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Molecular Identification Resolves Taxonomic Confusion in *Grammatophyllum speciosum* Complex (Orchidaceae)

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Abstract Molecular identification using nuclear ribosomal ITS sequence data revealed four genetic units in *Grammatophyllum speciosum* complex (Orchidaceae). Investigations of morphological characters support the species status of each unit and a provisional taxonomic revision of the complex is proposed. The following species are recognised in the complex: *G. speciosum* Blume, *G. wallisii* Rchb.f., *G. kinabaluense* Ames & C.Schweinf., *G. pantherinum* Rchb.f., and *G. cominsii* Rolfe.

Key words: DNA barcoding, *Grammatophyllum speciosum*, ITS, molecular identification, Orchidaceae, taxonomy.

Introduction

Grammatophyllum speciosum Blume, commonly called the Giant Orchid, is granted the title of the largest orchid of the world. The vegetative shoot grows to a length of 3.5 m, and the flowering stem grows to a length of 3 m. The total weight of the entire plant was estimated to be over more than 1000 kg based on a calculation of a Bornean plant (Lamb, 2011).

Although noticeable variations in reproductive characters exist among individuals, *Grammatophyllum speciosum* has been treated as a single entity in many floristic and taxonomic accounts. Meanwhile, several entities assignable to this species complex have been described so far. Interpretations on the status of these names are variable and inconsistent. However, a taxonomic revision of the *G. speciosum* complex has not been attempted. Such situations have been caused by limited opportunities for investigations due to its rarity, inaccessibility at canopy habitats, irregular and rare flowering nature (Seidenfaden, 1983; Yukawa, unpublished), wide distribution throughout Southeast Asia further to New Guinea and the Solomon Islands, and unmanageable size of the plant for collection.

Interpretations of species boundaries based on morphological characters alone frequently generate misleading results when only a limited number of samples are used because discontinuity of a character can become continuous if sampling intensity is increased. Under these circumstances, macromolecular (especially DNA) markers discriminating closely related species are powerful tools to uncover genetic units corresponding to species. To provide a framework for a taxonomic revision of *G. speciosum* complex, we used the internal transcribed spacer (ITS) regions of the 18S-26S nuclear ribosomal DNA because the regions have been shown to be an excellent barcoding marker at the species level in

Table 1. Materials used for DNA sequencing and a summary of the results

Sample code	Locality	Accession	Genotype	Identification
1	unknown	TBG 78805	A	*Grammatophyllum speciosum*
2	unknown	TNS 8500844	A	*Grammatophyllum speciosum*
3	unknown	TBG 153268	A	*Grammatophyllum speciosum*
4	Philippines	TBG 161752	B	*Grammatophyllum wallisii*
5	Malaysia, Sabah	TBG 161754	C	*Grammatophyllum kinabaluense*
6	Malaysia, Sarawak	TBG 161753	D	*Grammatophyllum pantherinum*
7	Solomon Islands	MBK 20101534	D	*Grammatophyllum pantherinum*
Outgroup	unknown	TBG 118874		*Grammatophyllum scriptum*

various groups of seed plants (e.g., China Plant BOL Group, 2011), including subtribe Cyrtopodiinae to which *Grammatophyllum* is assigned (Yukawa *et al.*, 2002). We investigated ITS diversity of all morphotypes in *G. speciosum* complex that were available for study so as to clarify genetic units. Furthermore, morphological characters of the samples used for the molecular analysis were examined. We also referred to protologues of all of the names in the complex and the type specimens where available. On the basis of these data, we propose a provisional taxonomic revision of the complex.

Materials and Methods

Observation and measurement of morphological characters was based on living plants, dried herbarium specimens, and spirit-preserved specimens in Tsukuba Botanical Garden, National Museum of Nature and Science, Kyoto Botanical Garden, and Kochi Prefectural Makino Botanical Garden.

