

Cephalanthera exigua rediscovered: new insights in the taxonomy, habitat requirements and breeding system of a rare mycoheterotrophic orchid

Henrik Æ. Pedersen, Santi Watthana, Mélanie Roy, Somran Suddee and Marc-André Selosse

H. Æ. Pedersen (henrikp@snm.ku.dk), Botanical Garden and Museum, Natural History Museum of Denmark, Univ. of Copenhagen, Gothersgade 130, DK-1123 Copenhagen K, Denmark. – S. Watthana, Queen Sirikit Botanic Garden, PO Box 7, Mae Rim, Chiang Mai 50180, Thailand. – M. Roy and M.-A. Selosse, Centre d'Ecologie Fonctionnelle et Evolutive, 1919 route de Mende, FR-34293 Montpellier cedex 05, France. – S. Suddee, Forest Herbarium (BKF), National Park, Wildlife and Plant Conservation Department, Chatuchak, Bangkok 10900, Thailand.

Five mycoheterotrophic species of *Cephalanthera* have been proposed from tropical Asia. Until recently, all of them were only known from the type specimens, for which reason it has been difficult to judge if some of them were actually conspecific. The recent discovery of two large populations of *C. exigua* in Thailand made it possible to describe the range of variation of this species in much more detail, and we then found it to be well distinguished from the others. A phylogenetic analysis based on nuclear rDNA ITS sequences corroborated the systematic position of *C. exigua* at the generic level. Examination of the previously neglected type of *C. pusilla* from Myanmar lead to the conclusion that this taxon of disputed generic affinity likewise belongs to *Cephalanthera*, and that it is a clearly distinct species. Finally, we accept that also *C. ericiflora* from Laos as well as *C. gracilis* and *C. calcarata* from Yunnan are probably distinct species. *Cephalanthera exigua* grows in hill evergreen forest, but we found little floristic similarity between its two localities in Thailand ($IS_S = 22.5\%$). Ectomycorrhizal trees of the families Fagaceae and Ulmaceae are probably the ultimate carbon source for *C. exigua* at both sites, as the mycorrhizal partners of this orchid (basidiomycetes of the family Thelephoraceae) obtain their carbon nutrition through ectomycorrhizal association with forest trees. Pollination experiments demonstrated that the nectarless flowers of *C. exigua* are not spontaneously autogamous. Based on the pollination syndrome and previously published observations on other species of the genus, we suggest that *C. exigua* has an insect-operated breeding system acting by deceit, and that the pollinators are probably solitary bees.

Orchids without chlorophyll are unable to photosynthesize, and those with strongly reduced levels of chlorophyll can only perform photosynthesis far below the compensation point. In both cases, the plants are therefore completely dependent on their mycorrhizae as a carbon source, even at their adult stages (Rasmussen 1995), and they are referred to as being mycoheterotrophic or holomycotrophic (Leake 1994). Orchid species with a permanently mycoheterotrophic way of life have evolved on several occasions in three of the five subfamilies of the Orchidaceae (Molvray et al. 2000). Therefore, it is hardly surprising that the limit between autotrophy and mycoheterotrophy is indistinct in several genera (Selosse and Roy 2009).

One such genus is *Cephalanthera* Rich. that comprises a total of ca 15 species in Europe, the Mediterranean, Asia and the western part of the USA. For *C. damasonium* (Mill.) Druce, *C. longifolia* (L.) Fritsch and *C. rubra* (L.)

Rich., it has recently been demonstrated that individuals with normal green leaves do not only obtain carbon through photosynthesis, but also to a high extent from their fungal symbionts; a strategy known as mixotrophy (Gebauer and Meyer 2003, Julou et al. 2005, Abadie et al. 2006). This obviously makes species of *Cephalanthera* predisposed for developing a mycoheterotrophic strategy, and indeed chlorophyll-deficient to chlorophyll-free individuals have been observed in each of the normally mixotrophic species *C. damasonium* (Renner 1938, 1943, Mairold and Weber 1950, Kohns and Schneider 1993, Julou et al. 2005), *C. longifolia* (Renner 1943, Abadie et al. 2006) and *C. rubra* (Burgeff 1954, J. P. Amardeilh et al. unpubl.).

In a few species of *Cephalanthera*, mycoheterotrophy has become fixed, and the permanent loss of chlorophyll has been accompanied by strong leaf reduction. The best known example is the sole North American representative of the genus, *C. austiniiae* (A. Gray) A. Heller; for good descriptions and illustrations, see MacDougal (1899, sub nom. *C. oregana* Rchb.f.), Luer (1975) and

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Coleman (1995). It is less widely known that five mycoheterotrophic species of *Cephalanthera* have been proposed from Myanmar (*C. pusilla* (Hook. f.) Seidenf.), Laos (*C. ericiflora* Szlach. & Mytnik; *C. exigua* Seidenf.) and Yunnan (*C. calcarata* S. C. Chen & K. Y. Lang; *C. gracilis* S. C. Chen & G. H. Zhu). Until recently, all of these species were only known from the type specimens. Therefore, it has been difficult to judge if they should be recognized as five distinct species, or whether improved knowledge of their range of variation would indicate that two or more of them are conspecific.

On 10 Apr 2001, staff members from Queen Sirikit Botanic Garden in Chiang Mai climbed Doi Pee Pan Nam, Ban Khun Lao at the Chiang Mai/Chiang Rai province border in northern Thailand. In hill evergreen forest at ca 1700 m a.s.l., dominated by trees of the families Fagaceae, Lauraceae and Theaceae, they found a hitherto unknown population of an unidentified mycoheterotrophic *Cephalanthera* – a new generic record for Thailand. A few years later, rumours were circulating that a population of similar plants had been found on Doi Inthanon, province of Chiang Mai. On 17 Feb 2008, Santi Watthana visited this locality together with the Assistant Head of Doi Inthanon National Park. They retrieved the population in hill evergreen forest at ca 1600 m a.s.l., and they confirmed that it belonged to the same unidentified species.

