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ORIGINAL ARTICLE

Pollen morphology of *Ellisiophyllum* and *Sibthorpia* (Plantaginaceae, tribe Sibthorpieae) and phylogenetics of the tribe

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Abstract

Pollen morphology of six species belonging to genera *Ellisiophyllum* and *Sibthorpia* (Plantaginaceae tribe Sibthorpieae) was studied using light and scanning electron microscopy. The data were analyzed in the light of the first phylogenetic analysis including all but one species of the tribe using DNA sequence data from nuclear ribosomal (ITS) and plastid *trn*L-F region. Pollen grains in representatives of this tribe are 3-colpate, occasionally 3-porate, suboblate to prolate; mainly medium-sized, rarely small. One major pollen type (3-colpate) is recognized in the tribe. Within this pollen type, six subtypes are distinguished based on their exine sculpture, pollen grain size, length of the apertures, and exine thickness. The obtained results confirm that pollen characters are useful for species identification. Palynomorphological data are consistent with the results of the molecular phylogenetic analyses. All studies support a sister relationship of the widespread European *Sibthorpia europaea* with the widespread South American *Sibthorpia repens* and a sister relationship of two insular species, the Balearic *Sibthorpia africana* and the Madeiran *Sibthorpia peregrina*. Pollen grains in the tribe Sibthorpieae have both reticulate exine sculpture characteristic for representatives of the Russelieae–Cheloneae–Antirrhineae clades of Plantaginaceae, and also nanoechinate sculpture, which is typical for the Veroniceae and Plantagineae clades of that family. Also, in *Sibthorpia repens*, we observe a possible transition from the colpate type to the porate type typical for taxa of *Plantago* and *Littorella*.

Keywords *Ellisiophyllum* · Evolution · Palynology · Phylogenetics · *Sibthorpia*

Introduction

The circumscription of the family Scrophulariaceae has greatly changed since the first report of its polyphyly (Olmstead and Reeves 1995), and members of the traditional Scrophulariaceae are now split among at least eight families representing monophyletic lineages. Polyphyly extends also to traditional subfamilies and tribes of the family, and thus, reevaluation of the importance of characters in genera of traditional Scrophulariaceae is necessary. The tribe Sibthorpieae Benth. was established by Bentham (1846) with eleven genera, two now belonging to Phrymaceae, three to Scrophulariaceae, and seven to Plantaginaceae. However, later systems combined these genera with *Digitalis* L., *Veronica* L., and related genera, placing them in Digitalideae (Wettstein 1891–1893), or subsumed *Sibthorpia* (with *Hemiphragma* Wall., *Scoparia* L. and *Capraria* L., the latter now in Scrophulariaceae sensu stricto) under Hemiphragmeae (Rouy 1909). Wettstein's system was followed by most authors, for example by Takhtajan (1987, 1997), who included them in the tribe Veroniceae. Fischer (2004) restricted Sibthorpieae to only two genera, *Ellisiophyllum* Maxim. and *Sibthorpia* L. and placed the tribe in subfamily Digitalidoideae. Molecular phylogenetic studies of *Ellisiophyllum* and *Sibthorpia* were first conducted by Albach et al. (2005) who confirmed that they are phylogenetically closely related to each other and unrelated to genera previously considered close to them. Sibthorpieae, as outlined now, thus includes only the genera *Ellisiophyllum*

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and *Sibthorpia* (Albach et al. 2005; Tank et al. 2006; Reveal 2012; Olmstead 2016).

The genus *Sibthorpia* includes five currently recognized species that occur in tropical America, the Azores, Madeira, Europe (two species), and African mountains (Hedberg 1955, 1975; Diaz-Miranda 1988; Mabberley 1997, 2017; Fischer 2004; Albach et al. 2005; Tank et al. 2006; Olmstead 2016). A comprehensive taxonomic treatment of *Sibthorpia* was published by Hedberg (1955). The morphological features of flowers, fruits, seeds, and chromosome numbers of the genus in general (Hedberg 1975) and in *Sibthorpia europaea* L. in particular (Juan et al. 1999) were investigated. Based on his investigations, Hedberg (1955) suggested that the Balearic *Sibthorpia africana* L. and the Madeiran *Sibthorpia peregrina* L. are sister species, which was supported by the same chromosome number (Hedberg 1975). In turn, he hypothesized that the Neotropical *Sibthorpia repens* (L.) Kuntze and the closely related *S. conspicua* Diels are tetraploid derivatives of the diploid European-African *S. europaea* (Hedberg 1955, 1975). To date, this phylogenetic hypothesis has not been tested in a phylogenetic analysis.

The genus *Ellisiophyllum* is represented by the only species, *E. pinnatum* (Benth.) Makino, which is distributed from India to Japan and Taiwan, and to eastern New Guinea (Hedberg 1975; Mabberley 1997, 2017; Fischer 2004; Olmstead 2016). The species was originally described by Bentham (1846) based on the specimen(s) collected by Wallich in Nepal or adjacent regions of India and listed in his handwritten catalog under No. 3915.

Earlier opinions on the proper phylogenetic position and relationships of *Ellisiophyllum* varied greatly. Wallich provisionally listed the species under the name *Mazus pinnatus* Wall. (nom. inval., nom. nudum), in a genus now placed in Phrymaceae, but Bentham validly published it as *Ourisia pinnata* Benth. (Bentham 1835; see also Hayata 1911; Meudt 2006, etc.). Later, Bentham (1846) described the genus *Hornemannia* Benth. for it, an illegitimate later homonym of *Hornemannia* Willd., and put the species in his order close to *Sibthorpia*. Maximowicz (1871) established the new genus *Ellisiophyllum* with one species, *E. reptans* Maxim. The names of the genus and its only species were simultaneously validated by one description (*descriptio generico-specifica*, Art. 38.5 of the ICN; Turland et al. 2018). Most probably Maximowicz was unaware of the identity (or at least similarity) of his newly described species with the species earlier described by Bentham as *Ourisia pinnata*, which is understandable, partly because these taxa were described from distant territories: Japan and Nepal (or India), respectively. Maximowicz (1871: 223) characterized his genus as being intermediate "inter Hydrophyllaceas et Polemoniaceas." It was consequently included in the family Hydrophyllaceae by Peter (1897). Hooker (1885), however, considered *Ellisiophyllum* to be a synonym of *Sibthorpia*. Hemsley (1899) disagreed with that generic placement and, being aware of the illegitimacy of Bentham's generic name *Hornemannia* but evidently not knowing about the availability of the name *Ellisiophyllum*, coined the replacement name *Mosleya* Hemsl. (to replace *Hornemannia* Benth.) and validated the combination *M. pinnata* (Benth.) Hemsl. Evidently, *Ellisiophyllum* has priority over *Mosleya* at the genus rank. Brand (1913: 185–186) definitely excluded *Ellisiophyllum* from Hydrophyllaceae and confirmed instead its placement in Scrophulariaceae ("Genus Scrophulariaceis attribuendum"). Recent molecular and other findings (see an overview above) firmly placed *Ellisiophyllum* and *Sibthorpia* in the extended and re-circumscribed Plantaginaceae.

With the gained certainty in the familial relationships and phylogenetic hypotheses available, it is timely to reinterpret trends in character evolution and investigate poorly known pollen characters in a phylogenetic framework. For example, very little information is available on pollen grains of representatives of Sibthorpieae. The morphological features of pollen grains of *S. europaea* (Juan et al. 1999) have been described. However, as far as we know, pollen grains of the monotypic (monospecific) genus *Ellisiophyllum* and the other species of *Sibthorpia* have not been investigated before.

The purpose of the present research was to study and analyze the phylogenetic relationships among members of the tribe Sibthorpieae using DNA sequence data and to compare them with data on morphological features of pollen grains of these taxa.

Materials and methods

DNA-based phylogenetic analysis

For the DNA-based part of the study, we have sampled four of the five species of *Sibthorpia* and the only species of *Ellisiophyllum*, with two or three samples of three of the species (Table 1). Only samples of *S. conspicua* were not available for DNA sequencing. Outgroups were chosen based on the analysis of Plantaginaceae by Albach et al. (2005) to ensure a wide variety of taxa and sufficient representation of the family (Table 1). DNA was isolated from about 20 mg of tissue from either living material, silica gel-dried or herbarium material with the NucleoSpin Plant II (Macherey and Nagel, Düren, Germany) or the DNeasy plant Mini Kit (Qiagen, Hilden Germany) following the provided protocol. The quality of the extracted DNA was checked on a 0.8% TBE-agarose-gel and the concentration measured spectrophotometrically with a GeneQuant RNA/DNA calculator (Pharmacia, Cambridge, UK).