Materials used in the DNA analysis are shown in Table 1. DNA was extracted from fresh or dried leaves. Nucleotide sequences were determined by amplifying the ITS regions of the 18S-26S nuclear ribosomal DNA via the polymerase chain reaction (PCR) using the primer combination 17SE/26SE (Sun *et al.,* 1994). Experimental methods follow those described in Topik *et al.* (2005) and Yukawa *et al.* (2005). Voucher specimens were deposited at TNS and MBK.

The ITS data set was aligned using CLUSTAL W implemented in MEGA 5.1 (Tamura *et al.*, 2011) and modified manually to minimize the number of gaps. All insertions/deletions were coded as missing data. *Grammatophyllum scriptum* was chosen as an outgroup taxon because this species occupies a sister group position relative to the *G. speciosum* complex (Yukawa *et al.*, 2002). The maximum parsimony (MP) and distance analyses were conducted using MEGA. MP trees were identified using heuristic searches with 100 random addition sequence replications and TBR branch-swapping algorithm. Distance trees were obtained using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with a Kimura two-parameter correction (Kimura, 1980). The bootstrap analysis (Felsenstein, 1985) with 1000 replicates was used for MP and NJ analyses to assess the relative strength of support for all branches. Prior to the maximum likelihood (ML) analysis, the most optimal model for the data set was estimated using jModelTest 2 (Darriba *et al.*, 2012) under the Akaike information criterion. We used GTR+G model to conduct ML analysis with phyML 3.0 (Guindon and Gascuel, 2003). The appropriate likelihood ratio test (aLRT) was used to evaluate branching support. The ML trees were visualized with MEGA.

Results and Discussion

Genotypes and phylogenetic relationships

Divergence between each pair of sequences is shown in Table 2. In total, four genotypes were recognised in the species complex. The ML tree of the *Grammatophyllum speciosum* complex derived from ITS sequences is shown in Fig. 1. Since the MP and NJ analyses showed the same phylogenetic relationships, the results are not

Table 2. Pairwise nucleotide divergence matrix based on ITS regions of nrDNA among *Grammatophyllum* samples

	Sample code	1	2	3	4	5	6	7	Outgroup
G. speciosum	1	—	—	—	—	—	—	—	—
G. speciosum	2	0	—	—	—	—	—	—	—
G. speciosum	3	0	0	—	—	—	—	—	—
G. wallisii	4	9	9	9	—	—	—	—	—
G. kinabaluense	5	7	7	7	16	—	—	—	—
G. pantherinum	6	10	10	10	19	13	—	—	—
G. pantherinum	7	10	10	10	19	13	0	—	—
G. scriptum	Outgroup	44	44	44	50	45	44	44	—

