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Evolution and loss of long-fringed petals: a case study using a dated phylogeny of the snake gourds, *Trichosanthes* (Cucurbitaceae)

Hugo J de Boer^{1*}, Hanno Schaefer², Mats Thulin³ and Susanne S Renner⁴

Abstract

Background: The Cucurbitaceae genus *Trichosanthes* comprises 90–100 species that occur from India to Japan and southeast to Australia and Fiji. Most species have large white or pale yellow petals with conspicuously fringed margins, the fringes sometimes several cm long. Pollination is usually by hawkmoths. Previous molecular data for a small number of species suggested that a monophyletic *Trichosanthes* might include the Asian genera *Gymnopetalum* (four species, lacking long petal fringes) and *Hodgsonia* (two species with petals fringed). Here we test these groups' relationships using a species sampling of c. 60% and 4759 nucleotides of nuclear and plastid DNA. To infer the time and direction of the geographic expansion of the *Trichosanthes* clade we employ molecular clock dating and statistical biogeographic reconstruction, and we also address the gain or loss of petal fringes.

Results: *Trichosanthes* is monophyletic as long as it includes *Gymnopetalum*, which itself is polyphyletic. The closest relative of *Trichosanthes* appears to be the sponge gourds, *Luffa*, while *Hodgsonia* is more distantly related. Of six morphology-based sections in *Trichosanthes* with more than one species, three are supported by the molecular results; two new sections appear warranted. Molecular dating and biogeographic analyses suggest an Oligocene origin of *Trichosanthes* in Eurasia or East Asia, followed by diversification and spread throughout the Malasian biogeographic region and into the Australian continent.

Conclusions: Long-fringed corollas evolved independently in *Hodgsonia* and *Trichosanthes*, followed by two losses in the latter coincident with shifts to other pollinators but not with long-distance dispersal events. Together with the Caribbean *Linnaeosicyos*, the Madagascan *Ampelosicyos* and the tropical African *Telfairia*, these cucurbit lineages represent an ideal system for more detailed studies of the evolution and function of petal fringes in plant-pollinator mutualisms.

Background

Deeply divided or fringed petal lobes are known from a range of angiosperm families, including Caryophyllaceae, Celastraceae, Cucurbitaceae, Myrtaceae, Orchidaceae, Saxifragaceae, and Tropaeolaceae [1]. While the origin and function of subdivided petals vary between groups, division of perianth edges is especially common among nocturnal hawkmoth-pollinated species (such as *Trichosanthes* [2], Figure 1), where the fringes, in combination with a light petal color, may enhance visibility and thus increase pollination success [3,4]. Experiments

have shown that diurnal and nocturnal hawkmoths are attracted by floral scent but also rely on visual clues to find and recognize flowers even at extremely low light intensity [5,6]. A preference for high contrasts might help them find their nectar sources, and it seems plausible that fringed petals enhance the sharp contrast between the petal margin and a dark background [4].

In Cucurbitaceae, long-fringed petals are known in five genera that occur in Madagascar, tropical Africa, the Caribbean, and East and Southeast Asia [7,8]. The largest of them is *Trichosanthes* with currently 90–100 species of mainly perennial, 3 to 30 m long climbers that are usually dioecious and have medium-sized fleshy fruits. Referring to the petal fringes, Linnaeus formed the genus name from the Greek words for 'hair' (genitive

* Correspondence: hugo.deboer@ebc.uu.se

¹Department of Systematic Biology, Uppsala University, Norbyvägen 18 D, Uppsala SE-75236, Sweden

Full list of author information is available at the end of the article

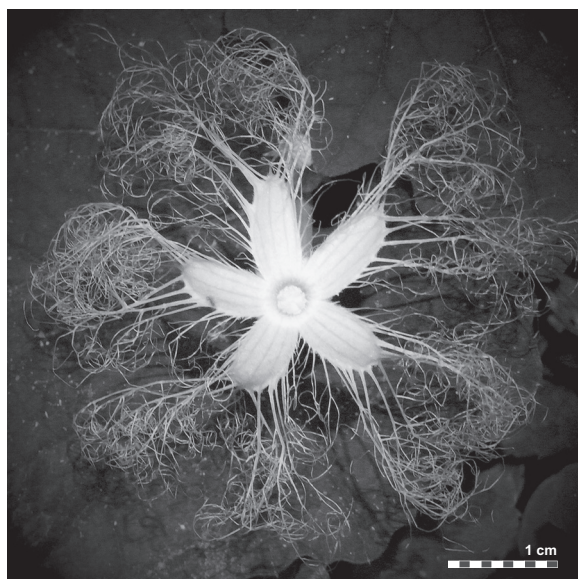


Figure 1 Fully expanded flower of *Trichosanthes pilosa* Lour. showing the characteristic feather-like fringes along the petal margins. Picture courtesy of Ken Ishikawa.

τριχός) and 'flower' (ἄνθος). *Trichosanthes* has its center of diversity in Southeast Asia, but ranges from India throughout tropical and subtropical Asia east to Japan, and southeast to New Guinea, Australia, and Fiji [9]. One species, the snake gourd, *T. cucumerina* L., is a widely cultivated vegetable in tropical and subtropical regions around the globe, and another 15 species are commonly used in Asian traditional medicine [10]. While floristic treatments are available for most of its range [9,11-16], a comprehensive revision of the nearly 300 names published in *Trichosanthes* is lacking (but see [17] for a synopsis).

Trichosanthes belongs in the tribe Sicyoeae, a group of 12 genera and c. 270 species that is supported by morphological and molecular data [18]. Based on a limited number of *Trichosanthes* species sequenced, it appeared that the genus might be paraphyletic, with the genera *Gymnopetalum* Arn. (four species; [19]) and *Hodgsonia* Hook.f. & Thomson (two species; [9]) possibly nested inside it [20]. Both share with *Trichosanthes* the white flowers, elongated receptacle-tubes, and free filaments. *Hodgsonia* also has long-fringed petals (Figure 2), but differs from *Trichosanthes* and *Gymnopetalum* in its much larger fruits (up to 25 cm across) and unusual seeds. The petal margins in *Gymnopetalum* are entire (Figure 2A, 2E) or in one species shortly fimbriate [9]. Geographically, *Gymnopetalum* and *Hodgsonia* largely overlap with the distribution area of *Trichosanthes* except for their absence from New Guinea and Australia,

and from much of the northeastern range of *Trichosanthes* (temperate China, Taiwan, Japan) [9].

Based on mainly fruit and seed characters, the 43 species of *Trichosanthes* occurring in the *Flora Malesiana* region have been grouped into six sections, the typical sect. *Trichosanthes* and sections *Cucumeroides* (Gaertn.) Kitam., *Edulis* Rugayah, *Foliobracteola* C.Y.Cheng & Yueh, *Involucraria* (Ser.) Wight, and *Asterosperma* W.J. de Wilde & Duyfjes [21,22]. The mainland Asian species, *T. truncata* C.B.Clarke, is in its own section, *Truncata* C.Y.Cheng & C.H.Yueh [23]. The four species of *Gymnopetalum* have been allocated to two sections that differ in flower morphology, the typical sect. *Gymnopetalum* with just one species from southern India and Sri Lanka and sect. *Tripodanthera* (M.Roem.) Cogn. with three southeast Asian and Malesian species [24].

Here we test the monophyly and phylogenetic placement of *Trichosanthes* using a broad sampling of some 60% of its species, including the type species of each section name, plus representatives of *Gymnopetalum*, *Hodgsonia*, and other Sicyoeae as well as more distant outgroups. The well-resolved phylogeny, combined with field observations on flower shape and color, allows us to test whether petal fringes in Old World Sicyoeae evolved just once as would be the case if *Gymnopetalum* and *Hodgsonia* were nested inside it [20] or multiple times as would be implied by these genera having separate evolutionary histories. A combination of molecular-dating and ancestral area reconstruction permits reconstructing the biogeographical history of the *Trichosanthes* clade.