The recent discoveries in Thailand made it possible to prepare a detailed description of the species concerned, and consequently to re-evaluate species delimitation in the genus. Furthermore, we got the opportunity to collect a flower for DNA-based phylogenetic analysis and to gather the first known data on habitat requirements and breeding system for an Asian mycoheterotrophic species of *Cephalanthera*.

Material and methods

Taxonomy and distribution

A detailed morphological description of the mycoheterotrophic *Cephalanthera* from Thailand was prepared through field examination of 36 individuals on Doi Pee Pan Nam on 3 Apr 2005, and in Feb–Mar 2008 it was ascertained that the description also covered the range of variation in the population on Doi Inthanon. Furthermore, plants were photographed in situ, and a few individuals were collected for herbarium documentation and for preparation of a detailed line drawing. To identify the species and to evaluate species delimitation in the complex in general, our description and illustrations were then compared with descriptions (and illustrations, whenever possible) in the protologues of *C. pusilla* (Hooker 1890–1894), *C. exigua* (Seidenfaden 1975), *C. calcarata* (Chen and Lang 1986), *C. gracilis* (Chen and Zhu 2002) and *C. ericiflora* (Szlachetko et al. 2008) and with all available herbarium specimens of these taxa (listed in the ‘Taxonomic synopsis’). Unfortunately, we did not succeed in getting the holotype of *C. calcarata* on loan from PE, and there was no time to access the type of *C. ericiflora* as this species was not described until our manuscript was

already under review. A dichotomous key to the accepted species was prepared together with a taxonomic synopsis.

Phylogenetic analysis

A phylogenetic analysis was performed to assess the overall phylogenetic position of the putative *Cephalanthera* from Thailand. A flower was collected from the population on Doi Pee Pan Nam and kept in silica gel (Chase and Hills 1991) until DNA was extracted using DNeasy™ Plant Mini Kit, according to the manufacturer’s instructions. Using primers ITS1P and ITS4, the nuclear rDNA internal transcribed spacer (ITS) was amplified and sequenced as in Roy et al. (2009), where also the primer sequences can be found. The sequence has been deposited at GenBank (FJ454868, Table 1). Downloaded ITS sequences for *Cephalanthera damasonium*, *C. longifolia*, *C. rubra* and representative species belonging to other genera of the Neottieae were also included in the analysis, whereas *Tropidia polystachya* Sw. and *Nervilia shirensis* Schltr. were used as outgroups (for GenBank accession no., see Table 1). Sequences alignment was performed under BioEdit v. 7.3.0 (Hall 1999) and corrected by eye. The phylogeny was computed by maximum likelihood using PhyML v. 2.4.4 (Guindon and Gascuel 2003). For this analysis, a general time-reversible model of DNA substitution was used (GTR; Lanave et al. 1984, Rodriguez et al. 1990), involving unequal base frequencies and six types of substitution. This model of DNA substitution was chosen using a series of hierarchical likelihood-ratio tests in Modeltest 3.7 (Posada and Crandall 1998). Base frequencies were estimated, and 10 000 bootstrap replicates were performed. The phylogenetic tree was visualized using Treeview 1.6.6 (Page 1996).

Ecology

The habitats of the newly discovered *Cephalanthera* populations on Doi Pee Pan Nam and Doi Inthanon were studied in 2005 and 2008, respectively. The forest accommodating the population on Doi Pee Pan Nam was characterized by identifying all the trees, and at both sites a

Table 1. List of species included in the phylogenetic analysis, with the GenBank accession numbers indicated.

| Taxon | GenBank accession no. |
|-----------------------------------------------|-----------------------|
| <i>Cephalanthera damasonium</i> (Mill.) Druce | AY146446 |
| <i>Cephalanthera exigua</i> Seidenf. | FJ454868 |
| <i>Cephalanthera longifolia</i> (L.) Fritsch | DQ182464 |
| <i>Cephalanthera rubra</i> (L.) Rich. | AY146445 |
| <i>Epipactis flava</i> Seidenf. | FJ454869 |
| <i>Epipactis helleborine</i> (L.) Crantz s.s. | AY351375 |
| <i>Epipactis leptochila</i> (Godfery) Godfery | FJ454870 |
| <i>Epipactis muelleri</i> Godfery | FJ454871 |
| <i>Epipactis palustris</i> (L.) Crantz | AY146448 |
| <i>Limodorum abortivum</i> (L.) Sw. | AY351378 |
| <i>Neottia ovata</i> (L.) Bluff & Fingerh. | BankIt1179998 |
| <i>Neottia smallii</i> (Wiegand) Szlach. | AF521058 |
| <i>Neottia nidus-avis</i> (L.) Rich. | AY351383 |
| <i>Nervilia shirensis</i> Schltr. | AF521066 |
| <i>Tropidia polystachya</i> Sw. | EU490674 |

rectangular plot (10 × 50 m) was demarcated for more detailed studies.

Within each plot, samples from all vascular plant species were collected, and herbarium specimens were prepared for later identification. Based on the identifications, Sørensen's (1948) presence community coefficient was calculated as: $IS_S = [c/2(A+B)] \times 100\%$, where c is the number of species common to both plots, whereas A and B are the total numbers of species recorded in the plots on Doi Pee Pan Nam and Doi Inthanon, respectively. In comparison to Jaccard's original index of similarity ($IS_J = [c/(A+B-c)] \times 100\%$), Sørensen's index expresses the actually measured coinciding species occurrences against the theoretically possible ones. As noted by Mueller-Dombois and Ellenberg (1974), this may be mathematically more satisfactory, as it includes a statistical probability term.

In both plots, all individuals of *Cephalanthera* were tagged during flowering in 2008 (17 Feb, 9 and 15 Mar on Doi Inthanon; 19 Mar and 9 Apr on Doi Pee Pan Nam). The number of flowers of each individual was counted, and flowers were checked for presence of nectar. When flowering had ceased, the number of fruits developed on each individual was scored (4 Jun on Doi Inthanon; 27 Jun on Doi Pee Pan Nam) in order to assess the natural levels of fruit set in the two populations.