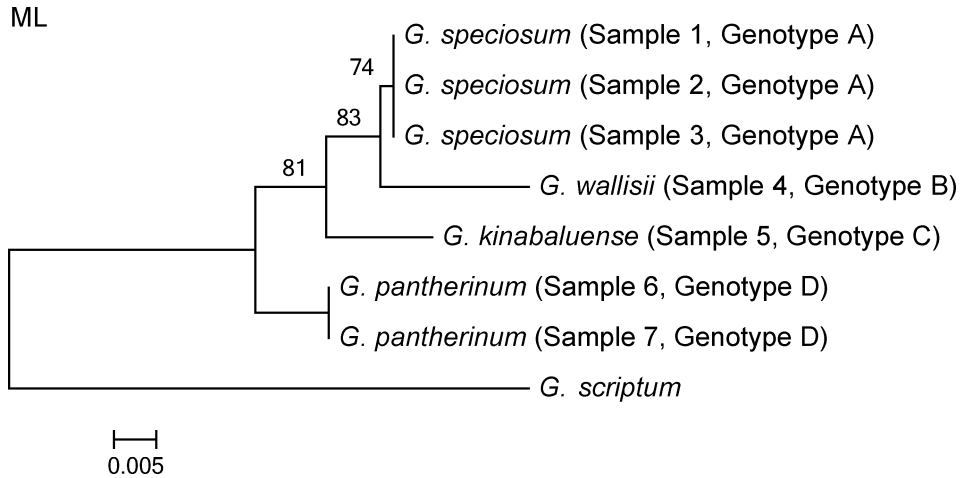

Fig. 1. Phylogenetic relationships of *Grammatophyllum speciosum* complex inferred from nrDNA ITS sequences. Sample codes and genotypes (see Table 1) are shown in parentheses. The tree is rooted by an outgroup taxon, *G. scriptum*. The tree was calculated using the maximum likelihood method and the topology was the same as the maximum parsimony and neighbor-joining estimates. The relative strength of support for all branches was assessed by the appropriate likelihood ratio test.

shown. Genotypes A and B form a monophyletic clade and C and D are successively grouped with this clade.

Identification of each genotype

Although there are no explicit criteria for the species delineation based on divergence in ITS sequences, China Plant BOL Group (2011) showed that, in a large data set comprising 1,757 species, 67.2% of seed plant species can be discriminated by ITS. We therefore hypothesised that each genotype discriminated by nucleotide substitutions in ITS represents a distinct species. Meanwhile, samples exhibiting the same genotype were postulated to represent the identical species. Based on these assumptions, we examined morphological characters of the samples and further referred to type specimens and protologues of relevant taxa in the complex.

Both vegetative and reproductive organs of genotype A matched completely with the protologue and associated figures of *Grammatophyllum speciosum* (Fig. 2A–C). Dimensions of vegetative and reproductive parts for this entity are the largest of all putative taxa within the complex. For example, the length of the vegetative stem, scape, and dorsal sepal reach 3.5 m, 3 m, and 65 mm, respectively, in this taxon. The labellum has the following distinct characters: the mid-lobe is densely villose; three or five keels