Results and discussion

Phylogenetic analyses and taxonomy

Phylogenies obtained under Bayesian or Maximum Likelihood (ML) optimization revealed no statistically supported incongruences, defined as nodes with Bayesian posterior probabilities (PP) >0.95 or ML bootstrap support >75. A Bayesian consensus tree is shown in Figure 2. It reveals that the genus *Trichosanthes* is paraphyletic because *Gymnopetalum* is embedded in it, while *Gymnopetalum* is polyphyletic because its four species do not group together. Instead, *G. tubiflorum* (Wight & Arn.) Cogn. groups with species from sections *Trichosanthes* and *Cucumeroides* (1.00 PP/84 ML support), while *G. orientale* W.J.de Wilde & Duyfjes, *G. chinense* (Lour.) Merr., and *G. scabrum* (Lour.) W.J. de Wilde & Duyfjes are sister to section *Edulis* (1.00 PP/86 ML). The *Trichosanthes*/*Gymnopetalum* clade (56 species sampled; 0.99 PP/62 ML support) is sister to *Luffa*, a genus of seven or eight species of which we included five. This sister group relationship, however, is only weakly supported (Figure 2). The genus *Hodgsonia* (two species with long-fringed flowers,

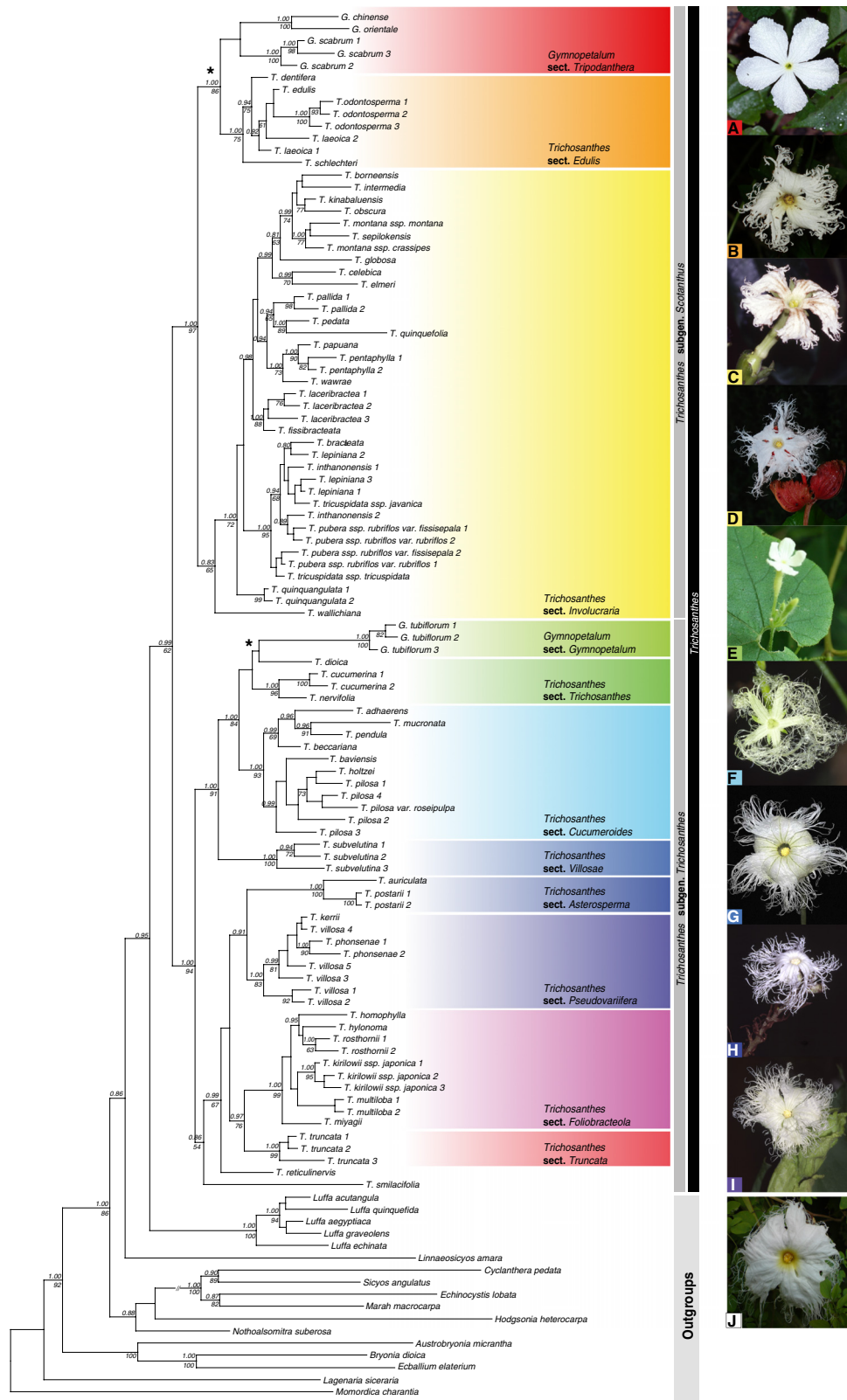


Figure 2 (See legend on next page.)

(See figure on previous page.)

Figure 2 Bayesian consensus tree with posterior probabilities (>0.80) and maximum likelihood bootstrap values (>60%) shown at the nodes. Photos on the right illustrate the floral morphology of the different sections and belong to the following species: **A)** *Gymnopetalum chinense*; **B)** *Trichosanthes odontosperma*; **C)** *Trichosanthes montana* ssp. *crassipes*; **D)** *Trichosanthes pubera* ssp. *rubriflos*; **E)** *Gymnopetalum tubiflorum*; **F)** *Trichosanthes beccariana*; **G)** *Trichosanthes subvelutina*; **H)** *Trichosanthes postarii*; **I)** *Trichosanthes villosa*. Pictures courtesy of W. J. de Wilde and B. Duyfjes (**A, C, D, F, H, I**), W. E. Cooper (**B**), N. Filipowicz (**E**), H. Nicholson (**G**), and P. Brownless (**J**). Inferred losses of petal fringes are marked by an asterisk.

one sampled here) is only distantly related to the *Trichosanthes/Gymnopetalum* clade.

Of the seven sections previously proposed in *Trichosanthes* (see *Background*), three are supported by the molecular results, namely sections *Asterosperma* (1.00 PP/100 ML; three species, two of them sampled here), *Cucumeroides* (1.00 PP/93 ML; seven species, five sampled), and *Edulis* (1.00 PP/75 ML; nine species, five sampled). Three other sections with more than one species (*Involucraria*, *Foliobracteola*, *Trichosanthes*) are not monophyletic in their current circumscriptions. To achieve a more natural classification, a revised infrageneric classification has been proposed including two new sections [17].

The biogeographic history of the *Trichosanthes* clade

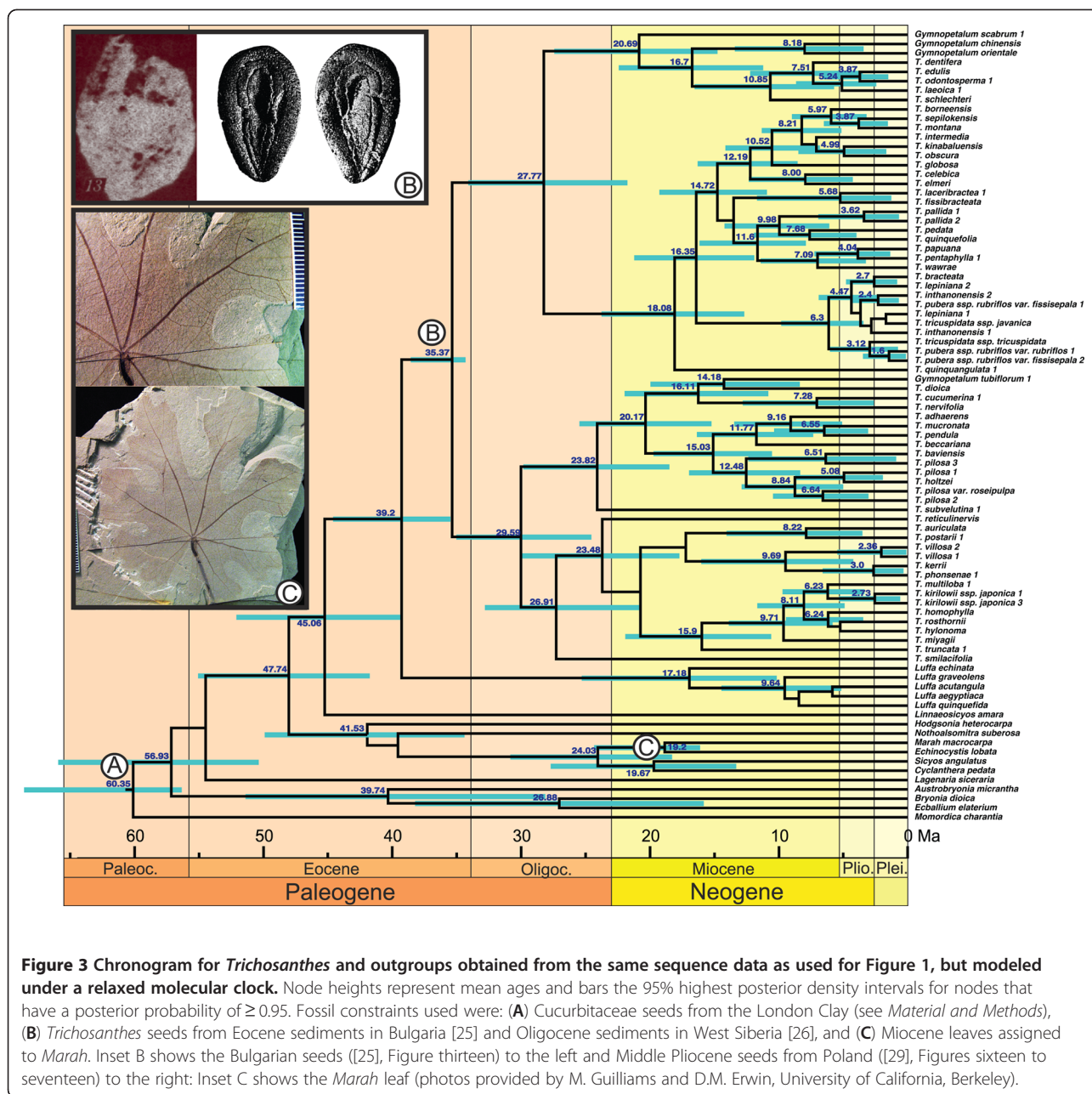
Based on a fossil-calibrated Bayesian relaxed molecular clock model, *Trichosanthes* originated during the Oligocene (Figure 3), an estimate influenced by our prior constraint of the crown node of the *Trichosanthes/Gymnopetalum* clade to 34 Ma. This constraint is based on *Trichosanthes*-like seeds from the Upper Eocene of Bulgaria [25] dating to c. 34 Ma and seeds from the Oligocene of West Siberia [26] dating to c. 23.8 Ma [27]. Seeds assigned to *Trichosanthes* have also been reported from Miocene and Pliocene sites in France, Germany, Italy, and Poland [28-30], and Pliocene *Trichosanthes*-like leaves are known from France [31]. The biogeographic analysis (Figure 4) inferred an East Asian origin of the genus (region C in Figure 4), but this inference is based only on the living species, while the just-discussed fossils indicate a more northern (Eurasian) range of *Trichosanthes* before the global climate cooling at the end of the Oligocene. Many other extinct elements of the European Oligocene, Miocene, and Pliocene floras, such as *Taxodium*, *Craigia*, *Fagus kraeuselii*, *Ilex*, and tropical Araceae, such as *Caladiosoma*, also have nearest living relatives in tropical Southeast Asia [31,32].