Table 1 Vouchers and GenBank accession numbers for the sequences used in the phylogenetic study

Species	Voucher	Locality	ITS	*trn*L-F
Scrophulariaceae				
Buddleja asiatica	unknown	unknown		AF380858
Buddleja marrubifolium	*Freeh* and *Johnson s.n.,ARIZ*	unknown	AF363671	
Oreosolen wattii	*Dickoree* 5182, GOET	China	AF509817	AF513357
Scrophularia californica	*Thulin* and *Gifri 8633, UPS*	USA		AF118802
Scrophularia peregrina	*Wolfe s.n.*, OS	USA	AF375146	
Plantaginaceae—Gratioloideae				
Gratiola officinalis	*Albach 490*, WU	Bulgaria	AY492106	AJ608591
Scoparia dulcis	*Carr* 10834, TEX	USA	AY492119	AY492190
Otacanthus sp.	*Albach s.n.*, WU	Cult. BG Bonn	AY492115	AY492189
Stemodia durantifolia	*Reina* et al. *98-198,* TEX	Mexico	AY492120	
Stemodia florulenta	*Nordenstam* and *Anderberg 967*, S	Australia		AJ608566
Mecardonia procumbens	*Denny* and *Harvey 449*, TEX	USA	AY492110	AY492184
Ourisia microphylla	*Meudt* and *López 036*, TEX	Chile	AY492116	AY492189
Basistemon silvaticus	*Nee 38032*, TEX	Bolivia	AY492096	AY492171
Melosperma andicola	*Arroyo* and *Humana 206607*, TEX	Chile	AY492112	AY492185
Plantaginaceae—Plantaginoideae				
Russelia equisetiformis	*Albach s.n.*, WU	Cult. BG Bonn	AY492118	AY492190
Tetranema roseum	*Smith College s.n.*, UCONN	Cult. Univ. Connecticut	AY492121	AY492192
Penstemon whippleanus	*Albach 661*, WU	Cult. BG Bonn	AY492117	AF034866
Keckiella breviflora	*Wilson 3487*, OS (ITS); *Ertter* and *Strachan 5011*, TEX (*trn*L-F)	USA	AF375161	AY492179
Lafuentea rotundifolia	*Martinez Ortega 889*, SALA	Spain	AF509816	AF513356
Antirrhinum majus	*Wolfe s.n*, OS (ITS); *Olmstead 846*, NY (*trn*L-F)	Cult	AF375150	AF482607
Callitriche cf. *brutia*	*Albach 491*, WU	Bulgaria: Rhodopes Mts	AY492097	AY492172
Callitriche japonica	*Murata 80198*, TNS	Japan	LC177722	
Callitriche muelleri	*Aspin s.n.*, AK288885	New Zealand	LC177728	
Hippuris vulgaris	*Albach s.n.*, WU	Cult. BG Bonn	AY492098	AY492178
Globularia salicina	*Chase 2547*, K	Cult. RBG Kew	AF313039	AF513358
Campylanthus salsoloides	*Panero* and *Ortega 6910*, TEX	Spain: Tenerife	AY492099	AY492171
Hemiphragma heterophylla	*Greason* and *Long 2512*, K	India: Sengur	AY492107	–
Erinus alpinus	*Albach 158*, WU	France: near Evian	AF313032	AF486417
Digitalis purpurea	*Meudt* and *Lopez 005*, TEX (ITS); UTEP54185 (*trn*L-F)	Chile (ITS); USA (trnL-F)	AY492102	AF034871
Plantago coronopus	*Ronsted 8*, C (ITS); *Chase 2763*, K (*trn*L-F)	Denmark(ITS); Great Britain (trnL-F)	AY101882	AF486419
Aragoa abietina	*González 3807*, COL	Colombia	AJ459404	MN709773
Veronica montana	*Albach 151*, WU	Germany	AF313014	AF486388
Picrorhiza kurrooa	*McBeath 2214*, K	Pakistan	AF509813	AF486414
Ellisiophyllum pinnatum	*Meudt s.n.*, TEX	Cult. U Texas ex Taiwan	AY492103	AY492176
Sibthorpia africana	*Albach s.n.*, WU	Cult. BG Bonn	–	OK073646
Sibthorpia europaea	Kew 1948-41901, K	Cult. RBG Kew	AF313035	AF513355
Sibthorpia europaea	*Pearman 28.4.2009*, MJG	England: near Mabe, Penryn	OK070754	OK073647
Sibhtorpia europaea	*Böhling 10069*, B (DNA 3597)	Greece: Crete, Sembronas	OK070755	–
Sibthorpia repens	*Cazalet* and *Pennington 5309*, B (DNA 3595)	Ecuador: Imbabura	OK070756	–
Sibthorpia repens	*Beaman 3955*, B (DNA 3594)	Guatemala	OK070757	OK073650
Sibthorpia peregrina	*Rustan* and *Sunding 18124*, B (DNA 3609)	Cult. BG Berlin ex Madeira	OK070758	OK073649
Sibthorpia peregrina	*Albach s.n.*, WU	Cult. BG Bonn	OK070759	OK073648

The nuclear ribosomal ITS region (hereafter ITS) and the plastid *trn*L intron, *trn*L 3´ exon and *trn*L-F spacer (hereafter *trn*L-F region) were amplified using primers ITS A (Blattner 1999) and ITS4 (White et al. 1990) for ITS, and the *trn*L-F region with primers c and f and sometimes including internal primers d and e (Taberlet et al. 1991). PCR reactions included 2–2.5 mM $MgCl_2$, 8 mM bovine serum albumin, 0.4 µm primer, 0.2 mM dNTP, 1U/µl Taq polymerase (New England Biolabs, Ipswich, MA, USA), 1 × polymerase buffer and 1–5 µl DNA for a final volume of 25 µl. ITS sequences were amplified with a program consisting of 2 min at 95 °C followed by 36 cycles of 1 min at 95 °C, 1 min at 50–55 °C, and 1.5–2 min at 72 °C with a final extension of 5 min at 72 °C on either a Mastercycler gradient (Eppendorf) or TProfessional Standard thermocycler (Biometra). The *trn*L-F region was amplified after 1 min denaturation at 95 °C followed by 35 cycles with 30 s at 95 °C, 30 s at 52 °C and 1 min at 72° with a final extension of 8 min at 72 °C. PCR products were cleaned using QIAquick PCR purification kits (Qiagen, Hilden, Germany) following the provided protocol. Sequencing reactions of 10 µl were carried out using 1 µl of the Taq DyeDeoxy Terminator Cycle Sequencing mix (Applied Biosystems, Foster City, CA, USA) and the same primers as for PCR. Sequences were generated by Sanger sequencing at commercial sequencing companies. All sequences are available from GenBank (Table 1). The data matrices are available at http://purl.org/phylo/treebase/phylows/study/TB2:S25825.

Sequences were manually aligned in Phyde v.0.9971 (Müller et al. 2010) and evaluated for the best model of evolution in jModeltest2 (Darriba et al. 2012). No indel coding was conducted due to the high variability of the ITS region across Plantaginaceae. Phylogenetic analyses were conducted in IQ-TREE (Trifinopoulos et al. 2016) using the GTR + Γ + I for ITS and GTR + Γ for *trn*L-F with 8 different rates and 1000 ultrafast bootstrap replicates.

Pollen analysis

Pollen grains of two species belonging to two genera of Sibthorpieae (*Ellisiophyllum* and *Sibthorpia*) were sampled in the herbarium of the Missouri Botanical Garden (MO; St. Louis, Missouri, U.S.A.). Pollen grains of four species of *Sibthorpia* were sampled in the herbarium of the Conservatoire et Jardin botaniques de la Ville de Genève (G, Genève, Switzerland). Pollen grains of two species of *Sibthorpia* were sampled in the National Herbarium of Ukraine (KW—herbarium of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kyiv, Ukraine). The specimens examined are listed in "Appendix" section. Herbarium acronyms are given following *Index Herbariorum* (Thiers 2008–onward).

