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1 **New highlights on Old World giant *Phragmites* (Poaceae) using leaf and floral bract**
2 **microscopic characters**

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12
13

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15

16 **1. Introduction**

17 The genus *Phragmites* Adans. (Poaceae) has a worldwide distribution from the tropics to the
18 cold-temperate regions. Its positive attributes as an ecosystem engineer in wetlands provide
19 essential ecological and economic services, such as wildlife habitat, erosion control, and
20 biomass production (Saltonstall and Meyerson, 2016). All species form dense stands, playing a
21 crucial role as carbon sinks over a long-time scale (Brix et al., 2001), and they are also known
22 for their phytoremediation abilities. Like other tall grasses, *Phragmites* spp. have followed
23 humans since the Paleolithic period due to their countless uses, *e.g.* energy source, construction,
24 fodder, weapons, music, fiber and medicine (Täckholm et al., 1973; Oyen, 2011). Due to their
25 usefulness, they were introduced into many adventive ranges, like the reed genus *Arundo* L.
26 (Hardion et al., 2014). The cosmopolitan *P. australis* (Cav.) Trin. ex Steud. has become a
27 "model organism for plant invasions" driven by its competitive abilities, particularly in North
28 America (Pyšek et al., 2019).

29 Although *Phragmites* are the subject of an extensive number of articles, their taxonomy
30 remains unclear. Thereby, the species number is widely debated, from only one species *P.*
31 *australis* (Täckholm et al., 1973, under the name *P. communis* Trin.) to seven only for Corsica
32 Island (Mabille, 1869) and for Russia (Tsvelev and Probatova, 2019), and up to 17 in the world
33 (Steudel, 1854). In recent studies (Clevering and Lissner, 1999; Scholz and Böhring, 2000;

34 Lambertini et al., 2006), the most cited taxa are *P. australis* (cosmopolitan), *P. karka* (Retz.)
35 Trin. ex Steud. (pantropical, except the American continent), *P. mauritanus* Kunth (tropical
36 Africa, Madagascar, Mascarene Islands), *P. japonicus* Steudel (N-Japan, E-Russia, NE-China),
37 and *P. frutescens* Scholz (Crete, Greece, SW-Turkey).

38 The poor taxonomic resolution of *Phragmites* results from many overlaps of quantitative
39 macroscopic traits, and the ambiguity of descriptive terms. For example according to Conert
40 (1961) or Clayton (1970), the upper glume is either lanceolate or acute in *P. karka*, and either
41 ovoid or acuminate in *P. mauritanus*. Long considered as distinct species, Clayton (1970)
42 suggests a subspecific status for *P. mauritanus* within *P. karka*, while some floras simply
43 include *P. mauritanus* within *P. karka*, without any infraspecific rank (e.g. Phillips, 1995).
44 Moreover for Veldkamp (1992), *P. karka* should be named *P. vallatoria* (Plunk. ex L.) Veldk.,
45 a name now considered as invalid (Kirkbride and Wiersema, 2007).

46 Surprisingly, despite its morphological differentiation, online taxonomic databases (e.g. Kew,
47 Paris) place *P. frutescens* in synonymy within *P. australis*. Described in 1996, *P. frutescens* is
48 a rare species with infrequent flowering, but morphologically more related to *P. mauritanus*
49 than to *P. australis* (Scholz and Böhling, 2000), as confirmed by AFLP analyses (Lambertini
50 et al., 2006: Fig. A1). In fact, the three taxa, *P. karka*, *P. mauritanus*, *P. frutescens*, form a
51 distinct genetic unit, with two unique cpDNA haplotypes (U, I:Table 1).

52 In his monograph, Conert (1961) lists 11 varieties within *P. australis*, while Clayton (1970)
53 recognizes only two taxa: the temperate subsp. *australis* (< 3 m) and the Mediterranean subsp.
54 *altissimus* (Benth.) Clayton (height and panicles taller). These two subspecies are adopted by
55 Feinbrun-Dothan (1986), but many floras include subsp. *altissimus* within *P. australis* (e.g.
56 Tutin, 1980; Fish et al., 2015), or place it under various names and ranks, such as var. *isiaca*
57 (Del.) Coss. (Täckholm et al., 1973; Hultén and Fries, 1986), but rarely as a species [Coste,
58 1937, as *Arundo maxima* Forsk.; Tsvelev and Probatova, 2019, as *P. altissimus* (Benth.) Mab.].
59 All genetic studies separate this taxon from the subsp. *australis*, but the individual groupings
60 differ according to methods and sampling, (AFLPs, haplotypes, SSRs: Lambertini et al., 2006,
61 2012; RAD-sequencing: Wang et al., 2021).

62 Finally, according to genetic studies, *P. japonicus* is separate from the *P. australis* subsp.
63 *australis* (hereafter *P. australis s.str.*), which includes two distinct phylogeographic groups: the
64 Afro-European unit (AE) and the Australian-E. Asian unit (AA) (Lambertini et al., 2006, 2012,
65 2020). In America, there are three native genetic units that highly differ from all Old World
66 taxa, based on AFLPs, haplotypes, microsatellites (Lambertini et al., 2006: Fig. A1; Saltonstall
67 and Meyerson, 2016) and RAD-sequencing (Wang et al., 2021).

68 In the absence of reliable macroscopic characters in *Phragmites*, we investigated the
69 microscopic traits of leaves and floral bracts. The high phylogenetic value of leaf epidermis and
70 anatomy traits is known since the founding work of Prat (1932) and has proven to be valuable
71 for the systematics of Poaceae (Watson et al., 1992; Kellogg, 2015), and for the identification
72 of South African *Phragmites* (Gordon-Gray and Ward, 1971). Such traits can be useful when
73 phylogenetic markers fail to resolve complex grass groups. The traits of floral bracts were also
74 found to have high discriminant value, phylogenetic significance (Liu et al., 2010; Hardion et
75 al., 2021), and are comparatively easy to use. Based on extensive sampling, including plants
76 previously analyzed in molecular studies, our aim here was to test the value of micro-
77 morphologic characters to distinguish Old World *Phragmites*, especially the problematic giant
78 reeds.

79

80 **2. Materials and Methods**

81 For a representative cover of the global distribution of taxa in the Old World, specimens from
82 several international herbaria, Aix-Marseille University collections, and twenty samples
83 previously studied in molecular analyses were investigated. The study was performed on 160
84 specimens of *Phragmites* [35 *P. karka*, 30 *P. mauritanus*, 11 *P. frutescens*, 44 *P. australis*
85 subsp. *altissimus*, 31 Afro-European (AE) *P. australis* subsp. *australis*, 5 Australian-E. Asian
86 (AA) *P. australis*, 4 *P. japonicus*], and one *Molinia caerulea* (L.) Moench., the sister group of
87 *Phragmites* (Hardion et al., 2021). All information on specimens are reported in the table A1.
88 Prior identifications were based on macroscopic characters observed on herbarium specimens,
89 except for the genetic unit AA *P. australis* for which no distinctive macro-morphological
90 character is yet known. These traits were chosen from original diagnosis, from some floras
91 (Coste, 1937; Clayton, 1970; Feinbrun-Dothan, 1986), and from the synthesis of Scholz and
92 Böhling (2000).