Collision between the Eurasian and Australian tectonic plates started in the Late Oligocene, about 25 Ma ago, and the Sahul Shelf (carrying New Guinea) and Sunda Shelf (Sumatra, Java, and Borneo) reached their present proximity only by the Late Miocene, some 10 Ma [33,34]. Mid-Miocene pollen records indicate a warm, moist climate and rainforest expansion on these newly forming islands [35], allowing groups adapted to humid

forest conditions, such as the liana clade *Trichosanthes*, to spread and diversify. Such plant groups would have benefited from land bridges that during times of sea level changes repeatedly connected New Guinea and Australia on the one hand, and Indochina, Sumatra, Java, and Borneo on the other. The lowest sea levels, during the last glacial maximum (LGM), were approximately 120 m below those of today, resulting in the complete exposure of the Sunda Shelf; even sea level reduction by just 40 m already connected Indochina, Sumatra, Java, and Borneo [35,36]. No land bridges, however, ever connected the islands on the Sunda Shelf with those in "Wallacea," that is, Sulawesi, the Moluccas, and the Lesser Sunda Islands, or the latter with New Guinea and Australia on the Sahul Shelf. In zoogeography, these two boundaries are known as Wallace's Line and Lydekker's line, but their significance as floristic boundaries is doubtful [37,38].

The most striking transoceanic disjunctions in *Trichosanthes* are numbered in Figure 4. They are (i) the disjunction between the Australian species *T. subvelutina* F.Muell. ex Cogn. and its sister clade on the Asian mainland and areas of the Sunda Shelf, dated to 23.8 (29.4-18.4) Ma; (ii) the disjunction between *T. edulis* Rugayah, *T. dentifera* Rugayah, *T. laeioica* C.Y.Cheng & L.Q.Huang, *T. schlechteri* Harms from New Guinea, and *T. odontosperma* W.E.Cooper & A.J.Ford from Australia on the one hand, and *Gymnopetalum chinense*, widespread in Asia as far East as Flores, and *G. orientale* in Sulawesi, the Lesser Sunda Islands, and the Moluccas on the other (this is dated to 16.7 (22.1-11.2) Ma, but the position of *G. scabrum* relative to *G. chinense* and *G. orientale* remains unclear; compare Figures 2, 3, and 4); and (iii) the disjunction between *T. wawrae* Cogn. from Thailand, peninsular Malaysia, Sumatra, and Borneo, and its sister clade *T. papuana* F.M.Bailey/*T. pentaphylla* F. Muell. ex Benth. from New Guinea and Australia, which dates to 7.1 (11.2-3.3) Ma.

Trichosanthes range expansion between New Guinea and Australia occurred during the Pliocene/Pleistocene, when these two regions were repeatedly connected due to the above-mentioned sea level changes [36]. Thus, the estimated divergence time of the Australian species *T. odontosperma* (a member of clade ii in Figure 4) from its New Guinean sister species, *T. edulis*, is 3.9 (6.4-1.6) Ma, while that of the sister species pair *T. papuana* from



the Aru Islands and New Guinea, and *T. pentaphylla* from Australia (clade iii in Figure 4) is 4.0 (7.1-1.4) Ma; considering their error ranges, these ages fall in the Pliocene/Pleistocene.

The geographic history of *T. pilosa* Lour. (including the synonyms *T. baviensis* Gagnep. and *T. holtzei* F.Muell. [16]), a widespread species here represented by seven samples from Queensland (Australia), Thailand, Vietnam, and Japan, cannot be inferred because the within-species relationships lack statistical support (Figure 2). Inferring the origin of the snake gourd, *T. cucumerina*, a vegetable cultivated in tropical and subtropical regions around the

globe (represented by a single sample from Sri Lanka) also would require population-level sampling. Both species have fleshy red fruits and small seeds, probably dispersed by birds.

Evolution and loss of petal fringes

The phylogeny obtained here implies that long-fringed corollas evolved independently in the Asian genera *Hodgsonia* and *Trichosanthes* and were lost in three of the four species formerly placed in the genus *Gymnometalum* (petals still bear c. 5 mm-long fringes in *G. orientale*). The two inferred losses (marked with an asterisk

