred from our analyses indicate that early Pliocene diversification may be as likely
374 as middle Pleistocene diversification and suggest that speciation within the afro-alpine
375 *Lychnis* may not be triggered solely by the climatic oscillations of the Plio-Pleistocene. The
376 rise of high mountains that provide suitable alpine habitats and conditions promoting dispersal

377 may have been one of the main drivers of diversification and range dynamics in African
378 *Lychnis*. Our previous results (Popp *et al.*, 2008) also favoured multiple dispersal hypotheses
379 including long-distance dispersal to West Africa.

380

381 **Origins and evolution of the afro-alpine flora - the *Lychnis* perspective**

382 The results presented here are consistent with Mercks's *et al.*, (2015) findings that underline
383 the importance of dispersal of lineages pre-adapted to alpine conditions and local speciation in
384 the process of generation of alpine species communities on isolated tropical mountains. Our
385 findings also corroborate at large Linder's (2014) suggestion that the afro-alpine flora is
386 among the youngest in Africa and contains a large number of taxa with Eurasian ancestry.
387 However, we found strong support for late Miocene-early Pliocene arrival and diversification
388 of *Lychnis*, challenging the idea that, as a whole, the afro-alpine flora is a product of recent
389 colonisation events and the climatic oscillations of the Plio-Pleistocene period (Livingstone,
390 1962; Hamilton, 1982; Mohammed & Bonnefille, 1998; Gottelli *et al.*, 2004; Koch *et al.*,
391 2006). We show that at least some alpine plants, such as *Lychnis*, may have colonised eastern
392 Africa as soon as the first alpine habitats were formed, or even some time before that.
393 Widespread species that are capable of surviving under a wide range of conditions, such as
394 present-day *L. kiwuensis* (found from 3100 to 1800 m), may have established in EARS first
395 and then diversified in the region as soon as the alpine habitat expanded with the formation of
396 higher mountains. The age inferred for the stem of the clade including *L. kiwuensis* in our
397 phylogeny is consistent with such a hypothesis (see also Popp *et al.*, 2008).

398 Patterns similar to those reported here have recently been described for the endemic
399 eastern African species of *Canarina* (Mairal *et al.*, 2015). Although *Canarina* belongs to the
400 montane forest zone, not the alpine zone, it has been hypothesized that it has migrated into
401 eastern Africa along routes similar to those suggested for *Lychnis*. This finding provides
402 further evidence for pre-Pleistocene colonisations of eastern Africa from Eurasia.

403 Pre-Pleistocene origins have also been suggested for several East African plant genera such as
404 *Isolona* and *Monodora* (Couvreur *et al.*, 2008) and *Saintpaulia* (Dimitrov *et al.*, 2012).

405 However, the latter have their origins in Africa and much of the afro-montane area that they
406 currently occupy (e.g., the Eastern Arc Mountains) has been under direct climatic influence
407 from the Indian Ocean and has remained stable despite the climatic oscillations of Pleistocene
408 (Fjeldså & Lovett, 1997; Hewitt, 2000). Because the distribution of the afro-alpine *Lychnis* in

409 eastern Africa has some overlap with these stable areas, their importance in the process of
410 speciation in *Lychnis* cannot be ruled out and needs to be further investigated.

411 Our results provide further support for a Eurasian origin of at least parts of the afro-
412 alpine flora and suggest that such elements were recruited at different times. *Arabis alpina*
413 seems to have dispersed twice to eastern Africa during the Pleistocene, the second time very
414 recently (Koch *et al.*, 2006), although this has so far not been corroborated using fossil-
415 calibrated molecular data. Here we show that dispersals to eastern African mountains from
416 Eurasia have also happened during the time periods preceding the Pleistocene. Our results
417 also corroborate the importance of dispersals and diversification within Africa in shaping the
418 present afro-alpine plant communities, where local diversification in several genera has
419 resulted in high numbers of afro-alpine endemics (e.g. Gehrke & Linder, 2009). As a result,
420 although less diverse than the afro-montane flora of the Eastern Afromontane Biodiversity
421 Region, the contemporary afro-alpine flora is unique in its origins and species composition. In
422 addition, afro-alpine plants are adapted to cope with harsh environmental conditions and often
423 have specialised life forms. Some features of these life forms (e.g. giant rosettes, large
424 tussocks, densely pubescent leaves) apparently evolved multiple times in plants established at
425 high altitudes on different tropical mountains (Hedberg, 1964). The combination of unique
426 life form traits, unique evolutionary history and high degree of endemism underlines the
427 importance of the afro-alpine habitat as a reservoir of unique yet fragile diversity.