On Doi Inthanon, 17 individuals of *Cephalanthera* were covered with nylon net while still in bud. Upon anthesis, the altogether 38 flowers were experimentally treated to test for fruit set resulting from spontaneous self-pollination ($n = 19$), induced self-pollination ($n = 11$) and induced cross-pollination ($n = 8$). Following the experimental treatments, the nets were replaced immediately, and the fruit set was assessed on 4 Jun.

Results

Taxonomy and distribution

Careful examination of the type specimen of *C. exigua*, together with information from the protologue, revealed a very close similarity to our Thai plants. The only differences were a few measurements (of the bracts, labellum and column) lying either on or slightly below the lower limits in our description. Furthermore, judging from Seidenfaden's (1975) description and line drawing, the epichile of *C. exigua* differed in being boat-shaped, rounded and devoid of ornaments. The smaller column length indicated by Seidenfaden (1975) for *C. exigua* could be explained by the circumstance that Seidenfaden, unlike us, probably did not include the anther in this measurement. The rather small dimensions of bracts and labellum in *C. exigua* were probably due to moderate shrinkage of the type during the drying process. Finally, we decided that the difference in epichile morphology between our Thai material and the type of *C. exigua* (as interpreted by Seidenfaden) was probably an artefact. This view was based on the very poor condition of the epichile on the only flower of the type specimen; we could not ourselves establish the epichile morphology of the type in detail.

Examination of the types of *C. gracilis* and *C. pusilla* and of a specimen previously identified as *C. calcarata* by P. Ormerod (Ching 22569 K), together with information from the protologues of these taxa as well as *C. ericiflora*, did not reveal a correspondingly close match with our Thai material. Thus, *C. pusilla* had a completely different labellum morphology; *C. ericiflora* had sepals and petals with subcaudate reflexed apices, different ornaments on the labellum and an apiculate epichile; *C. calcarata* had a more distinctly set-off spur and a differently shaped epichile; and *C. gracilis* had longer inflorescences, smaller flowers and a hypochile that was saccate in its middle to distal (rather than basal) part.

Comparing *C. pusilla*, *C. calcarata*, *C. gracilis* and *C. ericiflora* to each other, we found *C. pusilla* to have a labellum morphology that was strikingly different from that in the other three taxa. Judging from the only two specimens available to us, *C. calcarata* was clearly distinct from *C. gracilis* due to its basal and more distinctly set off spur and due to its differently shaped epichile. Finally, the original description and illustration of *C. ericiflora* suggest that this species is distinguished from the others in characters pertaining to the sepals, petals and labellum (cf. key below).

Phylogenetic analysis

An ITS sequence was successfully obtained from *C. exigua*, and the phylogenetic analysis strongly supported its position within the genus *Cephalanthera* (Fig. 1), thus confirming the generic identification based on morphological features. As could be expected, *C. exigua* is nested in the clade of mixotrophic *Cephalanthera*.

Ecology

The composition of the vascular plant flora in the plots on Doi Pee Pan Nam and Doi Inthanon, respectively, is shown in Table 2 (with additional tree species found outside the plot on Doi Pee Pan Nam listed in a footnote). Because much of the collected material was in a vegetative state, not all specimens could be identified to species (or even genus), but in all cases it was possible to establish the number of species from each family. The flora was slightly more diverse in the plot on Doi Inthanon (0.086 species per m²) than in the plot on Doi Pee Pan Nam (0.056 species per m²). Furthermore, the floristic composition was markedly dissimilar between the two plots ($IS_S = 22.5\%$).

In the plot on Doi Inthanon, a total of 126 *Cephalanthera* individuals (all with solitary flowering shoots) produced from 1–3 flowers per inflorescence. The altogether 183 flowers developed 8 fruits (0–2 per inflorescence), corresponding to a natural relative fruit set of 4.4%. In the plot on Doi Pee Pan Nam, a total of 133 individuals produced from 1–4 flowers per inflorescence. The altogether 281 flowers developed 31 fruits (0–2 per inflorescence), corresponding to a natural relative fruit set of 7.7%. No nectar production was detected in the flowers.

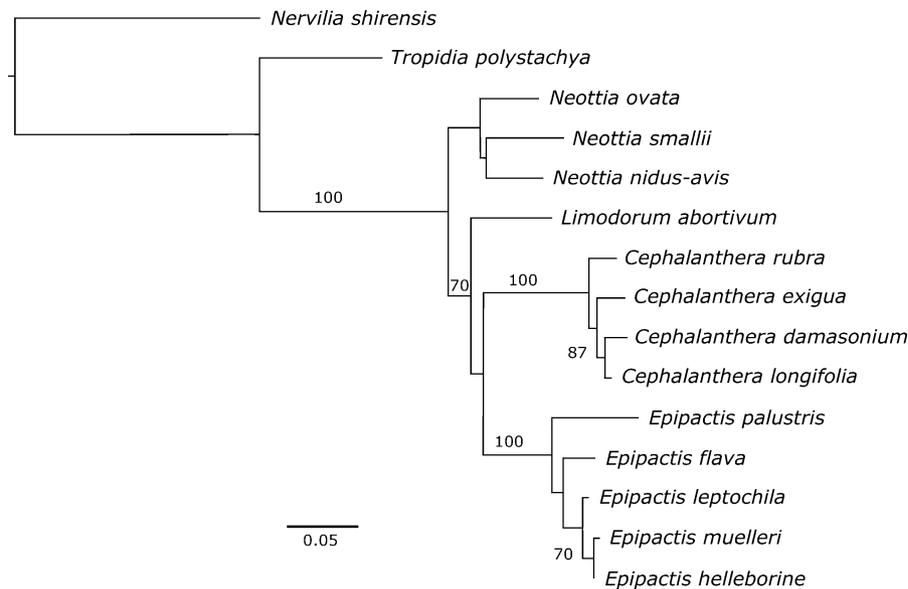


Figure 1. Phylogenetic tree showing the position of *Cephalanthera exigua* within the Neottieae. Phylogeny based on ITS using the maximum likelihood method (GTR model). Numbers on branches indicate bootstrap values above 70% (over 10 000 replicates); the scale bar shows 0.05 substitutions per site.