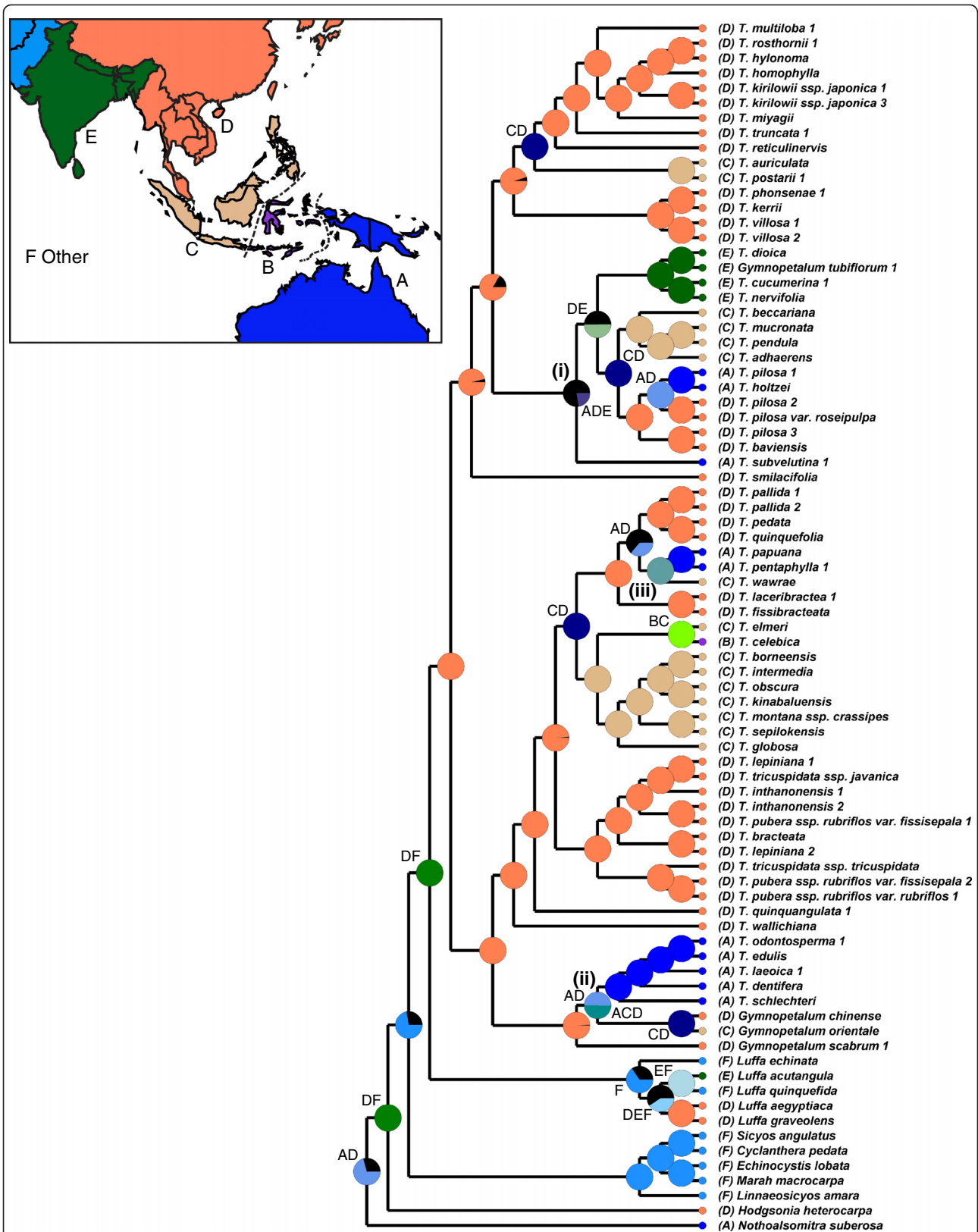


Figure 4 (See legend on next page.)

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Figure 4 Ancestral range reconstruction for *Trichosanthes* and outgroups inferred on 8000 output trees resulting from the Bayesian dating analysis and distribution ranges for all species. Letters in the legend correspond to the colored distribution ranges in the map (inset), and letters adjacent to taxon names correspond to the geographic origin of the sampled plant. Wallace's Line is shown as a broken line between Borneo and Sulawesi, Lydekker's Line is shown as a broken line between New Guinea and the Moluccas. The three numbered clades and inferred transoceanic disjunctions are discussed in the text.

in Figure 2) coincide with shifts from nocturnal to diurnal flowering times (HS personal observation of *G. scaberrimum* and *G. chinense* in Cambodia, Jan. 2010, and China, Sept. 2005; N. Filipowicz, Medical University of Gdansk, personal observation of *G. tubiflorum* in India, Nov. 2010), and it therefore seems likely that there is a shift from predominantly nocturnal sphingid pollinators to diurnal bee or butterfly pollinators. The loss of fringes does not coincide with long-distance dispersal events to insular habitats (where hawkmoths might be absent), and the trigger for the pollinator shifts so far is unknown.

The adaptive function of the corolla fringes in pollinator attraction requires experimental study. An innate preference for radial patterns [39] and high contrasts might help hawkmoths find their nectar sources [5,6], and one possible explanation for the evolution of fringed petals is that they help create such a radial pattern and sharper contrasts between the petals and a dark background [4]. In a diurnal, hawkmoth-pollinated *Viola* species, more complex corolla outlines correlate with higher fruit set [40] but it remains to be tested if this is also the case in the nocturnal *Trichosanthes*-hawkmoth system. Another untested possibility is that the fringes with their highly increased surface area and exposed position might be involved in scent production (B. Schlumpberger, Herrenhaeuser Gardens, Hannover, pers. comm., Feb. 2012) or produce a waving motion, which has been shown to increase pollinator attraction in other systems [41]. Anatomical studies of the petal tissue of *Trichosanthes*, wind tunnel experiments with naive hawkmoths, and detailed field observations are required to test these possibilities.

Conclusions

Molecular evidence supports the inclusion of *Gymnopetalum* into a then monophyletic *Trichosanthes* [17]. Our molecular phylogenies reveal that long-fringed petals evolved independently in *Hodgsonia* and *Trichosanthes*/*Gymnopetalum*, followed by two losses of corolla fringes in the latter clade, most likely associated with pollinator shifts. Molecular dating and a biogeographic analysis indicate an Oligocene initial diversification of *Trichosanthes* in mainland Asia. The lineage then diversified and spread in Malaysia (the Malesian biogeographic region) during the late Miocene and Pliocene, reaching the Australian continent several times.

Methods

Morphology

Herbarium specimens from A, BRI, CNS, E, GH, K, KUN, KYO, L, LE, M, MO, P, S, UC, UPS and US were obtained on loan or studied during herbarium visits. Determination of herbarium material was verified using identification keys [9,11-16,19,42]. All species in *Trichosanthes* have corolla fringes, and these are absent in three of the four *Gymnopetalum* species, except *G. orientale*, which can have short-fimbriate petal margins (fringes up to 5 mm length).

Sampling, DNA extraction and amplification

We included six DNA regions, namely the nuclear ribosomal ITS region (ITS1-5.8S-ITS2), the chloroplast genes *rbcL* and *matK*, the *trnL* and *trnL-trnF* intron and spacer, and *rpl20-rps12* spacer. Data for *rbcL* and the *trnL* region were taken from previous studies [7,18,20,43,44]. Only plant samples for which two or more markers were successfully sequenced were included in the analyses, and the combined dataset included one of the two species of *Hodgsonia*, all four of *Gymnopetalum*, and 52 of *Trichosanthes*, representing approximately 60% of the accepted species in the latter genus. Type species of all sections were included: *Gymnopetalum tubiflorum* (Wight & Arn.) Cogn. (*G. sect. Gymnopetalum*), *Gymnopetalum chinense* (Lour.) Merr. (*G. sect. Tripodanthera*), *Trichosanthes postarii* W.J.de Wilde & Duyfjes (*T. sect. Asterosperma*), *Trichosanthes pilosa* Lour. (*T. sect. Cucumeroides*), *Trichosanthes edulis* Rugayah (*T. sect. Edulis*), *Trichosanthes kirilowii* Maxim. (*T. sect. Foliobracteola*), *Trichosanthes wallichiana* (Ser.) Wight (*T. sect. Involucraria*), *Trichosanthes villosa* Blume (*T. sect. Pseudovariifera*), *Trichosanthes cucumerina* L. (*T. sect. Trichosanthes*), *Trichosanthes truncata* C.B. Clarke (*T. sect. Truncata*), *Trichosanthes subvelutina* F. Muell. ex Cogn. (*T. sect. Villosae*). Species names and their authors, specimen voucher information, and GenBank accession numbers for all sequenced markers (including 262 new sequences) are summarized in Table 1.

Total DNA was extracted using the Carlson/Yoon DNA isolation procedure [45] and a Mini-Beadbeater (BioSpec Products) to pulverize the plant material. Extracts were purified using the GE Illustra GFX™ PCR DNA and Gel Band Purification Kit following the standard protocol.

