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Unveiling the taxonomic identity of *Vanilla sprucei* (Orchidaceae, Vanilloideae): its phylogenetic position and a new record for Brazil

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ABSTRACT

The pantropical *Vanilla* is the most species-rich genus among Vanilloideae (Orchidaceae). With 38 species, Brazil is the center of diversity for *Vanilla*. Several of the Brazilian taxa are considered obscure, as species boundaries are often unclear when studies are performed exclusively on dried specimens. This is the case of *Vanilla sprucei* Rolfe, a rare taxon with ephemeral flowers that has been referred to Brazil based on a specimen collected in the Colombian Amazonia. We present data confirming its occurrence in Brazil, together with a taxonomic evaluation of this poorly-known species including a detailed, illustrated morphological description. An epitype for *V. sprucei* based on Brazilian material is designated. The geographical distribution of this taxon is presented. The phylogenetic position of *V. sprucei* within *Vanilla* and its relationships with Neotropical congeners are discussed based on a phylogenetic hypothesis. *Vanilla sprucei* emerges at a basal node among the non-membranaceous, Neotropical *Vanilla* clade. The inclusion of *V. sprucei* in the molecular data matrix reinforces the *V. trigonocarpa* group as polyphyletic. For this reason, further infrageneric rearrangements will be necessary. Preliminary evaluation of the species´ conservation status according to IUCN criteria indicate a global status of Endangered, with subpopulations in Brazil assessed as Critically endangered.

KEYWORDS: Brazilian flora, geographic expansion, hemiepiphytes, taxonomy, white-sand vegetation.

Revelando a identidade taxonômica de *Vanilla sprucei* (Orchidaceae, Vanilloideae): sua posição filogenética e um novo registro para o Brasil

RESUMO

Vanilla é o gênero mais rico em espécies dentro da subfamília Vanilloideae (Orchidaceae). Com 38 espécies, o Brasil é o centro de diversidade de *Vanilla*. Apesar de sua diversidade, alguns táxons brasileiros têm sido considerados obscuros, pois os limites entre espécies são dificultados quando os estudos são realizados exclusivamente em espécimes herborizados. Esse é o caso de *V. sprucei*, táxon raro de flores efêmeras que tem sido referido ao Brasil com base em espécime coletado na Amazônia colombiana. Aqui, uma avaliação taxonômica desta espécie pouco conhecida é apresentada e uma descrição morfológica detalhada é fornecida. Uma ilustração de *V. sprucei* baseada em espécimes brasileiros vivos é mostrada, e a distribuição geográfica deste táxon é apresentada. Um epítipo para *V. sprucei* é designado aqui. A posição filogenética de *V. sprucei* dentro de *Vanilla* e suas relações entre congêneres neotropicais são discutidas com base em uma hipótese filogenética. *Vanilla sprucei* emerge em uma posição basal entre o clado não membranáceo de *Vanilla* Neotropical. A inclusão de *V. sprucei* na matriz de dados moleculares reforça o grupo *V. trigonocarpa* como polifilético. Por esta razão, novos rearranjos infragenéricos serão necessários. Uma avaliação preliminar sobre o status de conservação de *V. sprucei* indica que a espécie se enquadra na categoria Em Perigo, com subpopulações no Brasil avaliadas como Criticamente Ameaçadas.

PALAVRAS-CHAVE: Ambientes de areia branca, epífita, flora brasileira, floresta de campinarana, hemiepífita, taxonomia, taxonomia.

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INTRODUCTION

With ca. 100–115 species, the pantropical *Vanilla* Mill. is the most species-rich genus among the vanilloid orchids (*e.g.* Cameron 2003; Soto-Arenas and Cribb 2010; Soto-Arenas and Dressler 2010; Govaerts *et al.* 2023). The vast majority of species occur in tropical areas, while a few taxa show a subtropical distribution (Soto-Arenas and Cribb 2010). This genus is widely distributed throughout the Neotropics, with some species occurring in Southeastern Asia, Africa, Pacific islands, and Caribbean (Soto-Arenas and Cribb 2010; Soto-Arenas and Dressler 2010; Karremans *et al.* 2020; Govaerts *et al.* 2023).

