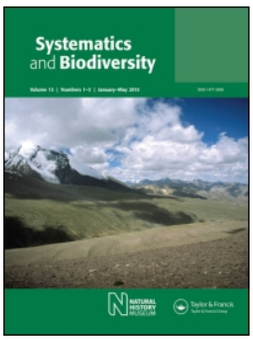

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Research Article

Integrative analyses of *Nervilia* (Orchidaceae) section *Linervia* reveal further undescribed cryptic diversity in Thailand

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The delimitation of cryptic species is necessary to accurately classify and appropriately conserve biodiversity. Integrative analyses can be incisive in detecting and circumscribing cryptic diversity, especially in species complexes whose members are delineated by minor or overlapping morphological variation. We adopt an integrative approach to assess species relationships and resolve species boundaries in the taxonomically difficult *Nervilia adolphi/punctata* species alliance of *N.* sect. *Linervia*, an Old World complex of reduced, one-flowered terrestrial orchids that is both species-rich and poorly known in tropical and warm temperate Asia. We sampled 12 of the 27 known species of the alliance in Asia, including all four species reported from Thailand and a further 20 plants collected in that country that could not be satisfactorily identified using morphology alone. Phylogenetic analyses using one nuclear (ITS) and two plastid (*mat*K and *trn*L-F) markers confirmed both *N.* sect. *Linervia* and the alliance itself as monophyletic, and corroborated 11 of the 12 sampled species; *N. punctata* proved polyphyletic, with the Thai samples referred to this Indonesian species falling sister to the Himalayan *N. mackinnonii*. The 20 unidentified Thai samples formed three distinct, strongly supported clades. STACEY, a Bayesian coalescence approach to species delimitation, resolved the same three clusters, but provided evidence suggesting that one comprised two distinct sub-clades. Building on this genetic evidence, we identify subtle morphological differences and invoke a diagnosable species concept to circumscribe three previously unrecognized cryptic species from Thailand. This objective approach to species delimitation validates ostensibly minor morphological differences as a basis for differentiating species within the alliance, paving the way for a global analysis of species boundaries throughout the genus as a whole.

Key words: cryptic species, integrative analyses, morphology, *Nervilia adolphi/punctata* species alliance, phylogenetic analysis, section *Linervia*, species complex, species delimitation, STACEY

Introduction

Cryptic species pose a recalcitrant challenge to the classification of biodiversity (Bickford et al., 2007). Despite being phylogenetically distinct, their morphological similarity to other members of the group to which they belong masks their recognition and undermines the accurate circumscription of better-known relatives (Fernandez, Shevock, Glazer, & Thompson, 2006; Pfenninger, Nowak, Kley, Steinke, & Streit, 2007). This can confound understanding of speciation, biogeography, and community assembly (Molbo, Machado, Sevenster, Keller, & Herre, 2003; Newmaster & Ragupathy, 2009; Okuyama & Kato, 2009), as well as debase attempts to appropriately conserve, manage or utilize them and their congeners (Davidson-Watts, Walls, & Jones, 2006; Pringle et al., 2005; Whittall, Hellquist, Schneider, & Hodges, 2004). The prevalence and causes of morphological stasis in speciation are therefore important to taxonomists, ecologists, and conservationists alike.

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In an analysis of the occurrence of cryptic animal species, Pfenninger and Schwenk (2007) found evidence for their uniform distribution across all major metazoan taxa and biomes, suggesting that the phenomenon is both phylogenetically and geographically ubiquitous. By comparison, reports of cryptic plant species are sparse in the literature (Bickford et al., 2007) and, as a result, their geographic and phylogenetic prevalence remains untested. This under-representation of cryptic taxa amongst plants might be considered surprising, especially if morphological resemblance amongst genetically distinct and reproductively isolated entities alone is taken as the core definition (leaving aside sympatry, a sibling relationship, and recent divergence as additional criteria; summarized by Bickford et al., 2007), and that failure to correctly distinguish them therefore represents no more than a deficiency in human perception (Pfenninger & Schwenk, 2007). Recognition of cryptic plant species increasingly relies upon the detection of unexpectedly wide genetic distance amongst multiple samples assigned to the same taxon using molecular markers suitable for the lineage under study (Newmaster & Ragupathy, 2009; Okuyama & Kato, 2009). The theoretical prediction that numerous plant species are likely to be paraphyletic (Rieseberg & Brouillet, 1994) therefore suggests that many cryptic plant species still await discovery, as has been borne out by a number of empirical studies (e.g., Goodwillie & Stiller, 2001; Syring, Farrell, Businsky, Cronn, & Liston, 2007).

Undescribed plant diversity is thought to occur predominantly in the world's biodiversity hotspots (Joppa, Roberts, Myers, & Pimm, 2011) where poorly sampled and little studied plant groups are most concentrated (Prance, Beentje, Dransfield, & Johns, 2000). In addition to the prediction that this as-yet-unknown diversity is likely to be rare and locally restricted (Joppa, Roberts, & Pimm, 2010), it seems reasonable to expect that some will also prove to be cryptic, as integrative analyses are undertaken on hitherto only poorly known groups. Tropical species complexes whose members are delineated by minor or overlapping morphological variation therefore represent promising foci for the discovery of undetected cryptic taxa.

The utility of an integrated approach in identifying cryptic taxa within species complexes has been demonstrated by several studies. Heinrichs et al. (2015) combined sequence data with a re-examination of taxonomic characters to circumscribe two previously unrecognized species in the morphologically conservative, pantropical bryophyte genus *Lepidolejeunea* (Lejeuneaceae). Similarly, Liu, Möller, Gao, Zhang, and Li (2011) used DNA barcodes to discern polyphyletic lineages in Eurasian yews (*Taxus* spp., Taxaceae), each of which was found to correspond to geographically distinct morphological units. Whilst caution has been urged in the use of divergence amongst DNA sequences alone to support the recognition of cryptic species (Brower, 2006; Okuyama & Kato, 2009), inferring reproductive isolation on the basis of corroborating phylogeographic disparities (Carstens & Satler, 2013; Fernandez et al., 2006; Shaw, 2000), morphological differences (Newmaster & Ragupathy, 2009; Ragupathy, Newmaster, Murugesan, & Balasubramaniam, 2009; Whittall et al., 2004), hybrid sterility (Okuyama & Kato, 2009) or genome-wide divergence (Després, Gielly, Redoutet, & Taberlet, 2003; Gale, Maeda, Chen, & Yukawa, 2010; Gurushidze, Fritsch, & Blattner, 2008) can be compelling. Bayesian coalescent approaches to species delimitation have also proved insightful (Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012; Toprak et al., 2016).

Species complexes have been blamed for a proliferation of ambiguous species descriptions in the Old World terrestrial orchid genus *Nervilia* (Orchidaceae: Epidendroideae; Gale, Li, Kinoshita, & Yukawa, 2015; Pettersson, 1991; Seidenfaden, 1978). This genus of approximately 70 species is characterized by a hysteranthous annual cycle (Pridgeon, Cribb, Chase, & Rasmussen, 2005), in which the single, deciduous leaf emerges only once the single, ephemeral scape has faded, rendering plants inconspicuous in the field and complete specimens rare in herbaria. Options for morphological analysis are particularly limited in the so-called '*Nervilia adolphi/punctata* species alliance', a complex of approximately 35 reduced, one-flowered species in *N.* section *Linervia* that all possess an entire, slender, white and purple-marked labellum and a glabrous, angular-cordate leaf (Gale & Phaxaysombath, 2017; Govaerts et al., 2017). Many species are known only from type material, but several key characters (such as floral presentation, lip ornamentation, and leaf mottling) are effectively lost in preserved specimens. Integrative analyses of this complex in East Asia have helped refine species boundaries and identify cryptic species by uncovering significant genetic and cytological divergence amongst otherwise morphologically similar plants (Eum, Gale, Yukawa, Lee, & Lee, 2011; Gale et al., 2010, 2015).

Recent taxonomic treatments of the genus in China (Chen & Gale, 2009) and Thailand (Gale & Watthana, 2014) now provide a framework for critical appraisal of species numbers and relationships in tropical and warm temperate Asia, where the *N. adolphi/punctata* species alliance is most diverse, with 27 currently accepted names. In the present study, we subjected all four known members of the alliance in Thailand, as well as a further 12 species of *N.* sect. *Linervia* from neighbouring countries, to combined molecular phylogenetic and morphological analyses to test species concepts and prospect for undescribed diversity towards an improved understanding of the evolution, ecology, and conservation needs of the genus in the region.

Materials and methods

Study taxa

The genus *Nervilia* is distributed from sub-Saharan Africa to Australasia and the South-west Pacific Islands, but is most diverse in tropical Asia (Gale et al., 2015; Pettersson, 1991). However, a recent slew of new species descriptions in this region (Averyanov, 2011; Gale & Phaxaysombath, 2017; Gale et al., 2015, 2016; Gale, Rueangruea, & Suddee, 2014; Gale, Suddee, & Watthana, 2013; Hsu, Chung, & Kuo, 2012; Jalal, Kumar & Rawat, 2012; Lin, 2014; Lin & Chang, 2013) suggests that taxonomic understanding of the genus remains incomplete. Species boundaries are particularly unclear amongst members of the one-flowered *N. adolphi/punctata* species alliance in *N.* sect. *Linervia*, with minor variation in leaf and floral morphology sometimes masking wide phylogenetic divergence (Gale et al., 2015). Two or more species can occur sympatrically, making the matching of flowering and leafing specimens collected at different times in the season problematic (Gale et al., 2010).

To circumvent this problem, Thai material used in the present study was sampled during the leafing phase and, as far as possible, cultivated until it flowered the following year. This allowed us to study the morphology of the reproductive and vegetative shoots of the same individual and deposit correctly matched specimens at the herbaria indicated in Table S1 (see supplemental material online, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/xx.xxxx/xxxxxxxx.xxxx.xxxxxx). Plants were examined under a confocal microscope and critically compared with material available at BCU, BKF, E, HITBC, HN, IBSC, K, KAG, KATH, KUN, KYO, L, MAK, MBK, QBG, RYU, TAI, TAIF, TI, TNS, and TUCH. Particular attention was paid to labellum outline, disc ornamentation and leaf mottling, which have been highlighted as important characters for species delimitation (Gale & Phaxaysombath, 2017; Gale et al., 2015; Pettersson, 1991). Identifications were made using the keys and descriptions given in Backer and Bakhuizen van den Brink (1968), Chen and Gale (2009), Gale and Watthana (2014), Gale and Wu (2008), Gale et al. (2015), Gale, Yukawa, and Kuroiwa (2007), Hooker (1890), Hsieh, Gale, Lee, Yeh, Leou, and Yeh (2013), Hsu et al. (2012), King and Pantling (1896), Pearce and Cribb (2002), Seidenfaden (1978), and Su (2000).