Concerning the bagging experiments, the levels of relative fruit set were 0% resulting from spontaneous self-pollination, 72.7% resulting from induced self-pollination and 87.5% resulting from induced cross-pollination.

Discussion

Species delimitation and systematic affinities

Based on our comparisons with descriptions, illustrations, and available herbarium specimens of Asian mycoheterotrophic orchids previously assigned to *Cephalanthera*, we confidently identify our *Cephalanthera* material from the Thai mountains Doi Inthanon and Doi Pee Pan Nam as *C. exigua* (Fig. 2–3). This represents a new generic and specific record for Thailand, and it considerably widens the known geographic range of this species, which was previously considered endemic to Laos. Furthermore, though considerably less well-substantiated, we recognize the Yunnanese *C. calcarata* and *C. gracilis* and the Laotian *C. ericiflora* as three distinct species.

When first reporting his find of the present *C. exigua* from Laos, Kerr (1933) identified his plant as *Galeola pusilla* Hook. f., the basionym of *Cephalanthera pusilla*. When Tang and Wang (1951) transferred the latter species to *Aphyllorchis* Blume, they compared Kerr's plant with the protologue (Hooker 1890–1894) and Hooker's unpublished sketches, and they felt that Kerr was right in considering the two plants as conspecific. A herbarium specimen of *C. pusilla* (Kurz 336) is present at CAL and must be regarded as the type. The sheet carries an originally three-flowered individual. Two flowers are extant on the specimen, whereas the third has been detached and dissected, and the various parts of this flower are now kept in a capsule on the sheet. The same sheet also carries Hooker's original sketches of the plant, including floral details. A drawing at K (cited by Seidenfaden 1975, who

was apparently unaware of the type specimen at CAL) is an accurate copy of these sketches. One of the floral sketches shows a labellum much unlike any known species of *Cephalanthera*, and it was probably this sketch that convinced Tang and Wang (1951) that *Galeola pusilla* should rather be referred to *Aphyllorchis* – only the basal auricles typical of an *Aphyllorchis* labellum are missing on the sketch. Indeed, if it could be demonstrated that the absence of these structures on the sketch were due to the very base of the labellum being left on the column, the idea of placing this species in *Aphyllorchis* would appear sensible. However, during a visit to CAL in 2008, H. Æ. Pedersen got an opportunity to boil and examine all parts of the dissected flower from the type specimen. This examination revealed that the labellum had been sketched in its entirety by Hooker, but it also revealed that Hooker's sketch is inaccurate. In reality, the labellum morphology suggests a *Cephalanthera* rather than an *Aphyllorchis*, and this indication was supported by the completely sessile ovary of all three flowers (all known species of *Aphyllorchis* have a shortly pedicelled ovary). Still, the shallowly (rather than deeply) three-lobed condition of the labellum in *Cephalanthera pusilla* at once distinguishes this species from the other mycoheterotrophic species of *Cephalanthera* in Asia, including *C. exigua*.

In the phylogenetic analysis that included *C. exigua*, *C. damasonium*, *C. longifolia*, *C. rubra* and representative species of *Epipactis* Zinn, *Limodorum* Boehm. and *Neottia* Guett. (Fig. 1), *Cephalanthera exigua* is sister to the species pair *C. damasonium*/*C. longifolia*, and *C. rubra* is sister to the clade consisting of these three species. Apart from the 87% bootstrap support of the *C. damasonium*/*C. longifolia* clade, however, the internal relationships in *Cephalanthera* are poorly supported. Still, the *Cephalanthera* clade as a whole is well supported (100%). This strengthens the perception of *Cephalanthera* as being monophyletic (Pridgeon et al. 2005), and it corroborates the systematic

Table 2. List of vascular plant taxa occurring in the study plots on Doi Pee Pan Nam (A) and Doi Inthanon (B), northern Thailand. Specimens found (+), not found (-).