93 Courtesy of herbarium curators, we collected four mature spikelets on each sample, and a
94 small piece from the middle part of the two terminal leaves of the primary axis, because they
95 possess the most differentiated epidermis (Prat, 1932). To avoid damages on type specimens,
96 two spikelets (taken in the bag of fragments) and an imprint of leaf epidermis with clear nail
97 polish were taken. The material was re-hydrated in distilled water for ca. one week. For each
98 spikelet, glumes, lemmas and paleas of the two first basal flowers were separated by cutting at
99 their base. For each leaf, the adaxial epidermis was prepared by scraping away the abaxial face
100 and mesophyll cells with a razor blade. Floral bracts, leaf margin and epidermis were stained

101 with iodine green solution for 3 min and mounted in glycerol. For anatomy, about 10 transversal
102 sections per leaf were performed in lateral part (out of the central rib), cleared with sodium
103 hypochlorite, washed in distilled water, stained with carmine green and mounted in glycerol.

104 In the spikelet, only lemmas and paleas, which are more differentiated than glumes in reeds,
105 were studied in detail. Classical discriminant traits were studied, as well as several new
106 characters, such as marginal teeth of leaves and paleas, and floral bract coloring. A total of 56
107 characters were retained: 23, 12 and 21 for the leaf epidermis, anatomy, and spikelet,
108 respectively (Table A2). The micromorphological terminology follows Prat (1932) and its
109 revisions (Metcalf, 1960; Watson et al., 1992; ICPT, 2019). All specimens were studied using
110 a light microscope (LM; ca.10 observations for each trait), and some samples were observed
111 under scanning electron microscope (SEM). All traits were coded as discrete states (Table A2a-
112 c). Before each analysis, datasets were converted into binary variables, with one new variable
113 for each state of initial variables, using the *dummies* R-package in the R environment (R Core
114 Team, 2022). Data were then analyzed in correspondence and hierarchical clustering analyses
115 with Jaccard binary dissimilarities using the *ade4* R-package (Thioulouse et al., 2018).

116

117 **3. Results**

118 Based on 21 epidermal characters of lemmas and paleas, the hierarchical clustering analysis
119 (HCA) highlights two main clusters (Fig. 1a). The clade A strictly includes the African and
120 Mediterranean *P. mauritanus*, *P. frutescens* and *P. australis* subsp. *altissimus*, while the clade
121 B includes the remaining taxa: *P. karka*, *P. japonicus* and *P. australis* s.str. The clade A mainly
122 differs from clade B by glumes and lemmas being green and spread out, vs brownish and rolled
123 up (Fig. 2a vs 2b), and lemma hooks larger and pointed to all directions, vs small (<30 µm) and
124 pointed to the top (Fig. 3c-d vs 3a-b). In addition, within each clade, palea characters distinguish
125 the different taxa (Fig. 3g-h and e-f). The correspondence analysis (CA) clearly confirms these
126 two clades, with the first three axes representing 59.8% of the total variance (26.4%, 12.5% and
127 10.9%, respectively; Fig. A2a). The main distinctive traits of the two clades A and B, and taxa
128 are summarized in a key (Box 1).

129 The HCA of the 23 leaf epidermal characters also highlights two main clusters that mainly
130 match with previous ones, excepted for the inverted position of *P. australis* subsp. *altissimus*
131 and *P. karka* between the two clades (Fig. 1b). The clade I includes the three highest (up to 8
132 m) and thermophilic species, *P. karka*, *P. mauritanus*, and *P. frutescens*, while the clade II
133 includes the more temperate and smaller taxa, *P. australis* subsp. *altissimus*, subsp. *australis*,

134 and *P. japonica*. The clade I differs from clade II in its simpler epidermal structure: ribs with
135 large base and long-pointed prickles (vs small in clade II), intercostal zones with bicellular
136 microhairs (vs absent), many cork-cells (vs rare), rare hooks (vs frequent) (Fig. 3i-j vs 3k-m),
137 and weakly ornate leaf margin (vs more ornate) (Fig. 2c-e vs 2f-j). In our preparations, the
138 iodine green staining of floral bracts lasts several years, but that of leaf teeth fades after a few
139 weeks. The CA confirms the two clades I and II, with the three first axes representing 78% of
140 the total variance (54.1%, 14.9% and 9%, respectively; Fig. A2b). The main traits are reported
141 in the Box 2.

142 The coupled analysis of floral and leaf epidermis shows a better discrimination of taxa (Fig.
143 4), notably for *P. frutescens*, the Afro-European (AE) and Australian-E. Asian (AA) *P.*
144 *australis*. The three first axes of the CA represent 50.6% of the total variance (24.2%, 15.6%
145 and 10.8%, respectively). The two S-African hybrids genetically attested (Canavan et al., 2018),
146 KC2 (*P. mauritanus* x AE *P. australis*), and KC3 (*P. mauritanus* x *P. altissimus*), have a mix
147 of parental traits, and the CA placed them far from their parents, especially from *P. mauritanus*.

148 Leaf anatomy shows the main structural characteristics of *Phragmites*, especially the shape
149 and size of bulliform cells (BC), with the round bottom forming a gutter of regular adaxial
150 groups between the vascular bundles (Fig. 5). The CA separates the giant reed taxa (> 3m) from
151 the smaller ones along the axis 1, as well as the different taxa of each group (Fig. 6) and shows
152 the polymorphism of AA *P. australis*. The three first axes represent 66% of the total variance
153 (32%, 22% and 12%, respectively). The anatomical key based on the HCA distinguishes all the
154 Old World taxa (Box 3).

155 **4. Discussion**

156 *4.1. Taxonomy*

157 The objective of this study was to delimit well supported Old World *Phragmites* taxa. As
158 expected, microscopic characters have more discriminant resolution than macroscopic ones,
159 and shed a new light on this problematic genus. Both leaf and spikelet traits show a strong
160 individualization of four giant taxa: *P. mauritanus*, *P. karka*, and more surprisingly *P.*
161 *frutescens* and *P. australis* subsp. *altissimus*. Given the stability and the high taxonomic value
162 of these micro-characters, we consider them as four distinct species. For the subsp. *altissimus*,
163 which possesses highly specific traits, the most appropriate name should be *P. altissimus*
164 (Benth.) Mabilie (1869).

165 The floras of S-Africa only report *P. australis* and ignore *P. altissimus* (Fish et al., 2015),
166 while both taxa co-exist. Our results show that the S-African “*P. australis*” illustrated by
167 Gordon-Gray and Ward (1971), and genetically studied (Canavan et al. 2018) is in fact *P.*
168 *altissimus*. Our sampling does not show obvious micro-characters differences between S-
169 African and Mediterranean *P. altissimus*, in agreement with DNA microsatellites analyses
170 (Lambertini et al., 2012). Within *P. australis s.l.* of the Danube Delta, Hanganu et al. (1999)
171 found two distinct taxa called “giant reeds” ($2n = 96$), and “fine reeds” ($2n = 48$), which likely
172 correspond to *P. altissimus* and *P. australis s.str.*, respectively. As suggested by the map of
173 Hultén and Fries (1986) and the presence of the exclusive 200-bp waxy band (Lambertini et al.,
174 2012), the distribution of *P. altissimus* is larger than expected, including African,
175 Mediterranean, and Middle East regions up to Armenia and Kashmir. *Phragmites frutescens* is
176 sometimes sympatric with *P. altissimus* and morphologically close to *P. mauritanus*, but it is
177 restricted to the Mediterranean Basin, and it presents distinct specific traits, especially in leaf
178 anatomy.

179 Unlike the giant reeds, the taxonomy of Old World small reeds (< 3 m), *P. japonicus* and *P.*
180 *australis s.str.* (AE, AA), appears more challenging. Except leaf anatomy, the other characters
181 distinguish these three taxa less clearly than genetic studies (Lambertini et al., 2006, 2020; Liu
182 et al., 2020; Wang et al., 2021). Further investigations are needed, based on a broader sampling
183 in Australia and Asia, to clarify their morphological distinction and geographic distribution.