Taxon sampling and outgroup selection

In total, 45 samples belonging to *N.* sect. *Linervia* from tropical and warm temperate Asia were included as ingroup taxa (Table S1, see supplemental material online). Three of these represented two species, *N. crociformis* (Zoll. & Moritzi) Seidenf. and *N. cumberlegei* Seidenf. & Smitinand, not referable to the *N. adolphi/punctata* species alliance on account of their fimbriate labellum apex and reniform, sparsely setulose leaf, but which were included to assess the monophyly of the alliance and the section. The other 42 samples conformed to the concept of the *N. adolphi/punctata* species alliance, including 11 that represented seven species of Taiwan, South China, Japan, and Nepal (*N. alishanensis* T.C.Hsu, S.W.Chung & C.M.Kuo, *N. futago* S.W.Gale & T.Yukawa, *N. lanyuensis* S.S.Ying, *N. mackinnonii* (Duthie) Schltr., *N. macroglossa* (Hook.f.) Schltr., *N. nipponica* Makino, *N. taiwaniana* S.S.Ying) sequenced in previous studies (Eum et al., 2011; Gale et al., 2015). The remaining 31 accessions represented species newly sequenced for this study, including all four known members of the alliance in Thailand: *N. infundibulifolia* Blatt. & McCann, *N. khaoyaica* Suddee, Watthana & S.W.Gale, *N. punctata* (Blume) Makino and *N. umphangensis* Suddee, Rueangr. & S.W.Gale. For the purposes of this study, we regarded Thai plants of *N. punctata* sampled here as '*N. cf. punctata*' on account of small differences identified between them and the taxon originally described from Indonesia (Blume, 1849), as highlighted elsewhere (Gale & Watthana, 2014; Table S2, see supplemental material online). We also included 20 samples from northern, eastern, and southern Thailand that could neither be keyed out using Gale and Watthana (2014) nor satisfactorily matched with any known species from elsewhere in the region. The remaining samples were from South China (*N. infundibulifolia*), Vietnam (*N. muratana* S.W.Gale & S.K.Wu) and Indonesia (*N. punctata*). Multiple accessions of taxa were included where possible to account for infra-specific variation. Based on the phylograms of Gale et al. (2015), we included two samples of *N. plicata* (Andrews) Schltr. (a two-flowered species of *N.* sect. *Vinerlia*), plus two of *N. mekongensis* S.W.Gale, Schuit. & Suddee and one of *N. shirensis* (Rolfe) Schltr. (multi-flowered species of *N.* sect. *Nervilia*) as outgroup taxa.

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from fresh or silica dried leaf samples using either a modified 2 × CTAB procedure (Doyle & Doyle, 1987) or a QIAGEN DNeasy® plant DNA Kit (Hiden, Germany) according to the manufacturer's instructions. Previous molecular phylogenetic studies of *Nervilia* have shown the internal transcribed spacer (ITS) of nuclear ribosomal DNA, the plastid maturase K gene (*mat*K, including part of the flanking *trn*K introns) and the plastid *trn*L intron to be sufficient for discriminating members of the *N. adolphi/punctata* species alliance (Eum et al., 2011; Gale et al., 2015). In the present study, the ITS1 and ITS2 spacers plus 5.8S gene were amplified using the 5F and 4R primers of White, Bruns,

Lee, and Taylor (1990) or Sun, Skinner, Liang, and Hulbert (1994), *mat*K was amplified using the 19F primer of Molvray, Kores, and Chase (2000) and the 2R primer of Johnson and Soltis (1994), or in three sections using the primers of Hidayat, Yukawa, and Ito (2005), and the *trn*L intron and *trn*L-*trn*F intergenic spacer (hereinafter *trn*L-F) were amplified using the *c*, *d*, *e*, and *f* primers of Taberlet, Geilly, Pautou, and Bouvet (1991).

PCR was performed in a total reaction mixture of 50 μL containing 25 μL of 2 × DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Thailand), 21 μL of nuclease-free water, 1 μL of 4.0% bovine serum albumin (BSA), 1 μL of each primer (20 mmol/L), and 2 μL template DNA. The PCR profile consisted of an initial 3 min pre-melt at 94°C and 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C and a 1 min extension at 72°C, followed by a final extension of 10 min at 72°C. For ITS, the volume of nuclease-free water was reduced to 20 μL and 1 μL DMSO was added. Most of the successfully amplified products were cleaned using FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Thermo Fisher Scientific, Thailand); some of the ITS PCR products were excised from the gel and cleaned with a GeneJET Gel Extraction kit (Thermo Fisher Scientific, Thailand). Cleaned PCR products were sequenced using the same primers used in the initial amplifications, except in the case of *mat*K for which we also used two internal primers *mat*K XF (Ford et al., 2009) and *mat*K 828F (5-CTATGGTTCCTCAAAGATCCT-3). Raw sequences were edited using Sequence Navigator (Applied Biosystems), and complementary sequences were assembled using AutoAssembler version 1.4.0 (Applied Biosystems). All sequences have been deposited in GenBank (Table S1, see supplemental material online).

Sequence alignment and phylogenetic analyses

Alignments were conducted using the MAFFT multiple alignment plugin in Geneious v6.1.6 (Drummond et al., 2011), with subsequent adjustment by eye. We excluded two polyA regions comprising 23 and 39 positions in the *trn*L-F and *mat*K genes, respectively. Sequence alignments have been submitted to TreeBASE (Study No. 21713). Because topological conflict may be encountered in combined analyses of plastid and nuclear markers due to the different inheritance modes of the two genomic compartments (Cafasso, Widmer, & Cozzolino, 2005; Corriveau & Coleman, 1988) or as a result of other biological phenomena (Pérez-Escobar, Balbuena, & Gottschling, 2016; van der Niet & Linder, 2008), we examined individual data partitions for potential differences. Firstly, a 'hard' incongruence test was performed by directly comparing respective topologies and support for each clade generated in the separate analyses, with bootstrap percentages (BP) of ≥85% (Chase et al., 2000) and posterior probabilities (PP) of ≥0.95 (Martínez-Azorín, Crespo, Juan, & Fay, 2011) being taken as evidence of strong support. Next, an incongruence length difference (ILD) test (Farris, Källersjö, Kluge, & Bult, 1995) was performed in PAUP* v4.0b10 (Swofford, 2003) using 1,000 replicates, each with 1,000 random addition sequence replicates and TBR branch swapping, to assess whether the individual *mat*K and *trn*L-F datasets, and the ITS and combined plastid (ptDNA) datasets, reflected similar potential phylogenies; a P value of <0.05 was considered significant (Darlu & Lecointre, 2002; Sullivan, 1996). Finally, because some studies have shown the ILD test to be prone to Type I errors (e.g., Yoder, Irwin, & Payseur, 2001), we also used PACo (Balbuena, Míguez-Lozano, & Blasco-Costa, 2013) to further assess topological congruence. Developed as a global-fit method to assess similarities between host and parasite phylogenies, PACo has recently been demonstrated to effectively quantify cophylogenetic structure between organelle and host nuclear systems (Pérez-Escobar et al., 2016). PACo was run in R with separate consensus MrBayes trees representing the plastid (*mat*K + *trn*L-F) and nuclear (ITS) datasets used as input; the number of permutations was set to 10,000, with a P value of <0.01 indicating that H_o ('similarity between trees not higher than expected by chance') should be rejected (Pérez-Escobar et al., 2016).

Phylogenetic analysis of individual and multilocus alignments were carried out using maximum parsimony (MP) in PAUP* v4.0b10 and Bayesian inference (BI) in MrBayes v3.2 (Huelsenbeck & Ronquist, 2003). For MP analyses, heuristic searches were conducted with 1,000 random addition replicates followed by tree bisection-reconnection branch swapping. All characters were unordered and equally weighted with gaps (including unavailable sequences) treated as missing data. Topological robustness was assessed using 1,000 bootstrap replicates. For BI analyses, each DNA region was assigned its own model of nucleotide substitution, as determined by the Akaike information criterion (AIC) in Modeltest v3.06 (Posada & Crandall, 1998). Four simultaneous Monte Carlo Markov Chains (MCMC) were run, with one tree sampled every 1,000 generations for 30,000,000 generations, starting with a randomly generated tree. Majority rule (>50%) consensus trees were constructed after removing the first 25% of sampled trees as burn-in.

Species delimitation using STACEY

A Bayesian coalescent method of species delimitation was conducted in STACEY (species tree estimation using DNA sequences from multiple loci; Jones, 2017), which

is an extension of DISSECT (Jones, Aydin, & Oxelman, 2015). STACEY analysis was implemented in BEAST v2.4.4 (Bouckaert et al., 2014; Drummond & Rambaut, 2007; Drummond, Ho, Phillips, & Rambaut, 2006) using the multilocus dataset (ITS+ptDNA) for all 45 ingroup samples; outgroup samples were removed to avoid rate differences and hidden substitutions between ingroup and outgroup species (B. Oxelman, personal communication). We performed two independent runs of 10 million generations of the MCMC chains, sampling every 1,000 generations. Convergence of the stationary distribution was checked by visual inspection of plotted posterior estimates using Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). After discarding the first 1,000 trees as burn-in, the samples were summarized in the maximum clade credibility tree using TreeAnnotator v1.6.1 (Drummond & Rambaut, 2007) with a posterior probability limit of 0.5 and summarizing of mean node heights. The results were visualized using FigTree v1.3.1 (Rambaut, 2009).

Results

Morphology

Twenty-eight plants sampled throughout Thailand were confirmed as belonging to the *N. adolphi/punctata* species alliance on account of their glabrous, angular-cordate leaf and/or their single flower comprising similar, elliptic-lanceolate tepals and a narrow labellum divided into a semi-tubular hypochile and a dilated, entire, predominantly white epichile. Eight were referred to one of the four species of the alliance known in the country (Table S1, see supplemental material online), viz. *N. infundibulifola* (two samples), *N. khaoyaica* (two samples), *N.* cf. *punctata* (two samples) and *N. umphangensis* (two samples). The identity of the remaining 20 samples from Chiang Rai (FT, NER14, NER15, NER18, NER26, NER28, NER29), Trang (NBK, NBL01, NBL02, NBM01, NBM02, NER02, NTK01, NTK02), Mae Hong Son (SERM01), Chiang Mai (SG1331), and Nakhon Ratchasima Provinces (NER01, NER24, NER27) was equivocal on account of indiscrete and overlapping variation in the following key characters (Table S2, see supplemental material online).