Table 1 Voucher information and GenBank accession numbers

| Species | No. | Voucher (Herbarium) | Origin of the sequenced material | ITS | <i>rpl20-rps12</i> IS | <i>matK</i> | <i>rbcl</i> | <i>trnL-trnF</i> IS | <i>trnL</i> intron |
|---|-----|--|--|--------------|-----------------------|--------------|--------------|---------------------|--------------------|
| <i>Austrobyronia micrantha</i> (F.Muell.) I.Telford | | <i>I. R. Telford</i> 8173 (CANB) | Australia, New South Wales | EF487546 | EF487567 | EF487559 | EF487552 | EF487575 | EF487575 |
| <i>Bryonia dioica</i> Jacq. | | (1) <i>S. Renner</i> 2187 (M) (2) <i>A. Faure</i> 66/76 (M) | (1) Switzerland, cult. BG Zürich (2) Algeria, Lamoriciere | (2) EU102709 | (1) DQ648157 | (1) DQ536641 | (1) DQ536791 | (1) DQ536791 | (1) DQ536791 |
| <i>Cyclanthera pedata</i> (L.) Schrad. | | <i>S. Renner</i> et al. 2767 (M) | Germany, cult. BG Mainz | HE661293 | DQ648172 | DQ536667 | DQ535749 | DQ536767 | DQ536767 |
| <i>Ecballium elaterium</i> (L.) A.Rich. ssp. <i>elaterium</i> | | (1) <i>M. Chase</i> 922 (K) (2) <i>S. Renner</i> et al. 2768 (M) | (1) UK, cult. RBG-K (2) Germany, cult. BG Mainz | (2) EU102746 | (1) AY968541 | (1) AY973019 | (1) AY973023 | (1) AY973006 | (1) AY973006 |
| <i>Echinocystis lobata</i> (Michx.) Torr. & A.Gray | | <i>S. Renner</i> et al. 2829 (M) | Germany, cult. BG Mainz | - | DQ648174 | DQ536673 | DQ535809 | DQ536814 | DQ536814 |
| <i>Gymnopetalum chinense</i> (Lour.) Merr. | | <i>H. Schaefer</i> 2005/661 (M) | China, Guangdong | HE661294 | EU155612 | EU155606 | EU155601 | EU155621 | EU155630 |
| <i>Gymnopetalum orientale</i> W.J. de Wilde & Duyfjes | | <i>M. van Balgooy</i> 7553 (L) | Indonesia, Bali | HE661301 | HE661468 | HE661397 | - | - | - |
| <i>Gymnopetalum scabrum</i> (Lour.) W.J. de Wilde & Duyfjes | 1 | <i>W. de Wilde & B. Duyfjes</i> 22269 (L) | Thailand, Central | HE661295 | DQ536556 | DQ536683 | DQ535754 | DQ536824 | DQ536824 |
| <i>Gymnopetalum scabrum</i> (Lour.) W.J. de Wilde & Duyfjes | 2 | <i>J. Maxwell</i> 16-11-2002 (CMU) | Thailand | HE661296 | HE661469 | HE661398 | - | - | - |
| <i>Gymnopetalum scabrum</i> (Lour.) W.J. de Wilde & Duyfjes | 3 | <i>C.H. Wong, J. Helm & J. Schultze-Motel</i> 2071 (LE) | China, Hainan | HE661297 | HE661470 | HE661399 | - | - | - |
| <i>Gymnopetalum tubiflorum</i> (Wight & Arn.) Cogn. | 1 | <i>N. Filipowicz & Z. Van Herwijnen</i> NF25a (M) | India, Kerala | HE661298 | HE661471 | HE661400 | - | - | - |
| <i>Gymnopetalum tubiflorum</i> (Wight & Arn.) Cogn. | 2 | <i>A. Alston</i> 1670 (UC) | Sri Lanka, Veragantota | HE661299 | HE661472 | HE661401 | - | - | - |
| <i>Gymnopetalum tubiflorum</i> (Wight & Arn.) Cogn. | 3 | <i>G.H.K. Thwaites</i> CP1625 (K) | Sri Lanka | HE661300 | HE661473 | HE661402 | - | - | - |
| <i>Hodgsonia heteroclita</i> Hook.f. & Thomson | | (1) <i>P. Phonsena</i> 4705 (L) (2) <i>L. Loeffler</i> s.n. (M) | (1) Thailand, Nan (2) Bangladesh | (1) HE661302 | (1) HE661474 | (1) HE661403 | - | (2) EU155631 | - |
| <i>Lagenaria siceraria</i> (Molina) Standl. | | <i>M. Merello</i> 1331 (MO) | Ghana | HE661303 | HE661475 | HE661404 | AY935747 | AY935788 | AY968570 |
| <i>Linnaeosicyos amara</i> (L.) H.Schaefer. & Kocyan | | <i>M. Mejia, J. Pimentel & R. Garcia</i> 1877 (NY) | Dominican Republic | HE661304 | DQ536602 | DQ536741 | DQ535774 | DQ536873 | DQ536873 |
| <i>Luffa acutangula</i> (L.) Roxb. | | (1) <i>S. Renner</i> et al. 2757 (M), seeds from D. S. Decker-Walters & A. Wagner TCN 1130 (FTG) (2) <i>LX. Zhou</i> s.n., no voucher | (1) Germany, cult. BG Munich, seeds from India, Ahmadnagar, Maharashtra (2) China, cult. BG Guangzhou | (1) HE661305 | (1) HE661476 | (2) DQ536695 | (2) DQ535826 | (2) DQ536835 | (2) DQ536835 |

Table 1 Voucher information and GenBank accession numbers (Continued)

| | | | | | | | | |
|--|--|--|---------------|--------------|--------------|--------------|--------------|--------------|
| <i>Luffa aegyptiaca</i> Mill. (incl. <i>L. cylindrica</i> L.) | D.Z. Zhang 15 April 2003, no voucher | China, cult. BG Guangzhou | HE661306 | HE661477 | HE661405 | DQ535827 | DQ536836 | DQ536836 |
| <i>Luffa echinata</i> Roxb. | G. Schweinfurth 555 (M) | Egypt | HE661307 | HE661478 | HE661406 | - | EU436357 | EU436357 |
| <i>Luffa graveolens</i> Roxb. | S. Renner & A. Kocyan 2758 (M), seeds from D. Decker-Walters 1543 (FTG 121855) | Germany, cult. BG Munich, seeds from India, USDA PI540921 | HE661308 | EU436334 | EU436409 | EU436385 | EU436358 | EU436358 |
| <i>Luffa quinquefida</i> (Hook. & Arn.) Seemann | (1) R. Berhaut 7308 (M) | (1) Senegal | (2) HQ201986 | (1) EU436335 | (2) DQ536697 | - | (1) EU436359 | - |
| | (2) S. Renner & A. Kocyan 2754 (M), seeds from D. S. Decker-Walters TCN 1440 (FTG 118010) | (2) Germany, cult. BG Munich, seeds originally from Louisiana, USA | | | | | | |
| <i>Marah macrocarpa</i> (Greene) Greene | (1) M. Olson s.n. (MO) | (1) USA, Sonoran Desert | (2) AF11906-7 | (1) DQ536566 | (2) AY968453 | (2) AY968524 | (1) AY968387 | (1) AY968571 |
| | (2) D. Arisa & S. Swensen 1009 (RSA) | (2) USA, Sonoran Desert | | | | | | |
| <i>Momordica charantia</i> L. | S. Renner et al. 2775 (M) | Germany, cult. BG Munich | HE661309 | DQ491013 | DQ491019 | DQ535760 | DQ501269 | DQ501269 |
| <i>Nothoalsomitra suberosa</i> (F.M.Bailey) I.Telford | I. Telford 12487 (NE) | Australia, SE Queensland | HE661310 | DQ536575 | DQ536709 | DQ535762 | DQ536844 | DQ536844 |
| <i>Sicyos angulatus</i> L. | M. Chase 979 (K) | North America | HE661311 | DQ648189 | DQ536732 | DQ535847 | DQ536777 | DQ536777 |
| <i>Trichosanthes adhaerens</i> W.J. de Wilde & Duyfjes | S. Lim, J. J. Postar & G. Markus SAN 143273 (L) | Malaysia, Borneo, Sabah | HE661312 | HE661479 | - | - | - | - |
| <i>Trichosanthes auriculata</i> Rugayah | A. Kalat, I. Abdullah, & J. Clayton BRUN 17016 (L) | Borneo, Brunei | HE661313 | HE661480 | HE661407 | - | - | - |
| <i>Trichosanthes baviensis</i> Gagnep. | N.M. Cuong 1248 (P) | Vietnam | HE661314 | HE661481 | - | - | - | - |
| <i>Trichosanthes beccariana</i> Cogn. ssp. <i>beccariana</i> | W. de Wilde et al. SAN 142229 (L) | Malaysia, Borneo, Sabah | HE661315 | HE661482 | HE661408 | - | - | - |
| <i>Trichosanthes borneensis</i> Cogn. | C. Argent et al. 93127 (E) | Indonesia, Borneo, Kalimantan Timur | HE661316 | HE661483 | - | - | - | - |
| <i>Trichosanthes bracteata</i> (Lam.) Voigt | T. Haegele 20 (M) | India, Kochin | HE661317 | HE661484 | EU155608 | EU155602 | EU155622 | EU155632 |
| <i>Trichosanthes celebica</i> Cogn. | W. de Wilde & B. Duyfjes 21903 (L) | Indonesia, Sulawesi | HE661318 | HE661485 | HE661409 | - | - | - |
| <i>Trichosanthes cucumerina</i> L. | 1 H. Schaefer 2007/327 (M) | Germany, cult. BG Munich | HE661319 | EU155614 | EU155609 | EU155603 | EU155623 | EU155633 |
| <i>Trichosanthes cucumerina</i> L. | 2 N. Lundqvist 11380 (UPS) | Sri Lanka | HE661320 | HE661486 | HE661410 | - | - | - |
| <i>Trichosanthes dentifera</i> Rugayah | J.H.L. Waterhouse 445-B (L) | Papua New Guinea, Bougainville Is. | HE661321 | HE661487 | - | - | - | - |
| <i>Trichosanthes dioica</i> Roxb. | O. Polunin, W. Sykes & J. Williams 5925 (E) | Nepal | HE661322 | HE661488 | HE661411 | - | - | - |
| <i>Trichosanthes edulis</i> Rugayah | W. Avé 4076 (L) | Indonesia, Irian Jaya | HE661323 | HE661489 | HE661412 | - | - | - |