The methods used in the present study are essentially the same as we used earlier (Mosyakin and Tsymbalyuk 2015a, b, 2017). Pollen morphology was studied using light microscopy and scanning electron microscopy. For light microscopy (LM) studies (Biolar, × 700), the pollen was acetolyzed following Erdtman (1952), mounted on slides with glycerinated gelatin and analyzed and photomicrographed using light microscopy. Pollen morphometric features of 20 properly developed pollen grains from each specimen were measured on the acetolyzed pollen grains, and the measurements included the following parameters: polar axis (*P*), equatorial diameter (*E*), mesocolpium diameter, exine thickness, and 10 measurements of the apocolpium diameter, the width and length of apertures were performed. The *P*/*E* ratio was calculated in order to determine pollen shape. For all the quantitative characters, descriptive statistics was applied and the range (minimum and maximum values), arithmetic mean and standard deviation were calculated (Tables 2 and 3). The slides were deposited in the Palynotheca (reference pollen collection) at the National Herbarium of Ukraine (KW) (Bezusko and Tsymbalyuk 2011).

For scanning electron microscopy (SEM) studies (JEOL JSM-6060LA), dry pollen grains were treated with 96%-ethanol; then, these samples were sputter-coated with gold and investigated at the Center of Electron Microscopy of the M.G. Kholodny Institute of Botany. Terminology used in descriptions of pollen grains mainly follows the glossaries by Punt et al. (2007) and Halbritter et al. (2018).

Evolution of pollen characters was analyzed with the ancestral character state model using the package phytools (Revell 2012) in RStudio v. 1.4 (RStudio Team 2021) and R version 4.0.3 (R Development Core Team 2020) using the ITS species tree restricted to Sibthorpieae.

Results

DNA-based phylogenetic analysis

The ITS dataset included 38 sequences with a final alignment of 832 characters with 352 potentially parsimony informative, whereas the *trn*L-F region included 34 sequences with 1137 characters with 254 potentially parsimony informative. The optimal tree from the maximum likelihood analyses of each dataset separate are shown in Figs. 1 and 2. Analyses of ITS and *trn*L-F region were congruent for relationships within the Sibthorpieae. Relationships among the outgroups are inconclusive because of incongruence among markers. Noteworthy is the difference among both datasets regarding the closest relatives of Sibthorpieae. However, in both cases Sibthorpieae branch deeply within Plantaginaceae. In turn, the Sibthorpieae clade itself is strongly supported to

Table 2 Pollen morphometric characters (all measurements given as µm; mean ± standard deviation, range min–max)

Taxon	Polar axis	Equatorial diameter	*P*/*E*	Mesocolpium	Apocolpium	Colpi/pores length	Colpi/pores width	Exine thickness
Ellisiophyllum pinnatum	37.50 ± 3.43 30.59–42.56	28.79 ± 2.86 25.27–34.58	1.31 ± 0.18 0.96–1.63	20.21 ± 0.99 18.62–22.61	5.98 ± 0.66 5.32–6.65	30.98 ± 3.62 26.60–37.24	3.96 ± 1.48 2.39–6.65	2.28 ± 0.31 1.59–2.66
Sibthorpia peregrina	31.50 ± 3.88 23.94–42.56	27.84 ± 3.72 21.28–37.24	1.14 ± 0.19 0.87–1.56	22.14 ± 1.84 19.95–26.60	6.31 ± 1.50 3.99–9.31	26.79 ± 5.10 18.62–37.24	3.72 ± 0.86 2.66 –5.32	1.40 ± 0.14 1.06–1.59
Sibthorpia africana	40.56 ± 1.99 37.24–45.22	34.31 ± 2.93 26.60–39.90	1.19 ± 0.11 0.96–1.40	25.73 ± 1.64 21.28 – 29.26	7.71 ± 0.99 6.65–9.31	29.52 ± 3.35 26.60–35.91	5.18 ± 1.56 2.66–7.98	2.46 ± 0.23 1.99–2.66
Sibthorpia conspicua	21.21 ± 1.94 18.62–25.27	21.01 ± 1.66 18.62–23.94	1.01 ± 0.11 0.77–1.28	14.16 ± 1.13 13.30–15.96	6.38 ± 0.79 5.32–7.98	13.43 ± 0.93 11.97–14.63	4.45 ± 1.57 1.99–6.65	1.40 ± 0.26 1.06–1.99
Sibthorpia europaea	20.14 ± 0.63 18.62–21.28	19.41 ± 0.88 17.29–21.28	1.03 ± 0.04 1.00–1.15	11.57 ± 1.03 09.31–13.30	6.25 ± 0.60 5.32–6.65	13.16 ± 1.25 10.64–14.63	3.65 ± 1.18 2.66–6.65	1.70 ± 0.27 1.33–1.99
Sibthorpia repens	21.61 ± 1.77 18.62–26.60	24.53 ± 1.85 19.95–26.60	0.88 ± 0.05 0.80–1.00	15.89 ± 1.90 13.30–18.62	14.49 ± 1.62 11.97–17.29	9.57 ± 2.12 6.65–13.30/ 7.84 ± 2.552 5.32–13.30	2.79 ± 0.39 2.66–3.99/ 5.71 ± 2.52 2.66–10.64	1.56 ± 0.27 1.33–1.99

Table 3 Pollen morphological characters

Taxon	Apertures	Polar view	Equatorial view	Colpi/pores	Colpus membrane	Exine sculpture	Columellae
Ellisiophyllum pinnatum	3-colpate	Trilobate	Elliptic	Long, acute or blunt ends	Rugulate-nanoechinate	Rugulate-nanoechinate, nanoechinate	Distinct
Sibthorpia peregrina	3-colpate	Slightly trilobate, circular-triangular	Elliptic	Long, acute or indistinct ends	Granulate-nanoechinate	Nanoechinate-perforate, nanoechinate	Indistinct
Sibthorpia africana	3-colpate	Circular-triangular, slightly trilobate	Elliptic	Long, acute ends	Granulate	Rugulate-perforate	Distinct
Sibthorpia conspicua	3-colpate	Slightly trilobate, circular-triangular	Elliptic, circular	Medium-length, acute ends	Psilate-granulate	Reticulate	Distinct
Sibthorpia europaea	3-colpate	Slightly trilobate, trilobate	Elliptic, circular	Medium-length, acute or indistinct ends	Granulate	Perforate, microreticulate	Distinct
Sibthorpia repens	3-colpate and 3-porate	Circular, circular-triangular	Elliptic, circular	Brevicolpi, indistinct ends, pores lolongate	Psilate-granulate	Microreticulate	Distinct

be monophyletic by analyses of both ITS and *trn*L-F region (Figs. 1, 2; 100% and 99% bootstrap support (BS), respectively) with *Ellisiophyllum pinnatum* sister to *Sibthorpia* in both analyses (100% BS). Within *Sibthorpia,* all species sampled by multiple individuals are monophyletic. Amplification of *S. africana* was unsuccessful for ITS but is sister to *S. peregrina* in the analysis of the *trn*L-F region (99% BS). *Sibthorpia europaea* and *S. repens* are sisters (100% BS).