Labellum outline. The seven plants from Chiang Rai Province possessed a relatively short, oblong-obovate labellum that was broadest across its hypochile auricles and truncate at the apex of the epichile (Fig. 1.3). These features affiliated it with *N. falcata* (King & Pantl.) Schltr., a poorly known species of north-east India; however, in that species, the hypochile auricles are triangular-falcate and acute, whereas those of the Chiang Rai plants were triangular-ovate and obtuse. The labellum of the eight plants collected from two populations in Trang

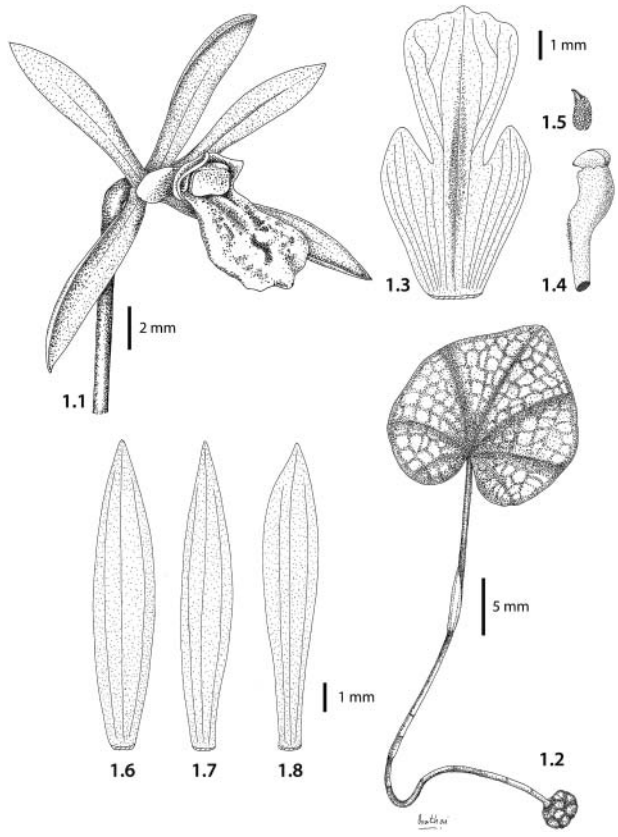

Fig. 1. *Nervilia marmorata* S.W.Gale, Suddee & Duangjai. 1.1. Flowering scape. 1.2. Plant in leaf with subterranean tuber. 1.3. Labellum. 1.4. Column. 1.5. Pollinia. 1.6. Dorsal sepal. 1.7. Lateral sepal. 1.8. Petal. All parts drawn by Orathai Kerdkaew from *Duangjai 250314* (1.1, 1.3–1.8) and *Suddee 4910* (1.2).

Province resembled that of the recently described *N. pangteyana* Jalal, Kumar & G.S.Rawat from the western Himalayas, with both being broadest across the epichile, but the former differed from the latter in having a much broader, obtuse-rounded epichile (Figs 2.5–2.6). A third entity represented by plants from Mae Hong Son Province and Chiang Mai Province possessed a labellum that was as broad or broader across its epichile, as across its short, rounded hypochile auricles (Fig. 3.4). In this respect, this entity differed from virtually all known species in Asia, with only the Himalayan *N. macroglossa* having a similarly shaped labellum; however, the labellum of that species is significantly larger.

Disc ornamentation. The disc of the seven plants from Chiang Rai Province was glabrous apart from a single, narrow, central band of short, soft hairs that arose midway along the hypochile and extended to the basal third of the epichile (Fig. 1.3), making it similar to the disc of *N. taiwaniana* and *N. macroglossa*. In contrast, the labellum of the eight plants from the two populations in Trang Province bore a broad raised ridge that arose from near the

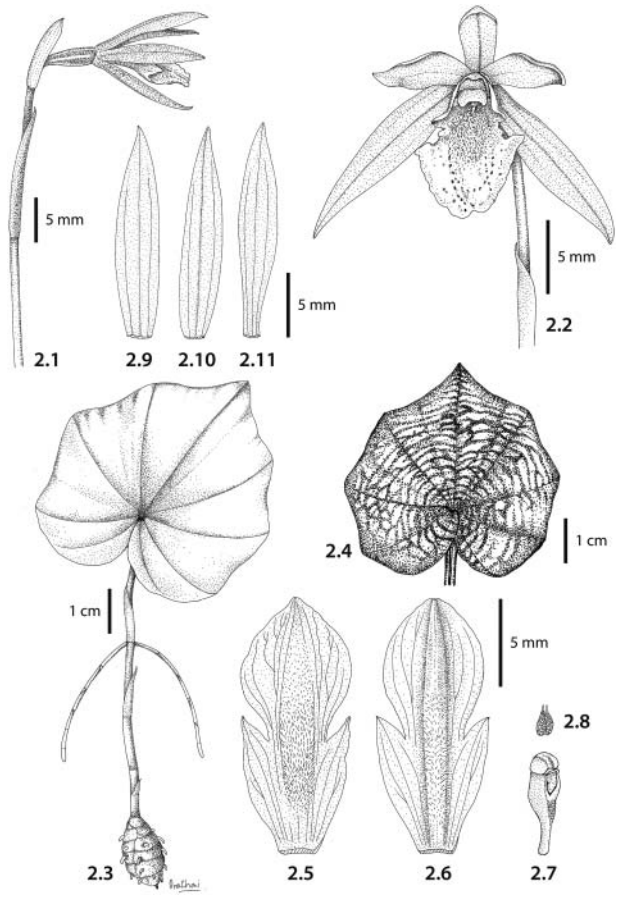

Fig. 2. *Nervilia trangensis* S.W.Gale, Suddee & Duangjai. 2.1. Flowering scape. 2.2. Front view of flower. 2.3. Plant with plain leaf, with subterranean tuber and lateral runners. 2.4. Mottled leaf form. 2.5., 2.6. Labellum. 2.7. Column. 2.8. Pollinia. 2.9. Dorsal sepal. 2.10. Lateral sepal. 2.11. Petal. All parts drawn by Orathai Kerdkaew from *Suddee 4647* (2.1, 2.2, 2.5–2.11), *Po-iam 02* (2.3) and *Po-iam 03* (2.4).

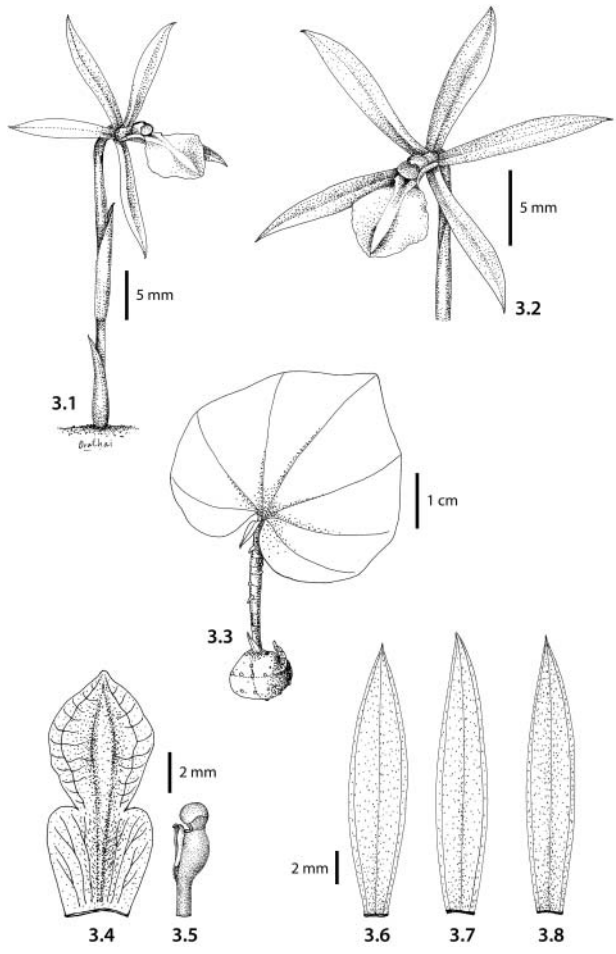

Fig. 3. *Nervilia viridis* S.W.Gale, Watthana & Suddee. 3.1. Flowering scape. 3.2. Front view of flower. 3.3. Plant in leaf showing subterranean tuber. 3.4. Labellum. 3.5. Column. 3.6. Dorsal sepal. 3.7. Lateral sepal. 3.8. Petal. All parts drawn by Orathai Kerdkaew from *Nualgnam 01*.

base of the hypochile and extended almost to the apex of the epichile and which was pubescent predominantly between the hypochile auricles (Figs 2.5–2.6). A similar broad, elongated keel has been described in the East Asian *N. alishanensis*, *N. futago*, and *N. lanyuensis*. The labellum of plants from populations in Mae Hong Son and Chiang Mai Provinces bore two parallel ridges that arose midway along the hypochile to form a narrow, pubescent channel that extended to the base of the epichile and which then converged into a single elongate-triangular, shortly papillose ridge that terminated at the apex of the epichile (Fig. 3.4), similar to that of the Indonesian *N. punctata*. However, the unidentified plants differed from these taxa in other qualitative respects (Table S2, see supplemental material online).

Leaf mottling. The adaxial surface of the leaf of seven plants from Chiang Rai Province was either light to dark green with silver mottling, or purple and green with silver mottling; the abaxial surface was mauve streaked light green along the main veins (Figs 4.4–4.5). Plants at the two populations in Trang Province exhibited wide variation in the colour of their leaf, with that of some individuals being uniformly dark green (Fig. 5.4), rendering them indistinguishable from the leaf of most members of the *N. adolphi/punctata* species alliance, whilst that of others was uniformly purple (Fig. 5.5) and so practically inseparable from the purple-leaved form of *N. khaoyaica*, and that of yet others was green with varying degrees of reticulate silver mottling (Fig. 5.6). In Asia, silver mottling similar to that observed in both the Chiang Rai plants and some of the Trang plants has otherwise been reported only in *N. taiwaniana*, a species considered endemic to Taiwan. Finally, leaf colouration of plants from the populations in Chiang Mai and Mae Hong Son Provinces (Fig. 6.4) was uniformly green, making it difficult to