Table 1 Voucher information and GenBank accession numbers (Continued)

| | | | | | | | | | |
|--|---|---|-------------------------------------|----------|----------|----------|----------|----------|----------|
| <i>Trichosanthes elmeri</i> Merr. | | <i>E.F.J. Campbell</i> 43 (E) | Malaysia, Borneo, Sabah | HE661324 | HE661490 | - | - | - | - |
| <i>Trichosanthes globosa</i> Blume | | <i>W. de Wilde</i> et al. SAN 144003 (L) | Malaysia, Borneo, Sabah | HE661325 | HE661491 | HE661413 | - | - | - |
| <i>Trichosanthes holtzei</i> F.Muell. | | <i>B. Gray</i> 7482 (CNS) | Australia, N Queensland | HE661326 | HE661492 | HE661414 | - | - | - |
| <i>Trichosanthes homophylla</i> Hayata | | <i>Y.-C. Kao</i> 499 (GH) | Taiwan | HE661327 | HE661493 | HE661415 | - | - | - |
| <i>Trichosanthes hylonoma</i> Hand.-Mazz. | | <i>Wuling Mt Exp</i> 1646 (KUN) | China | HE661328 | HE661494 | HE661416 | - | - | - |
| <i>Trichosanthes intermedia</i> W.J. de Wilde & Duyfjes | | <i>V. Julaihi</i> et al. S 76602 (L) | Malaysia, Borneo, Sarawak | HE661329 | HE661495 | - | - | - | - |
| <i>Trichosanthes inthanonensis</i> Duyfjes & Pruesapan | 1 | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 3930 (L) | Thailand, Chiang Mai | HE661330 | HE661496 | HE661417 | - | - | - |
| <i>Trichosanthes inthanonensis</i> Duyfjes & Pruesapan | 2 | <i>K. Pruesapan</i> et al. 67 (L) | Thailand, Kanchanaburi | HE661331 | HE661497 | HE661418 | - | - | - |
| <i>Trichosanthes kerrii</i> Craib | | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 3959 (L) | Thailand, Nan | HE661333 | HE661498 | - | - | - | - |
| <i>Trichosanthes kinabaluensis</i> Rugayah | | <i>J. Postar</i> et al. SAN 144260 (L) | Malaysia, Borneo, Sabah | HE661334 | EU155615 | HE661419 | - | EU155624 | EU155634 |
| <i>Trichosanthes kirilowii</i> Maxim. var. <i>japonica</i> (Miq.) Kitam. | 3 | <i>H. Takahashi</i> 20711 (GIFU) | Japan | HE661335 | DQ536603 | DQ536742 | DQ535855 | DQ536874 | DQ536874 |
| <i>Trichosanthes kirilowii</i> Maxim. var. <i>japonica</i> (Miq.) Kitam. | 1 | <i>K. Kondo</i> 05090401e (KYO) | Japan | HE661332 | HE661499 | HE661420 | - | - | - |
| <i>Trichosanthes kirilowii</i> Maxim. var. <i>japonica</i> (Miq.) Kitam. | 2 | <i>K. Deguchi, K. Uchida, K. Shiino & H. Hideshima</i> s.n. (KYO) | Japan | - | HE661500 | HE661421 | - | - | - |
| <i>Trichosanthes laceribractea</i> Hayata | 1 | <i>S. Fujii</i> 9623 (KYO) | Japan | HE661336 | HE661501 | HE661422 | - | - | - |
| <i>Trichosanthes laceribractea</i> Hayata | 2 | <i>S. Fujii</i> 9978 (KYO) | Japan | HE661337 | HE661502 | HE661423 | - | - | - |
| <i>Trichosanthes laceribractea</i> Hayata | 3 | <i>Liang Deng</i> 7090 (KUN) | China | HE661338 | HE661503 | - | - | - | - |
| <i>Trichosanthes laeoica</i> C.Y.Cheng & L.Q.Huang | 1 | <i>M. Coode</i> et al. NGF 32585 (E) | Papua New Guinea, Eastern Highlands | HE661339 | HE661504 | - | - | - | - |
| <i>Trichosanthes laeoica</i> C.Y.Cheng & L.Q.Huang | 2 | <i>P. Katik</i> LAE 77807a (BRI) | Papua New Guinea | HE661340 | HE661505 | - | - | - | - |
| <i>Trichosanthes lepiniana</i> (Naud.) Cogn. | 1 | <i>J.D.A. Stainton</i> 8522 (E) | Nepal | HE661341 | HE661506 | HE661424 | - | - | - |
| <i>Trichosanthes lepiniana</i> (Naud.) Cogn. | 2 | <i>Shanzu Wen</i> 85 (KUN) | China | HE661342 | HE661507 | HE661425 | - | - | - |
| <i>Trichosanthes lepiniana</i> (Naud.) Cogn. | 3 | <i>H. de Boer</i> HB49, coll. 1865 (P) | France, cult BG Paris | HE661343 | HE661508 | - | - | - | - |

Table 1 Voucher information and GenBank accession numbers (Continued)

| | | | | | | | | | |
|--|---|---|-------------------------|----------|----------|----------|---|----------|----------|
| <i>Trichosanthes miyagii</i> Hayata | | <i>T. Yamazaki</i> 310 (KYO) | Japan | HE661344 | HE661509 | HE661426 | - | - | - |
| <i>Trichosanthes montana</i> Rugayah ssp. <i>crassipes</i> W.J. de Wilde & Duyfjes | | <i>J. Postar</i> et al. SAN 144259 (L) | Malaysia, Borneo, Sabah | HE661346 | EU155616 | HE661427 | - | EU155625 | EU155635 |
| <i>Trichosanthes montana</i> Rugayah ssp. <i>montana</i> | | <i>W. de Wilde</i> et al. 22279 (L) | Indonesia, Java | HE661345 | HE661510 | - | - | - | - |
| <i>Trichosanthes mucronata</i> Rugayah | | <i>W. de Wilde</i> & <i>B. Duyfjes</i> SAN 139459 (L) | Malaysia, Borneo, Sabah | HE661347 | HE661511 | HE661428 | - | - | - |
| <i>Trichosanthes multiloba</i> Miq. | 1 | <i>S. Tsugaru</i> , <i>G. Murata</i> & <i>T. Sawada</i> s.n. (KYO) | Japan | HE661348 | HE661512 | HE661429 | - | - | - |
| <i>Trichosanthes multiloba</i> Miq. | 2 | <i>S. Fujii</i> 9957 (KYO) | Japan | HE661349 | HE661513 | HE661430 | - | - | - |
| <i>Trichosanthes nervifolia</i> L. | | <i>B. Jonsell</i> 3828 (UPS) | Sri Lanka | HE661350 | HE661514 | HE661431 | - | - | - |
| <i>Trichosanthes obscura</i> Rugayah | | <i>K.M. Wang</i> 1581 (L) | Borneo, Brunei | HE661351 | HE661515 | - | - | - | - |
| <i>Trichosanthes odontosperma</i> W.E.Cooper & A.J.Ford | 1 | <i>H. Schaefer</i> 2007/09 (M) | Australia, Queensland | HE661352 | EU037013 | HE661432 | - | EU037011 | EU037010 |
| <i>Trichosanthes odontosperma</i> W.E.Cooper & A.J.Ford | 2 | <i>B. Gray</i> 9147 (UPS) | Australia, Queensland | HE661353 | HE661516 | HE661433 | - | - | - |
| <i>Trichosanthes odontosperma</i> W.E.Cooper & A.J.Ford | 3 | <i>I. Telford</i> 11285 (CNS) | Australia, Queensland | HE661354 | HE661517 | HE661434 | - | - | - |
| <i>Trichosanthes pallida</i> Duyfjes & Pruesapan | 1 | <i>P. Phonsena</i> , <i>W. de Wilde</i> & <i>B. Duyfjes</i> 4658 (L) | Thailand, Phetchaburi | HE661355 | HE661518 | HE661435 | - | - | - |
| <i>Trichosanthes pallida</i> Duyfjes & Pruesapan | 2 | <i>P. Phonsena</i> , <i>W. de Wilde</i> & <i>B. Duyfjes</i> 3981 (L) | Thailand, Phetchaburi | HE661356 | HE661519 | HE661436 | - | - | - |
| <i>Trichosanthes papuana</i> F.M.Bailey | | <i>W. Takeuchi</i> & <i>D. Ama</i> 17069 (L) | Papua New Guinea | HE661357 | HE661520 | HE661437 | - | - | - |
| <i>Trichosanthes pedata</i> Merr. & Chun | | <i>Jiangiang Li</i> 239 (KUN) | China | HE661358 | HE661521 | HE661438 | - | - | - |
| <i>Trichosanthes pendula</i> Rugayah | | <i>J. Postar</i> et al. 144100 (L) | Malaysia, Borneo, Sabah | HE661359 | EU155617 | HE661439 | - | EU155626 | EU155636 |
| <i>Trichosanthes pentaphylla</i> F.Muell. ex Benth. | 1 | <i>W. Cooper</i> 2094 (CNS) | Australia, Queensland | HE661360 | HE661522 | HE661440 | - | - | - |
| <i>Trichosanthes pentaphylla</i> F.Muell. ex Benth. | 2 | <i>W. Cooper</i> 2061 (CNS) | Australia, Queensland | HE661361 | HE661523 | HE661441 | - | - | - |
| <i>Trichosanthes phonsenae</i> Duyfjes & Pruesapan | 1 | <i>P. Phonsena</i> , <i>W. de Wilde</i> & <i>B. Duyfjes</i> 4419 (L) | Thailand, Phetchaburi | HE661362 | HE661524 | HE661442 | - | - | - |
| <i>Trichosanthes phonsenae</i> Duyfjes & Pruesapan | 2 | <i>P. Phonsena</i> , <i>W. de Wilde</i> & <i>B. Duyfjes</i> 3980 (L) | Thailand, Phetchaburi | HE661363 | HE661525 | HE661443 | - | - | - |