Vanilla species are commonly hemiepiphytes or nomadic vines (Zotz *et al.* 2021), while a few species are terrestrial, lithophytic or occasionally epiphytic. The genus is characterized by flexible and commonly climbing stems, alternate leaves, free sepals and petals, petals usually with a longitudinal keel, labellum partially fused with the column, and fleshy fruit with sclerified seeds. The flowers of *Vanilla* species can be white, greenish-white, cream, or yellow, and they usually last less than 12 hours (Cameron 2003; Soto-Arenas and Cribb 2010; Pansarin and Pansarin 2014; Pansarin 2022; Pansarin and Menezes 2023).

Most *Vanilla* species are pollinated by animal vectors, mainly Euglossini bees (*e.g.* Pansarin and Pansarin 2014; Pansarin 2023), and more rarely birds (*e.g.* Pansarin and Ferreira 2022). Mammals and birds are involved in seed dispersal (*e.g.* Pansarin 2021; Pansarin and Suetsugu 2022). With regard to pollination, *Vanilla* species have been associated with nectar deception (*e.g.* Pridgeon *et al.* 2003; Chaipanich *et al.* 2020), however recent studies have shown that the pollen transfer of a number of Neotropical species involves the production of flower rewards (*e.g.* Pansarin 2022, 2023). *Vanilla* is the most economically important orchid genus because some species, mainly *V. planifolia* Andrews and related species are the source of natural vanilla flavor (Rain 2004).

The systematics and taxonomy of *Vanilla* have been the subject of debate (Rolfe 1896; Portères 1954; Cameron 1999; Soto-Arenas 1999; Cameron 2003; Soto-Arenas 2003; Cameron and Molina 2006; Bouetard *et al.* 2010; Pansarin 2010; Soto-Arenas and Cribb 2010; Soto-Arenas and Dressler 2010; Karremans *et al.* 2020; Pansarin and Menezes 2023; Pansarin 2024). The most accepted infrageneric classification for *Vanilla* has been based on both molecular and morphological data (Soto-Arenas 2003; Soto-Arenas and Cribb 2010; Bouetard *et al.* 2010). *Vanilla* is currently divided in two distinct subgenera: (1) subgen. *Vanilla*; and (2) subgen. *Xanata* Soto-Arenas & P.J. Cribb. The latter is divided into two sections: *i.e.* sect. *Xanata* Soto-Arenas & P.J. Cribb and sect. *Tethya* Soto-Arenas & P.J. Cribb. However, the discovery of new species (mainly endemic) from Brazil and the inclusion of taxa not represented in previous phylogenetic analyses suggest that infrageneric realignments will be necessary (Pansarin and Menezes 2023). Nonetheless, *Vanilla* encompasses three well-supported lineages, two of which occur in the Neotropics (Bouetard *et al.* 2010; Pansarin and Menezes 2023). The Neotropical *Vanilla* with membranaceous leaves emerges as sister to a large clade that includes two subclades: an Old-World/Caribbean clade, and a well-supported clade including the non-membranaceous Neotropical *Vanilla* (Pansarin and Menezes 2023).

With 38 species Brazil is the center of diversity among Neotropical *Vanilla* (Flora e Funga do Brasil 2023). However, recent discoveries (*e.g.* Kock *et al.* 2013; Ferreira *et al.* 2020; Carvalho *et al.* 2023; Pansarin and Menezes 2023) and synonymizations (*e.g.* Karremans *et al.* 2020) must be considered in further inventories. Thirteen *Vanilla* species are referred to the Brazilian Amazon (e.g. Krahl *et al.* 2020; Karremans *et al.* 2020; Engels and Koch 2021; Flora e Funga do Brasil 2023), including *Vanilla sprucei* Rolfe (see Cogniaux 1904-1906; Hoehne 1945; Flora e Funga do Brasil 2023; Govaerts *et al.* 2023). The occurrence of *V. sprucei* in Brazil has been subject of debate, as there is no herbarium material that confirms the occurrence of this poorly-known taxon in Brazilian forests. In addition, *V. sprucei* is considered an obscure species, possibly an earlier name for *V. trigonocarpa* Hoehne (see Karremans *et al.* 2020).