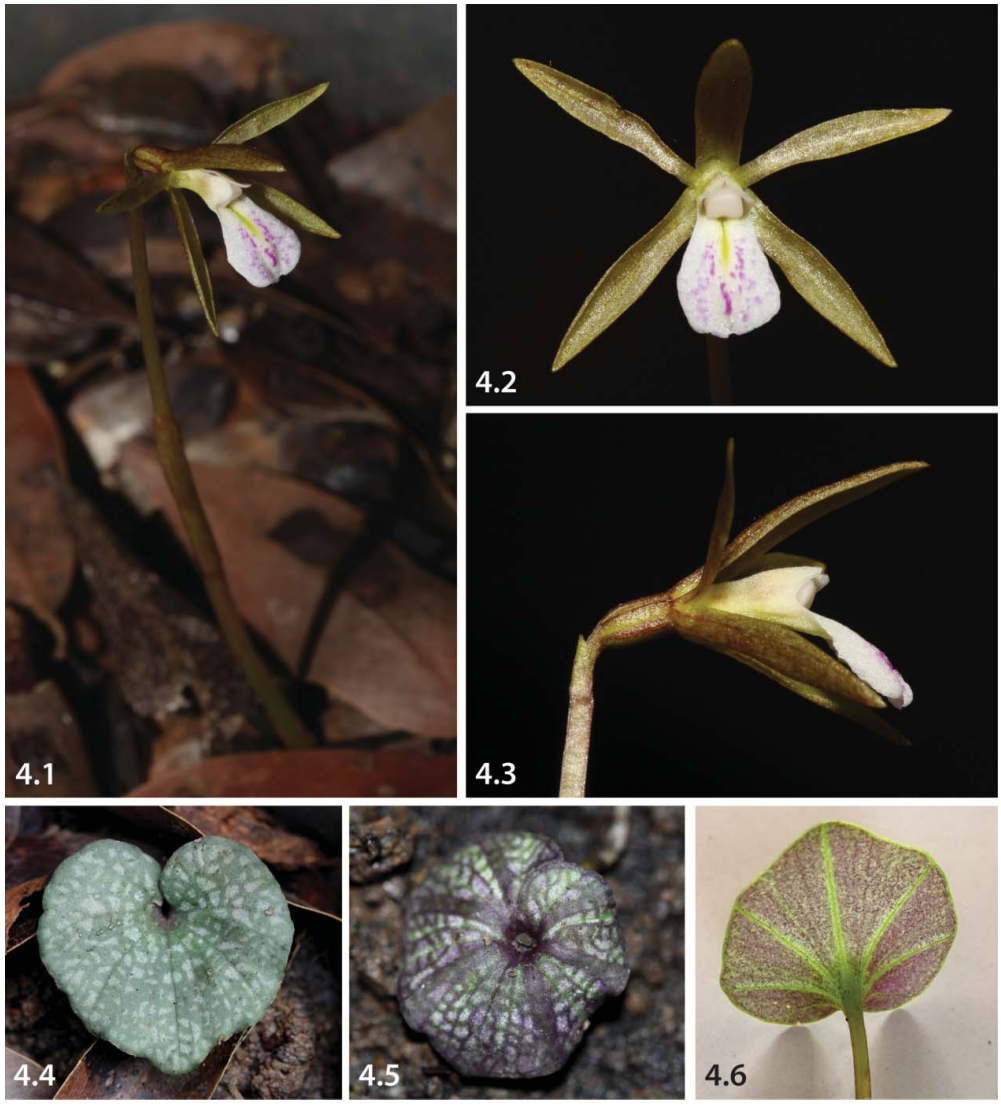

Fig. 4. *Nervilia marmorata* S.W.Gale, Suddee & Duangjai. 4.1. Flowering scape. 4.2. Front view of flower. 4.3. Lateral view of flower. 4.4. Green and silver-mottled leaf form. 4.5. Purple, green, and silver-mottled leaf form. 4.6. Purple abaxial leaf surface with green venation. Photographed in cultivation from *Duangjai 250314* (4.1–4.3) and *Suddee 4647* (4.4–4.6).

decisively match them with many other members of the alliance in South, South-east and East Asia. Plants sampled at a population in Nakhon Ratchasima Province which failed to flower in cultivation also had uniformly green leaves, precluding formal identification on the strength of this character alone (Fig. 6.5).

Molecular phylogeny

ITS, *mat*K, and *trn*L-F sequence data were newly generated for 32 samples, chloroplast data (*mat*K and *trn*L-F) alone were generated for a further three samples, and ITS and *mat*K data were generated for a single sample (Table S1, see supplemental material online). Statistics relating to the aligned matrix for each region and for the combined datasets are given in Table 1.

Tree topologies generated for the ITS data partition using BI were approximately congruent with those using MP; the BI phylogram is shown in Fig. 7.1. The ingroup samples representing *N.* sect. *Linervia* were strongly supported as monophyletic (BP 100%, PP 1.00) and comprised two strongly supported clades: one containing *N. crociformis* and *N. cumberlegei* (BP 81%, PP 1.00) and the second representing the *N. adolphi/punctata* species alliance (BP 100%, PP 1.00). Within the second clade, there was strong support for a sister relationship between *N. nipponica* and *N. taiwaniana* (BP 99%, PP 1.00). The monophyly of duplicate samples representing the species *N. alishanensis*, *N. nipponica*, *N. taiwaniana*, *N. futago,* and *N. umphangensis* was confirmed with moderate to strong support. The two Thai samples of *N.* cf. *punctata* (NSC05-01 and NSC05-02) were also monophyletic (BP

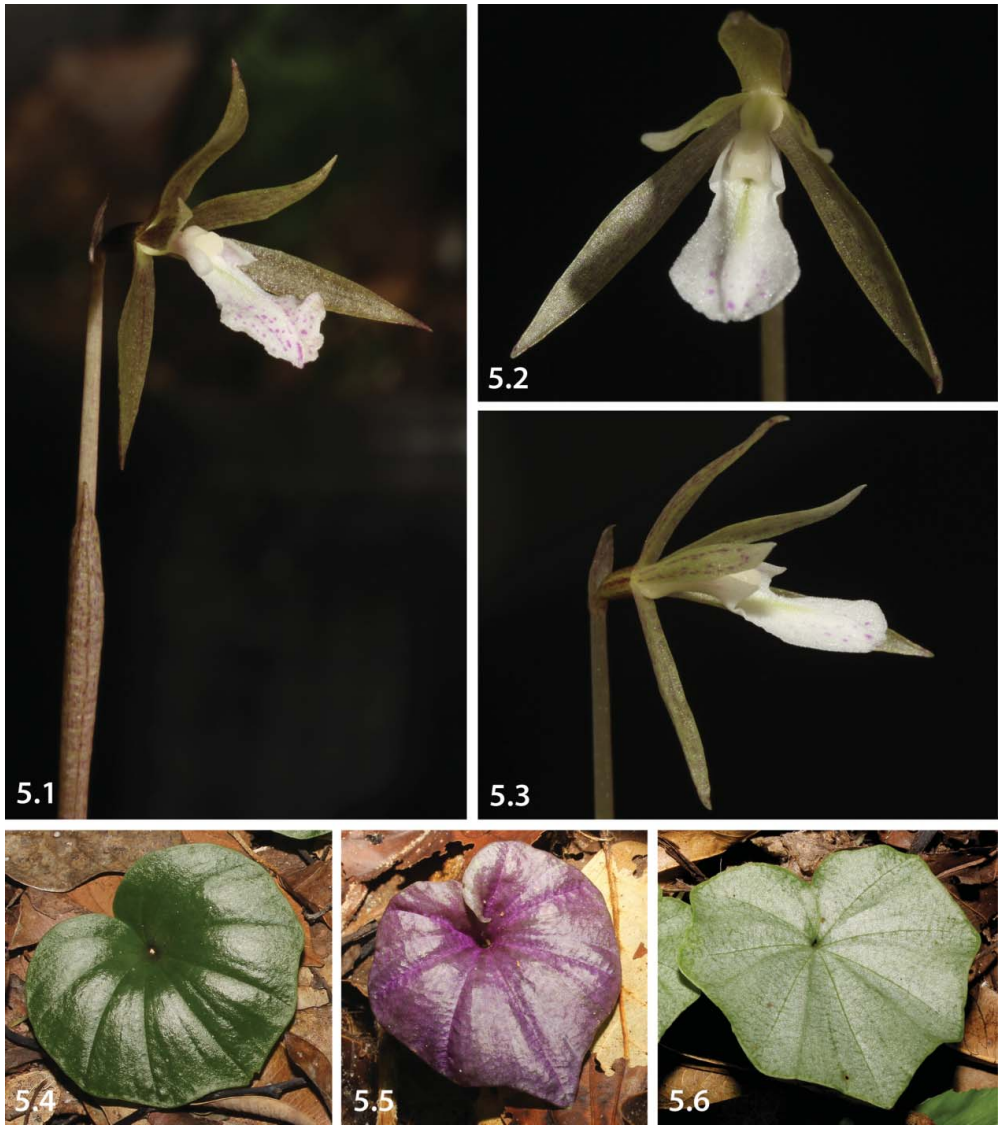

Fig. 5. *Nervilia trangensis* S.W.Gale, Suddee & Duangjai. 5.1. Flowering scape. 5.2. Front view of flower. 5.3. Lateral view of flower. 5.4. Plain green leaf form. 5.5. Purple leaf form. 5.6. Green and silver-mottled leaf form. Photographed in cultivation from *Duangjai 290317* (5.1) and *Suddee 4647* (5.2 & 5.3), and in habitat from *Duangjai 120416* (5.4), *Duangjai 290317* (5.5) and *Suddee 5072* (5.6).

95%, PP 1.00), but did not group with the Indonesian sample of this species (NPT). Four unidentified samples representing populations in Nakhon Ratchisima (NER01, NER24, NER27) and Chiang Mai (SG1331) were also strongly supported as monophyletic (BP 100%, PP 1.00), as were all seven unidentified samples obtained from Chatuchak Market but originally collected in Chiang Rai (FT, NER14, NER15, NER18, NER26, NER28, NER29; BP 98%, PP 1.00). These seven samples formed a strongly supported clade with *N. infundibulifolia* plus seven unidentified samples from Trang (NBK, NBL01, NBL02, NBM01, NBM02, NER02, NTK01; BP 92%, PP 1.00), but relationships amongst these entities were not resolved well.

Visual node-by-node comparisons of trees generated for the *mat*K and *trn*L-F datasets individually revealed no major topological disparities for nodes of BP $\geq$85% and PP $\geq$0.95, and the ILD homogeneity test for either region gave a *P* value of 0.559, indicating no significant incongruence; the two ptDNA regions were therefore combined. Tree topologies generated by BI and MP analyses were consistent; the BI phylogram is shown in Fig. 7.2. This phylogram provided better resolution at the species level than did the ITS phylogram. All ingroup samples were again strongly supported as monophyletic (BP 100%, PP 1.00) and again formed two distinct clades. The two Thai *N.* cf. *punctata* samples were resolved as distinct from the Indonesian *N. punctata* but strongly supported as