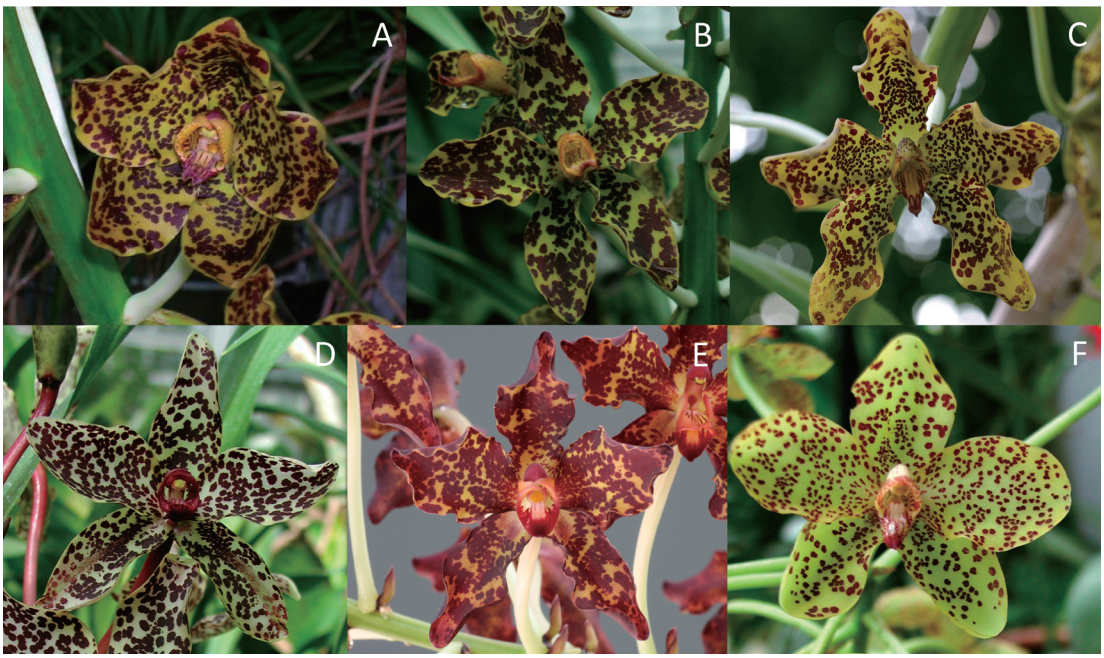

Fig. 2. Flowers of *Grammatophyllum speciosum* complex examined in this study. A. *G. speciosum* (sample 1, genotype A). B. *G. speciosum* (sample 2, genotype A). C. *G. speciosum* (sample 3, genotype A). D. *G. wallisii* (sample 4, genotype B). E. *G. kinabaluense* (sample 5, genotype C). F. *G. pantherinum* (sample 6, genotype D). Photo courtesy: Minoru Isobe (A), Jun'ichi Nagasawa (B, D, F), Kazuhiro Suzuki (E).

develop weakly and the two keels adjoining the central one extend to the base. The column foot forms a cup-like structure that extends into two vertical, parallel, ovate-triangular lobes. Development of this character gives rise to a firmly attached labellum that maintains its original position throughout flowering. In contrast, the labellum in the other entities in this complex becomes pendulous at later stages of anthesis because the structure connecting the labellum and column is rather fragile. The flower colour of plants belonging to genotype A is also characteristic; the sepals and petals are greenish-yellow and marked with orange-brown or reddish-brown, randomly dispersed spots of various sizes.

Genotype B collected in the Philippines agrees with the protologue of *Grammatophyllum wallisii* Rchb.f., which was described on the basis of material also collected in the Philippines (Fig. 2D). Reichenbach (1877) noted that *G. wallisii* could be separated from *G. speciosum* by its flower colour of white with olive-green spots. Furthermore, a drawing of a flower on the holotype sheet deposited in the Reichenbach Herbarium, Naturhistorische Museum, Vienna, does not depict ovate-triangular lobes on the column foot which otherwise characterise *G. speciosum*. These differences warrant the recognition of *G. wallisii* as a separate species.

Morphological characters of genotype C, originating from Sabah, East Malaysia, match well with the protologue of *Grammatophyllum kinabaluense* Ames & C.Schweinf., an obscure species described from a collection made in Sabah (Fig. 2E). A drawing is provided here (Fig. 3) because no illustration has previously been published for this species. Although the examined plant had larger flower dimensions than those given in the protologue of *G. kinabaluense* (e.g., length of dorsal sepal 41–45 mm versus 34 mm), diagnostic characters such as the shape of keels and claw of the labellum are consistent between the two. More specifically, three, closely approximate, semi-orbicular keels of similar