Table 1 Voucher information and GenBank accession numbers (Continued)

| | | | | | | | | | |
|---|---|---|--|----------|----------|----------|----------|----------|----------|
| <i>Trichosanthes pilosa</i> Lour. | 1 | <i>H. Schaefer</i> 2007/17 (M) | Australia, Queensland | HE661364 | EU155620 | EU155611 | - | EU155629 | EU155639 |
| <i>Trichosanthes pilosa</i> Lour. | 2 | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 3913 (L) | Thailand, Chiang Mai | HE661365 | HE661526 | HE661444 | - | - | - |
| <i>Trichosanthes pilosa</i> Lour. | 3 | <i>H. Takahashi</i> 20755 (GIFU) | Japan | - | DQ536604 | DQ536743 | DQ535856 | DQ536875 | DQ536875 |
| <i>Trichosanthes pilosa</i> Lour. | 4 | <i>H. Schaefer</i> 2007/09 (M) | Australia, Queensland | HE661366 | HE661528 | HE661445 | - | - | - |
| <i>Trichosanthes pilosa</i> var. <i>roseipulpa</i> W.J. de Wilde & Duyfjes | | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 4694 (L, holotype) | Thailand, Nan | HE661367 | HE661529 | HE661446 | - | - | - |
| <i>Trichosanthes postarii</i> W.J. de Wilde & Duyfjes | 1 | <i>J. Postar</i> et al. SAN 144066 (L, isotype) | Malaysia, Borneo, Sabah | HE661368 | EU155618 | HE661447 | - | EU155627 | EU155637 |
| <i>Trichosanthes postarii</i> W.J. de Wilde & Duyfjes | 2 | <i>J. Postar</i> et al. SAN 144098 (L) | Malaysia, Borneo, Sabah | HE661369 | HE661530 | HE661448 | - | - | - |
| <i>Trichosanthes pubera</i> Blume ssp. <i>rubriflos</i> (Cayla) Duyfjes & Pruesapan var. <i>fissisepala</i> Duyfjes & Pruesapan | 1 | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 4451 (L) | Thailand, Chiang Mai | HE661370 | HE661531 | HE661449 | - | - | - |
| <i>Trichosanthes pubera</i> Blume ssp. <i>rubriflos</i> (Cayla) Duyfjes & Pruesapan var. <i>fissisepala</i> Duyfjes & Pruesapan | 2 | <i>K. Pruesapan</i> et al. 56 (L) | Thailand, Kanchanaburi | HE661371 | HE661532 | HE661450 | - | - | - |
| <i>Trichosanthes pubera</i> Blume ssp. <i>rubriflos</i> (Cayla) Duyfjes & Pruesapan var. <i>rubriflos</i> | 1 | <i>R. Zhang</i> 1 (M) | China, cult. South China BG, Guangzhou | HE661372 | DQ536560 | DQ536688 | DQ535819 | DQ536828 | - |
| <i>Trichosanthes pubera</i> Blume ssp. <i>rubriflos</i> (Cayla) Duyfjes & Pruesapan var. <i>rubriflos</i> | 2 | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 3907 (L) | Thailand, Saraburi | HE661373 | HE661533 | HE661451 | - | - | - |
| <i>Trichosanthes quinquangulata</i> A.Gray | 1 | <i>P. Phonsena, W. de Wilde & B. Duyfjes</i> 4416 (L) | Thailand, Phetchaburi | HE661374 | HE661534 | HE661452 | - | - | - |
| <i>Trichosanthes quinquangulata</i> A.Gray | 2 | <i>N. Koonthudthod</i> et al. 326 (L) | Thailand, Phetchaburi | HE661375 | HE661535 | HE661453 | - | - | - |
| <i>Trichosanthes quinquefolia</i> C.Y.Wu | | <i>K. Nanthavong</i> et al. BT 705 (L) | Laos, Khammouan | HE661376 | HE661536 | HE661454 | - | - | - |
| <i>Trichosanthes reticulineris</i> C.Y.Wu ex S.K.Chen | | <i>X.F. Deng</i> 131 (IBSC) | China, Guangdong | HE661377 | DQ536605 | DQ536744 | DQ535857 | DQ536876 | DQ536876 |
| <i>Trichosanthes rosthornii</i> Harms | 1 | <i>Jingliang Chuan</i> 5654 (KUN) | China | HE661378 | HE661537 | HE661455 | - | - | - |
| <i>Trichosanthes rosthornii</i> Harms | 2 | <i>A. Henry</i> 1626 (LE) | China, Hubei | HE661379 | HE661538 | - | - | - | - |
| <i>Trichosanthes schlechteri</i> Harms | | <i>W. Takeuchi & D. Ama</i> 15663 (LAE) | Papua New Guinea | HE661380 | EU155619 | EU155610 | EU155605 | EU155628 | EU155638 |
| | | <i>J. Postar</i> et al. SAN 151201 (L) | Malaysia, Borneo, Sabah | HE661381 | HE661539 | - | - | - | - |

Table 1 Voucher information and GenBank accession numbers (Continued)

| | | | | | | | | | |
|---|---|---|----------------------------|----------|------------|----------|----------|----------|----------|
| <i>Trichosanthes sepilokensis</i> Rugayah | | | | | | | | | |
| <i>Trichosanthes smilacifolia</i> C.Y.Wu | | Qiwu Wang 85620 (KUN) | China | HE661382 | HE661540 | - | - | - | - |
| <i>Trichosanthes subvelutina</i> F.Muell. ex Cogn. | 1 | I. Telford 9778 (CANB) | Australia, Queensland | HE661383 | HE661541 | HE661456 | - | - | - |
| <i>Trichosanthes subvelutina</i> F.Muell. ex Cogn. | 2 | F. Davies 1541 (CANB) | Australia, Queensland | HE661384 | HE661542 | HE661457 | - | - | - |
| <i>Trichosanthes subvelutina</i> F.Muell. ex Cogn. | 3 | N. Nicholson 3110 (BRI) | Australia, New South Wales | HE661385 | HE661543 | HE661458 | - | - | - |
| <i>Trichosanthes tricuspidata</i> Lour spp. <i>javanica</i> Pruesapan & Duyfjes | | P. Phonsena, W. de Wilde & B. Duyfjes 4414 (L) | Thailand, Phetchaburi | - | HE661592 | HE661591 | - | - | - |
| <i>Trichosanthes tricuspidata</i> Lour. ssp. <i>tricuspidata</i> | | P. Phonsena, W. de Wilde & B. Duyfjes 4007 (L) | Thailand, Nakhon Sawan | HE661386 | HE661544 | HE661459 | - | - | - |
| <i>Trichosanthes truncata</i> C.B.Clarke | 1 | P. Phonsena, W. de Wilde & B. Duyfjes 3917 (L) | Thailand, Chiang Mai | HE661387 | HE661545 | HE661460 | - | - | - |
| <i>Trichosanthes truncata</i> C.B.Clarke | 2 | P. Phonsena, W. de Wilde & B. Duyfjes 4490 (L) | Thailand, Chiang Mai | HE661388 | HE661546 | HE661461 | - | - | - |
| <i>Trichosanthes truncata</i> C.B.Clarke | 3 | P. Phonsena, W. de Wilde & B. Duyfjes 6329 (L) | Thailand, Chiang Mai | HE661389 | HE661547 | HE661462 | - | - | - |
| <i>Trichosanthes villosa</i> Blume | 1 | P. Phonsena, W. de Wilde & B. Duyfjes 4669 (L) | Thailand, Chiang Mai | - | EU037006 | EU037007 | EU037005 | EU037009 | EU037008 |
| <i>Trichosanthes villosa</i> Blume | 2 | P. Phonsena, W. de Wilde & B. Duyfjes 6331 (L) | Thailand, Chiang Mai | HE661390 | : HE661548 | HE661463 | - | - | - |
| <i>Trichosanthes villosa</i> Blume | 3 | P. Phonsena, W. de Wilde & B. Duyfjes 4449 (L) | Thailand, Chiang Mai | HE661391 | HE661549 | HE661464 | - | - | - |
| <i>Trichosanthes villosa</i> Blume | 4 | P. Phonsena, W. de Wilde & B. Duyfjes 4000 (L) | Thailand, Phetchaburi | HE661392 | HE661550 | - | - | - | - |
| <i>Trichosanthes villosa</i> Blume | 5 | K. Pruesapan et al. 60 (L) | Thailand, Kanchanaburi | HE661393 | HE661551 | HE661465 | - | - | - |
| <i>Trichosanthes fissibracteata</i> C.Y.Wu ex C.Y.Cheng & Yueh | | Shaowen Yu 974 (KUN) | China, Yunnan | HE661394 | HE661552 | HE661466 | - | - | - |
| <i>Trichosanthes wallichiana</i> (Ser.) Wight | | A. Henry 9432 (LE) | China, Yunnan | HE661395 | HE661553 | - | - | - | - |
| <i>Trichosanthes wawrae</i> Cogn. | | B. Gravendeel et al. 631 (L) | Indonesia, Java | HE661396 | HE661554 | HE661467 | - | - | - |