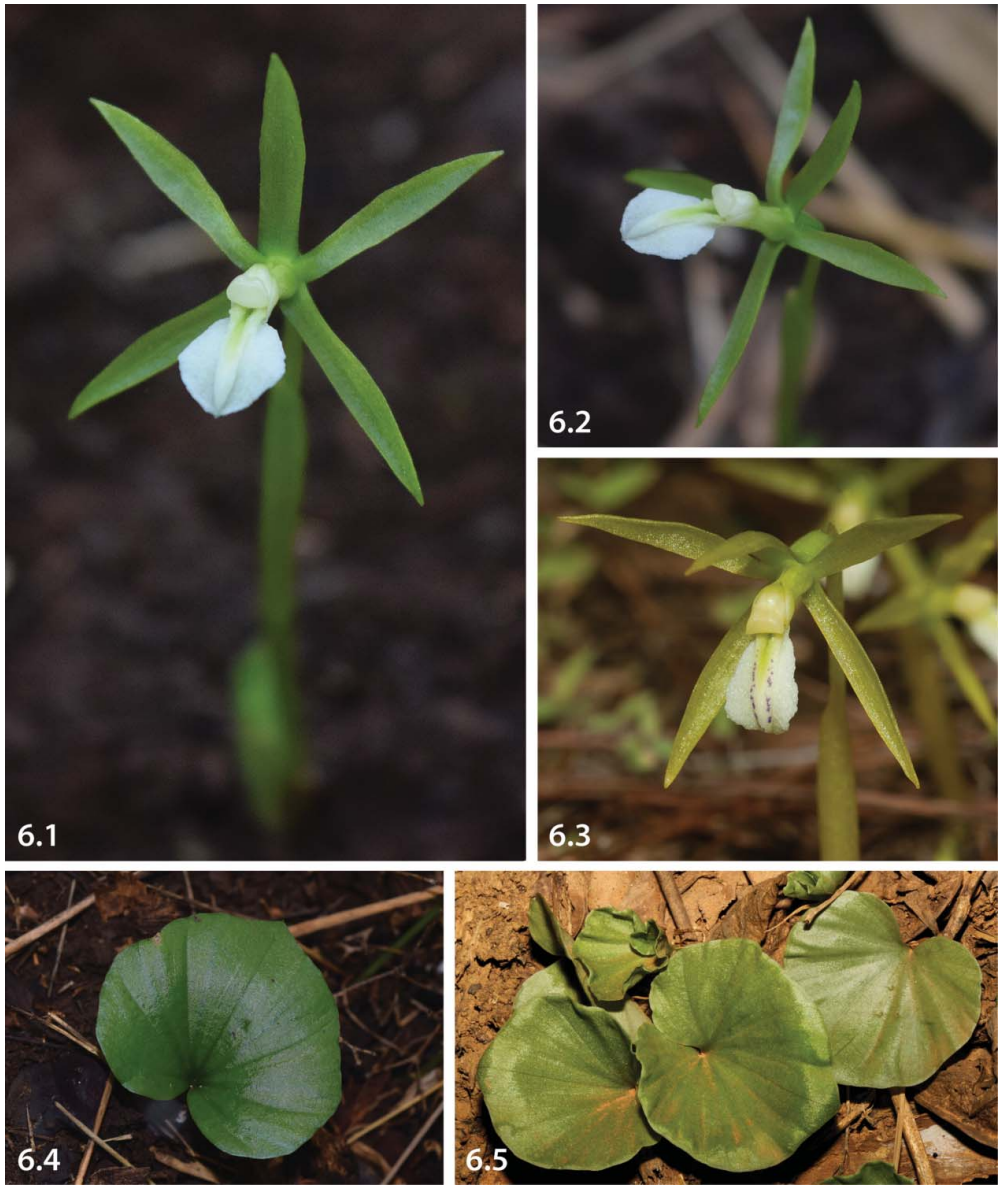

Fig. 6. *Nervilia viridis* S.W.Gale, Watthana & Suddee. 6.1. Flowering scape. 6.2. Lateral view of flower. 6.3. Front view of flower. 6.4. Leaf. 6.5. Leaves of '*N. sp. indet.*', which formed a closely related sister clade in the present study. Photographed in habitat from *Nualgnam 01* (6.1, 6.2, 6.4), *Watthana & Momkaew 4199* (6.3) and *Duangjai 020815* (6.5).

monophyletic (BP 95%, PP 1.00) and as sister to *N. mackinnonii* (BP 98%, PP 1.00). All other named species represented by two or more samples were each supported as monophyletic. Five samples representing unidentified populations in Chiang Mai (SG1331), Mae Hong Son (SERM01), and Nakhon Ratchisima (NER01, NER24, NER27) together formed a strongly supported clade (BP 100%, PP 1.00), as did the eight samples from Trang (NBK, NBL01, NBL02, NBM01, NBM02, NER02, NTK01, NTK02; BP 89%, PP 0.84) and the seven samples from Chiang Rai (FT, NER14, NER15, NER18, NER26, NER28, NER29; BP 97%, PP 1.00).

Visual node-by-node comparisons revealed the placement of all taxa to be congruent between the nrDNA (ITS) and ptDNA (*mat*K+*trn*L-F) phylograms for nodes with support values of BP $\geq$85% and PP $\geq$0.95. An ILD partition homogeneity test for the datasets resulted in $P = 0.075$ implying no significant incongruence, and PACo detected a significant global cophylogenetic signal ($m^2_{XY} = 0.315$, $P<0.0001$); the two datasets were therefore combined. Tree topologies generated by BI and MP analyses were consistent; the BI phylogram is shown in Fig. 8. This combined analysis resulted in greater resolution as compared with the two separate analyses.

Table 1. Statistics relating to the DNA datasets analysed in this study.

Parameter	ITS	*mat*K	*trn*L-F	Combined ptDNA (*mat*K and *trn*L-F) datasets	Combined ITS and ptDNA datasets
No. ingroup samples	41	44	45	45	45
No. outgroup samples	5	5	5	5	5
Aligned length	708	1,770	1,082	2,852	3,560
No. variable characters	41	74	30	104	145
No. parsimony-informative characters	240	219	146	365	605
Tree length	446	362	205	569	1023
Consistency index (CI)	0.791	0.845	0.917	0.868	0.828
Retention index (RI)	0.909	0.938	0.963	0.945	0.927
Rescaled consistency index (RC)	0.720	0.793	0.883	0.821	0.767
Best-fit model determined by AIC in Modeltest	GTR + G	K81uf + G	K81uf + G	TVM + G	TVM + I + G

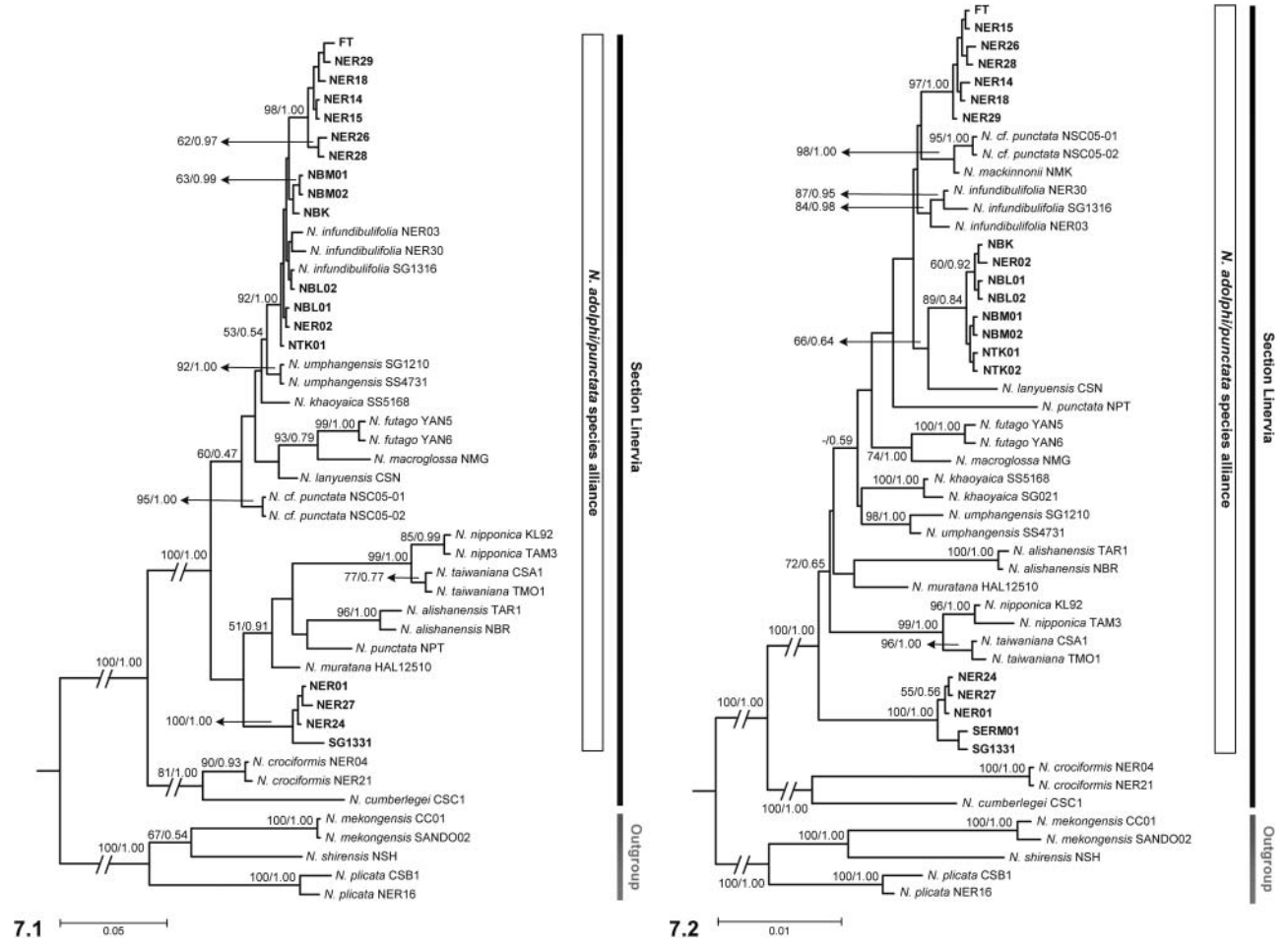

Fig. 7. Majority rule BI phylograms of ITS (7.1) and ptDNA (*mat*K+*trn*L-F) (7.2) data. Numbers at nodes indicate bootstrap percentages from MP analysis and posterior probabilities from BI analysis, respectively; '-' indicates nodes that collapsed in MP analysis. The unidentified Thai samples are shown in bold.