| Family | Species | A | B |
|------------------|-------------------------------------------------------------|---|---|
| Trees* | | | |
| Aceraceae | <i>Acer laurinum</i> Hassk. | - | + |
| Caprifoliaceae | <i>Viburnum</i> sp. | + | - |
| Clusiaceae | <i>Calophyllum</i> sp. | - | + |
| Fabaceae | <i>Archidendron lucidum</i> I. C. Nielsen | - | + |
| Fagaceae | <i>Castanopsis acuminatissima</i> (Blume) A. DC. | + | - |
| Fagaceae | <i>Castanopsis</i> sp. | + | - |
| Fagaceae | <i>Lithocarpus</i> sp. 1 | - | + |
| Fagaceae | <i>Lithocarpus</i> sp. 2 | + | - |
| Fagaceae | <i>Quercus aliena</i> Blume | - | + |
| Juglandaceae | <i>Engelhardtia spicata</i> Blume | + | - |
| Lauraceae | <i>Cinnamomum</i> sp. | - | + |
| Lauraceae | <i>Litsea</i> sp. | - | + |
| Lauraceae | <i>Neolitsea</i> sp. | + | + |
| Lauraceae | Gen. et sp. indet. 1 | - | + |
| Lauraceae | Gen. et sp. indet. 2 | - | + |
| Lauraceae | Gen. et sp. indet. 3 | + | - |
| Magnoliaceae | <i>Magnolia floribunda</i> (Finet & Gagnep.) Figlar | + | - |
| Meliaceae | <i>Heynea trijuga</i> Roxb. ex Sims | - | + |
| Moraceae | <i>Ficus parietalis</i> Blume | - | + |
| Myricaceae | <i>Myrica esculenta</i> Buch.-Ham. | + | - |
| Myrtaceae | <i>Syzygium angkae</i> (Craib) P. Chantaranothai & J. Parn. | + | + |
| Myrtaceae | <i>Syzygium</i> sp. | + | + |
| Theaceae | <i>Camellia connata</i> (Craib) Craib | - | + |
| Theaceae | <i>Camellia oleifera</i> Abel. | + | - |
| Theaceae | <i>Gordonia dalglieshiana</i> Craib | - | + |
| Theaceae | <i>Schima wallichii</i> (DC.) Korth. | + | - |
| Ulmaceae | <i>Gironniera</i> sp. | + | + |
| Xanthophyllaceae | <i>Xanthophyllum</i> sp. | - | + |
| Shrubs | | | |
| Acanthaceae | <i>Strobilanthes</i> sp. | - | + |
| Melastomataceae | <i>Melastoma</i> sp. | + | + |
| Myrsinaceae | <i>Ardisia</i> sp. 1 | - | + |
| Myrsinaceae | <i>Ardisia</i> sp. 2 | - | + |
| Myrsinaceae | <i>Maesa</i> sp. | + | - |
| Rubiaceae | <i>Ixora</i> sp. | - | + |
| Rubiaceae | <i>Lasianthus</i> sp. | + | - |
| Rubiaceae | <i>Psychotria</i> sp. 1 | - | + |
| Rubiaceae | <i>Psychotria</i> sp. 2 | + | + |
| Rutaceae | <i>Melicope pteleifolia</i> (Champ. ex Benth.) Hartley | - | + |
| Climbers | | | |
| Apocynaceae | Gen. et sp. indet. | - | + |
| Combretaceae | <i>Combretum</i> sp. | - | + |
| Myrsinaceae | <i>Embelia pulchella</i> Mez | - | + |
| Myrsinaceae | <i>Embelia sessiliflora</i> Kurz | + | + |
| Piperaceae | <i>Piper</i> sp. | - | + |
| Rosaceae | <i>Rubus</i> sp. 1 | - | + |
| Rosaceae | <i>Rubus</i> sp. 2 | + | - |
| Smilacaceae | <i>Smilax lanceifolia</i> Roxb. | - | + |
| Smilacaceae | <i>Smilax ovalifolia</i> Roxb. | + | - |
| Smilacaceae | <i>Smilax rigida</i> Wall. ex Kunth | + | - |
| Herbs | | | |
| Balsaminaceae | <i>Impatiens</i> sp. | + | - |
| Convallariaceae | <i>Aspidistra</i> sp. | + | - |
| Convallariaceae | <i>Ophiopogon</i> sp. | - | + |
| Cyperaceae | <i>Carex baccans</i> Nees | - | + |
| Cyperaceae | <i>Scleria</i> sp. | + | - |
| Dennstaedtiaceae | <i>Pteridium aquilinum</i> Kuhn | - | + |
| Gentianaceae | <i>Gentiana hessliana</i> Hosseus | - | + |
| Lamiaceae | <i>Pogostemon</i> sp. | + | - |
| Phormiaceae | <i>Dianella ensifolia</i> (L.) DC. | - | + |
| Poaceae | <i>Bambusa</i> sp. | - | + |
| Poaceae | <i>Thysanolaena maxima</i> Kuntze | - | + |
| Pteridaceae | <i>Pteris bella</i> Tagawa | - | + |

Table 2 (continued)

| Family | Species | A | B |
|---------------|---------------------------------------------|---|---|
| Violaceae | <i>Viola curvistylis</i> Boissieu & Gagnep. | + | – |
| Zingiberaceae | <i>Amomum</i> sp. 1 | + | + |
| Zingiberaceae | <i>Amomum</i> sp. 2 | – | + |

*Additional tree taxa registered outside the plot on Doi Pee Pan Nam. – Araliaceae: *Schefflera* sp.; Asteraceae: *Vernonia* sp.; Daphniphyllaceae: *Daphniphyllum* sp.; Elaeocarpaceae: *Elaeocarpus* sp.; Ericaceae: *Rhododendron arboreum* Sm., *R. moulmeynense* Hook.f.; Euphorbiaceae: *Glochidion* sp.; Fabaceae: *Archidendron clyperaria* (Jack) I. C. Nielsen; Fagaceae: *Trigonobalanus doichangensis* (A. Camus) Forman; Proteaceae: *Helicia* sp.; Rutaceae: *Euodia triphylla* DC.; Symplocaceae: *Symplocos macrophylla* Wall. ex DC. subsp. *sulcata* (Kurz) Noot.; Theaceae: *Anneslea fragrans* Wall., *Eurya nitida* Korth., *Gordonia dalglieshiana* Craib.

position of *C. exigua* at the generic level. It is not yet clear whether mycoheterotrophy arose more than once in the genus; in the slightly more comprehensive phylogeny of Pridgeon et al. (2005), the mycoheterotrophic *C. austiniiae* assumed a position similar to *C. exigua* in our analysis. In a larger phylogeny that includes representatives of *Aphylorchis* and *Cephalanthera* (Roy et al. unpubl.), the former is clearly distinct, and the monophyly of *Cephalanthera* is strongly supported.

Occurrence, habitat requirements and conservation of *Cephalanthera exigua*

Judging from label information on Kerr's type from Laos and from our own field studies in Thailand, *C. exigua* generally grows in hill evergreen forest from 1500–1700 m a.s.l. It seems to require places with deep leaf litter, but may occur both under heavy shade and in only partly shaded spots. The compositions of the vascular plant floras in our plots on Doi Pee Pan Nam and Doi Inthanon are given in Table 2. As the floras are markedly dissimilar ($IS_S = 22.5\%$), *C. exigua* does not seem to have particularly strict habitat requirements.