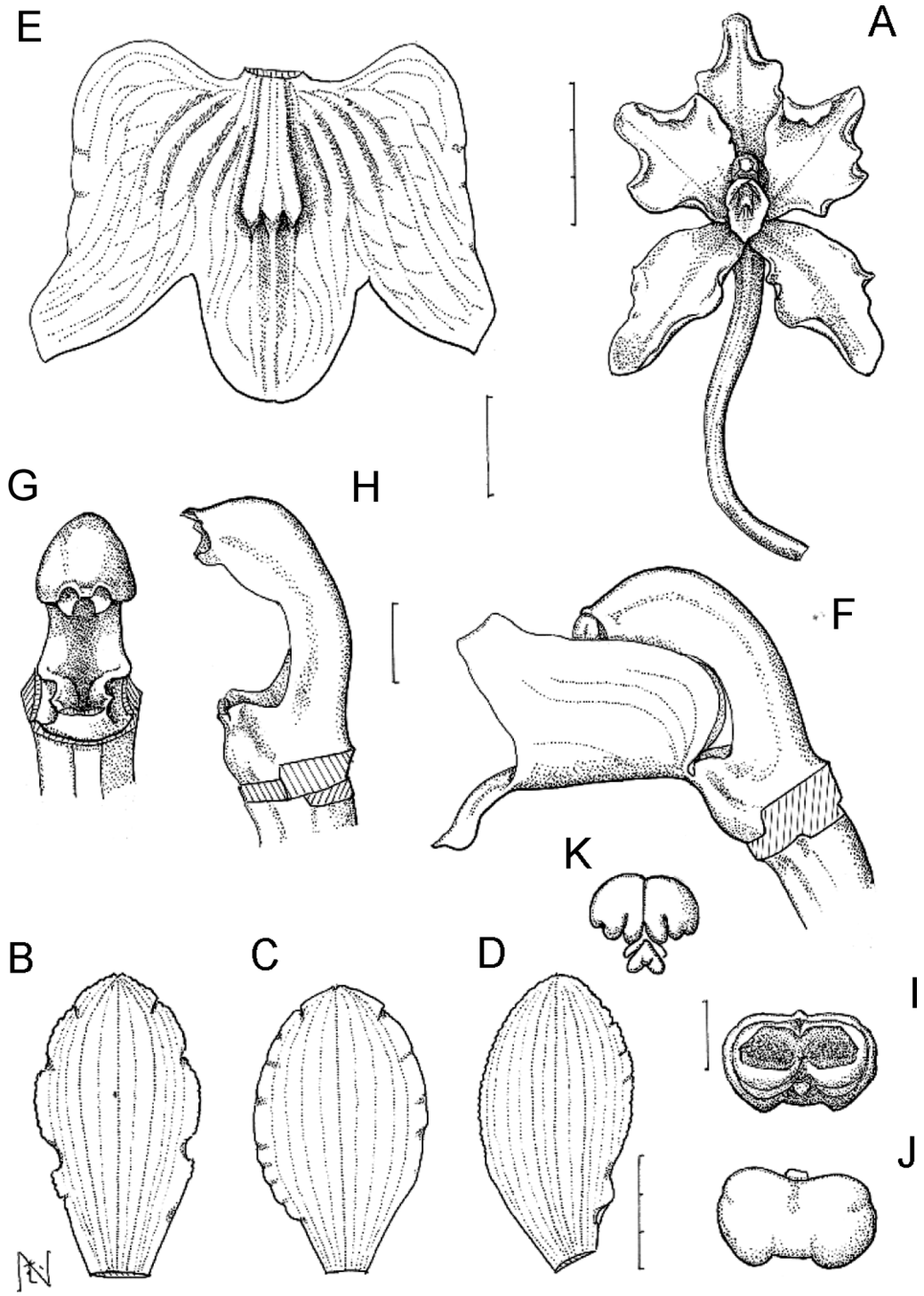

Fig. 3. *Grammatophyllum kinabaluense* Ames & C.Schweinf. A. Flower, front view. B. Dorsal sepal. C. Petal. D. Lateral sepal. E. Labellum. F. Labellum and column, side view. G. Column, from below. Operculum and pollinarium detached. H. Column, side view. Operculum and pollinarium detached. I. Operculum, ventral view. J. Operculum, dorsal view. K. Pollinarium. Drawn from sample 5 (collection from Malaysia, Sabah; TBG 161754) by Mutsuko Nakajima. Scale bar = 30 mm (A), 15 mm (B–D), 5 mm (E–H) or 1 mm (I–K).

length are present in the centre of the labellum. The keels do not extend either to the apex or to the base of the labellum. The claw of the labellum is very small and conduplicate, and is adnate to the basal, extended part of the column. The shape of a cup-like structure at the base of the column is rectangular in lateral view (Fig. 3). The mid-lobe of the labellum is glabrous. The colour of the sepals and petals is more intensely marked with maroon-red than in *G. speciosum*. The labellum is stained with sanguine-red. Flower odour was negligible in our material, whilst *G. speciosum* and *G. pantherinum* possess a sweet fragrance. This combination of characters is not found in the other entities in the complex.