General description of pollen grains of *Ellisiophyllum*

Pollen grains are monads, radially symmetrical, isopolar, tricolpate. *Ellisiophyllum* pollen is medium-sized ($P = 30.59–42.56$ µm, $E = 25.27–34.58$ µm). According to P/E ratio, pollen grains are oblate-spheroidal to prolate ($P/E = 0.96–1.63$) in shape. Outline of pollen grains in equatorial view is elliptic. Outline of pollen

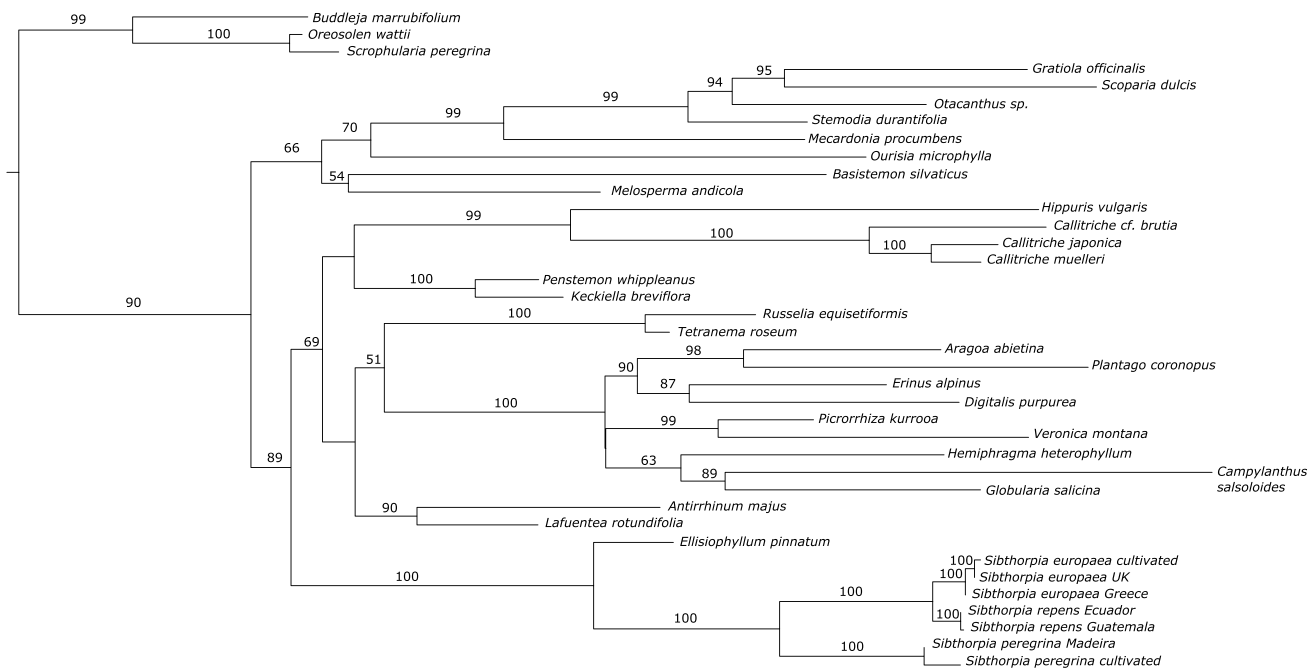


Fig. 1 Maximum likelihood tree from the analysis of the nuclear ribosomal ITS dataset. Numbers above the branches indicate maximum likelihood bootstrap support above 50%

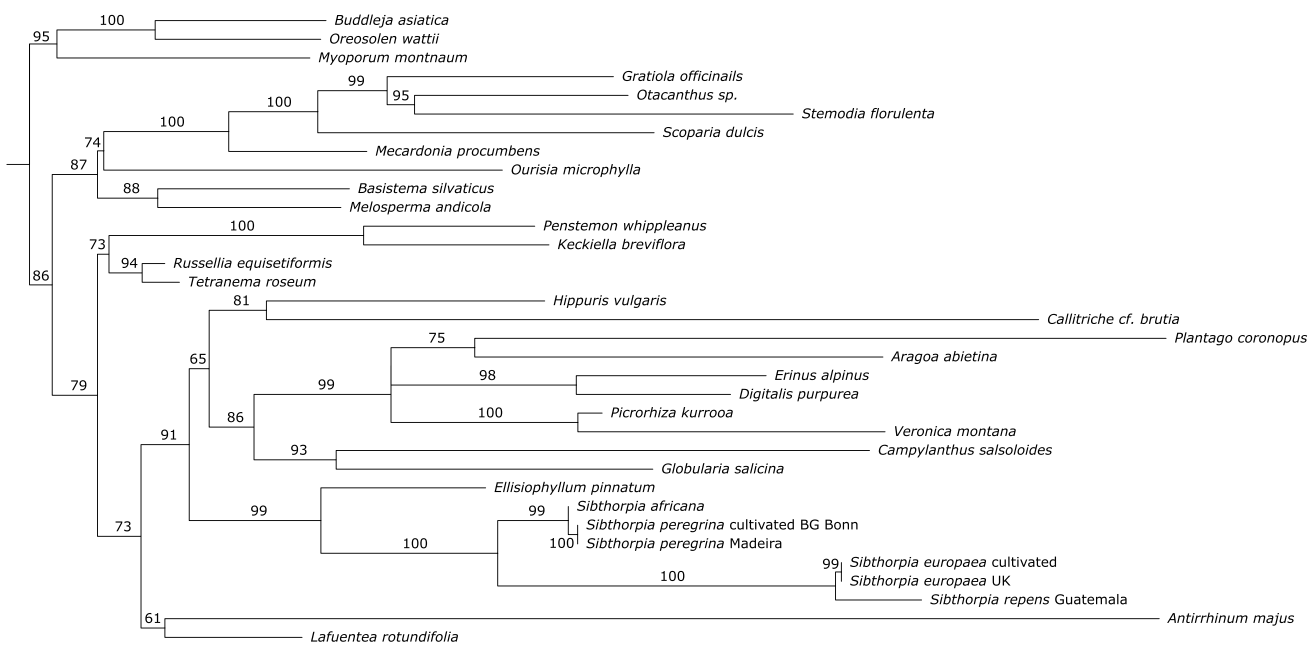


Fig. 2 Maximum likelihood tree from the analysis of the plastid *trn*L-F-dataset. Numbers above the branches indicate maximum likelihood bootstrap support above 50%

grains in polar view is trilobate (Table 3). Colpi are long (26.60–37.24 µm), with distinct, more or less straight, sometimes thickened margins (Tables 2 and 3). Colpus membranes are rugulate-nanoechinate (Fig. 3c). Exine is 1.59–2.66 µm thick (Table 2). Sexine is thicker than nexine. Tectum is nearly equal to infratectum, columellae distinct. Exine sculpture is rugulate-nanoechinate, nanoechinate (Fig. 3b, c).