428

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436

437

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615

617 Table 1 Geographical information of the study material

Database number	Population/individual number	Taxon	Country	Locality	Altitude (m)	Latitude	Longitude
O-DP-13583	AFR522-4 [‡]	<i>L. abyssinica</i> LGS	Ethiopia	Arsi: Mt Chilalo	3000	7.9269	39.1795
O-DP-13592	AFR528-5 [‡]	<i>L. abyssinica</i> LGS	Ethiopia	Arsi: Mt Kaka	3400	7.3663	39.2020
O-DP-13596	AFR533-1 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Arsi: Betw. Kofele and Asassa, 15 km from Kofele	2700	7.1735	38.8746
O-DP-13600	AFR536-4 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Bale Mts	3200	6.9328	39.9460
O-DP-13604	AFR540-1 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Bale Mts: Betw. Park Head Quarter and Goba, 2 km from Head Quarter	3100	7.1056	39.8015
O-DP-13608	AFR545-1 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Shoa, Entoto	3000	9.0895	38.7648
O-DP-13616	AFR548-4 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Arsi: Betw. Shashemene and Kofele, 12.5 km from Shashemene	2300	7.1492	38.6990
O-DP-13619	AFR560-2 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Betw. Agere Maryam and Dilla, 23 km from Agere Maryam, Sidamo	2300	5.8181	38.2650
O-DP-13621	AFR570-1 [‡]	<i>L. abyssinica</i> SGS	Ethiopia	Keffa, Masha	2200	7.7693	35.4745
O-DP-29620	ET098-2	<i>L. abyssinica</i> LGS	Ethiopia	Simen Mts: Close to Gich Camp Site	3570	13.2666	38.1078
O-DP-30300	ET271-2	<i>L. abyssinica</i> LGS	Ethiopia	Simen Mts: Gich Camp Site	3650	13.2697	38.1059
O-DP-30734	ET391-2	<i>L. abyssinica</i> LGS	Ethiopia	Simen Mts: Silki	3760	13.3333	38.2333
O-DP-31231	ET509-2	<i>L. abyssinica</i> LGS	Ethiopia	Simen Mts: Silki	3600	13.3203	38.2351
O-DP-33637	ET1336-2	<i>L. abyssinica</i> LGS	Ethiopia	Mt Choke	3960	10.6420	37.8357
O-DP-33712	ET1351-2	<i>L. abyssinica</i> LGS	Ethiopia	Mt Choke	3940	10.6560	37.8257
O-DP-33907	ET1391-2	<i>L. abyssinica</i> LGS	Ethiopia	Mt Choke	3920	10.6575	37.8220
O-DP-34660	ET1526-1	<i>L. abyssinica</i> SGS	Ethiopia	Gara Muleta	3140	9.2195	41.7867
O-DP-34661	ET1526-2	<i>L. abyssinica</i> SGS	Ethiopia	Gara Muleta	3140	9.2195	41.7867
O-DP-34662	ET1526-3	<i>L. abyssinica</i> SGS	Ethiopia	Gara Muleta	3140	9.2195	41.7867

	AFR582-1	<i>L. abyssinica</i> SGS	Cameroon	Bamenda High lands	N/A	N/A	N/A	
	AFR582-2	<i>L. abyssinica</i> SGS	Cameroon	Bamenda High lands	N/A	N/A	N/A	
	AFR611-1	<i>L. abyssinica</i> SGS	Cameroon	Bamenda High lands	N/A	N/A	N/A	
	AFR611-2	<i>L. abyssinica</i> SGS	Cameroon	Bamenda High lands	N/A	N/A	N/A	
O-DP-27285	KN0443-2	<i>L. crassifolia</i>	Kenya	Cherangani Hills: Tululuwa		3180	1.1770	35.5184
O-DP-27499	KN0493-2	<i>L. crassifolia</i>	Kenya	Aberdare Mts: Mt Kinangop area		3090	-0.5425	36.7175
O-DP-27812	KN0563-2	<i>L. crassifolia</i>	Kenya	Aberdare Mts: Mutumbio Gate		3090	-0.5425	36.7175
	180610-1	<i>L. flos-cuculi</i>	Sweden	Roadside, Håby, Munkedal, Västra Götaland	N/A		58.4890	11.5890
O-DP-41977	UG2599-2	<i>L. kigesiensis</i> subsp. <i>kigesiensis</i>	Uganda	Echuya Forest: Kanaba Swamp		2300	-1.2553	29.8093
O-DP-41982	UG2600-2	<i>L. kigesiensis</i> subsp. <i>kigesiensis</i>	Uganda	Echuya Forest: Kanaba Swamp		2300	-1.2553	29.8093
O-DP-13628	AFR542-4 [‡]	<i>L. kigesiensis</i> subsp. <i>ragazziana</i>	Ethiopia	Bale Mts: Betw. Park Head Quarter and Goba, 2 km from Head Quarter		3100	7.1055	39.8015
O-DP-13629	AFR546-1 [‡]	<i>L. kigesiensis</i> subsp. <i>ragazziana</i>	Ethiopia	Shoa, Entoto		3000	9.0950	38.7662
O-DP-13637	AFR534-3 [‡]	<i>L. kiwuensis</i> subsp. <i>erlangeriana</i>	Ethiopia	Arsi: Betw. Kofele and Asassa, 15 km from Kofele		2700	7.1735	38.8746
O-DP-13648	AFR550-4 [‡]	<i>L. kiwuensis</i> subsp. <i>erlangeriana</i>	Ethiopia	Arsi: Betw. Shashemene and Goba, 26 km from Shashemene		2600	7.0985	38.7579
O-DP-13654	AFR555-1 [‡]	<i>L. kiwuensis</i> subsp. <i>erlangeriana</i>	Ethiopia	Betw. Agereslam and Aleta Wondo, 17 km from Agereslam, Sidamo		2500	6.5749	38.4891
O-DP-13657	AFR527-4 [‡]	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Ethiopia	Arsi: Mt Chilalo		3100	7.9206	39.1840
O-DP-13662	AFR538-4 [‡]	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Ethiopia	Bale Mts: Rira Village		2800	6.7531	39.7204
O-DP-13664	AFR551-3 [‡]	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Ethiopia	Gamu-Gofa, Chench		2600	6.2296	37.5678
O-DP-13671	AFR561-2 [‡]	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Ethiopia	Between Teppi and Gore,		1800	7.3153	35.3692