A collection from Sarawak, East Malaysia, which was designated Genotype D, coincides with the concept of *Grammatophyllum pantherinum* Rchb.f. on the basis of a New Guinean specimen (Fig. 2F). This species exhibits a unique combination of characters not found in the remaining members of the *G. speciosum* complex. A cup-like structure at the base of the column is truncated. The sepals and petals are pale greenish- or brownish-yellow with red-brown spots. The spots are more evenly distributed than they are in *G. speciosum*. The flower size is intermediate between *G. speciosum* and *G. kinabaluense*. For instance, the dorsal sepal is 47–50 mm in length. High sequence divergence in the ITS region (Table 2) and distinct morphological characters endorse independent status for *G. pantherinum*. The other sample of Genotype D (Sample 7), collected in the Solomon Islands, has not yet bloomed. We have tentatively identified it as *G. pantherinum* because 100% identity of ITS sequence data indicates the conspecificity of the two samples.

Taxonomic summary of *Grammatophyllum speciosum* complex

Due to the limited number of samples available for this study, and the fact that we have so far been unable to consult several type specimens, we cannot provide a conclusive view on the taxonomy of the *Grammatophyllum speciosum* complex at this stage. However, we propose a revision of the complex because the distinct genetic units identified in our molecular analysis were supported by our morphological reappraisal of plants, and these are in turn consistent with taxonomic concepts that have been proposed elsewhere.

We demonstrate that the two-step approach, i.e. evaluation of morphological characters subsequent to genotyping, is useful for accurate identification and taxonomic circumscription, especially where sampling densities are low.

Grammatophyllum speciosum Blume, Bijdr. Fl. Ned. Indië: 378, t. 20 (1825). TYPE: Java, Buitenzorg, *Blume* s. n. (holotype L).

Pattonia macrantha Wight, Icon. Pl. Ind. Orient. **5**: 21, t. 1750 (1851). TYPE: Peninsular Malaysia, Malacca, *Griffith* 5518 (holotype K; isotype C, S!).

Grammatophyllum fastuosum Lindl., Paxton's Fl. Gard. **2**: 159 (1852). TYPE: Peninsular Malaysia, Malacca, *Griffith* s.n. (holotype K).

Grammatophyllum macranthum (Wight) Rchb.f., Xenia Orchid. **2**: 16 (1862).

Of the legitimate names in the complex, we confirmed that protologues of *Grammatophyllum fastulosum* and *Pattonia macrantha* are identical to characters pertaining to *G. speciosum*. Names such as *G. giganteum* and *G. sanderianum* have been often cited as synonyms of *G. speciosum*. However, these names have not been validly published.

The distribution of this taxon in Myanmar, Thailand, Laos, the Malay Peninsula, Sumatra, Borneo and Java are well documented. However, although many accounts also cite its distribution in the Philippines, Sulawesi, Maluku, New Guinea and the Solomon Islands, we do not have any direct evidence of this. For example, figures and photographs identified as *Grammatophyllum speciosum* by Lewis and Cribb (1991) for the Solomon material, by O'Byrne (1994) and Millar (1999) for New Guinea material, and by Cootes (2011) for the Philippines material, actually rep-

resent *G. pantherinum*. Further studies are needed to confirm the true distribution range of *G. speciosum*.

Grammatophyllum wallisii Rchb.f., Linnaea **41**: 107 (1877). TYPE: Philippines, Luzon, Manilla, *Wallis* s.n. (holotype W!).

Although several taxonomic studies such as Ames (1924–1925), Seidenfaden (1983), Comber (2001) and Wood *et al.* (2011) placed *Grammatophyllum wallisii* as a synonym of *G. speciosum*, both molecular and morphological characters distinguish it from the other species in the complex. Holttum (1955) suggested its independent status on the basis of his observation of a fresh flower. The species is endemic to the Philippines.

Grammatophyllum kinabaluense Ames & C.Schweinf. in O.Ames, Orchidaceae **6**: 210 (1920). TYPE: Borneo, Sabah, Kiau, 900 m, *Clemens* 55. (holotype AMES; isotypes BM, E!, F!, K, NY!, S!, SING!, US!).