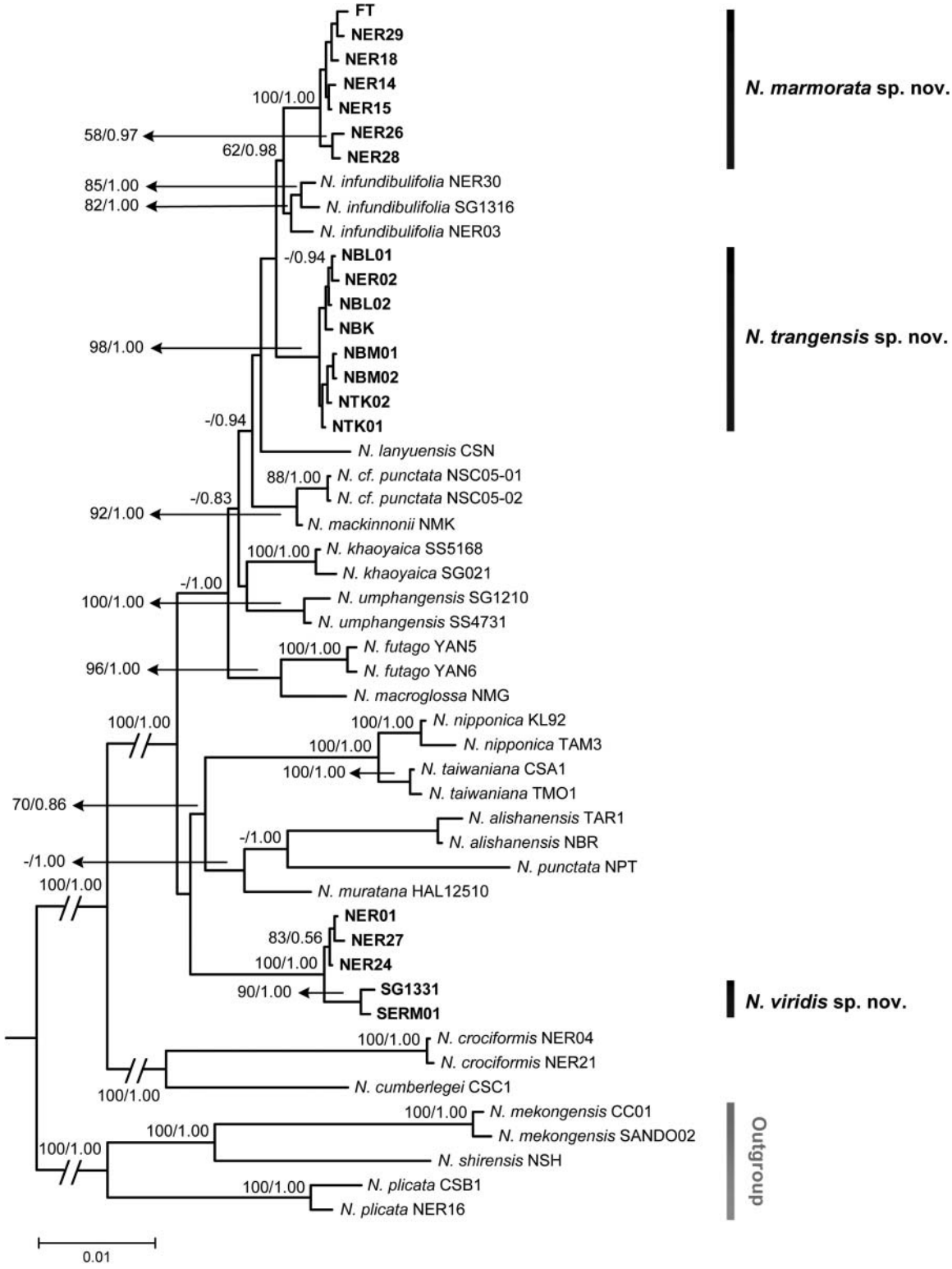

Fig. 8. Majority rule BI phylogram of combined ITS and ptDNA data. Numbers at nodes indicate bootstrap percentages from MP analysis and posterior probabilities from BI analysis, respectively; '-' indicates nodes that collapsed in MP analysis. The unidentified Thai samples are shown in bold, with the three new names proposed in this study indicated at right.

All ingroup taxa again fell into two strongly supported clades composed of the same samples as in the individual ITS and ptDNA trees. The Thai samples of *N.* cf. *punctata* were again resolved as distinct from the Indonesian sample, and as sister to *N. mackinnonii*. All other named species represented by two or more samples were otherwise strongly supported as monophyletic. The same five samples representing the unidentified populations in Chiang Mai, Mae Hong Son, and Nakhon Ratchisima again formed a strongly supported clade (BP 100%, PP 1.00), with distinct clustering amongst the three Nakhon Ratchisima samples (NER01, NER24, NER27) on the one hand, and the Chiang Mai (SG1331) and Mae Hong Son (SERM01) samples on the other. All eight samples from Trang (NBK, NBL01, NBL02, NBM01, NBM02, NER02, NTK01, NTK02) and seven samples from Chiang Rai (FT, NER14, NER15, NER18, NER26, NER28, NER29) were strongly supported as monophyletic (BP 98%, PP 1.00 and BP 100%, PP 1.00, respectively).

STACEY species delimitation

The SpeciesDelimitationAnalyser generated 1,256 clusterings from the MCMC runs, with a highest posterior probability of 0.05 PP for a classification comprising 13 clades and six singletons (i.e., 19 minimal clusters), and a zero posterior probability for any of the individuals within the 13 clades belonging to a different cluster or with any of the six singletons (Fig. 9). The two Thai *N.* cf. *punctata* samples (NSC05-01, NSC05-2) clustered with *N. mackinnonii*, not with the Indonesian *N. punctata*, with a similarity score of ≥0.7395891. Significant intraspecific variation was detected within *N. infundibulifolia*, with STACEY supporting delimitation of one singleton (NER03) plus one minimal cluster of two samples (NER30, SG1316), with similarity scores of 0.2509717–0.2537479 amongst them. All other named species were supported with an internal similarity score of ≥0.8173237 (Table S3, see supplemental material online). Although the unidentified samples from Chiang Mai, Mae Hong Son, and Nakhon Ratchisima formed a single strongly supported clade, two minimal clusters were detected within them, with SG1331+SERM01 receiving a similarity score of 0.9022765 and NER01+NER24+NER27 receiving a similarity score of ≥0.9250416, and with similarity scores in the range 0.1637979–0.1699056 between them. STACEY provided support for the delimitation of the eight unidentified samples from Trang (NBK, NBL01, NBL02, NBM01, NBM02, NER02, NTK01, NTK02) and of the seven unidentified samples from Chiang Rai (FT, NER14, NER15, NER18, NER26, NER28, NER29) as distinct minimal clusters with similarity scores of ≥0.7562465 and ≥0.6601888, respectively.

Taxonomic treatment

Nervilia marmorata S.W. Gale, Suddee & Duangjai sp. nov.
Figs 1, 4

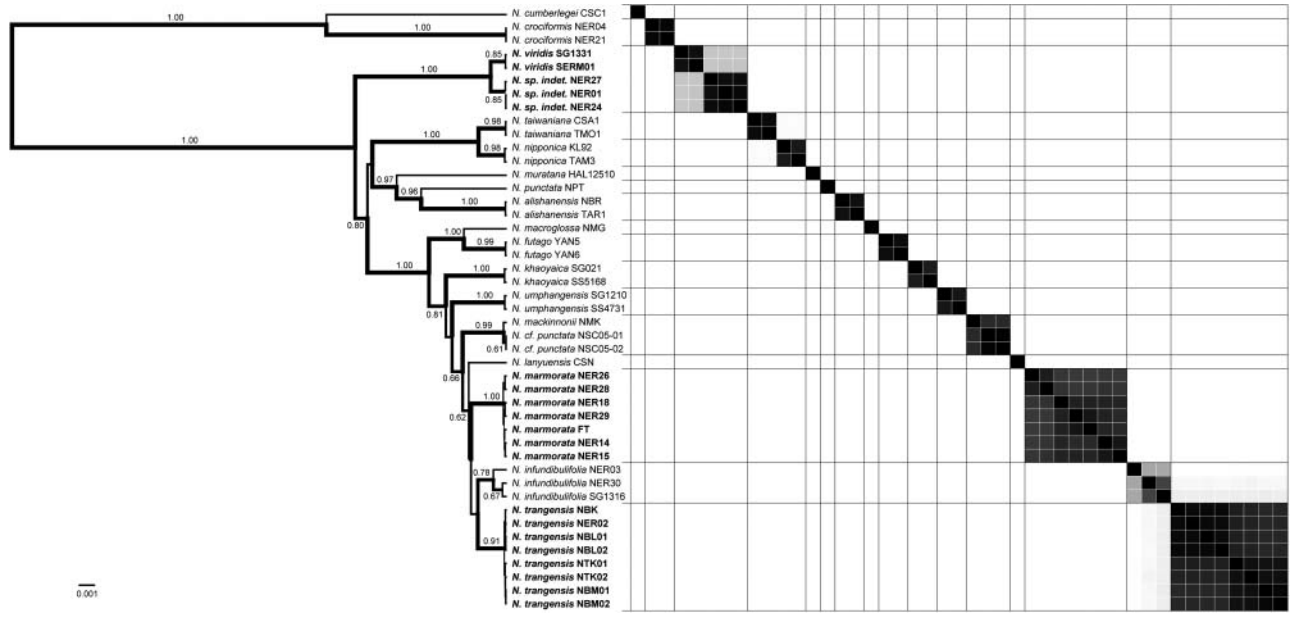

Fig. 9. Maximum sum of clade credibility SMC-tree based on combined ITS and ptDNA data from BEAST analysis and similarity matrix from STACEY. Posterior probabilities are shown at nodes and are represented by branch thickness. The colour of matrix squares indicate posterior probabilities for pairs of individuals belonging to each minimal cluster (white = 0, black = 1). The 20 Thai collections that could not be matched to any known species at the outset of the study are shown in bold.

Type: THAILAND. Chiang Rai Province: precise locality unknown, 25 March 2014, *Duangjai 250314* (holotype BKF!, flower); 15 July 2015, *Suddee 4910* (paratype BKF!, leaf).

Diagnosis: Morphologically allied to the north-east Indian *N. falcata* (King & Pantl.) Schltr. in terms of labellum outline, but differing in the shape of the hypochile auricles and disc ornamentation. Phylogenetically, this new species is related to *N. infundibulifolia* Blatt. & McCann and *N. trangensis* S.W.Gale, Suddee & Duangjai, both of which also occur in Thailand, but is distinguishable from these by its truncate epichile and mottled leaf.

Glabrous terrestrial herb up to 12 cm tall. Tuber whitish-beige, compressed globose, 4.5–7.2 mm long, 3.2–4.5 mm in diameter, 2–3-noded, with an uneven surface. *Subterranean stem* emerging from apical node of tuber, whitish-brown, 1.7– 5.5 cm long, 0.6–1.4 mm in diameter, several-noded, bearing a short, membranous, sheathing cataphyll at upper nodes, producing 1 or more segmented lateral runners 0.8–5.2 cm in length in the leafing phase that each give rise to a daughter tuber at the apex. Petiole-like stalk erect, 0.6–3.6 cm long, 1.2–1.5 mm in diameter, pale green flushed brown-purple, sulcate, with 1 brown, membranous, tightly sheathing cataphyll 0.4–0.7 cm long near the base. Leaf blade held a short way or well above ground level, cordate-polygonal, with 7 main veins diverging palmately, 1.8–2.8 cm long, 1.5–2.9 cm wide, deeply cordate at base, the basal lobes overlapping or not, apex acute or obtuse, margin flat, iridescent and light to dark green or purple mottled with silver above, mauve-purple streaked light green along the main veins below. Scape 9.0–10.5 cm tall, 1.5 mm in diameter, greenish-brown, bearing 1 membranous sheathing cataphyll *c*. 1.6 cm long, 1-flowered; floral bract enclosing the pedicel at base, *c*. 4.0 mm long, *c*. 1.6 mm wide, acute. Flower erect in bud, nodding, opening widely, 16.8–17.5 mm long. Pedicel and ovary 4.8–5.4 mm long, greenish-brown. Sepals narrowly ovate-elliptic, acute, 3-veined, greenish-ochre. Dorsal sepal 11.7–12.0 mm long, 1.9–2.0 mm wide. Lateral sepals slightly oblique, 10.9–11.5 mm long, 1.8–2.0 mm wide. Petals narrowly obovate-elliptic, 9.4–10.0 mm long, 1.2–1.5 mm wide, acute, 3-veined, greenish-ochre. Labellum oblong-obovate, 10.0–10.8 mm long, not saccate or swollen at the base, divided at the middle into a semi-tubular hypochile and a broad epichile, white with purple spots on the epichile and a light green central strip near the middle; hypochile oblong-obovate, 4.2–4.5 mm long, 3.5–4.6 mm wide at its widest, margins rolled upwards and embracing the column, terminating in a pair of triangular-ovate, obtuse auricles 1.8–2.1 mm long; epichile obovate, 5.7–6.8 mm long, 3.4–4.3 mm wide, margin entire, truncate at the apex; disc glabrous apart from a narrow central band of short, soft hairs arising midway along the hypochile and extending to basal third of the epichile. Column clavate, 4.8–5.5 mm long, *c*. 0.8 mm in diameter at the base, dilating to *c*. 1.6 mm at the apex, white, with a patch of short hairs beneath the stigma; anther helmet-shaped, *c*. 1.4 mm in diameter; pollinium *c*. 1.5 mm long; rostellum slightly thickened and protruding at the apex of the stigma; stigma shield-shaped, slightly concave. Capsule not seen.