While developing a floristic survey of Brazilian *Vanilla*, a population of *V. sprucei* was found within the boundaries of the city of Manaus (state of Amazonas), northern Brazil. Here, a taxonomic evaluation of this species is presented and a detailed morphological description is provided. Based on the absence of illustrations and photographs for *V. sprucei* in the literature, an illustration of this species based on living specimens is provided. The geographic distribution of this uncommon taxon is presented based on herbarium material and new records from the Brazilian Amazon. In addition, based on the evidence that the protologue of *V. sprucei* is based on a single specimen, and that Rolfe (1896) indicated it "is not in very good state", an epitype for *V. sprucei* is here designated. The phylogenetic position of *V. sprucei* within *Vanilla* and its relationships with Neotropical congeners are discussed based on a phylogenetic inference.

MATERIAL AND METHODS

Study and collection area of *Vanilla sprucei*

Field work was carried out in a white-sand *forest* ("campinarana" vegetation) of the municipality of Manaus (2°51'22.02" S, 60°13'18.96" W,), State of Amazonas, Northern Brazil (Figure 1). The region is characterized by a poor soil and a closed-canopy forest with trees up to 15 meters (Anderson 1981; Luizão *et al.* 2007). The climate is humid tropical ('Afi'), according to Köppen's (1948) classification. The

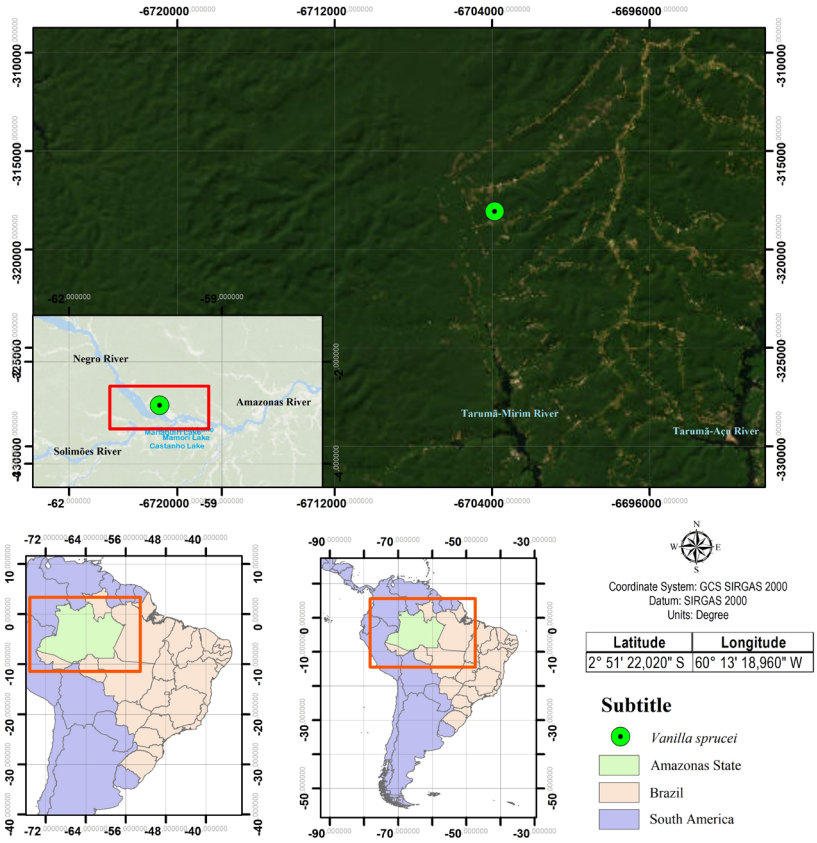

Figure 1. Study and collection area of *Vanilla sprucei* in the municipality of Manaus (State of Amazonas), Brazil.

temperature varies from 24 to 27º C and rarely is below 18º C (Braga 1977). Rainfall occurs throughout the year with a higher occurrence between November and June (Braga 1977; Luizão *et al.* 2007).

Taxonomic treatment

Vanilla sprucei specimens were collected and herborized according to the usual procedures described by Mori *et al.* (1989) for later incorporation into HUAM herbaria (acronyms according to Thiers 2023), including the epitype designated here. The species was identified by comparison with both the holotype (K001382262 – digital image!), and the protologue (Rolfe 1896). The taxonomic description was based on the examined specimens, and the morphological terminology followed Dressler (1993) and Harris and Harris (2001). Data on the flowering phenology were gathered in the collection area during the 2019-2020 flowering periods. A photographic plate and an ink drawing plate were made from the material collected due to the absence of illustrations and photographs for *V. sprucei*. A comparison was made between *V. sprucei* and *V. trigonocarpa*, as previous studies have suggested they are closely related (*e.g.* Soto-Arenas and Cribb 2010; Soto-Arenas and Dressler 2010; Karremans *et al.* 2020). The protologue (Hoehne 1944) and holotype of *V. trigonocarpa* (*A. Ducke s/n.*, RB19445 – digital image!) were also consulted.