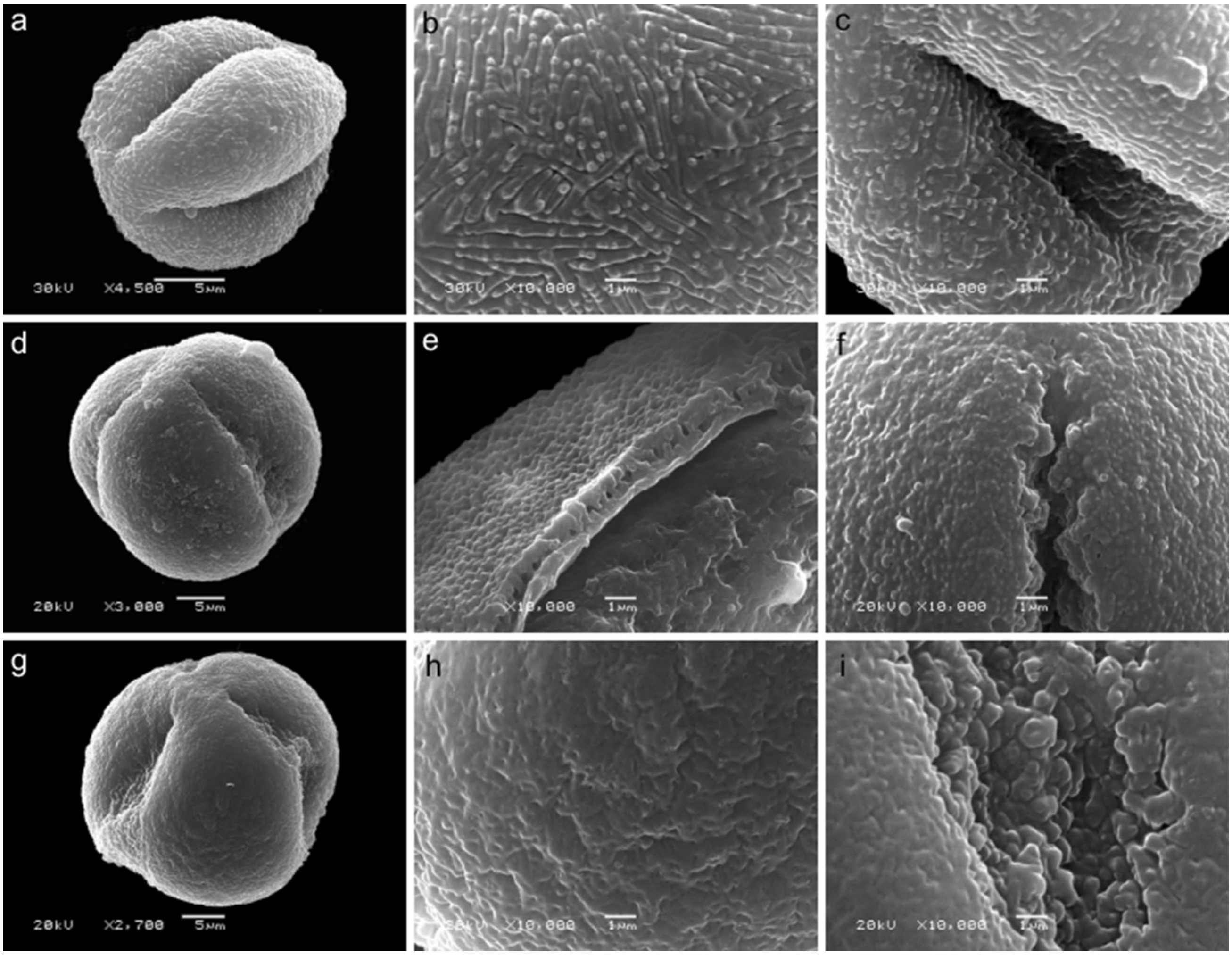


Fig. 3 Pollen grains of *Ellisiophyllum* and *Sibthorpia* (SEM). **a**–**c** *Ellisiophyllum pinnatum*: **a** equatorial view, **b** rugulate-nanoechinate sculpture, **c** colpus membrane rugulate-nanoechinate. **d**–**f** *Sibthorpia peregrina*: **d** equatorial view, **e** nanoechinate sculpture and broken pollen exine, columellae, **f** nanoechinate-perforate sculpture. **g**–**i** *Sibthorpia africana*: **g** equatorial view, **h** rugulate-perforate sculpture, **i** colpus membrane granulate

General description of pollen grains of *Sibthorpia*

Pollen grains are monads, radially symmetrical, isopolar, tricolpate, and rarely triporate. *Sibthorpia* pollen grains are small to medium-sized ($P = 18.62–45.22$ µm, $E = 18.62–39.90$ µm). According to P/E ratio, pollen grains are suboblate to prolate ($P/E = 0.77–1.56$) in shape. The smallest pollen grains were found in *S. conspicua*, *S. europaea* and *S. repens*, and the largest ones, in *S. peregrina* and *S. africana* (Table 2). Outline of pollen grains in equatorial view is elliptic and circular. Outline of pollen grains in polar view is slightly trilobate, trilobate, circular or circular-triangular. Colpi are long (18.62–37.24 µm), medium-length (10.64–14.63 µm) or short (6.65–13.30 µm), with distinct (in *S. africana* and *S. conspicua*), indistinct or distinct (*S. peregrina*), or indistinct (*S. europaea* and *S. repens*), uneven, rarely thickened (*S. africana* and *S. peregrina*) margins (Tables 2 and 3). Pores are lolongate, with indistinct, irregular margins (*S. repens*). Aperture membranes in the investigated species are psilate-granulate (in *S. conspicua* and *S. repens*), granulate (*S. africana* and *S. europaea*), or granulate-nanoechinate (*S. peregrina*). Exine thickness varies between 1.06 and 2.66 µm (Table 2). Sexine is thicker than nexine. Tectum is nearly equal to infratectum. Columellae are distinct in *S. africana*, *S. conspicua*, *S. europaea* and *S. repens*, or indistinct in *S. peregrina*. *Sibthorpia peregrina* has columellae short, simple, and densely arranged in mesocolpium (Fig. 3e). Exine sculpture is nanoechinate-perforate, nanoechinate, rugulate-perforate, perforate, microreticulate and reticulate (Table 3 and Figs. 3, 4).

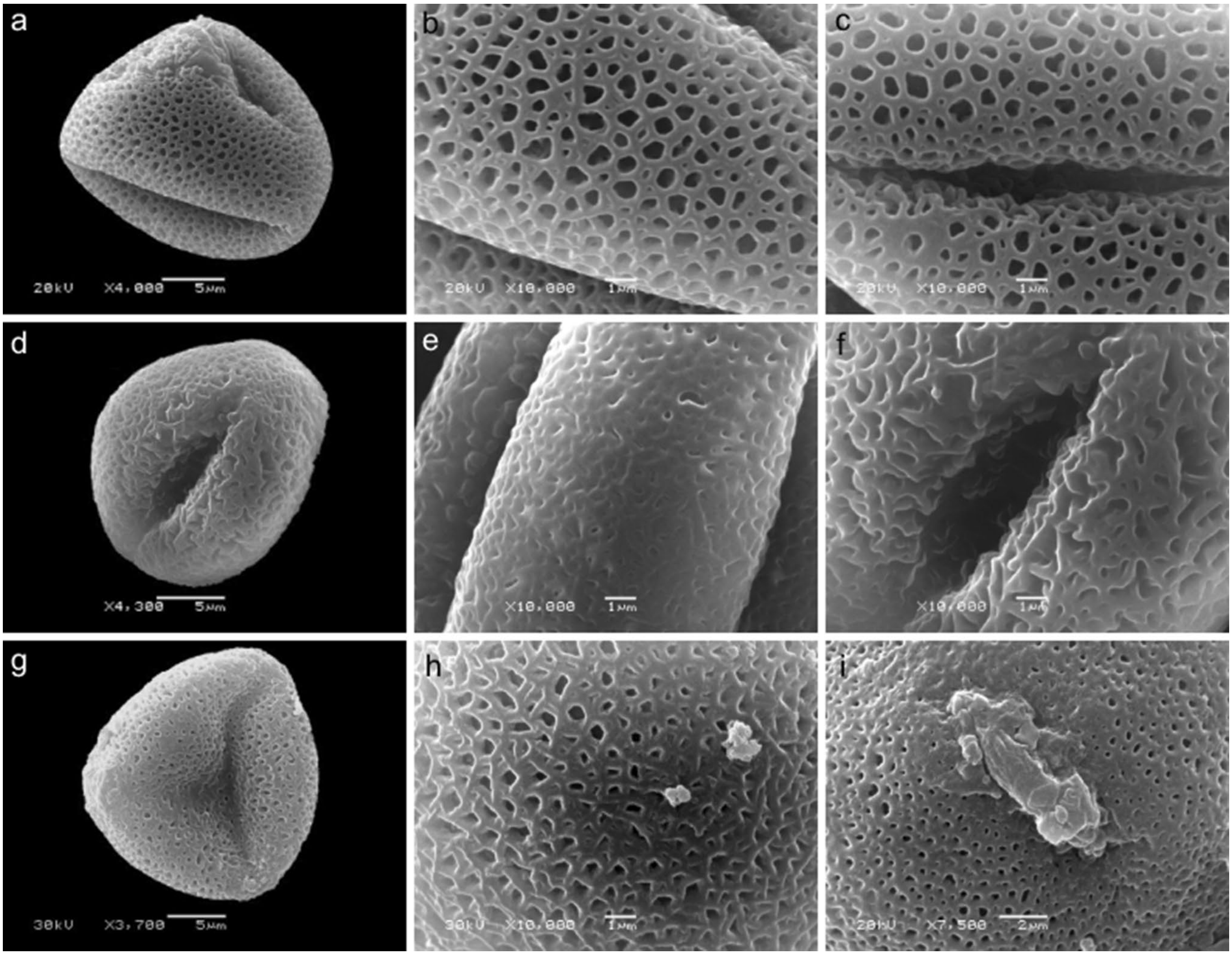


Fig. 4 Pollen grains of *Sibthorpia* (SEM). **a–c** *Sibthorpia conspicua*: **a** equatorial view, **b**, **c** Rreticulate sculpture. **d–f** *Sibthorpia europaea*: **d** equatorial view, **e** perforate sculpture, **f** microreticulate sculpture and colpus membrane granulate. **g–i** *Sibthorpia repens*: **g** polar view, **h**, **i** microreticulate sculpture, **i** pore membrane psilate-granulate