Polymerase chain reaction (PCR) amplification of purified total DNA was performed in 200 μ l reaction tubes with a total volume of 50 μ l. Each tube contained a mixture of 5 μ l reaction buffer (ABgene, 10x), 3 μ l MgCl₂ (25 mM), 1 μ l dNTP's (10 μ M), 0.25 μ l Taq-polymerase (ABgene; 5U/ μ l), 0.25 μ l BSA (Roche Diagnostics), 12.5 μ l of each primer (2 mM), 14.5 μ l Milli-Q water and 1 μ l template DNA. The ITS region was amplified using the primer pair ITS-P17 and ITS-26 S-82R [46] with the following PCR protocol 97°C 5 min., (97°C 30 s., 55°C 1 min., 72°C 1 min.) x 35, 72°C 10 min., 4°C ∞ ; *matK* with primers *matK*-2.1a [47] and *matK*-1440R [48], 95° 5 min., (95° 30 s., 50° 1 min., 72° 1 min.) x 35, 72° 10 min., 4° ∞ ; and *rpl20-rps12* using the primers *rpl20* and *rps12* [49], 95° 5 min., (95° 30 s., 53° 1 min., 72° 1 min.) x 35, 72° 10 min., 4° ∞ . Sequencing was performed by Macrogen Inc. (Seoul, South Korea) on an ABI3730XL automated sequencer (Applied Biosystems). The same primers as used in the PCR were used for the sequencing reactions.

Sequence alignment

Sequence trace files were compiled into contigs with the program Gap4 and edited using Pregap4 [50], both part of the Staden package [51]. Sequences were aligned manually in Se-Al [52]. The final matrix included *rpl20-rps12* (100% of taxa), ITS (96%), *matK* (84%), *trnL-F* spacer (31%), *trnL* intron (28%), and *rbcl* (20%). The three latter regions increased statistical support values at early-branching clades. Sequences were concatenated, and gap-coded using the Simmons and Ochoterena simple method [53] implemented in SeqState [54].

Phylogenetic analyses

Selection of best-fit models of nucleotide substitution for the nuclear and plastid data partitions relied on the Akaike Information Criterion (AIC and AICc) as implemented in JModelTest version 0.1.1 [55,56]. Likelihood calculations were carried out for 88 substitution models on an ML-optimized tree. The best-fitting model for the combined data was the general time-reversible (GTR) model, with a proportion of invariable sites (I) and rate variation among sites (G) with four rate categories. Maximum likelihood tree searches and bootstrapping of the combined data (using 1000 replicates) relied on RAxML version 7.2.6 [57] on the CIPRES cluster [58].

Bayesian tree searching used MrBayes [59] on the CIPRES cluster [58]. The combined data were analyzed using three partitions (nuclear, plastid, gap data), allowing partition models to vary by unlinking gamma shapes, transition matrices, and proportions of invariable sites. Markov chain Monte Carlo (MCMC) runs started from independent random trees, were repeated twice, and extended for 10 million generations, with trees sampled

every 1000th generation. We used the default priors in MrBayes, namely a flat Dirichlet prior for the relative nucleotide frequencies and rate parameters, a discrete uniform prior for topologies, and an exponential distribution (mean 1.0) for the gamma-shape parameter and branch lengths. Convergence was assessed by checking that the standard deviations of split frequencies were <0.01; that the log probabilities of the data given the parameter values fluctuated within narrow limits; that the convergence diagnostic (the potential scale reduction factor given by MrBayes) approached one; and by examining the plot provided by MrBayes of the generation number versus the log probability of the data. Trees saved prior to convergence were discarded as burn-in (10 000 trees) and a consensus tree was constructed from the remaining trees.

The data matrix and trees have been deposited in TreeBASE (www.treebase.org; study number 12339).

Divergence time estimation

Divergence times were estimated using the Bayesian relaxed clock approach implemented in BEAST version 1.6.2 [60]. Searches used a Yule tree prior, the GTR+G substitution model, and 50 million MCMC generations, sampling every 1000th generation. Six monophyletic groups were defined based on the results of our phylogenetic analyses and previously published phylogenies [18,20,44]. Tracer version 1.5 [61] was used to check that effective sampling sizes had all reached >200, suggesting convergence of the chains. TreeAnnotator, part of the BEAST package, was then used to create a maximum clade credibility tree, with the mean divergence ages shown for all nodes with >95% highest posterior density.

Calibration relied on Cucurbitaceae fossils assigned to particular nodes (labeled A--C in Figure 3), using a gamma prior distribution with the fossil age as the offset and shape and scale parameter chosen to add a 95% CI of c. 10 Ma older than the fossil. (A) The root node, that is, the most recent common ancestor of *Momordica* and *Trichosanthes*, was constrained to 55.8 Ma with a shape parameter of 1.0 and a scale of 1.0, based on seeds from the Paleocene/Eocene Felpham flora representing the oldest Cucurbitaceae and dated to c. 55.8 Ma [62]. (B) The crown node of the *Trichosanthes/Gymnopetalum* clade was constrained to 34 Ma with a shape parameter of 1.0 and a scale of 3.4, based on *Trichosanthes* seeds from the Upper Eocene of Bulgaria [25] dated to c. 34 Ma and seeds from the Oligocene of West Siberia [26] dated to c. 23.8 Ma [27]. (C) The divergence of *Marah* and *Echinocystis* was set to 16 Ma with a shape parameter of 1.0 and a scale of 3.35, based on leaves and a fruit representing *Marah* from the Miocene of Stewart Valley, Nevada (M. Guillems and D. M.

Erwin, University of California, Berkeley, in preparation; the fruit comes from the Fingerrock Wash site, dated to c. 16 Ma, the leaf from the Savage Canyon Formation, dated to c. 14.5 Ma). Absolute ages were taken from the geologic time scale of Walker and Geissman [63]. We also tested lognormal and exponential prior distributions, which gave very similar age estimates (results not shown).

Biogeographical analysis

Biogeographic reconstruction relied on statistical dispersal-variance analysis using S-DIVA version 2.0 [64] as implemented in RASP, which carries out parsimony inference on the chain of trees obtained from an MCMC search [65,66], in our case the 8000 post burn-in Bayesian trees resulting from the BEAST dating analysis. S-DIVA averages the frequencies of an ancestral range at a node in ancestral reconstructions over all trees, with alternative ancestral ranges at a node weighted by the frequency of the node [64]. Range information for all species was compiled from taxonomic treatments [9,11,13-16], and the coded distribution areas were: A) Australia and New Guinea, B) Wallacea, C) Insular Sunda Malesia, D) Mainland Southeast Asia, E) India and adjacent countries, F) Africa, Europe and the New World.

Authors' contributions

HB conceived the study, carried out the molecular genetic analyses, and drafted the manuscript. HS participated in the design of the study and data analysis, and also contributed field observations. SR and MT participated in the design and coordination of the study, and SR also helped with clock calibration and writing. All authors read and approved the final manuscript.

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Author details

¹Department of Systematic Biology, Uppsala University, Norbyvägen 18 D, Uppsala SE-75236, Sweden. ²Harvard University, Department of Organismic and Evolutionary Biology, 22 Divinity Avenue, Cambridge, MA 02138, U.S.A. ³Department of Systematic Biology, Uppsala University, Norbyvägen 18 D, Uppsala SE-75236, Sweden. ⁴University of Munich (LMU), Systematic Botany and Mycology, Menzinger Str. 67, Munich 80638, Germany.

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