Geographic distribution and conservation

The geographical distribution of *Vanilla sprucei* was based on a detailed analysis of the information contained in Rolfe (1896), Cogniaux (1904-1906), Hoehne (1945) and Soto-Arenas and Cribb (2010), and records available in Brazilian (EAFM, HAMAB, HUAM, IAN, INPA, MG, UEC, SP and SPFR) and international (COL, F, MO, NY, P and US) herbaria. Herbaria acronyms follow Thiers (2023).

To construct the geographic distribution map through the QGIS software (Version 3.28 Firenze), occurrence coordinates were entered in the online platform Geospatial Conservation Assessment Tool (GeoCAT - http://geocat.kew.org/). In this map, the coordinates of the new occurrences in Brazil (municipality of Manaus, Amazonas State), including the material deposited in the HUAM, INPA and NY herbaria were included.

Also, from the GeoCAT online platform, the Extent of Occurrence (EOO) and the Area of Occupation (AOO) were calculated for the assessment of the global and national (Brazil) conservation status. The AOO calculation was based on a defined 2 km grid as recommended by the International Union for Conservation of Nature (IUCN) (Bachman *et al.* 2011; IUCN 2022). The conservation status of the species was assessed according to the methodology and the IUCN Red List Categories and Criteria (IUCN 2022).

Taxon sampling for phylogenetic analysis

A total of 43 *Vanilla* accessions (38 species) were analyzed and are referred to here as the ingroup. *Lecanorchis multiflora* J.J. Sm. was selected as an outgroup according to previous phylogenetic studies on *Vanilla* (*e.g.* Pansarin and Ferreira 2022; Pansarin and Menezes 2023). A data matrix was built based on sequences available in the GenBank database in addition to new ones obtained during this study. A list of ingroup and outgroup species, vouchers and GenBank accession numbers is given in supplementary material, Table S1.

DNA extraction, amplification and sequencing

Phylogenetic analyses were performed in the Laboratory of Molecular Biology and Plant Systematics at FFCLRP-USP. DNA of *V. sprucei* from the study area was extracted from fresh leaves according to a modified CTAB method (Doyle and Doyle 1987). The amplifications were carried out using 50 µL PCR volumes. Relaxation of the DNA strands was achieved by the addition of a 5M betaine solution to the PCR preparations. Primers of the nuclear ribosomal transcribed spacer region (ITS), including the 5.8S gene (Sun *et al.* 1994) were used for amplification and sequencing. *Taq* DNA polymerase was added to the PCR mixture at 80 ºC following a 10 min period of denaturation at 99 ºC in the thermocycler. Thirty-five cycles were run according to the following program: denaturation, 1 min, 94 ºC; annealing, 45 sec, 64 ºC; extension, 1 min, 72 ºC; final extension, 5 min, 72 ºC. Amplified PCR products were purified using GFX PCR columns (GE Health Care). Sequencing reactions were prepared using Big Dye 3.1 (ABI), purified PCR products and the same aforementioned primers. Samples were dehydrated and re-suspended with loading dye. Sequences were obtained using an Applied Biosystems automated sequencer model 3100. Sequence Navigator and Autoassembler (Applied Biosystems) software was used for sequence editing and assembly of complementary and overlapping sequences. DNA sequences were aligned using the BioEdit version 5.0.9 software (Hall 1999). The sequence alignment is available *upon request* from the last author.

Phylogenetic analyses

A data matrix of ITS containing 44 taxa was used for phylogenetic analyses. Maximum parsimony analysis (MP) was run with PAUP* version 4.0b5 (Swofford 2001) software. A heuristic search was conducted with 1000 replicates of random taxon entry additions, MULTREES option, and the tree bisection-reconnection (TBR) swapping algorithm, holding 10 trees per replicate and saving all the shortest trees. Support for clades was assessed using 1,000 bootstrap replicates (Felsenstein 1985). Bootstrap support (BS) values above 50% were calculated and mapped above the branches of the consensus tree. For bootstrap support levels, we considered bootstrap percentages of 50–70% as weak, 71–85% as moderate, and >85% as strong (Kress *et al.* 2002).