184 4.2. Anatomy

185 Leaf anatomy shows the most discriminant traits, particularly the bulliform cells (BC). The
186 huge flabellate reed BC are unique among Poaceae (Chen et al., 2020), and called “*Phragmites-*
187 *type*” as wetland markers in paleoecology. These are the only traits that easily separate *P.*
188 *frutescens* (BC protruding) and *P. japonicus* (BC wider than deep) from all others. Anatomical
189 analyses produce original groupings, and clearly distinguish three taxa within Old World *P.*
190 *australis s.str.* (Box 3). The two temperate to cold small reeds (AE *P. australis*, and *P.*
191 *japonicus*) possess thin leaves, and small fan-shaped BC, penetrating ca. 1/2 of the blade. In
192 contrast, thick leaves and deep BC, penetrating ca. 2/3 of the blade, separate the thermophilic
193 taxa (the giant reeds and AA *P. australis*) from the first two taxa. This group includes the
194 tropical pair *P. karka* - *P. mauritanus*, and the three other subtropical taxa: the Mediterranean
195 pair *P. altissimus* - *P. frutescens*, and the humid subtropical AA *P. australis*.

196 In Poaceae, Cyperaceae and Juncaceae, BC have a crucial role in leaf rolling, and water
197 storage, in response to drought and salt stress. All *Phragmites* live in wetlands, but AE *P.*
198 *australis* has a better salt tolerance than AA *P. australis* (Lambertini et al., 2020), and *P.*
199 *altissimus* (Hanganu et al., 1999). Therefore, the strong anatomical differentiation of *Phragmites*
200 taxa could be an adaptation to eco-climatic conditions.

201 4.3. Staining and waxy genes

202 In iodine green solution, floral bracts, and leaf margin teeth of *P. frutescens*, *P. mauritanus*
203 and *P. altissimus* turned green, while those of other taxa remain unstained. These results are
204 consistent with the banding pattern of the waxy genes: green colour corresponds to the presence
205 of 100 bp band in *P. mauritanus* and *P. frutescens*, 200 bp band in *P. altissimus*, and unstained
206 parts match to the lack of waxy bands in the other taxa (Lambertini et al., 2012; Canavan et al.,
207 2018).

208 Waxy plants, notably cereals, are of great interest for their countless industrial uses. Iodine
209 solutions are used to detect waxy genes, they colour the starch of pollen and endosperm
210 differently: waxy plants rich in amylopectin turn reddish, whereas non-waxy plants with only
211 amylose turn blue (Bates et al., 1943). If in herbarium specimens, these organs are rarely in
212 good condition, our results show that the spikelet and leaf staining can easily detect waxy plants.
213 Similar observations were cited for waxy rice leaves (Abé, 1985). This phenomenon might
214 result from the relationship between the presence of amylopectin and a better permeability of
215 cell walls (Michailova et al., 2001). The spreading out or rolling up of glumes and lemmas in
216 glycerol can also be explained by starch types: water soluble amylopectin, hydrophilic with
217 alcohol, would cause spreading out bracts, while water insoluble amylose, hydrophobic with
218 alcohol, would induce rolling up bracts. Waxy rice (glutinous) has been used for millennia in
219 Asia, due to its better digestibility, early ripening, and tolerance to temperature variation and
220 drought (Abé, 1985). Waxy corn also has a high resistance to pathogenic fungi (Walden, 1978),
221 which can explain our leaf observations: taxa without waxy genes are often infected with rusts,
222 while waxy taxa are almost always healthy. Biochemical differences could also explain the
223 distinct uses of species, e.g. in African traditional medicine (Oyen, 2011).

224 4.4. Ploidy levels and hybrids

225 In karyological studies of *Phragmites*, the authors considered $2n = 48$ as the tetraploid level,
226 with a basic chromosome number $n = 12$, even if $n = 6$ is more likely for the Arundinoideae,
227 particularly for *Molinia* spp. with $2n = 36$ (Conert, 1961; Hardion et al., 2011). According to

228 the main syntheses (Gorenflot, 1976; Connor et al., 1998; Clevering and Lissner, 1999; Ishii
229 and Kadono, 2001; Hansen et al., 2007), the *Phragmites* include: (1) four stable species with
230 $2n = 48$: *P. karka*, *P. mauritanus*, *P. frutescens* and *P. japonicus*; (2) the AE *P. australis* with
231 $2n = 48$ (rarely $2n = 96$); and (3) two greatly variable taxa: AA *P. australis* with $2n = 96$ (120,
232 264), and *P. altissimus* with $2n = 48$ (72) in Senegal and S. Europe, 96 (144) in S. Africa, S.
233 Mediterranean, Danube Delta, and Middle East.

234 Some herbarium samples were difficult to identify, as evidenced by the various names
235 attributed by different botanists (Table A1). Notably, two plants previously named “*P. karka*”
236 showed mixed characters: (i) the Cameroonian P2 with spikelet traits of *P. mauritanus* and,
237 leaf epidermis and anatomy of *P. karka*, and (ii) the Japanese P11 with spikelet traits and
238 anatomy of AA *P. australis* and leaf epidermis of *P. japonicus*. Both could be hybrids of these
239 close related taxa, or rather the expression of strong polymorphism, because the true S-African
240 hybrids KC2 (*P. mauritanus* x AE *P. australis*: 100 bp band), and KC3 (*P. mauritanus* x *P.*
241 *altissimus*: 100-200 bp bands), get away from their parent taxa in CA. In hybridizations between
242 waxy and non-waxy taxa (P2: *P. mauritanus* x *P. karka*, or KC2), floral bracts and leaf margins
243 always turned green. This could explain some hybrids with 200 bp waxy band (S-Africa,
244 Danube Delta), but related to AE *P. australis* in some genetic traits (Lambertini et al., 2012;
245 Canavan et al., 2018).

246 The proportion of natural hybrids is challenging, given the strong polymorphism of reeds due
247 to the high polyploidy resulting from a reticulated evolution (Lambertini et al., 2012; Liu et al.,
248 2020; Wang et al., 2021). Like most polyploids (Richard, 1997), *Phragmites* spp. have weak
249 genetic barriers that explain the occurrence of some hybrids. Based on a large *P. japonicus* ($2n$
250 $= 48$) and AA *P. australis* ($2n = 96, 120$) sampling, Ishii and Kadono (2001) argue that hybrids
251 are very rare due to: (i) mismatched flowering periods between species, (ii) temporal
252 reproductive isolation related to the short flowering period, (iii) short distance of anemophilous
253 pollination, and (iv) distinct ploidy levels without intermediate numbers. According to Scholz
254 and Böhling (2000) and many floras, clear asynchronous flowering of sympatric taxa applies to
255 all *Phragmites*, e.g. the early flowering of *P. mauritanus* vs *P. karka*, AE *P. australis* vs *P.*
256 *altissimus*, and AA *P. australis* vs *P. japonicus*. In addition, the pollen of Mediterranean “*P.*
257 *australis*” hybrids ($2n = 72$) is entirely sterile (Gorenflot et al., 1990). Lastly, in the wild, hybrid
258 formation is also strongly limited by the almost exclusive vegetative growth of dense long-lived
259 clones (Clevering and Lissner, 1999), often distinct geographic areas and eco-climatic
260 preferences (geo-climatic barriers; Lambertini et al., 2012).