				Illubabor			
O-DP-13674	AFR566-1 [‡]	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Ethiopia	Keffa, Masha	2200	7.7633	35.4745
O-DP-39472	UG2001-2	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Uganda	Virunga Mts: Mt Mgahinga	2690	-1.3600	29.6348
O-DP-39477	UG2002-2	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Uganda	Virunga Mts: Mt Mgahinga	2350	-1.3528	29.6201
O-DP-40181	UG2167-2	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Uganda	Virunga Mts: Mt Muhavura, Kabaragnuma Swamp	3060	-1.3675	29.6713
O-DP-40186	UG2168-2	<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	Uganda	Virunga Mts: Mt Muhavura, Kabaragnuma Swamp	3060	-1.3675	29.6713
	UP16925*	<i>L. lagrangei</i>	Morocco	Friedhof in Oulad-el-Arbi, südlich Asilah, auf Küstenland	N/A	35.4626	-6.0317
	UP16926*	<i>L. lagrangei</i>	Morocco	Habib, near the road from Arba-Ayacha (Soko Arbaa) to Dar Chaoui	N/A	35.4700	-5.8000
O-DP-27519	KN0497-2	<i>L. rotundifolia</i>	Kenya	Aberdare Mts: Mt Satima area	3690	-0.3218	36.6407
O-DP-36976	TZ0016-2	<i>L. rotundifolia</i>	Tanzania	Mt Kilimanjaro: Shira Plateau near Mt Simba	3640	-3.0343	37.2430
O-DP-37105	TZ0043-2	<i>L. rotundifolia</i>	Tanzania	Mt Kilimanjaro: Shira Plateau	3540	-3.0056	37.2416
O-DP-13679	AFR554-4 [‡]	<i>L. scottii</i>	Ethiopia	Gamu-Gofa, Mt Guge	3000	6.1992	37.3333
O-DP-13680	AFR554-5 [‡]	<i>L. scottii</i>	Ethiopia	Gamu-Gofa, Mt Guge	3000	6.1992	37.3333
O-DP-13682	AFR554-1 [‡]	<i>L. scottii</i>	Ethiopia	Gamu-Gofa, Mt Guge	3000	6.1992	37.3333
	UP16917*	<i>L. subintegra</i>	Greece	Ioanninon, above Metsovo, N of the road to the Katara pass	N/A	39.7760	21.1750

618 Leaf material for *Lychnis* was collected in the field and dried in silica gel for most accessions; herbarium material was used in a few cases (indicated with *).

619 The assignment of two genome size variants (LGS - Large genome size, SGS - small genome size) to *L. abyssinica* samples were determined based on flow

620 cytometry analyses (for samples from Popp *et al.* (2008) study marked with [‡]) and based on their phylogenetic position (for samples collected and analysed in

621 this study). N/A indicates lack of detailed geographical information.