Maximum Likelihood (ML) analysis was run using the MEGA X tool (Kumar *et al.* 2018) with bootstrapping for 3,000 *replicates. The analysis* was based on the Kimura 2-parameter model (Kimura 1980). Initial trees for the heuristic search were obtained automatically by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value.

Bayesian Inference (BI) was conducted using the MrBayes program, version 3.1 (Ronquist and Huelsenbeck 2003). The optimal model of sequence evolution for each partition was selected using MEGA X (Kumar *et al.* 2018) and Bayesian Information Criterion (BIC). The software selected the HKY+G as the best evolution model for the ITS region. Four Markov chains were run simultaneously for three million generations, with parameters sampled every 100 generations. The consensus tree was calculated after removal of the first 3,000 trees, which were considered to be "burn-in". Posterior probability (PP) values above 0.5 were calculated and mapped below the branches of the consensus tree. The infrageneric classification for *Vanilla* followed (Soto-Arenas and Cribb 2010).

RESULTS

Taxonomy and distribution

Vanilla sprucei Rolfe (1896: 461). **Type:** COLOMBIA. Vaupés River, Upper Amazon, no day/XI/1852, fl., *R. Spruce 2727* (holotype: K, barcode K00138262! [digital image]).

Epitype (designated here): BRAZIL. **Amazonas:** Manaus, Ramal Vale do Novo Amanhecer, 2°51'22.02"S, 60°13'18.96" W, on *Mauritia flexuosa*, 12/XII/2019, fl., *A. H. Krahl et al. 1371* (HUAM012580!). (Figures 2a–h, 3a–j and 4a–b).

Notes: On basis on the evidence that the protologue of *V. sprucei* is based on a single specimen, and that Rolfe (1896) indicated it "is not in very good state", an epitype is here designated. In fact, we checked the holotype and found that the flower, the main structure for identification, is not in a good state of conservation.

Diagnosis: Species easily recognizable from remaining *Vanilla* by its elliptic to oblanceolate leaves, by its one-day flowers with greenish-yellow sepals and petals and white labellum, by its narrowly spatulate sepals, by its linear petals and by its trilobate labellum with a penicillate callus just above the anther and stigma, lateral lobes rounded, and apical lobe ovate with undulate margin and emarginated apex.

Description of the epitype: Hemiepiphyte herb, ca. 10–20 m long. **Stem** 0.3–0.5 cm in diameter, cylindrical, glabrous, green; internodes 2.9–8.3 cm long. **Leaves** 11.5–17.5 × 3.7–5.2 cm, alternate, chartaceous to slightly fleshy, light green, pseudopetiolate; pseudo-petiole 0.5–1.0 cm long., canaliculated; leaf blade elliptic to oblanceolate, longer