261 4.5. Evolution and biogeography

262 Micromorphological studies are not only descriptive, but they can also be used in phylogeny
263 (Liu et al., 2010). Floral bract and leaf epidermis characters define different clades, implying
264 that each category of traits has a distinct evolutionary significance. The main leaf traits of the
265 clade I (*P. karka*, *P. mauritanus*, *P. frutescens*) are (i) ribs with huge prickles and oblong silica-
266 bodies, and (ii) intercostal zone without hooks, but with straight wall long cells, many bicellular
267 microhairs and cork-cells. Comparisons of descriptions and illustrations (Metcalf, 1960;
268 Watson et al., 1992) show that these traits are also present in the earliest groups of the BEP
269 clade in the phylogenetic classification of Poaceae (Soreng et al., 2017), *i.e.* the first three basal
270 tribes of the Oryzoideae. The *Phragmites* share other characters with ancestral states, since this
271 genus is the only one of the PACMAD clade having arm cells in the mesophyll, like the basal
272 Oryzoideae and Bambusoideae (Kellogg, 2015).

273 The species of the clade I (*P. karka*, *P. mauritanus*, *P. frutescens*) form a morphological unit
274 characterized by their height (8-10 m), large panicles, deciduous leaf sheaths, and leaf blades
275 that are rough to the touch. Epidermis comparison between *Phragmites* and its sister group
276 *Molinia* (Hardion et al., 2021) shows that *M. caerulea* shares a maximal similarity with *P.*
277 *karka*: 85 and 78% for flower and leaf traits, respectively. Thus, *P. karka* retains the most
278 number of ancestral traits across *Phragmites* spp., in accordance with its basal position in the
279 AFLP tree (Lambertini et al., 2006: Fig. A1). In addition, *P. karka* and *P. mauritanus* have a
280 large distribution in tropical warm riparian habitats, like the earliest Poaceae (Kellogg, 2015).
281 Opposingly, the taxa of clade II (*P. altissimus*, *P. australis s.str.*, and *P. japonica*) exhibit
282 derived traits and are distributed in subtropical and temperate to cold zones. On the other hand,
283 spikelet traits draw on two other evolutionary lineages, divergent from *P. karka* with an
284 increasing differentiation. The clade B includes the non-waxy taxa (brownish bracts), with
285 lightly ornamented lemma and palea: *P. karka*, AE *P. australis*, *P. japonicus* ($2n = 48$), and
286 lastly AA *P. australis* ($2n = 96$). The clade A includes the waxy taxa (green bracts), with
287 increasingly ornate paleas: *P. mauritanus*, *P. frutescens* ($2n = 48$), and *P. altissimus* ($2n = 48$,
288 96).

289 In conclusion, we have highlighted several valuable micro-characters allowing for the
290 identification of four Old World giant *Phragmites*, which match well with the previous genetic
291 clustering. Future investigations will focus on the small reeds, particularly those from Asia and
292 Australia, and the macroscopic characters of Old World *Phragmites*.

293 **CRedit authorship contribution statement**

294 R. Verlaque: Conceptualization, species identification, morphological investigation, and writing. L.
295 Hardion: Data analyses, figures, and writing. K. Canavan, C. Lambertini and B. Vila: Field work,
296 documents, and writing. M. Verlaque: tables, figures, and writing.

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302

303 **Appendix A. Supporting information**

304 Supplementary data associated with this article can be found in the online version at

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423 Legends of figures

424 **Figure 1:** Hierarchical clustering analyses: **1a.** Floral bract epidermis; **1b.** Leaf epidermis; **1c.**
425 Leaf anatomy. Clade A: Floral bracts green and spread out; Clade B: Floral bracts brownish
426 and rolled up; Clade I: ribs with large prickles and bicellular microhairs; Clade II: ribs with
427 small prickles, without bicellular microhairs.

428 **Figure 2: Lemmas.** (a) Clade A, green and spread out: *P. mauritanus* (B24); (b) Clade B,
429 brownish and rolled up: *Phragmites karka* (P9). **Leaf margin:** teeth rounded tipped, similar,
430 low: (c) *P. mauritanus* (L1); (d-i): teeth large and small, pointed, erect, and more spaced: (d)
431 *P. karka* (B11); (e) *P. frutescens* (C196); (f) *P. japonicus* (C177); (g) Afro-European *P.*
432 *australis* (MS24); (h) Australian-E-Asian *P. australis* (MP7); (i) *P. australis* subsp. *altissimus*:
433 (MS26); (j) detail of basal internal ornament of large teeth (MS20). Scale = 100 µm.

434 **Figure 3: Lemma epidermis, Clade B,** hooks pointed to the top: (a) *Phragmites karka* (B13),
435 (b) AE *P. australis* (MS27). **Clade A,** hooks pointed in all directions: (c) *P. mauritanus* (B24),
436 (d) *P. australis* subsp. *altissimus* (MS1). **Palea apex, Clade B,** few ornamented: (e) *P. karka*

437 (B13); **(f)** *P. japonicus* (MP10, similar in AE *P. australis*). **Clade A**, well ornamented, large
438 teeth pointed in all directions: **(g)** *P. mauritanus* (B24); **(h)** *P. australis* subsp. *altissimus*
439 (MS2): entire palea with marginal long hairs. **Leaf characters** (abaxial face): **Clade I**, ribs with
440 large prickles and bicellular microhairs: **(i)** *P. karka* (B39, similar in *P. mauritanus*); **(j)** *P.*
441 *frutescens* (MS8) smaller prickles. **Clade II**, ribs with small prickles, without bicellular
442 microhairs: **(k)** AE *P. australis* (MS29, similar in AA); **(l)** *P. japonicus* (C177) many obvious
443 hooks; **(m)** *P. australis* subsp. *altissimus* (C174) blunt prickles. R = rib; p = prickle; b =
444 bicellular microhair; h = hook. Scales: (a-g) = 40 µm; (h) = 250 µm; (i-m) = 20 µm.

445 **Figure 4:** Hierarchical clustering analysis of floral bracts and leaves epidermal characters.
446 Hybrids: KC2 (*P. mauritanus* x AE *P. australis*) and KC3 (*P. mauritanus* x *P. altissimus*).

447 **Figure 5: Leaf anatomy** (transversal section). **(a)** *Phragmites karka* (P6); **(b)** *P. mauritanus*
448 (P14); **(c)** *P. frutescens* (MS6); **(d)** *P. australis* subsp. *altissimus* (MP16); **(e)** Australian-E.
449 Asian *P. australis* (MS16); **(f)** Afro-European *P. australis* (MS13); **(g)** *P. japonicus* (C177).
450 BC = bulliform cells, ad = adaxial face, ab = abaxial face, arrow = obvious underlying colorless
451 cells, I = primary vascular bundles (elliptic or circular), separated by 3-4 secondary bundles
452 (=II, always circular). Scale = 50 µm.

453 **Figure 6:** Hierarchical clustering analysis of leaf anatomic characters.

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456 **Box 1:** Key of spikelet epidermal characters.

457 **Box 2:** Key of leaf epidermal characters.

458 **Box 3:** Key of leaf anatomy characters.

459

460 **Box 1: Key of spikelet epidermal characters** (specific traits in italics)

461 1. Glumes and lemmas green and spread out*; lemma hooks: many large (> 30µm), pointed in all directions: **clade A**

462 2. *Paleas densely ornate*: margins with *large teeth* (70-140µm), *pointed in all directions*, and some *long hairs* (up to 1.5
463 mm); apex *retuse with many hooks*: ***P. australis* subsp. *altissimus***

464 2'. *Paleas less ornate*: margins with smaller teeth (<100µm), and without long hairs

465 3. Hooks of lemmas and paleas large (30-150µm); palea apex bifid: ***P. mauritanus***

466 3'. Hooks and margin teeth of lemmas and paleas smaller (<40µm); palea apex retuse-truncated: ***P. frutescens***

467 1'. Glumes and lemmas brownish and rolled up*; lemma hooks: smaller (< 30µm) and pointed to the top: **clade B**

468 4. Lemmas with *straight long-cell walls* and many hooks; palea: margins with small teeth (< 50 µm), *veins stopped long*
469 *before the top*, apex *hyaline generally rounded*: ***P. karka***

470 4'. Lemmas with *sinuous long-cell walls* and *rare hooks*; palea margins with larger teeth (> 50µm), veins going to the top,
471 apex not hyaline, generally truncated: **small reeds** including ***P. australis* subsp. *australis*** (AE, AA) and ***P. japonicus***.