622 Table 2 DNA sequence information of the study material

Taxon	Population number	GenBank accession number				
		<i>matK</i>	<i>rps16</i>	<i>psbE-petL</i>	ITS	<i>RPB2</i>
<i>L. abyssinica</i>	21568		EF602350†	EF602323†	EF602379†	EF602406†
	21571		EF602351†	EF602324†	EF602380†	EF602407†
	21591		EF602352†	EF602325†	EF602381†	EF602408†
	AFR522-4		EF602353†	EF602326†	-	EF602409†
	AFR528-5		EF602354†	EF602327†	-	EF602410†
	AFR533-1		EF602355†	EF602328†	EF602382†	EF602411†
	AFR536-4		EF602356†	EF602329†	EF602383†	EF602412†
	AFR540-1		EF602357†	EF602330†	EF602384†	EF602413†
	AFR545-1		EF602358†	EF602331†	EF602385†	EF602414†
	AFR548-4			-	EF602386†	EF602415†
	AFR560-2	KT581604‡	EF602360†	EF602333†	EF602387†	EF602416†
	AFR570-1		EF602361†	EF602334†	EF602388†	EF602417†
	ET0098-2	KT581605‡				
	<i>L. chalcedonica</i>			-	X86894	AJ634068
<i>L. coronaria</i>			EF674193	FJ376841	X86891	AJ634069
			-	-	AY857966	-
			-	-	SCU30953	-
			-	-	SCU30979	-
<i>L. crassifolia</i>	21567		EF602362†	EF602335†	EF602389	EF602418†
	KN0493-2	KT581609‡				
<i>L. flos-cuculi</i>			Z83163	EF602320†	X86893	AJ634070
			-	-		AJ634071
			-	-	-	FJ376910
			-	-	SFU30957	-

	180610-1	KT581613 [‡]				
<i>L. flos-jovis</i>			Z83166	EF602321 [†]	X86892	-
			-	-	AY936261	-
			-	-	EF407940	-
<i>L. kigesiensis</i> subsp. <i>kigesiensis</i>	21595		EF602366 [†]	EF602339 [†]	EF602393 [†]	EF602422 [†]
	UG2599-2	KT581606 [‡]				
<i>L. kigesiensis</i> subsp. <i>ragazziana</i>	AFR542-4	KT581607 [‡]	EF602367 [†]	EF602340 [†]	EF602394 [†]	EF602423 [†]
	AFR546-1		EF602368 [†]	EF602341 [†]	EF602395 [†]	EF602424 [†]
<i>L. kiwuensis</i> subsp. <i>erlangeriana</i>	AFR534-3		EF602363 [†]	EF602336 [†]	EF602390 [†]	EF602419 [†]
	AFR550-4	KT581611 [‡]	EF602364 [†]	EF602337 [†]	EF602391 [†]	EF602420 [†]
	AFR555-1		EF602365 [†]	EF602338 [†]	EF602392 [†]	EF602421 [†]
<i>L. kiwuensis</i> subsp. <i>kiwuensis</i>	21598		-	-	EF602396 [†]	-
	AFR527-4		EF602370 [†]	EF602342 [†]	EF602397 [†]	EF602425 [†]
	AFR538-4		EF602371 [†]	EF602343 [†]	EF602398 [†]	-
	AFR551-3		EF602372 [†]	EF602344 [†]	EF602399 [†]	EF602426 [†]
	AFR561-2		EF602373 [†]	EF602345 [†]	EF602400 [†]	EF602427 [†]
	AFR566-1		EF602374 [†]	EF602346 [†]	EF602401 [†]	EF602428 [†]
	UG2168-2	KT581612 [‡]				
<i>L. lagrangei</i>	LAG01		-	-	EF602377 [†]	EF602404 [†]
	LAG02		EF602349 [†]	EF602322 [†]	EF602378 [†]	EF602405 [†]
	UP16925	KT581615 [‡]				
<i>L. rotundifolia</i>	21573		EF602375 [†]	EF602347 [†]	EF602402 [†]	EF602429 [†]
	TZ0043-2	KT581610 [‡]				
<i>L. scottii</i>	AFR554-1		EF602376 [†]	EF602348 [†]	EF602403 [†]	EF602430 [†]
	AFR554-4	KT581608 [‡]				
<i>L. subintegra</i>	UP16917	KT581614 [‡]				
<i>L. coronaria</i>		FJ589507	-	-	-	-