Table 4 Pollen subtypes

Taxon	Subtypes	*P*	*E*	Colpi	Exine sculpture	Exine thickness	Figures
E. pinnatum	I	30.59–42.56	25.27–34.58	Long	Rugulate-nanoechinate, nanoechinate	1.59–2.66	3b, c
S. peregrina	II	23.94–42.56	21.28–37.24	Long	Nanoechinate-perforate, nanoechinate	1.06–1.59	3f
S. africana	III	37.24–45.22	26.60–39.90	Long	Rugulate-perforate	1.99–2.66	3h, i
S. conspicua	IV	18.62–25.27	18.62–23.94	Medium-length	Reticulate	1.06–1.99	4b, c
S. europaea	V	18.62–21.28	17.29–21.28	Medium-length	Perforate, microreticulate	1.33–1.99	4e, f
S. repens	VI	18.62–26.60	19.95–26.60	Short	Microreticulate	1.33–1.99	4h, i

The data obtained demonstrated that the pollen grains of Sibthorpieae differ in their shape, outline, and size, length and width of the colpi, exine thickness, exine sculpture, and aperture membranes between species. This confirms that pollen grain characteristics are useful for species identification. Pollen grains of the studied species can be included in one type (3-colpate). This type in Sibthorpieae contains six subtypes segregated according to the exine sculpture, grain size, length of apertures, and thickness of the exine (Table 4).

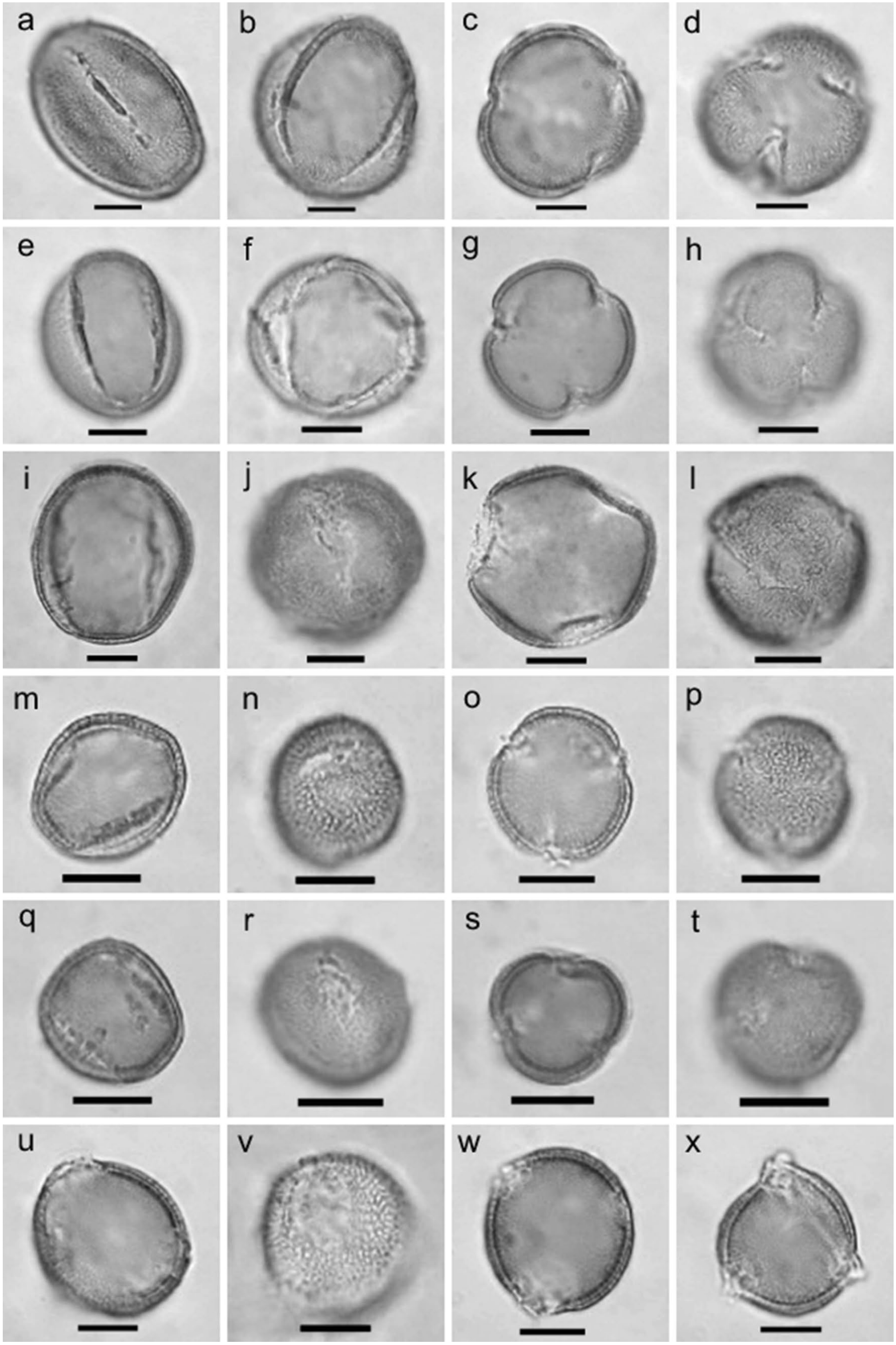
Discussion

The phylogenetic analyses based on both ITS (Fig. 1) and plastid *trn*L-F region (Fig. 2) are congruent with the hypothesis of Hedberg (1955) that *S. europaea* is sister to *S. repens* while *S. africana* is sister to *S. peregrina*. Hedberg (1955) hypothesized these relationships based on marked difference in seed and pollen size between the two species pairs, and later (Hedberg 1975) also added base chromosome numbers and crossability between the species as the characters supporting that phylogenetic scheme, which agrees with our analyses (Fig. 6). Species of *S. africana* and *S. peregrina* have the basic chromosome number $x = 10$ and larger pollen grains (Table 2; Fig. 6), while in *S. europaea*, *S. repens* and *S. conspicua* the basic chromosome number is $x = 9$. The pollen grains of these three species have smaller sizes as compared to pollen of *S. africana* and *S. peregrina* (Hedberg 1955; Juan et al. 1999; Table 2). Also, pollen grains of *S. europaea*, *S. repens* and *S. conspicua* all have perforate to reticulate exine ornamentation (Fig. 4) and also agree in their general shape and outline despite that *S. repens* is tetra- to octoploid compared to *S. europaea* based on known chromosome numbers (Hedberg 1975).

These results suggest that a long-distance dispersal event occurred across the Atlantic Ocean relatively recently, and that migration was unidirectional, from Europe to America. Thus, *Sibthorpia* adds to the known examples of Mediterranean–American disjunctions (Raven 1973). Similar to most other examples, in that case, the phylogenetic relationships suggest a Mediterranean origin of the group. However, the *Sibthorpia* case has notable differences as compared to other examples of similar disjunctions. A number of studies have demonstrated a Miocene origin of the Madrean–Tethyan type of disjunctions between California and the Mediterranean region (e.g., Wen and Ickert-Bond 2009; Vargas et al. 2014) contributing to the evolution of the typical Mediterranean floras in both regions. Others have shown even more recent origins (within the last 500.000 years) of disjunctions between both regions in plants living in deserts (e.g., Coleman et al. 2003; Meyers and Liston 2008; Martín-Bravo et al. 2009). *Sibthorpia europaea* and *S. repens*, however, do not occur in typical Mediterranean, at least seasonally arid environments but instead are mostly confined to moist and shady places of montane forests (Hedberg 1955). Additionally, they differ from other examples in their more widespread occurrence in the New World, from Mexico southward to Argentina. The timing of the disjunctions is uncertain since molecular dating in Sibthorpieae is problematic due to the scarcity of fossils in the predominantly herbaceous family, the nucleotide substitution rate heterogeneity among species, and the incongruence among the outgroup taxa (Albach et al. 2005).