Based on data collected from Doi Pee Pan Nam, Roy et al. (unpubl.) found *C. exigua* to form mycorrhiza with basiodiomycetes of the family Thelephoraceae. This family forms other mycorrhizal associations (i.e. ectomycorrhizae) with forest trees that provide them their organic carbon (Selosse and Roy 2009). Like all other mycoheterotrophic Neottieae studied so far (Taylor et al. 2002, Abadie et al. 2006), *C. exigua* thus relies indirectly on trees as a carbon source. Species of the Thelephoraceae are regarded as non-specific ectomycorrhizal partners of forest trees (Smith and Read 2008). At both sites of *C. exigua* studied in Thailand, the vascular plant flora turned out to include several ectomycorrhizal tree species of the families Fagaceae and Ulmaceae (Table 2). Among the members of these families, only an unidentified species of *Gironniera* (Ulmaceae) was found (scattered) at both study sites. At the site on Doi Pee Pan Nam, *Castanopsis acuminatissima* (Blume) A. DC. (Fagaceae) was found to be particularly common, and several species of *Castanopsis* (D. Don) Spach have previously been reported to form ectomycorrhizae with representatives of the Thelephoraceae (Tam and Griffiths 1993).

The indirect nutritional dependency of *C. exigua* on ectomycorrhizal trees, as represented by members of the Fagaceae and Ulmaceae at the two sites in Thailand, should be taken into consideration when setting up conservation measures for this very rare species. Thus, at each locality of *C. exigua*, the trees forming ectomycorrhizae with

Thelephoraceae should be specifically protected, and the habitat should be generally protected against fires and soil disturbance.

Breeding system and fruit set in *Cephalanthera exigua*

With its white, zygomorphic, more or less horizontal flowers, its shortly spurred hypochile and its reasonably flat epichile (offering a landing platform), *C. exigua* fits the bee pollination syndrome of van der Pijl and Dodson (1966). Furthermore, the brown markings on the nectarless labellum may be interpreted as deceptive nectar guides, whereas the ruminant ridge of the hypochile and/or the papillae of the epichile may represent pseudopollen. Only the apparently scentless condition of the flowers does not fit the bee pollination syndrome (although the fact that the flowers are scentless to humans does not necessarily imply that they are scentless to insects as well). Deceit pollination by solitary bees is well-known from other species of *Cephalanthera*. Thus, Dafni and Ivri (1981) and Vöth (1999) observed *C. longifolia* to be pollinated by three species of *Andrena* (Andrenidae), *Halictus* sp. and *Lasioglossum laevigatum* (Halictidae), whereas Nilsson (1983), Nazarov and Ivanov (1990) and Vöth (1999) observed *C. rubra* to be pollinated by three species of *Chelostoma* (Megachilidae) and *Dufourea dentiventris* (Halictidae). In contrast, *C. damasonium* (with almost closed flowers even at the peak of flowering) is obligately autogamous (Scacchi et al. 1991, Claessens and Kleynen 1995, Vöth 1999), and van der Cingel (2001) interpreted the mycoheterotrophic *C. austiniiae* as facultatively autogamous.

The complete lack of fruit set in the 19 bagged, experimentally untouched flowers demonstrates that *C. exigua* is not spontaneously autogamous. On the other hand, the high and fairly equal levels of fruit set resulting from induced self-pollination (72.7%) and induced cross-pollination (83.3%) clearly indicate that this species is genetically self-compatible. The very low levels of natural fruit set (4.4% on Doi Inthanon, 7.7% on Doi Pee Pan Nam) support that the nectarless flowers of *C. exigua* are pollinated by insects, and the figures fit the general level of 0.0–43.0% relative fruit set in nectarless tropical orchids (compared to 17.8–41.0% in nectar-producing species of this group), confer Neiland and Wilcock (1998). Incidentally, the natural level of mean relative fruit set has been reported to vary from 17.3–62.4% in *C. longifolia* (Dafni and Ivri 1981) and from 0.0–30.1% in *C. rubra* (Nilsson 1983).



Figure 2. *Cephalanthera exigua* Seidenf. (A) habit, (B) bract, (C) flower, (D) flower (sepals and petals removed), (E) dorsal sepal, (F) lateral sepal, (G) petal, (H) labellum, (J) column. Double-line scale = 1 cm. Single-line scale = 1 mm. (A)–(J) Watthana 1338 C. Henrik Æ. Pedersen del.

Based on the lack of nectar, the patterns of fruit set and the pollination syndrome (supported by previous anthecological studies of other *Cephalanthera* species), we conclude that *C. exigua* has an insect-operated breeding system acting by deceit, and that the pollinators are probably small solitary bees.

Taxonomic synopsis

Key to the Asian mycoheterotrophic species of *Cephalanthera*

1. Labellum shallowly three-lobed in its distal part, not distinctly differentiated in hypochile and epichile 5. *C. pusilla*
2. Sepals and petals with subcaudate, reflexed apices. Epichile broadly rounded, strongly apiculate 4. *C. ericiflora*
 - Sepals and petals with obtuse to acuminate, straight to slightly recurved apices. Epichile rounded to acute, not apiculate 3
3. Hypochile with a conical, obtuse, distinctly set off spur at base; epichile transversely elliptic, rounded 3. *C. calcarata*
 - Hypochile gradually widened into a rounded sac; epichile ovate to cordate, obtuse 4
4. Inflorescence up to 4 cm long (measured from the node of the lowermost flower). Sepals at least 11 mm long.



Figure 3. *Cephalanthera exigua* Seidenf. Thailand, northern floristic region, Chiang Mai, Doi Pee Pan Nam, 1700 m a.s.l., 23 Mar 2005, photo: Henrik Æ. Pedersen.

Hypochile saccate at base. Column (including anther) at least 8 mm long 1. *C. exigua*
 – Inflorescence more than 4 cm long (measured from the node of the lowermost flower). Sepals up to 10 mm long. Hypochile saccate in its middle part. Column (including anther) up to 6 mm long . . . 2. *C. gracilis*

***Cephalanthera exigua* Seidenf. (1975, p. 71) (Fig. 2, 3)**

Type: Laos, Xiangkhoang, ‘Phu Muten’, 1500 m a.s.l., 20 Apr 1932, Kerr 01024 (holotype: K!).