<i>L. flos-jovis</i>	AY936313	-	-	-	-
<i>Polycarpon tetraphyllum</i>	AY936287	-	-	-	-
<i>Scleranthus perennis</i>	AY514847	-	-	-	-
<i>Arenaria serpylloides</i>	FJ404826	-	-	-	-
<i>Moehringia macrophylla</i>	FJ404852	-	-	-	-
<i>Stellaria media</i>	FJ404877	-	-	-	-
<i>Lepyrodiclis holosteoides</i>	FJ404840	-	-	-	-
<i>Minuartia nuttallii</i>	FJ404847	-	-	-	-
<i>Bufonia paniculata</i>	FJ404827	-	-	-	-
<i>Schiedea globosa</i>	DQ907818	-	-	-	-
<i>Geocarpon minimum</i>	FJ404836	-	-	-	-
<i>Gypsophila paniculata</i>	FJ404838	-	-	-	-
<i>Petrorhagia saxifraga</i>	FJ404857	-	-	-	-
<i>Dianthus armeria</i>	FJ404832	-	-	-	-
<i>Arenaria bryophylla</i>	FJ404815	-	-	-	-
<i>Arenaria gypsophiloides</i>	FJ404818	-	-	-	-
<i>Agrostemma githago</i>	FJ589503	-	-	-	-
<i>Petrocoptis pyrenaica</i>	FJ589508	-	-	-	-
<i>Silene hawaiiensis</i>	SHB0001*	-	-	-	-
<i>Silene uniflora</i>	FJ589565	-	-	-	-
<i>Silene douglasii</i>	EF547238	-	-	-	-
<i>Silene noctiflora</i>	EF547240	-	-	-	-
<i>Silene repens</i>	FJ589552	-	-	-	-
<i>Silene schafta</i>	FJ404873	-	-	-	-
<i>Silene ciliata</i>	FJ589519	-	-	-	-
<i>Silene muscipula</i>	FJ589543	-	-	-	-
<i>Silene antirrhina</i>	FJ589512	-	-	-	-
<i>Silene armena</i>	FJ589514	-	-	-	-
<i>Silene acaulis</i>	EF547235	-	-	-	-

<i>Silene campanula</i>	AY936311	-	-	-	-
<i>Silene yemensis</i>	FJ589567	-	-	-	-
<i>Silene sordida</i>	FJ589559	-	-	-	-
<i>Silene odontopetala</i>	FJ589546	-	-	-	-

623 DNA sequences retrieved from GenBank mainly from Popp *et al.* (2008) study ([†]), and sequences generated in the present study (GenBank accession
624 number[‡])

625
626 Table 3 Specific information of the five DNA regions (total number of characters) used in the present study

	<i>matK</i> (1566bp)			<i>rps16</i> (666bp)			<i>psbE-petL</i> (1253bp)		
	Total dataset	<i>Lychnis</i>	Afro-alpine <i>Lychnis</i>	Total dataset	<i>Lychnis</i>	Afro-alpine <i>Lychnis</i>	Total dataset	<i>Lychnis</i>	Afro-alpine <i>Lychnis</i>
Number of terminals	46	14	9	57	57	52	57	57	52
Conserved sites	778	1211	1254	616	616	632	1160	1160	1189
Variable sites	741	85	41	41	41	25	90	90	56
Parsimony informative sites	429	44	26	28	28	22	69	69	53

627

	ITS (602bp)			<i>RPB2</i> (1842bp)		
	Total dataset	<i>Lychnis</i>	Afro-alpine <i>Lychnis</i>	Total dataset	<i>Lychnis</i>	Afro- alpine <i>Lychnis</i>
Number of terminals	35	35	26	29	29	24
Conserved sites	500	500	539	1567	1567	1686
Variable sites	100	100	58	231	231	117
Parsimony informative sites	63	63	37	134	134	83

628

629

630 Table 4 Mean, median, 95% highest posterior density (HPD) interval in million years, and effective sample sizes (ESS) for nodes A-G in the species tree (Fig.
631 3)

Node	Mean	Median	95% HPD	ESS
A	5.13	5.07	3.05 – 7.41	3019
B	4.09	4.03	2.30 – 5.92	2848
C	2.54	2.51	1.46 – 3.74	2914
D	1.37	1.33	0.60 – 2.18	5884
E	0.51	0.49	0.17 – 0.89	5322
F	1.83	1.79	0.92 – 2.80	3497
G	4.46	4.41	2.51 – 6.60	4036

632

633 Table 5 Mean, median, and 95% highest posterior density (HPD) interval in million years for the tMRCA of each species inferred from ITS, *RPB2*, *matK*, and
634 the combined *psbE-petL/rps16* co-estimated in the multilocus species tree.