Ecology and distribution: Reportedly occurring in dry Dipterocarp forest and semi-deciduous forest on limestone in northern Thailand.

Phenology: Flowering in March, in leaf from April until October.

Conservation status: Like the recently described *Bulbophyllum anodon* and *B. dasystachys* (Vermeulen, Phelps, & Thavipoke, 2014), this new orchid has come to light through the wild plant trade in Thailand. Claimed by the vendor to have been collected in Chiang Rai Province for sale at Chatuchak Market in Bangkok, the trade in this case has apparently not been in breach of CITES, under which the international trade in all orchids is strictly regulated (Phelps & Webb, 2015). However, its collection is nevertheless likely to exert a detrimental impact on the viability of the species in the wild, especially because *Nervilia* populations are often small, isolated and prone to demographic stochasticity (Gale et al., 2010), and because intact limestone habitats are now highly threatened throughout the region (Li, Gale, Kumar, Zhang, & Fischer, in press). In the absence of any data on the occurrence of this species in the wild, an assessment of its conservation status is presently not possible and it is therefore deemed Data Deficient (DD; IUCN, 2012).

Etymology: Meaning marbled in Latin, ‘marmor’ is a reference to this species’ attractively mottled leaf.

Specimens examined: THAILAND. Chiang Rai Province: precise locality unknown, 25 March 2014, *Duangjai 250314* (BKF!); 15 July 2015, *Suddee 4910* (BKF!).

Nervilia trangensis S.W. Gale, Suddee & Duangjai sp. nov.
Figs 2, 5

Type: THAILAND. Trang Province: Na Yong District, Khao Chong Waterfall, 79 m, 17 March 2014, *Suddee 4647* (holotype BKF!, flower); Yan Ta Khao District, Thung Khai Botanic Garden, 17 m, 23 June 2015, *Po-iam 02* (paratype BKF!, leaf).

Diagnosis: Morphologically allied to the Western Himalayan *N. pangteyana* Jalal, Kumar & G.S.Rawat in terms of labellum outline, but distinguished by the broad pubescent ridge on its disc and the non-saccate base of its hypochile. This species is phylogenetically related to *N.*

infundibulifolia Blatt. & McCann, but differs in its much broader, elliptic epichile and in the colouration of its leaf.

Glabrous terrestrial herb up to 11 cm tall. Tuber whitish-beige, irregularly globose to elongated ovoid-fusiform, 8.5–30.2 mm long, 5.0–8.9 mm in diameter, 3–11-noded, with an irregularly warty surface. Subterranean stem emerging from apical node of tuber, whitish-brown, up to 9.4 cm long, 1.8–2.8 mm in diameter, several-noded, bearing a short, membranous, sheathing cataphyll at each of the upper nodes, producing 2 or more segmented lateral runners 5.0 cm in length or longer in the leafing phase that each give rise to a daughter tuber at the apex. Petiole-like stalk erect, 1.7–4.5 cm long, 1.5–2.1 mm in diameter, pale green, sulcate, with 1 brown, membranous, tightly sheathing cataphyll 1.4–2.3 cm long at base. Leaf blade held a short distance above ground level, cordate-polygonal, with 7 or 9 main veins diverging palmately, somewhat cornutely toothed at the tips of the main veins in some individuals, 4.2–6.7 cm long, 4.2–6.7 cm wide, deeply cordate at base, the basal lobes overlapping in some individuals, apex acute, margin flat, uniformly green, sometimes silver-mottled, or purple, glossy. Scape 5.6–9.0 cm tall, 1.5–2.1 mm in diameter, beige-brown, bearing 1 or 2 membranous sheathing cataphylls 2.2–3.3 cm long, 1-flowered; floral bract enclosing the pedicel at base, 4.1–5.9 mm long, 1.9–3.1 mm wide, acute. Flower erect in bud, nodding, usually opening widely, 19.8–24.2 mm long. Pedicel and ovary 5.6–7.5 mm long, brown with purple streaks. Sepals narrowly ovate-elliptic, acute, 3-veined, beige-brown with purple streaks. Dorsal sepal 16.1–20.3 mm long, 3.4–4.0 mm wide. Lateral sepals slightly oblique, 15.1–16.8 mm long, 2.3–3.3 mm wide. Petals narrowly ovate-elliptic, 13.0–14.4 mm long, 2.5–2.7 mm wide, acute, 3-veined, beige-brown with purple streaks. Labellum oblong-obovate, 13.5–15.4 mm long, not saccate or swollen at the base, divided by a narrow waist at the middle into a semi-tubular hypochile and a broad epichile, white with a few faint violet spots towards the apex and a faint light green central strip near the middle; hypochile obovate, 6.0–7.1 mm long, 6.2–7.0 mm wide at its widest, margins rolled upwards and embracing the column, terminating in a pair of triangular-ovate, acute auricles 1.8–2.0 mm long; epichile broadly elliptic, 7.7–8.2 mm long, 6.4–7.4 mm wide, the margin entire but slightly undulate, obtuse-rounded at the apex; disc shortly papillose throughout, with a broad central raised ridge arising from near the base of the hypochile and extending to the apex of the epichile, the ridge pubescent between the 2 auricles. Column clavate, 5.2–6.0 mm long, *c*. 1.0 mm in diameter at the base, dilating to *c*. 2.0 mm at the apex, white, with a patch of short hairs beneath the stigma; anther helmet-shaped, *c*. 1.3 mm in diameter; pollinium *c*. 1.2 mm long; rostellum slightly thickened and forming a low ridge at the apex of the stigma; stigma shield-shaped, slightly concave. Capsule not seen.

Ecology and distribution

Growing in shade in lowland evergreen forest. Confirmed at two localities in Trang Province in Peninsular Thailand, at both of which all three leaf forms (uniformly green, green and silver-mottled, and uniformly purple) were observed. Wide variation in leaf colouration of this sort is unusual amongst *Nervilia* species, but has been reported in the African *N. adolphi* Schltr. (Pettersson, 1991). Mottling in *N. trangensis* could be attributable to vegetative crypsis, with the plants' understorey, forest habitat and ephemeral, summer-active vegetative shoot conforming to the predictions of the 'camouflage hypothesis' (Givnish, 1990).

Phenology: Flowering in March and April, in leaf from May until November.

Conservation status: The two populations of *N. trangensis* confirmed in the present study lie within protected areas approximately 30 km from one another. We observed up to 50 emergent plants in each population. It is plausible that uniformly green-leafed forms of this species have been confused with other known members of the *N. adolphi*/*punctata* species alliance that lack distinctive leaf colouration, such as *N.* cf. *punctata* (which the present study suggests may not be the appropriate name for Thai plants), causing it to be under-reported. Surveys of suitable lowland forest habitats in southern Thailand, as well as Peninsular Malaysia, are needed to better gauge its true distribution and abundance. For now, it is considered Data Deficient (DD; IUCN, 2012).

Etymology: Named after the province in Thailand in which this new species was discovered.

Specimens examined: THAILAND. Trang Province: Na Yong District, Khao Chong Waterfall, 79 m, 17 March 2014, *Suddee 4647* (BKF!); 31 March 2016, *Suddee 5072* (BKF!); 12 April 2016, *Duangjai 120416* (BKF!); 29 March 2017, *Duangjai 290317* (BKF!); Yan Ta District, Thung Khai Botanic Garden, 17 m, 23 June 2015, *Po-iam 02* (BKF!); 17 August 2015, *Po-iam 03* (BKF!); 30 March 2017, *Duangjai 300317* (BKF!).

Nervilia viridis S.W. Gale, Watthana & Suddee sp. nov.
Figs 3, 6

Type: THAILAND. Mae Hong Son Province: Pang Mapha District, Lum Nam Pai Wildlife Sanctuary, 432 m alt., 8 June 2016, *Nualngam 01* (holotype BKF!, flower); 26 August 2016, *Nualngam 03* (paratype BKF!, leaf).

Diagnosis: Morphologically allied to the Indonesian *N. punctata* (Blume) Makino in terms of disc ornamentation, but differing in the absence of projecting hypochile auricles and in having a truncate or obtuse epichile. This

new species appears to be phylogenetically distinct from known members of the *N. adolphi*/*punctata* species alliance that have been sequenced to date.

Glabrous terrestrial herb up to 8.0 cm tall. Tuber pale cream-white or beige, globose to subglobose, 6.1–13.4 mm in diameter, 2–4-noded, with short, stubby roots at the nodes. Subterranean stem emerging from apical node of tuber, cream-white, up to 2.7 cm long, several-noded, with a short, membranous, cataphyll at each node, producing lateral runners *c*. 2.3 cm long in the leafing phase which each give rise to a daughter tuber at the apex. Petiole-like stalk erect, 0.7–1.6 cm long, 1.8–2.2 mm in diameter, light green, with one brown, membranous, loosely sheathing cataphyll 0.6–1.8 cm long near the base. Leaf blade held close to ground level, cordate, with 7 main veins diverging palmately, 2.3–6.0 cm long, 2.8–8.0 cm wide, shallowly to deeply cordate at the base, the basal lobes not overlapping, acute or obtuse at the apex, the margin undulate, both surfaces light green. *Scape* 4.3–7.5 cm tall, *c*. 1.5 mm in diameter, pale yellowish-green, bearing 2 membranous sheathing cataphylls 2.2–2.4 cm long; floral bract enclosing the pedicel at base, oblong-ovate, 0.4–1.2 mm long, *c*. 0.8 mm wide, acute, yellowish-green. Flower nodding, opening widely, 10.5–12.5 mm long. Pedicel and ovary 2.7–5.0 mm long, light green. Sepals and petals narrowly oblong-elliptic, 3-veined, acute, light green. Dorsal sepal 12.2–20.0 mm long, 2.7–4.6 mm wide. Lateral sepals 11.6–18.4 mm, 2.3–3.8 mm wide. Petals slightly oblique, 11.8–18.6 mm long, 2.2–3.7 mm wide. Labellum obovate, 9.2–14.7 mm long, shallowly saccate at base, divided near the middle into a tubular hypochile and a broad epichile, white with a central band of light green near the base, with or without two parallel lines of faint purple spots on the epichile; hypochile oblong, 4.5–7.1 mm long, 4.6–5.2 mm wide, margins rolled upwards and entirely enclosing the column, terminating in a pair of short, obtuse-rounded auricles less than 1.0 mm long; epichile elliptic-obovate, 4.6–7.4 mm long, 5.0–7.4 mm wide, margin entire, truncate-rounded to obtuse at the apex; disc glabrous, with 2 ridges arising midway along the hypochile, forming a narrow, pubescent channel that extends to the base of epichile, the ridges then converging to form a broad, raised, triangular, shortly papillose ridge that extends to the apex of the epichile. Column clavate, 4.4–7.7 mm long, straight, *c*. 1.5 mm in diameter, dilating to 2.2 mm diameter at the apex, white flushed light green at the base, with a patch of short hairs below the stigma; anther helmet-shaped, *c*. 2 mm in diameter; pollinium 1.4–2.2 mm long; rostellum thickened and protruding; stigma broad, shield-shaped, slightly raised. Capsule not seen.