Plant white (sometimes with a faint greenish tinge in backlight), rhizomatous with a few, unbranched, slender fusiform roots; flowering shoots 3.0–14.0 cm tall. Stem slender, 1–2 mm in diameter; internodes 1.2–4.4 cm. Leaves 1–2 (excluding 4–6 underground cataphylls), each of them reduced to a 0.6–1.0 cm long sheath and a boat-shaped, (ovate-)triangular, acute to obtuse lamina (0.3–0.9 × 0.2–0.6 cm). Inflorescence 0.9–4.0 cm long, 1- to 4-flowered (rarely 5-flowered), often somewhat secund. Floral bracts narrowly lanceolate-oblong, acute, 12.0–13.9 × 3.0–4.2 mm, 5- to 7-veined from the base. Flowers sessile, resupinate, suberect (to spreading). Sepals white, somewhat spreading, glabrous and smooth; dorsal sepal lanceolate, subacuminate, 3- to 5-veined from the base, 11.0–21.0 × 3.9–5.7 mm; lateral sepals lanceolate, slightly oblique, somewhat channelled towards the apex, acuminate, 3- to 4-veined from the base, 12.0–20.1 × 4.1–6.1 mm. Petals white, porrect, glabrous and smooth, narrowly elliptic-oblong, slightly recurved at the apex, obtuse, 3-veined from the base, 9.3–15.5 × 3.1–5.2 mm. Labellum white with pale brown ornaments, pale brown inner surface of the hypochile and a pale brown crescent-

shaped band near the apex of the epichile; immovably attached to the column, porrect, differentiated in hypochile and epichile; hypochile saccate with erect, falcately triangular, obtuse to subacute sidelobes and a median ruminate ridge, glabrous and smooth (except for the slightly papillose apices of the sidelobes), 2.9–4.6 mm long, 9.5–14.0 mm across the sidelobes when flattened; sac 2.0–3.5 mm long (measured along the back wall); epichile cordate with upcurved sides and downcurved apex, obtuse, densely papillose and carrying 3–5 longitudinal keels on the ventral side, 4.3–8.3 × 4.9–9.9 mm. Column semiterete, straight to slightly incurved, 8.0–9.6 mm long including the anther; anther versatile, obliquely ellipsoidal, obtuse to subacute, 2-loculate with white, strongly elongate, mealy pollinia, 2.7–2.8 mm long; rostellum well-developed, protruding below the anther; receptive part of stigma concave with protruding margins. Ovary terete, striated, 11.6–12.6 mm long.

Additional specimen examined

Thailand, northern floristic region, Chiang Mai, Doi Inthanon, 1600 m a.s.l., 17 Feb 2008 (Wattana 2714 QBG!), Doi Pee Pan Nam, 1700 m a.s.l., 10 Apr 2001 (Wattana 1338 BKF! C! QBG!), 3 Apr 2005 (Suddee et al. 2181 BKF!).

***Cephalanthera gracilis* S. C. Chen & G. H. Zhu (2002, p. 600)**

Type: China, Yunnan, Binchuan county, 1 May 1911, Ducloux 7171 (holotype: P!).

***Cephalanthera calcarata* S. C. Chen & K. Y. Lang (1986, p. 271)**

Type: China, Yunnan, Yangbi county, Ji Dan Shan west of Cang Shan, near Miao Ju Ping, 7 May 1929, Ching 22569 (holotype: PE, isotype: KUN).

Additional specimen examined

China, Yunnan, Yangbi county, above Yangbi, 2600 m a.s.l., 9 May 1981 (Sino-British Expedition to Cangshan 1981 0397 K!).

***Cephalanthera ericiflora* Szlach. & Mytnik (in Szlach. et al. 2008, p. 213)**

Type: Laos, Viangchan, ‘Pu Tat’, 1200 m a.s.l., 22 Apr 1932, Kerr s.n. (holotype: BM).

***Cephalanthera pusilla* (Hook. f.) Seidenf. (1975, p. 72)**

Basionym: *Galeola pusilla* Hook. f. (1890–1894, p. 89 [published 1890]).

Based on the same type: *Aphyllorchis pusilla* (Hook. f.) Tang & F. T. Wang (1951, p. 66) p.p.

Type: Myanmar, Bago, Pegu, ‘on the Pookee ridges’, sine anno, Kurz 336 (holotype: CAL!; drawing at K!).