472 * After staining with iodine green and mounting in glycerin.

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474 **Box 2: Key of leaf epidermal characters** (specific traits in italics)

475 1. Ribs with large (> 65 µm) long-pointed prickles; intercostal zone (IZ) with bicellular microhairs, cork-cells and rare hooks:
476 **clade I**

477 2. Ribs with *oblong silica bodies*; IZ with *superposed cork-cells*; *marginal teeth: rounded tip, quite similar, low, close*
478 *together*, green-colored: ***P. mauritanus***

479 2'. Ribs with saddle-shaped silica bodies; IZ with solitary cork-cells; marginal teeth: pointed tip, higher, irregular, the
480 largest (64-120 µm) separated by several small teeth:

481 3. Ribs with many large prickles; marginal teeth few colored, the largest (< 290 µm) few spaced: ***P. karka***

482 3'. Ribs with *rare smaller prickles*; marginal teeth green, irregular, the largest (290-450 µm) well-spaced: ***P. frutescens***

483 1'. Ribs with smaller (< 60µm) short-pointed prickles; IZ without microhairs, rare cork-cells and many hooks: **clade II**

484 4. Rib with *blunt tip prickles, flat silica bodies*; IZ with *many short-cell pairs*; marginal teeth green, the largest (0.5-1 mm),
485 *with obvious basal internal cells, well-spaced*: ***P. australis* subsp. *altissimus***

486 4'. Rib prickles with a small terminal tip (< 8µm), thick silica bodies; IZ with rare short-cell pairs; marginal teeth not or few
487 colored, the largest shorter, without obvious basal internal cells, less spaced: **small reeds**

488 5. IZ with small hooks: ***P. australis* subsp. *australis*** (AA and AE)

489 5'. IZ with many large hooks: ***P. japonicus*** and P11 (AA).

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Box 3: Key of leaf anatomy characters (specific traits in italics) (Figure 5)

1. Blade thin (< 190 μm , on primary ribs); bulliform cells (BC) small, penetrating ca. 1/2 of the blade, without underlying colorless cells; all bundles circular:
 2. Blade undulated, *BC semicircular* ($\varnothing < 70 \mu\text{m}$), straight sided; small hooks: **Afro-European *P. australis*** (AE)
 - 2'. Blade nearly flat, *BC wider than deep* (90 x 70 μm); many *obvious hooks*: ***P. japonicus***
- 1'. Blade mostly thicker (> 190 μm); BC deeper than wide, penetrating ca. 2/3 of the blade:
 3. BC (45-60 x 70-95 μm); blade undulated, abaxial face without hooks.
 4. BC with *obvious underlying colorless cells*, straight sides; primary bundles elliptic: ***P. karka***
 - 4'. BC with inconspicuous underlying colorless cells, sides flared toward the top; bundles circular: ***P. mauritanus***
 - 3'. BC bigger, sides flared toward the top, with inconspicuous colorless cells:
 5. *Blade flat*, without hooks; *BC (75-96 x 100-140 μm) protruding in upper part*; all bundles circular: ***P. frutescens***
 - 5'. Blade undulated, with small hooks; BC not protruding; primary bundles elliptic:
 6. Blade thick (220-280 μm), well undulated; BC big (70-96 x 85-110 μm): ***P. australis* subsp. *altissimus***
 - 6'. Blade thinner (170-230 μm), few undulated; BC smaller (50-65 x 65-90 μm): **Australian-E. Asian *P. australis*** (AA)

The genus *Phragmites* includes taxa that are very difficult to distinguish macroscopically, and even genetically. To better discriminate these taxa, microscopic traits, such as the epidermis of floral bracts and leaves, and leaf anatomy, were investigated. In the Old World, four distinct giant reeds species (> 3 m) were delineated: two tropical species, *P. karka* and *P. mauritanus*, and two Mediterranean species, *P. altissimus* and *P. frutescens*. Previously included within *P. australis sensu lato*, the rehabilitation of *P. altissimus* and *P. frutescens* is supported by relevant distinctive characteristics. Conversely, the small reeds (< 3 m) from temperate to cold regions form a distinct group. Although this group is still difficult to subdivide with epidermis traits, three taxa can be separated using leaf anatomy: both the Afro-European and the Australian-E. Asian *P. australis sensu stricto*, and *P. japonicus*. Using iodine green solution, we link our morphological analyses with previous genetic clustering, the green coloration of floral bracts and margin leaf teeth matches with the presence of nuclear waxy gene bands in *P. frutescens* and *P. mauritanus* (100 bp), and *P. altissimus* (200 bp), while *P. karka*, *P. australis* subsp. *australis* and *P. japonicus* have no coloration nor waxy band. While leaf anatomy features seem to be correlated with eco-climatic conditions, the epidermal traits delimited other clades. For leaf epidermis *P. karka*, *P. mauritanus*, and *P. frutescens* present the more ancestral characters, whereas *P. australis*, *P. japonicus*, and *P. altissimus* possess derived traits. Lastly, floral bract epidermis traits separate two divergent evolutionary lineages from the basal *P. karka*.