Ecology and distribution: Growing amongst clumps of *Dendrocalamus* bamboo in mixed deciduous forest dominated by *Tectona grandis*. Known from populations in Mae Hong Son and Chiang Mai Provinces in northern Thailand. Vegetatively similar plants collected from a population in Nakhon Ratchisima Province in eastern Thailand formed a closely related sister clade in the present study, but STACEY analysis indicated that they are phylogenetically distinct; the flower of these plants has not yet been seen, and so at present these plants cannot be circumscribed.

Phenology: Flowering in May and June, in leaf from June until October.

Conservation status: The two populations of this species confirmed in the present study lie around 130 km distant from one another, with up to 30 emergent plants observed at either site. Both populations are within protected areas. More research is needed to clarify this species' delimitation with respect to the unidentified plants from Nakhon Ratchisima, so that surveys can then be undertaken to better determine the distribution of both. For now, *N. viridis* is considered Data Deficient (DD; IUCN, 2012).

Etymology: Meaning green in Latin, 'viridis' is a reference to the colour of this species' perianth.

Specimens examined: THAILAND. Mae Hong Son Province: Pang Mapha District, Lum Nam Pai Wildlife Sanctuary, 432 m alt., 8 June 2016, *Nualngam 01* (BKF); 15 June 2016, *S. Nualngam 02* (BKF); 26 August 2016, *Nualngam 03* (BKF!); Chiang Mai Province: Chiang Dao District, 19 July 2014, *Watthana & Momkaew 4199* (QBG!).

Discussion

Integrative analyses delineate species boundaries

Several qualitative, mostly indiscrete morphological attributes of the 20 equivocal Thai collections drew similarities with a number of known members of the alliance, either from Thailand or elsewhere in tropical or temperate Asia. However, minor discrepancies made it difficult to categorically refer the plants to any particular species or determine if they represented novel infraspecific variation. Judging the weight of morphological characters in resolving species boundaries in the genus *Nervilia* is problematic. On the one hand, previous integrative studies have demonstrated that subtle differences in floral morphology can mask wide genetic distances: Gale et al. (2015) correlated inconsistencies in the distribution of coloured hairs on the labellum and petals, and in the length of the floral bract, amongst otherwise grossly similar samples collected at the same sites in south-west Japan to two phylogeographically distinct clades that could be distinguished at the species level. Equally, leaf mottling was shown to be a useful character in differentiating long-confused East Asian taxa with

consistently different AFLP profiles (Gale et al., 2010). On the other hand, however, floral colour variation within a member of the *N. adolphi*/*punctata* species alliance in South Korea proved to be phylogenetically irrelevant (Eum et al., 2011), and the present study revealed that multiple samples with striking differences in leaf colouration occurring at two geographically separated populations in Trang Province (Fig. 5.4–5.6) otherwise exhibit a stable floral morphology and belong to the same strongly supported phylogenetic clade. This indicates that certain morphological characters may not be uniformly informative across different operational taxonomic units (OTUs) in the genus, as has been reported for other species complexes (Belyaeva & Taylor, 2009; Schmidt-Roach, Miller, Lundgren, & Andreakis, 2014).

This underscores the utility of combined data sources for resolving species boundaries in *Nervilia*. Phylogenetic analyses here confirmed the monophyly of the four known Thai members of the *N. adolphi*/*punctata* species alliance (*N. infundibulifolia*, *N. khaoyaica*, *N.* cf. *punctata*, and *N. umphangensis*), all previously delimited on the basis of morphological traits alone, as well as the monophyly of the alliance and the section to which it belongs. However, application of the name *N. punctata* to Thai plants is cast further into doubt by the different phylogenetic placement of the sample of *N. punctata* from Indonesia, from where the species was originally described (Blume, 1849). Instead, the Thai samples cluster with *N. mackinnonii*, a species of the Himalaya, South China, and Myanmar (Fig. 8), and STACEY analysis indicates that they are conspecific (Fig. 9, Table S3, see supplemental material online). Both Gale and Watthana (2014) and Seidenfaden (1978) have questioned the authenticity of records of *N. punctata* in Thailand, citing small differences in floral morphology between specimens from continental South-east Asia and the Malay Archipelago, but with a paucity of fresh material on which to base a critical comparison, the matter had remained unresolved. The results presented here leave little uncertainty that the two Thai samples (NSC05-01 and NSC05-02) ought to be referred to *N. mackinnonii*, but more extensive sampling of plants presently treated as *N. punctata* in Thailand and adjacent countries is needed to ascertain if this taxon really occurs in continental South-east Asia (Seidenfaden & Wood, 1992). Despite limitations to the phylogenetic methods employed here for resolving species boundaries (Brower, 2006; Sukumaran & Knowles, 2016), taken together our findings indicate that an objective approach to species delimitation that combines both traditional taxonomic and genetic data sources validates ostensibly minor morphological differences as a basis for differentiating species within the alliance.

STACEY analysis detected unexpectedly wide infraspecific variation within *N. infundibulifolia*: one sample from Kanchanaburi Province in Thailand (NER03) formed a singleton, and one sample from each of Kanchanaburi (NER30) and Yunnan Province in South-west China (SG1316) formed a closely related minimal cluster, with a similarity score between them that was significantly lower than that retrieved for samples belonging to any of the other supported species. Further sampling throughout the range of this widespread species in India, Bhutan, Laos, and China, as well as in Thailand, is needed to confirm whether it genuinely harbours cryptic OTUs, and whether any autapomorphies can be identified with which to distinguish them (e.g., Zomlefer, Whitten, Williams, & Judd, 2006).

Despite the ambiguity of the morphological inferences alone, our phylogenetic analyses revealed the 20 equivocal Thai samples to form three distinct and strongly supported clades (Fig. 8) that were also clearly resolved in STACEY analysis (Fig. 9). In the case of the clades comprising the eight samples from two populations in Trang Province and of the seven samples originally collected in Chiang Rai Province, STACEY identified both as unique minimal clusters, each with high internal genetic similarity (Table S3, see supplemental material online). Given that these do not match any of the four known members of the *N. adolphi*/*punctata* species alliance in Thailand nor any of the other eight species included from adjacent areas, it can be inferred that these undoubtedly correspond to two distinct species (*sensu* Toprak et al., 2016). In the case of the clade comprising the five unidentified plants sampled from three populations in Mae Hong Son, Chiang Mai, and Nakhon Ratchisima Provinces, STACEY supports the recognition to two internal minimal clusters. In terms of leaf morphology, these two clusters were inseparable from one another (Fig. 6.4–6.5). However, information on floral characters are lacking for the Nakhon Ratchisima plants because they failed to flower in cultivation. It is plausible that future critical comparison of flowering material will uncover subtle morphological differences that correspond with these two minimal clusters, in line with our observations that minor discrepancies can distinguish the unidentified plants examined in this study, as well as with previous studies that have delineated cryptic species using specific and often obscure autapomorphies in this (Gale et al., 2015) and other genera (Newmaster & Ragupathy, 2009; Ragupathy et al., 2009; Whittall et al., 2004).

Unveiling new cryptic diversity in the *N. adolphi/punctata* species alliance

Delimiting cryptic diversity in species complexes is necessary to ensure the accurate classification and appropriate conservation of biodiversity (Bickford et al., 2007). Although in extreme cases cryptic species may be distinguished using sequence data alone (i.e., by applying molecular species descriptions; Johnson et al., 2015), integrative analyses typically invoke a

phylogenetic species concept to recognize OTUs that exhibit discrete apomorphies and/or fixed, diagnosable characters (Davis & Nixon, 1992; de Queiroz, 2007; Zomlefer et al., 2006). More comprehensive sampling for phylogenetic and morphological analysis is required to identify reliable characters, if any, that may be used to consistently delimit species throughout the *N. adolphi/punctata* species alliance in Africa and Asia, not least because the addition of species not included in the present analysis could be expected to alter the phylogenetic relationships inferred here. For now, the combination of both the qualitative and genotypic differences observed amongst the 20 equivocal Thai samples, which formed three distinct OTUs, provide evidence that they constitute distinct species.

It is possible that these OTUs could be comparable to other known species not sampled in our study. However, comparison with morphologically affiliated taxa fails to satisfactorily assign identities. Thus, although the Chiang Rai plants (FT, NER14, NER15, NER18, NER26, NER28, NER29) had a labellum outline comparable to that of the north-east Indian *N. falcata*, that species can be distinguished by its curved, acute hypochile auricles and pubescent disc (King & Pantling, 1896). Similarly, although the labellum of the Trang plants (NBK, NBL01, NBL02, NBM01, NBM02, NER02, NTK01, NTK02) was found to be similar in outline to that of *N. pangteyana*, that of the latter differs in being glabrous, basally saccate and apically reflexed (Jalal, Kumar, & Rawat, 2012). Finally, although the plants from Mae Hong Son Province and Chiang Mai Province (SERM01, SG1331) were considered qualitatively similar in terms of labellum ornamentation to *N. punctata* (Backer & Bakhuizen van den Brink, 1968), they otherwise differ in labellum outline. Minor morphological variation of this sort has generated extensive confusion in the identification of species of the alliance (Chen & Gale, 2009; Gale & Watthana, 2014; Pettersson, 1991; Seidenfaden, 1978), especially in studies utilizing preserved material alone, but a growing body of evidence suggests it can conceal wide evolutionary divergence (Eum et al., 2011; Gale et al., 2010, 2015). The present study underscores the utility of an integrative approach in detecting cryptic taxa within the *N. adolphi/punctata* species alliance, as others have for species complexes elsewhere (e.g., Gurushidze et al., 2008; Heinrichs et al., 2015). Based on these findings, the following three new members of the alliance are proposed.

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Supplemental data

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