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References

- Abadie, J.-C. et al. 2006. *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and nonphotosynthetic individuals. – *Can. J. Bot.* 84: 1462–1477.
- Burgeff, H. 1954. Samenkeimung und Kultur europäischer Erdorchideen nebst Versuchen zu ihrer Verbreitung. – Gustav Fischer.
- Chase, M. W. and Hills, H. G. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. – *Taxon* 40: 215–220.
- Chen, S.-C. and Lang, K.-Y. 1986. *Cephalanthera calcarata*, a new saprophytic orchid from China [in Chinese]. – *Acta Bot. Yunnan.* 8: 271–274.
- Chen, S.-C. and Zhu, G.-H. 2002. *Cephalanthera gracilis* (Orchidaceae), a new species from China. – *Acta Bot. Yunnan.* 24: 600–602.
- van der Cingel, N. A. 2001. An atlas of orchid pollination. America, Africa, Asia and Australia. – A. A. Balkema.
- Claessens, J. and Kleynen, J. 1995. Die Systematik der europäischen Orchideen illustriert an Hand von Makro-Fotos. – *J. Eur. Orchid.* 27: 93–124.
- Coleman, R. A. 1995. The wild orchids of California. – Cornell Univ. Press.
- Dafni, A. and Ivri, Y. 1981. The flower biology of *Cephalanthera longifolia* (Orchidaceae) – pollen imitation and facultative floral mimicry. – *Plant Syst. Evol.* 137: 229–240.
- Gebauer, G. and Meyer, M. 2003. ¹⁵N and ¹³C natural abundance of autotrophic and mycoheterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. – *New Phytol.* 160: 209–223.
- Guindon, S. and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. – *Syst. Biol.* 52: 696–704.
- Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – *Nucl. Acids Symp. Ser.* 41: 95–98.
- Hooker, J. D. 1890–1894. The flora of British India VI. Orchideae to Cyperaceae. – L. Reeve and Co.
- Julou, T. et al. 2005. Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. – *New Phytol.* 166: 639–653.
- Kerr, A. F. G. 1933. A collection of orchids from Laos. – *J. Siam Soc., Nat. Hist. Suppl.* IX: 225–243.
- Kohns, P. and Schneider, P. 1993. *Cephalanthera damasonium* (Mill.) Druce var. *chlorotica* – ein Standort im Saarland. – *Orchidee (Hamburg)* 44: 31–32.
- Lanave, C. et al. 1984. A new method for calculating evolutionary substitution rates. – *J. Mol. Evol.* 20: 86–93.
- Leake, J. R. 1994. The biology of myco-heterotrophic ('saprophytic') plants. – *New Phytol.* 127: 171–216.
- Luer, C. A. 1975. The native orchids of the United States and Canada excluding Florida. – NY Bot. Gard.
- MacDougal, D. T. 1899. Symbiosis and saprophytism. – *Bull. Torrey Bot. Club* 26: 511–530, Pls 367–369.
- Mairold, F. and Weber, F. 1950. Notiz über *Cephalanthera Albinos*. – *Protoplasma* 39: 275–277.
- Molvray, M. et al. 2000. Polyphyly of mycoheterotrophic orchids and functional influences on floral and molecular characters. – In: Wilson, K. L. and Morrison, D. A. (eds), *Monocots: systematics and evolution*. CSIRO, pp. 441–448.
- Mueller-Dombois, D. and Ellenberg, H. 1974. Aims and methods of vegetation ecology. – John Wiley.
- Nazarov, V. V. and Ivanov, S. P. 1990. Učastie pčel roda *Chelostoma* Latr. (Hymenoptera, Megachilidae) v opylenii mimikrirujuščix vidov *Cephalanthera rubra* (Z.) Rich. i *Campanula taurica* Juz. v Krymu. – *Éntomologičeskoe Obozrenie* LXIX: 534–537.
- Neiland, M. R. M. and Wilcock, C. C. 1998. Fruit set, nectar reward, and rarity in the Orchidaceae. – *Am. J. Bot.* 85: 1657–1671.
- Nilsson, L. A. 1983. Mimesis of bellflower (*Campanula*) by the red helleborine orchid *Cephalanthera rubra*. – *Nature* 305: 799–800.
- Page, R. D. M. 1996. Treeview: an application to display phylogenetic trees on personal computers. – *Computer Appl. Biosci.* 12: 357–358.
- van der Pijl, L. and Dodson, C. H. 1966. Orchid flowers: their pollination and evolution. – Fairchild Trop. Gard., Univ. of Miami Press.
- Posada, D. and Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. – *Bioinformatics* 14: 817–818.
- Pridgeon, A. M. et al. (eds) 2005. *Genera Orchidacearum* 4. Epidendroideae, part one. – Oxford Univ. Press.
- Rasmussen, H. N. 1995. Terrestrial orchids from seed to mycotrophic plant. – Cambridge Univ. Press.
- Renner, O. 1938. Über blasse, saprophytische *Cephalanthera alba* und *Epipactis latifolia*. – *Flora* 132: 225–233.
- Renner, O. 1943. Notiz über blasse *Cephalanthera* und *Tozzia*. – *Flora* 136: 309–312.
- Rodriguez, F. et al. 1990. The general stochastic model of nucleotide substitution. – *J. Theor. Biol.* 142: 485–501.
- Roy, M. et al. 2009. Ectomycorrhizal *Inocybe* species associate with the mycoheterotrophic orchid *Epipogium aphyllum* but not with its asexual propagules. – *Ann. Bot. Oxford* 104: 595–610.
- Scacchi, R. et al. 1991. Effect of the breeding system on the genetic structure in three *Cephalanthera* spp. (Orchidaceae). – *Plant Syst. Evol.* 176: 53–62.
- Seidenfaden, G. 1975. Contributions to the orchid flora of Thailand VI. – *Bot. Tidsskr.* 70: 64–97.
- Selosse, M.-A. and Roy, M. 2009. Green plants that feed on fungi: facts and questions about mixotrophy. – *Trends Plant Sci.* 14: 64–70.
- Smith, S. E. and Read, D. J. 2008. *Mycorrhizal symbiosis* (3rd ed.). – Academic Press.
- Sørensen, T. 1948. A method of establishing groups of equal amplitude in plant sociology based on similarity of species content. – *Biol. Skr.* 5: 1–34, Fig. 1, Table 4 (1–6).
- Szlachetko, D. L. et al. 2008. *Cephalanthera ericiflora* Szlach. & Mytnik, sp. nov. (Orchidaceae, Neottioideae), a new species from Laos. – *Acta Soc. Bot. Poloniae* 77: 213–215.
- Tam, P. C. F. and Griffiths, D. A. 1993. Mycorrhizal associations in Hong Kong Fagaceae. – *Mycorrhiza* 2: 111–115.
- Tang, T. and Wang, F. T. 1951. Contributions to the knowledge of eastern Asiatic Orchidaceae II. – *Acta Phytotax. Sin.* 1: 23–102.
- Taylor, D. L. et al. 2002. Mycorrhizal specificity and function in myco-heterotrophic plants. – In: van der Heijden, M. G. A. and Sanders, I. R. (eds), *Mycorrhizal ecology*. Ecol. Stud. Analysis Synth. 157. Springer, pp. 375–413 (I–XXIV, 1–469).
- Vöth, W. 1999. Lebensgeschichte und Bestäuber der Orchideen am Beispiel von Niederösterreich. – *Stapfia* 65: 1–257.