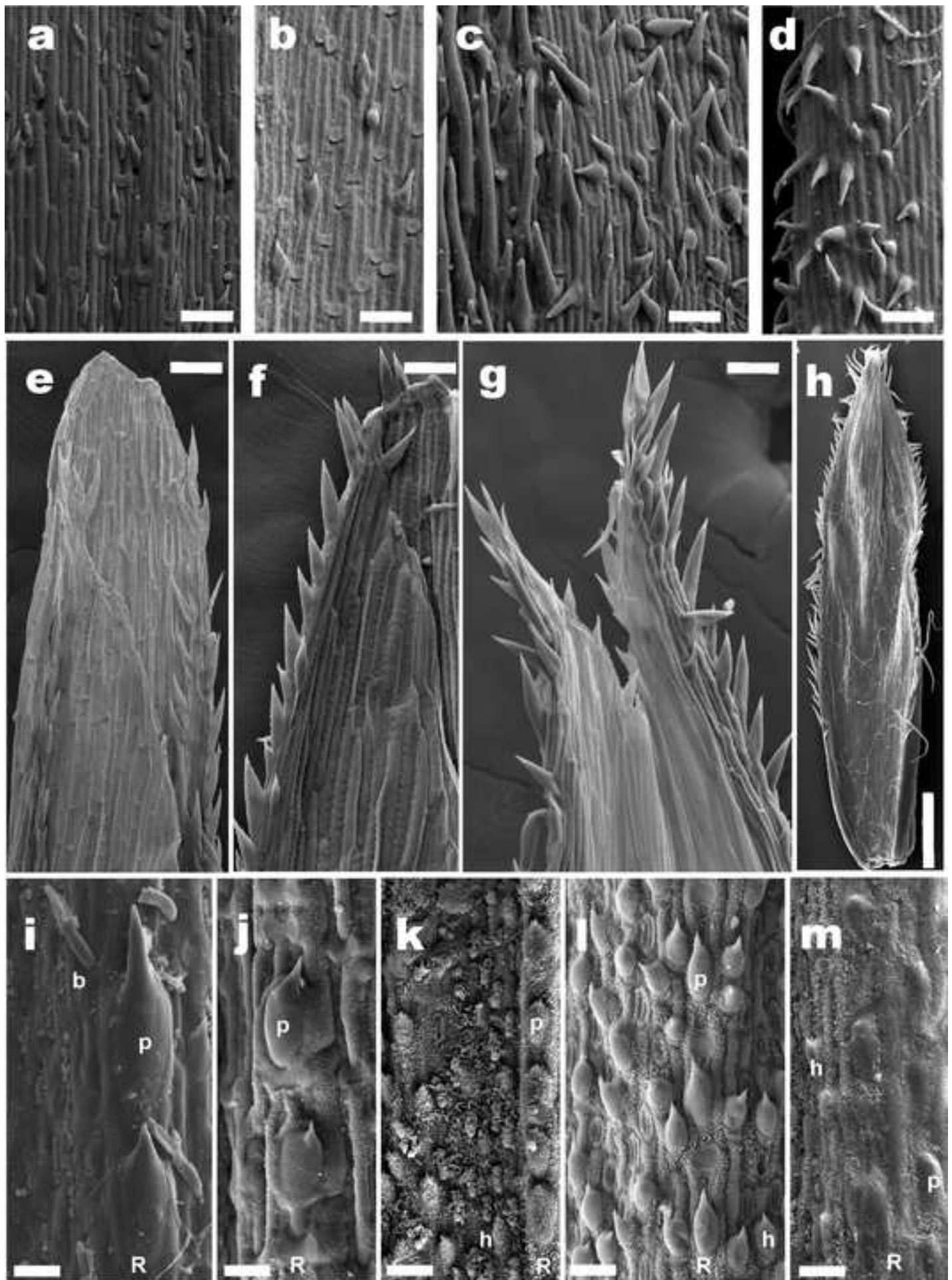


Figure 4

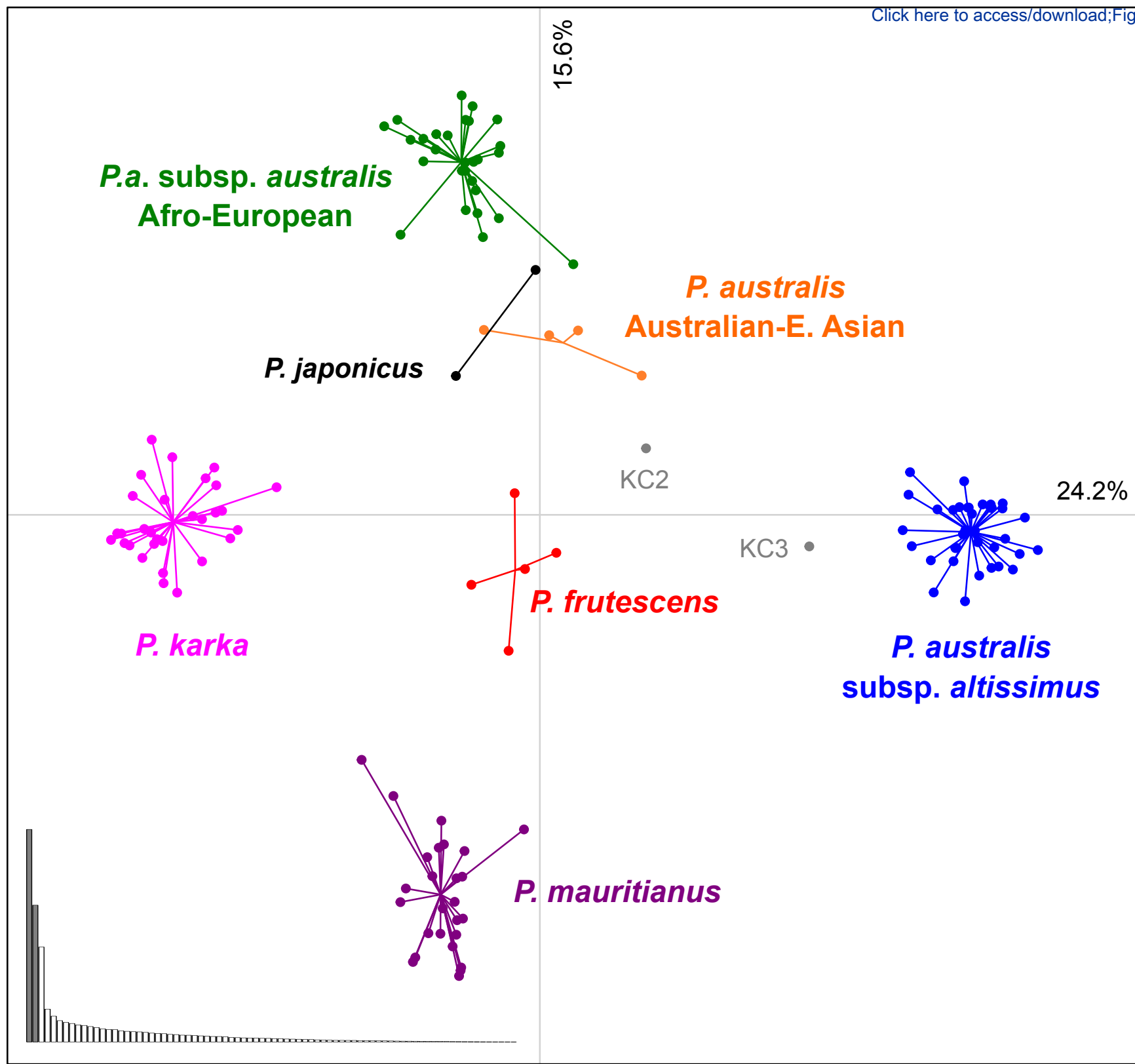


Figure 6