Species	ITS			<i>RPB2</i>			<i>matK</i>			<i>psbE-petL/rps16</i>		
	Mean	Median	95% HPD	Mean	Median	95% HPD	Mean	Median	95% HPD	Mean	Median	95% HPD
<i>L. abyssinica</i> SGS	0.28	0.25	0.07 – 0.54	0.43	0.40	0.13 – 0.80	NA	NA	NA	0.13	0.11	0.02 – 0.30
<i>L. kigesiensis</i>	0.24	0.21	0.01 – 0.53	0.15	0.13	0.01 – 0.37	0.09	0.07	0.00 – 0.23	0.08	0.06	0.00 – 0.19
<i>L. scottii</i>	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.28	0.24	0.03 – 0.64
<i>L. abyssinica</i> LGS	NA	NA	NA	NA	NA	NA	0.10	0.06	0.00 – 0.29	0.35	0.32	0.10 – 0.65
<i>L. rotundifolia</i>	NA	NA	NA	NA	NA	NA	0.08	0.05	0.00 – 0.26	0.16	0.13	0.01 – 0.39
<i>L. kiwuensis</i>	0.84	0.79	0.30 – 1.44	0.73	0.68	0.23 – 1.33	0.33	0.29	0.03 – 0.71	0.24	0.21	0.05 – 0.48
<i>L. crassifolia</i>	NA	NA	NA	NA	NA	NA	0.09	0.06	0.00 – 0.30	0.21	0.18	0.03 – 0.46

635 “NA” indicates that a species was represented by less than two sequences for a specific DNA region and no tMRCA was inferred. The inferred gene trees are
636 presented in Supplementary Information (Fig. 1a-d Suppl.). *Lychnis abyssinica* LGS and *L. abyssinica* SGS denote the large-genome-size and small-genome-
637 size variants of *L. abyssinica*, respectively.

638 **Figure Legends**

639

640 Fig. 1 Geographical distribution and sampling localities for the afro-alpine representatives of
641 *Lychnis*. a) Sampling areas in Eastern Africa, b) Total range of each species and subspecies
642 (solid or dashed lines) and sampling localities (symbols), modified from Popp *et al.* (2008)
643 and Ousted (1985).

644

645 Fig. 2 Maximum clade credibility tree and 95% HPD age interval estimates of nodes inferred
646 from 45 *matK* sequences representing the Caryophyllaceae subfamilies Alsinoideae and
647 Caryophylloideae, using *Polycarpon tetraphyllum* from subfamily Paronychioideae as an
648 outgroup. Internal node bars represent the 95% HPD age interval estimates for one fossil-
649 calibrated node (Node A) and the tMRCA for the genus *Lychnis* (Node B). Numbers
650 associated with nodes are posterior probabilities. Scale in million years.