As discussed above, *Grammatophyllum kinabaluense* is clearly distinct from the other members in the complex. The species is only recorded from Borneo.

Grammatophyllum pantherinum Rchb.f., Gard. Chron., n.s., **9**: 788 (1878). TYPE: New Guinea, precise locality uncertain, *Goldie* s.n. (holotype W!).

Grammatophyllum papuanum J.J.Sm., Bull. Dép. Agric. Indes Néerl. **45**: 11 (1911). TYPE: New Guinea, Salt spring on the Beguwri River, 160 m, *Gjellerup* 246. (holotype BO).

Grammatophyllum pantherinum is an insufficiently known entity, given its brief description. Schlechter (1915) first suggested its conspecificity with *G. papuanum*. Subsequently, Smith (1930) examined the holotype of *G. pantherinum* and stated that it is identical with his own *G. papuanum*. We follow Smith's interpretation because we also did not find any differences between the holotype of *G. pantherinum* and the protologue and associated illustrations of *G. papuanum*.

This entity is well demarcated from the remaining members in the complex as discussed above, though many studies such as Lewis and Cribb (1991), O'Byrne (1994), Millar (1999), Thomas and Schuiteman (1994), and Govaerts (2003) sunk it (under the name *G. papuanum*) as a synonym of *Grammatophyllum speciosum*. It is evident that *G. pantherinum* is distributed in New Guinea, the Solomon Islands, and Borneo because the former is the type locality and the latter two are the origin of the material used in this study.

Moreover, Smith (1928) recorded the distribution in Seram under the name *Grammatophyllum papuanum*. A photograph of "*G. speciosum*" in Cootes (2011) indicates its occurrence in the Philippines. Ridley (1924) mentioned a form of *G. speciosum* having finely spotted flowers, often with shorter rounded sepals and petals in his floristic account of Peninsular Malaysia. The features fit well with the concept of *G. pantherinum*. Consequently, this species may be widely distributed in the Malay Archipelago.

Another potential taxonomic problem exists in this species. *Grammatophyllum pantherinum* Zipp. ex Blume, which was described in 1849, is a synonym of *Vandopsis lissochiloides* (Gaudich.) Pfitzer. Since this name was not validly published, *G. pantherinum* Rchb.f. is the legitimate name, albeit its homonymous status.

Grammatophyllum cominsii Rolfe, Ann. Bot. (Oxford) **5**: 506 (1891). TYPE: Solomon Islands: San Christobal, *Comins* 57. (holotype K).

This is an overlooked taxon sometimes placed as a synonym of *Grammatophyllum speciosum* in Lewis and Cribb (1991) and Govaerts (2003). However, descriptions in the protologue are congruent with characteristics of *G. pantherinum* except for smaller flower size in the protologue of *G. cominsii* (e.g., length of dorsal sepal 38 mm). Moreover, the ITS sequence of a sterile sample from the Solomon Islands is identical to that of *G. pantherinum*. This entity may represent

a synonym *G. pantherinum*. Since the type specimen is not available for this study, we keep it as a separate entity at the moment.

Key to the taxa in the *Grammatophyllum speciosum* complex

A. Dorsal sepal 55–65 mm long; labellum keeping the original position throughout flowering; mid-lobe of labellum villose; column foot with ovate-triangular lobes ..... *G. speciosum*
A. Dorsal sepal less than 50 mm long; labellum drooping down at later stages of flowering; mid-lobe of labellum glabrous or pubescent; column foot without distinct lobes
  B. Sepals and petals ivory-white with dark maroon spots ..... *G. wallisii*
  B. Sepals and petals brownish- to greenish-yellow with reddish to purplish spots
    C. Sepals and petals intensely covered in irregular blotches; column foot with indistinct lobes ..... *G. kinabaluense*
    C. Sepals and petals evenly spotted; column foot truncate
      D. Dorsal sepal 47–50 mm long ..... *G. pantherinum*
      D. Dorsal sepal 38 mm long ..... *G. cominsii*

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