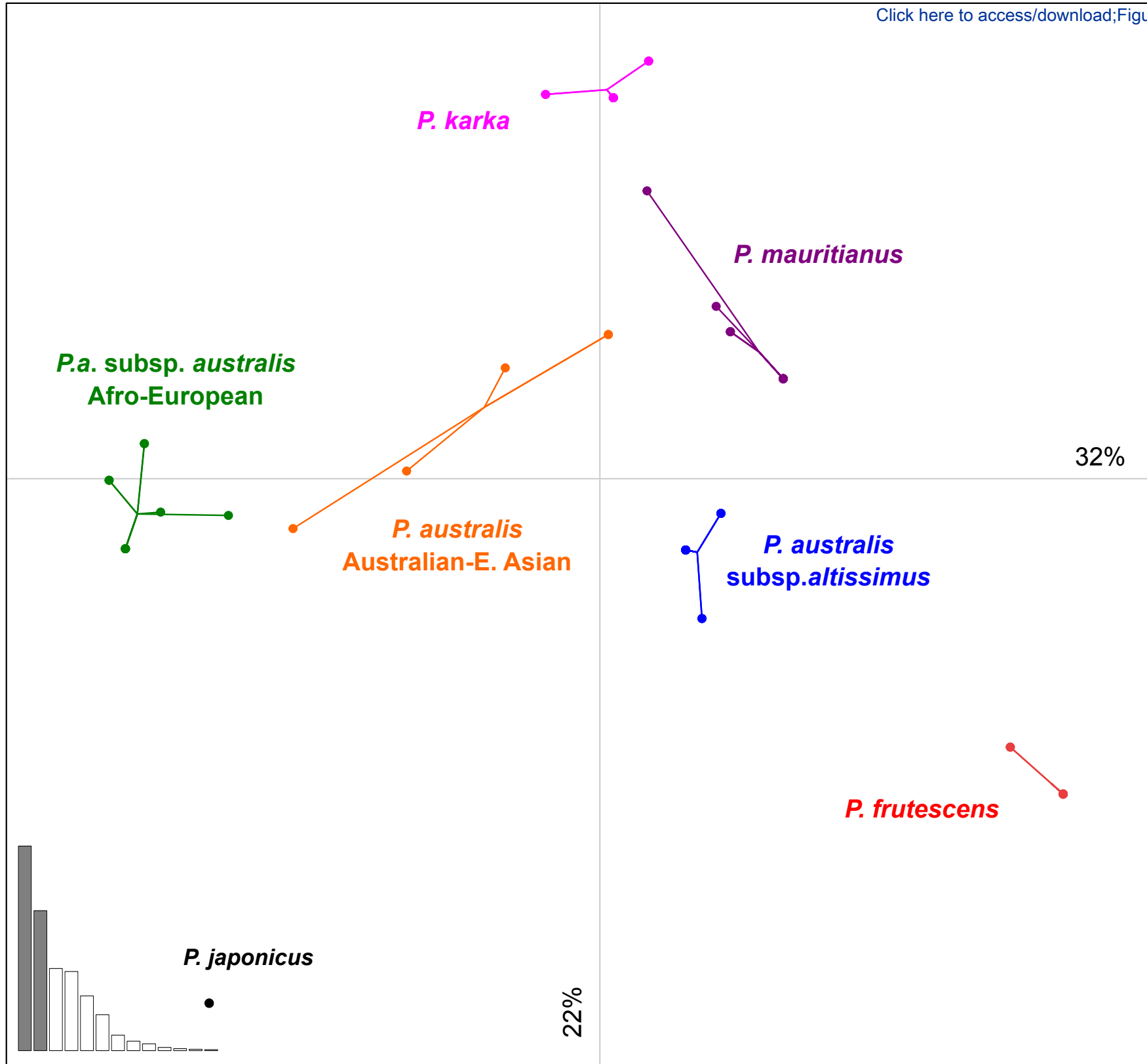
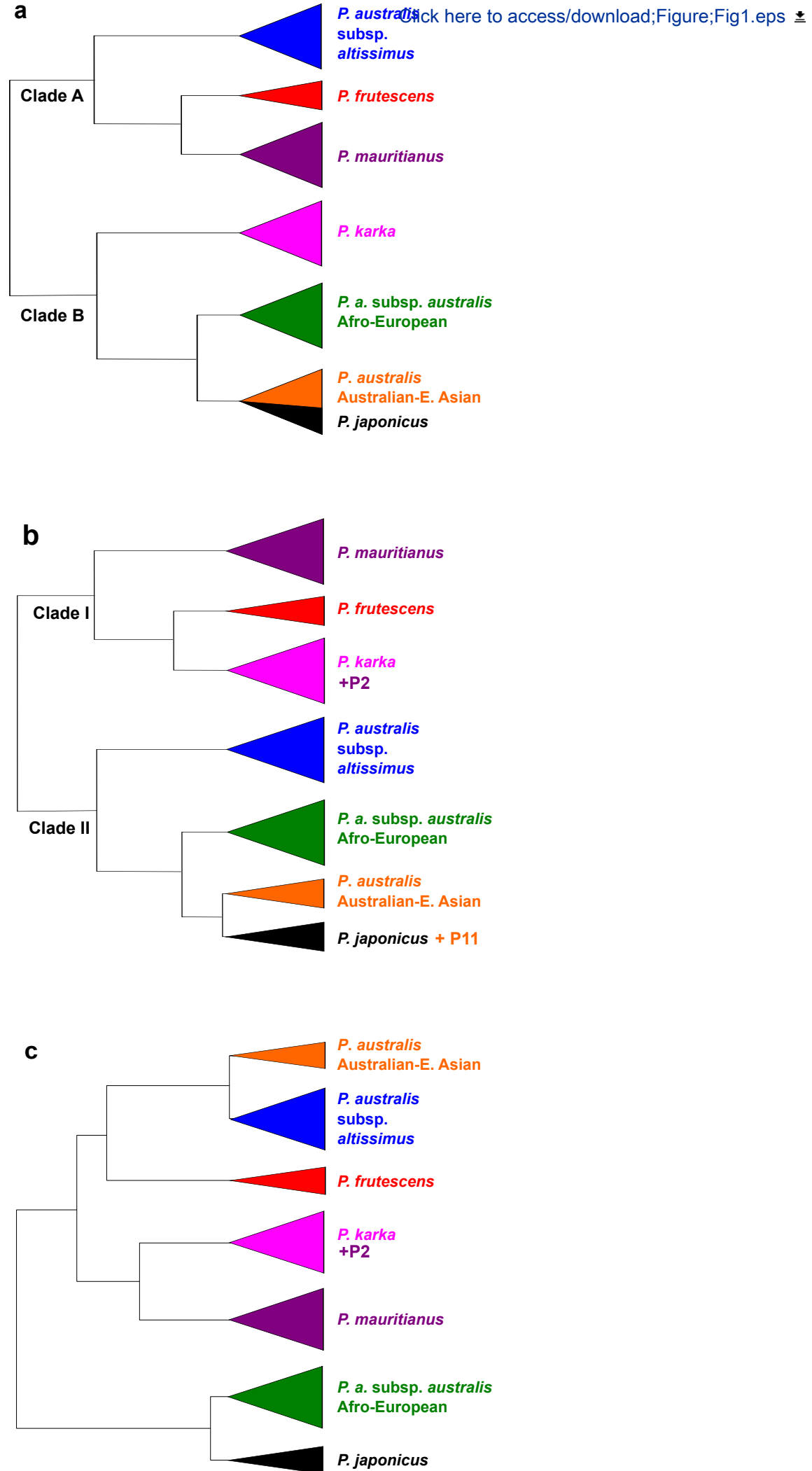
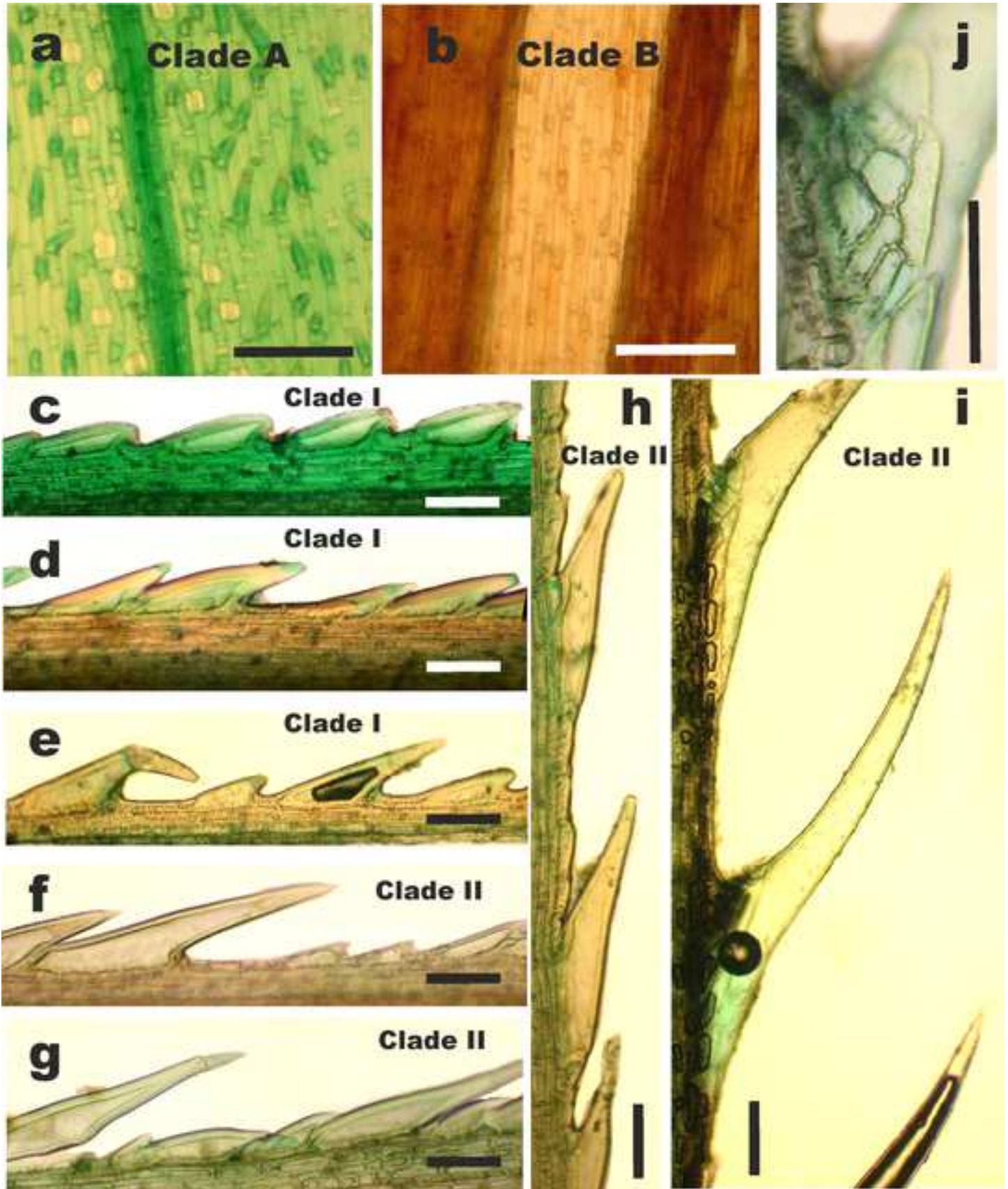