The sister-group relationship previously found between *Sibthorpia* and *Ellisiophyllum* (Albach et al. 2005) has been supported here with increased taxon sampling in *Sibthorpia* and is also supported by such pollen characters as the type of apertures, exine sculpture, shape, outline, size, and exine thickness (Tables 2, 3; Figs. 3, 4, and 5). Whereas comparison with *Ellisiophyllum* may help in explaining evolutionary trends in phenotypic characters, it adds even more complexity to the biogeographic scenario in the tribe. *Ellisiophyllum* shares with *S. europaea*/*S. repens* the base chromosome number of $x = 9$ (Borgmann 1964) and with the former the white color of the flower. It shares, however, with *S. africana* / *S. peregrina* the larger pollen (Table 2) and also the larger seeds (Hong et al. 1998). Also, pollen grains of *Ellisiophyllum* are similar to those in *S. africana* and *S. peregrina* by the type of apertures, shape, and outline. The exine sculpture is rugulate-nanoechinate, nanoechinate in *Ellisiophyllum* (Fig. 3b, c), nanoechinate-perforate, nanoechinate in *S. peregrina* (Fig. 3f), and rugulate-perforate in *S. africana* (Fig. 3h, i). Biogeographically, the Himalayan-to-East Asian distribution area suggests either another case of long-distance dispersal or, in this case more likely, a Himalayan-Mediterranean vicariance event similar to the one seen in the related Veroniceae (Surina et al. 2014). Based on ancestral character estimation, the larger pollen and seeds seem to be the ancestral condition (Figs. 6 and 7) and suggest an ancient Tethyan distribution of early evolved (ancestral) Sibthorpieae. However, this character evolution needs to be considered in the light of character evolution in the family.

Pollen grains in taxa of Sibthorpieae are characterized by a perforate to reticulate exine sculpture that is common in most of species of the Russelieae–Cheloneae–Antirrhineae clades of Plantaginaceae (Tsymbalyuk 2013, 2016; Tsymbalyuk and Mosyakin 2013, 2014). Also, in *Ellisiophyllum pinnatum* and *Sibthorpia peregrina,* the types of exine sculpture were observed (such as rugulate-nanoechinate, nanoechinate, nanoechinate-perforate), which are more typical for the Veroniceae–Plantagineae clade of the family (Hong 1984; Fernández et al. 1997; Martínez-Ortega et al. 2000; Saeidi-Mehrvarz and Zarrei 2006; Tsymbalyuk 2008; Mosyakin and Tsymbalyuk 2008; Sánchez-Agudo et al. 2009; Tsymbalyuk et al. 2011; Tsymbalyuk and Mosyakin 2013; Tsymbalyuk 2016; Halbritter 2015, 2016; Halbritter and Svojtka 2016a, b). In species of *Sibthorpia,* we observed a transition from the colpate type to the porate type; the latter is also typical for representatives of some taxa of *Veronica* L., and especially for *Littorella* Asch. and *Plantago* L., but this seems to be a parallel trend. Furthermore, pollen with a perforate and reticulate exine sculpture is hypothesized to be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◂**Fig. 5** Pollen grains of *Ellisiophyllum* and *Sibthorpia* (LM): **a–d** *E. pinnatum*, **e–h** *S. peregrina*, **i–l** *S. africana*, **m–p** *S. conspicua*, **q–t** *S. europaea*, **u–x** *S. repens.* **a**, **b**, **e**, **f**, **i**, **j**, **m**, **n**, **q**, **r**, **u**, **v** Equatorial view; **c**, **d**, **g**, **h**, **k**, **l**, **o**, **p**, **s**, **t**, **w**, **x** polar view. Scale bars: **a–x** = 10 µm

a plesiomorphic condition within Plantaginaceae. The porate pollen probably represents an apomorphy in this tribe. However, this requires a more robust phylogenetic hypothesis for relationships within the family.

We noted that there is not just a topological difference between DNA regions analyzed but also between our analysis and that of Albach et al. (2005) as well as between different types of analyses (preliminary parsimony and neighbor-joining analyses and maximum likelihood analyses). Based on our experience with the dataset, we especially assume that different alignments of highly variable regions of the ITS region are prone to cause different relationships. Plantaginaceae are congruently divided into two clades, Plantaginoideae and Gratioloideae, with Sibthorpieae being one of ten tribes in the former. The five tribes, Plantagineae, Veroniceae, Digitaleae, Globularieae and Hemiphragmeae, consistently form clades in phylogenetic analyses (Albach et al. 2005; Figs. 1 and 2) but the relationship between this PVDGH-clade and the other tribes, Cheloneae, Antirrhineae, Callitricheae, Russelieae, and Sibthorpieae, differs considerably between analyses. In the analyses of ITS and the plastid *rps*16 intron of Albach et al. (2005), Sibthorpieae