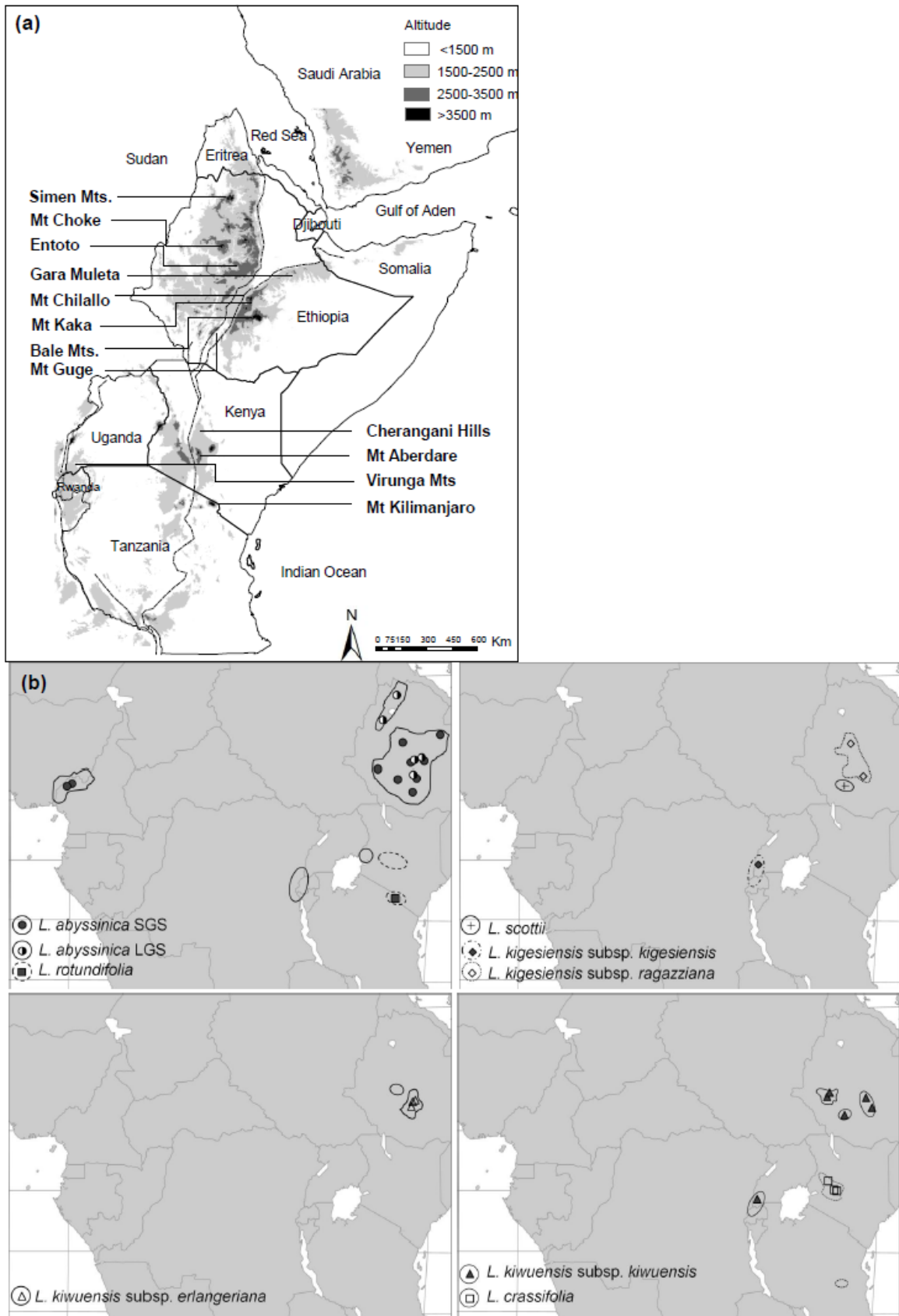
651

652 Fig. 3 Multilocus species tree for the genus *Lychnis* inferred from five DNA regions (*matK*,
653 *rps16*, *psbE-petL*, ITS and *RPB2*). Grey internal node bars represent the 95% HPD age
654 intervals, co-estimated with the multilocus species tree. Nodes A-G and their estimated ages
655 (see Table 4 for details) are discussed in the text. Numbers associated with nodes are posterior
656 probabilities. Scale in million years. White bars on terminal branches for the afro-montane
657 species represent the 95% HPD age interval ranges (boxes) and the species mean age co-
658 estimated for each DNA region (vertical bars in the boxes) in the multilocus species tree (see
659 Table 5 for details). *Lychnis abyssinica* LGS and *L. abyssinica* SGS denote the large-genome-
660 size and small-genome-size variants of *L. abyssinica*, respectively.