Figure 1





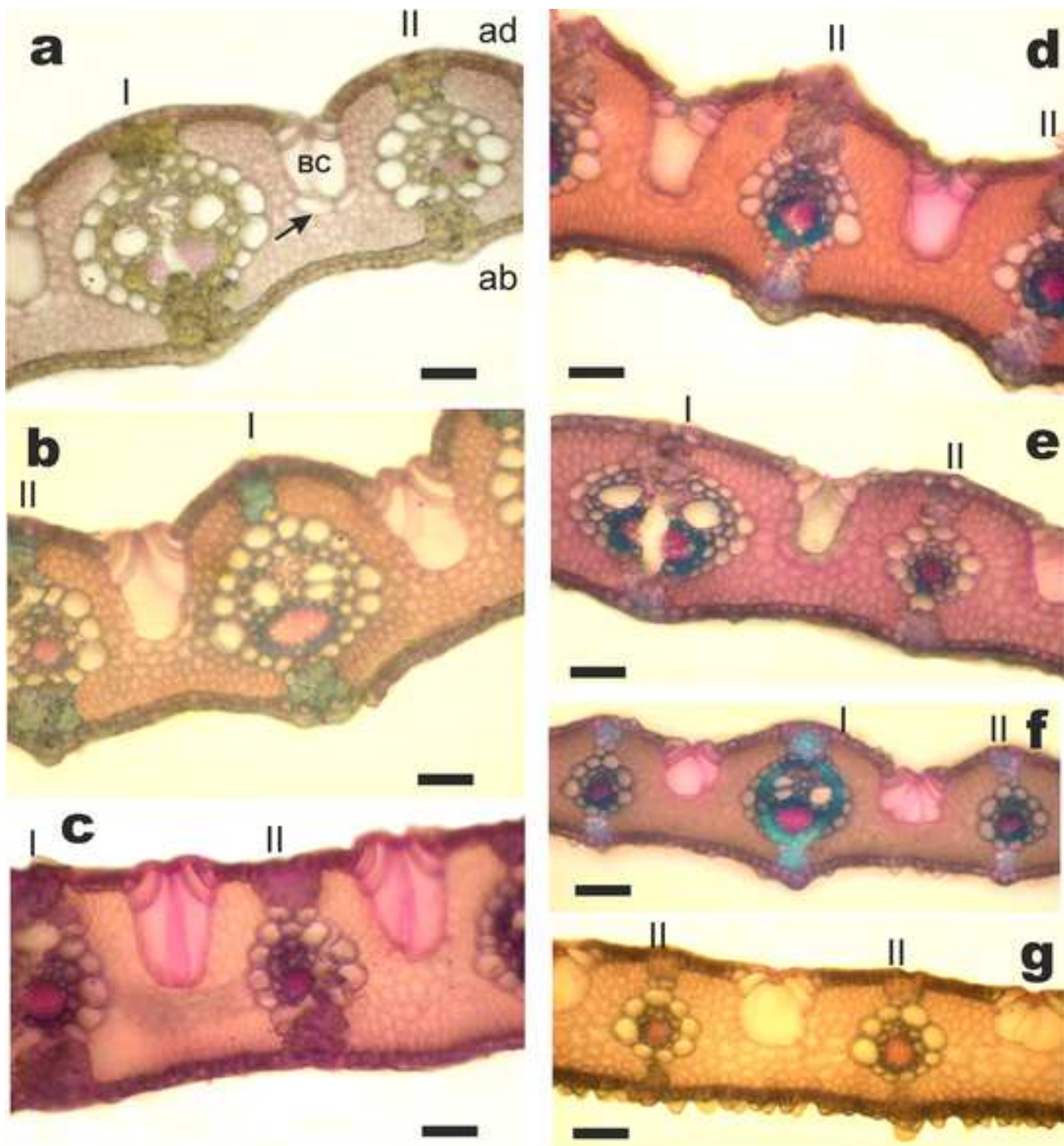


Table 1. Summary of genetic and phylogeographic groups of the Old World *Phragmites* taxa.

Taxa	AFLPs groups ^a	cpDNA groups ^b	cpDNA haplo-types ^{b, c}	Waxy bands ^b
Afro-European <i>P. australis</i>	<i>P. australis</i> core group	European <i>Phragmites</i>	L ¹ , M, O ^{1,2} , AD, AH, AI, AJ, AK, K	
Australian-E.Asian <i>P. australis</i>	<i>P. australis</i> 8x Australia-Asia	Far East - Australian <i>Phragmites</i>	P ^{1,2}	
<i>P. karka</i>	<i>P. vallatoria</i>	Tropical <i>Phragmites</i> (<i>P. karka</i>)	U ¹	
<i>P. mauritanus</i>	<i>P. mauritanus</i>	Tropical <i>Phragmites</i> (<i>P. mauritanus</i>)	I ¹	100 bp
<i>P. frutescens</i>	<i>P. frutescens</i>	Tropical <i>Phragmites</i> (<i>P. frutescens</i>)	I ¹ , U ¹	100 bp
<i>P. altissimus</i>	<i>P. australis</i> Africa	Mediterranean <i>Phragmites</i>	M ¹ , AL	200 bp
<i>P. japonicus</i>	<i>P. japonicus</i>	<i>P. japonicus</i>	AM	

^a Lambertini et al., 2006; ^b Lambertini et al., 2012; ^c Lambertini et al., 2020

¹ includes cpDNA microsatellite variants (ex. M1, M2, M3, M4 Lambertini et al., 2012)

² includes Haplotype O-related and Haplotype P-related haplotypes (Lambertini et al., 2020)