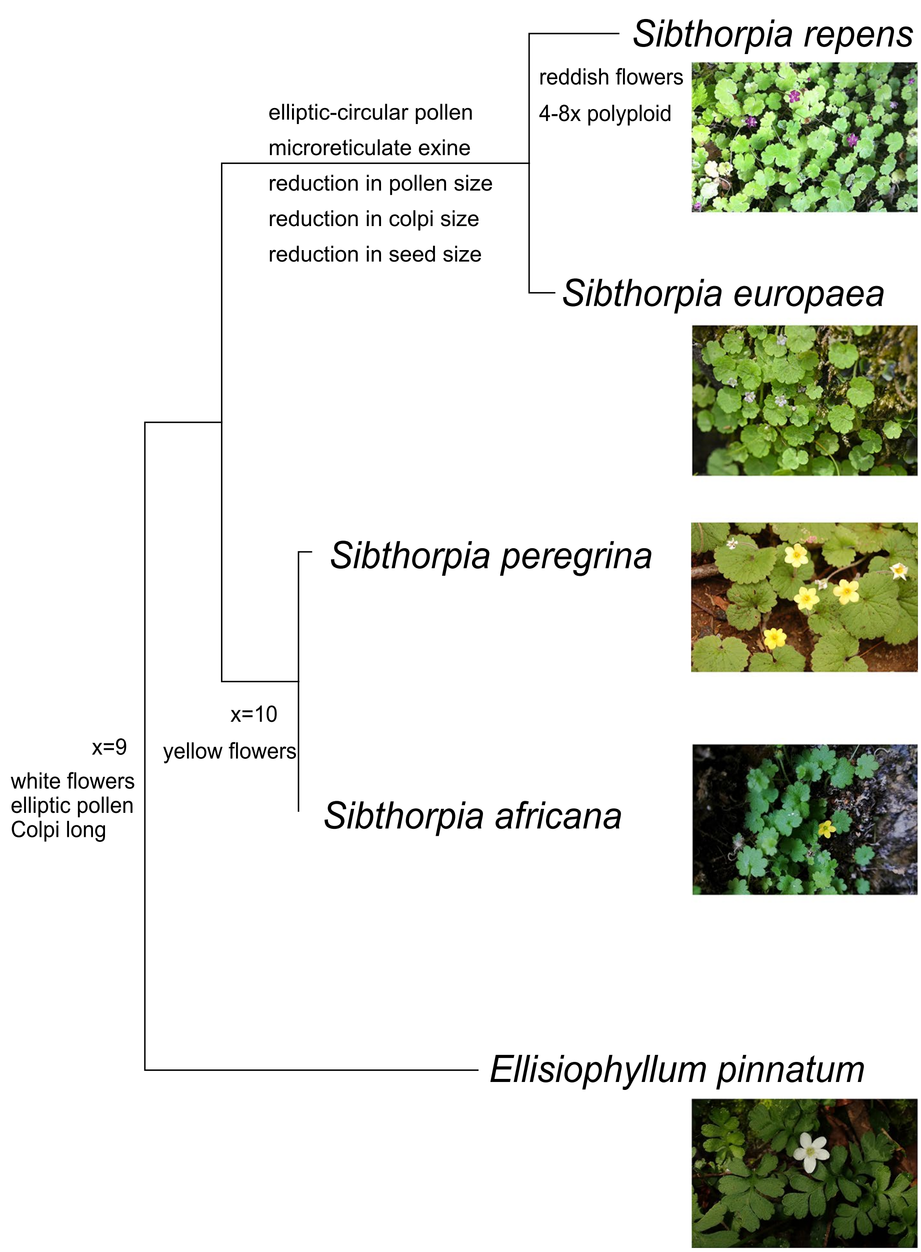
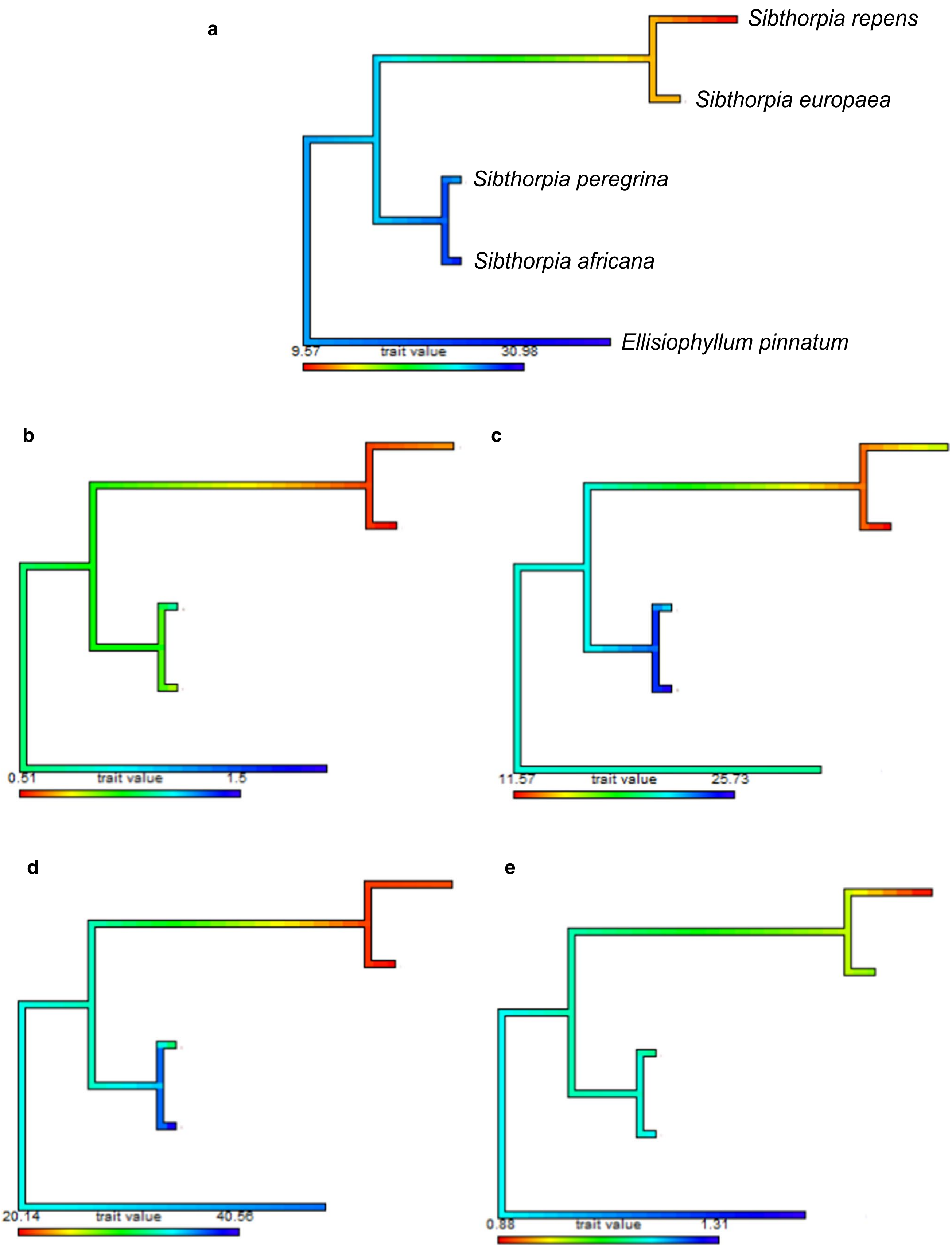


Fig. 6 Overview of relationships among species of Sibthorpieae and major innovations written on the branches on which they occurred based on phylogenetic analysis of character evolution. Photos of *Ellisiophyllum pinnatum* by Liu Jim Food, *Sibthorpia africana* by Miquel Capó Servera, *Sibthorpia peregrina* by Tim Waters, *Sibthorpia europaea* by Fotis Samaritakis, *Sibthorpia repens* by Armando Villegas


a
Sibthorpia repens
Sibthorpia europaea
Sibthorpia peregrina
Sibthorpia africana
Ellisiophyllum pinnatum
9.57
trait value
30.98
b
0.51
trait value
1.5
c
11.57
trait value
25.73
d
20.14
trait value
40.56
e
0.88
trait value
1.31

◂**Fig. 7** ContMaps of quantitative characters generated using the package phytools (Revell 2012) in RStudio v. 1.4 (RStudio Team 2021) and R version 4.0.3 (R Development Core Team 2020) using the ITS species tree restricted to Sibthorpieae: **a** colpus length, **b** seed length, **c** mesocolpium length, **d** pollen size, **e** *P*/*E* ratio

are even sister to Gratioloideae but this was not confirmed here, although in ITS it is sister to all Plantaginoideae. Based on the uncertainty in relationships between tribes of Plantaginaceae and the large variation of pollen and seed characters in the family, we will await a more robust phylogenetic hypothesis for relationships in the family to conduct a family wide analysis of pollen and seed characters.

Conclusions

The present study provides the first characterization of pollen grains of *Ellisiophyllum*. Images using scanning electron microscopy (SEM) were obtained for the first time for *Ellisiophyllum* and *S. peregrina*, *S. africana*, *S. conspicua*, and *S. repens*, which allowed more detailed descriptions of pollen characters in this group. We found variation in pollen grains morphology in Sibthorpieae, confirming its eurypalynous nature. Palynomorphological data support the placement of *Ellisiophyllum* and *Sibthorpia* in the well-defined tribe Sibthorpieae based on shared peculiarities such as shape, outline, size, exine thickness, exine sculpture, and the tricolpate type of pollen grains. The results of the current study expand the palynomorphological data for Sibthorpieae in particular and Plantaginaceae in general and will also contribute to future phylogenetic and taxonomic studies in this group.

Appendix

Ellisiophyllum pinnatum (Benth.) Makino – [China], 19 Jun 1997, *C.H. Li* [*Li Cehong*] *500* (MO).

Sibthorpia africana L. – [Spain, Islas Baleares], Mallorca, road from Puigpuñent [Puigpunyent] to Galatxo [Galatzó], calcareous rocks before the pass, ca. 750 m a. s. l., 4 Jun 1985, *A. Charpin, P. Hinz, D. Manon* and *J. Rossello 287620* (G).

Sibthorpia europaea L. – [Spain or France, precise location illegible], *Dufour* [no. *125* or *925*, the first digit barely legible] (KW-TURCZ: Turczaninow historical herbarium).

Sibthorpia conspicua Diels – [Argentina] Prov. de Salta, Dpto. Capital, Filo de cerros between Castellanos and San Lorenzo, 6–10 km to the east of Quebrada S. Lorenzo, 2000–2400 m a. s. l., in shady, humid forest, *L. Novara, S. Bruno* and *V. Novara 10143* (G).

Sibthorpia peregrina L. – [Portugal, Autonomous Region of Madeira] Pico das Pedras, 900 m a. s. l., 27 Aug 1992, *Walter Strasser s.n.* (G). [Ukraine, Ternopil Region], Culta Crem. [cultivated in Kremenets Botanical Garden by W.S.J.G. Besser] Herb. *W. Besser s.n.* (KW-BESS: Besser historical herbarium).

Sibthorpia repens (L.) Kuntze – [Mexico] Las Cruces 3350 m a. s. l., Temascaltepec, fir forest by the water, 9.21.32 [21 Aug 1932], *Hinton* et al. *1717* (MO). [Venezuela] Estado Merida: Paramo El Batallon [Páramo del Batallón], 2800–2900 m a. s. l., in humid places, 18 Nov 1976, *L. Bernardi, A. Charpin* and *F. Jacquemoud 232582* (G).

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Availability of data and material All DNA sequence data are freely available from GenBank after publication. All other data are included in the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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