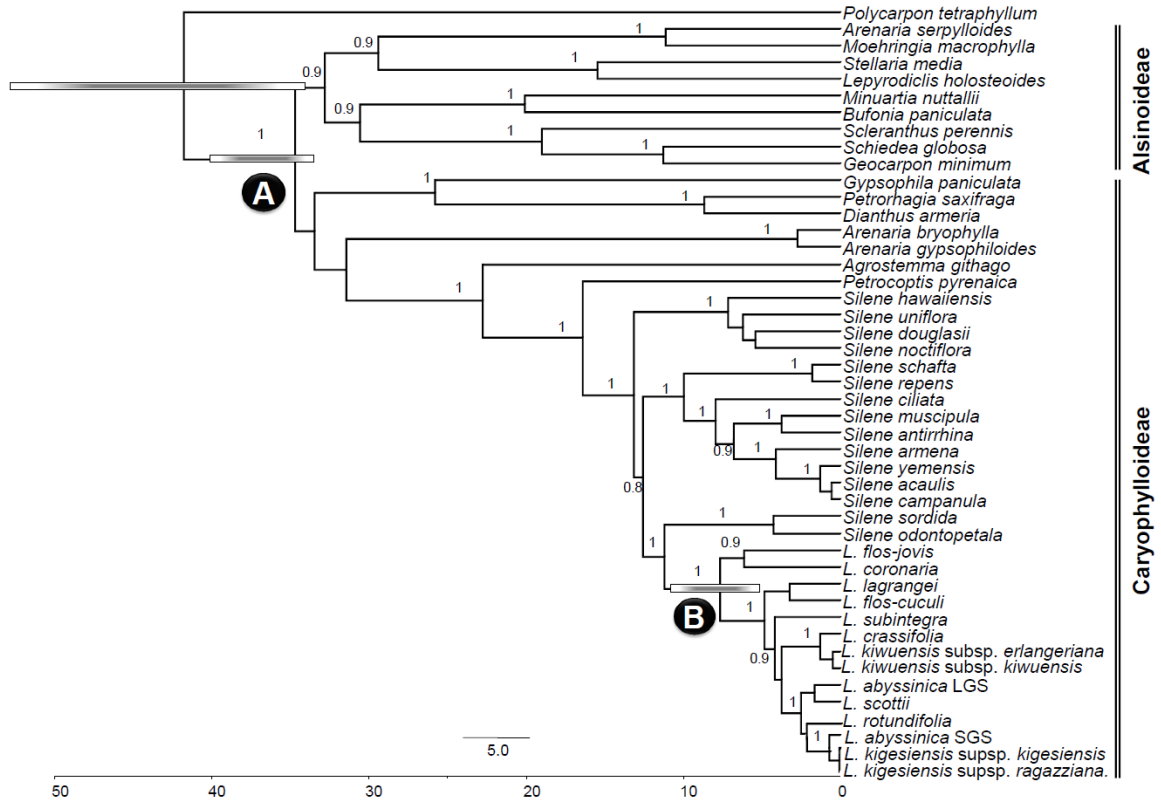
661

662 Supplementary Fig. 1S Single-locus trees for the genus *Lychnis*, with 95% HPD age interval
663 estimates for nodes inferred from sequences of *rps16/psbE-petL* (a), *matK* (b), ITS (c), and
664 *RPB2* (d). Node bars represent 95% HPD age intervals. Nodes A-G and their estimated ages
665 (see Table 5 for details) are discussed in the text. Numbers associated with nodes are posterior
666 probabilities. Scale bar indicates million years. *Lychnis abyssinica* LGS and *L. abyssinica*
667 SGS denote the large-genome-size and small-genome-size variants of *L. abyssinica*,
668 respectively. Sample of *L. abyssinica* LGS without accession number indicates missing data.

669



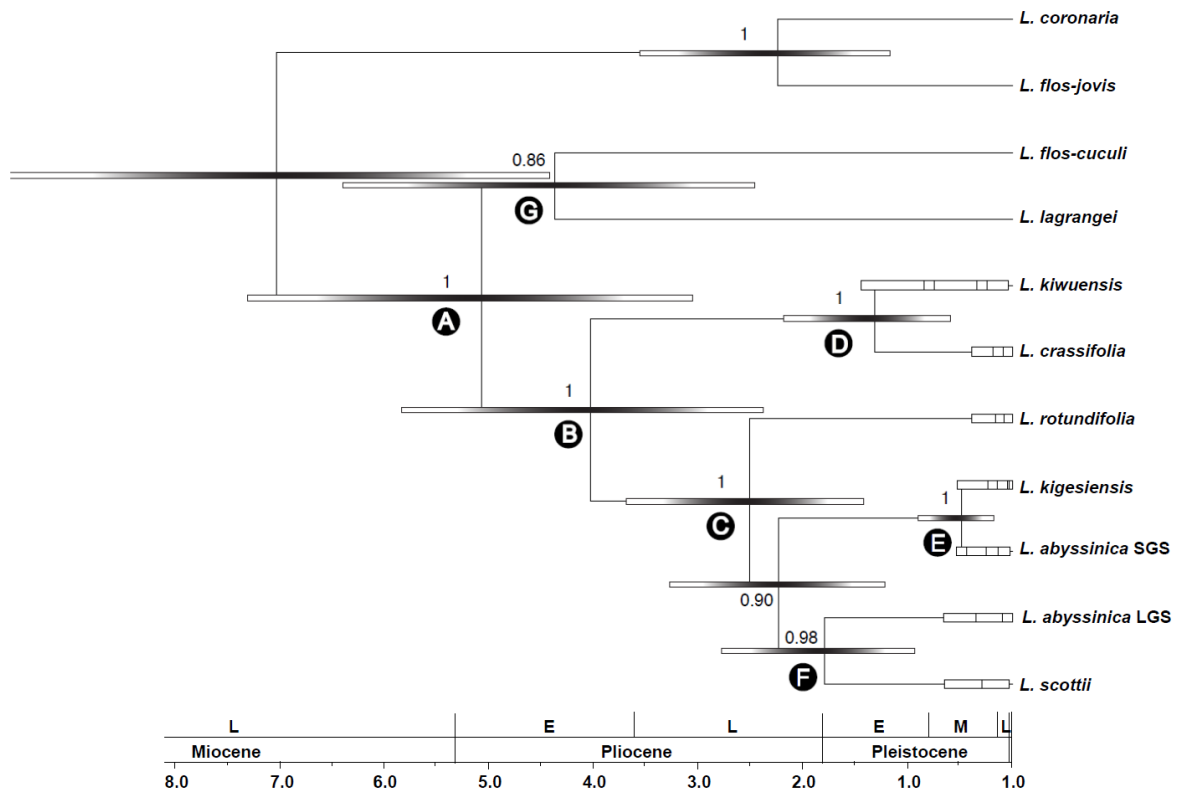
673 **Fig 2**



674

675

676 **Fig 3**



677

678