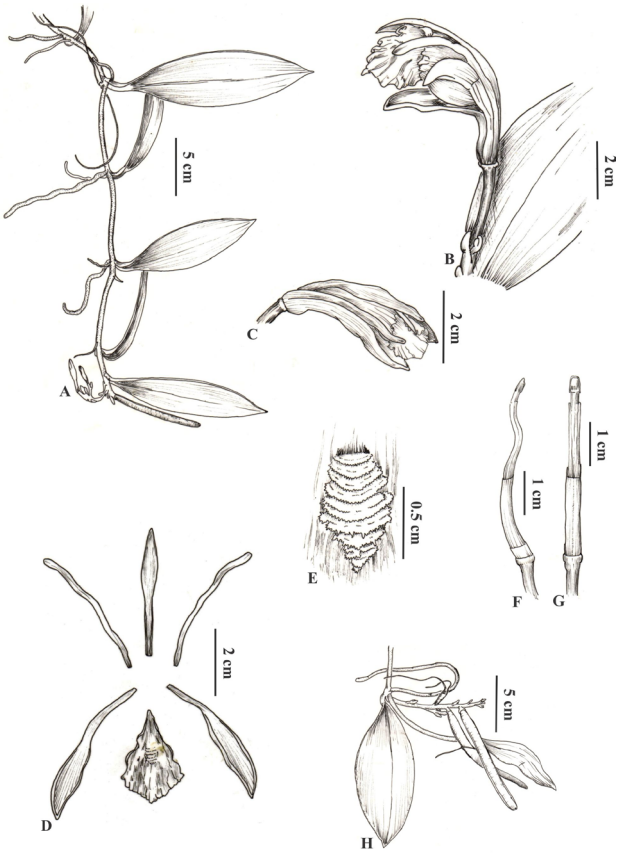

Figure 2. *Vanilla sprucei.* A – Habit; B and C – Flowers; D – Perianth parts; E – Penicillate callus; F – Column in side view; G – Column in ventral view; H – Fruits. Drawing by M.M. Corrêa.

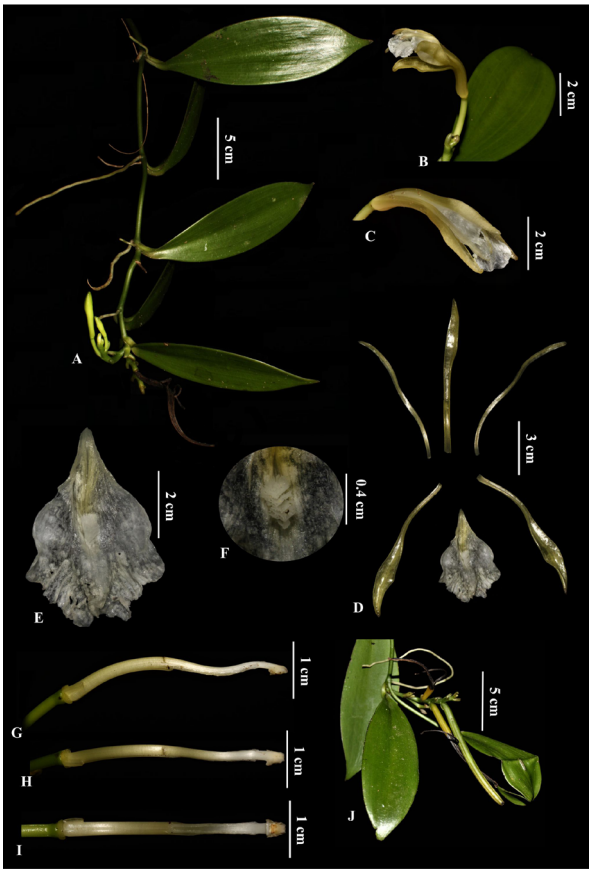

Figure 3. *Vanilla sprucei.* A – Habit; B and C – Flowers; D – Perianth parts; E – Labellum; F – Penicillate callus; G – Column in side view; H – Column in dorsal view; I – Column in ventral view; J – Fruits.

Figure 4. Epitype of the *Vanilla sprucei* deposited at HUAM (HUAM012580 barcode). Photos by the HUAM Herbarium team.

than internodes, margin entire, base acute, apex acuminate. **Inflorescence** 8.6–10.1 cm long., racemose, apical or axillary, with 4–7 flowers opening in succession; peduncle 3.1–4.9 cm long, erect, light green; floral bracts 0.5–0.7 × 0.3–0.5 cm, ovate, fleshy, glabrous, light green, margin entire, apex obtuse. **Flowers** lasting six hours with greenish-yellow sepals and petals and whitish labellum. Flowers tubular, resupinate, pedicellate; pedicel + ovary 2.0–2.5 cm long., cylindrical, glabrous, light green; dorsal sepal 6.0–6.4 × 0.5–0.6 cm, narrowly oblanceolate, symmetrical, glabrous, concave with an involute entire margin, base acute, apex acute; lateral sepals 5.9–6.1 × 0.5–0.6 cm, narrowly oblanceolate, asymmetrical, glabrous, concave with an involute margin, slightly falcate near the apex, base acute, apex acute; petals 5.9–6.0 × 0.1–0.2 cm, linear to narrowly oblong, symmetrical, glabrous, flat, margin entire, base oblong, apex acute; lip 3.8–4.7 × 2.1–2.4 cm, entire to subtrilobed, glabrous, partially fused to the column margins ¾ in length from the base to the apex, with a penicillate callus in the central portion, just below the anther and stigma; margin entire and sinuate; apex sub-obtuse and reflex, cushion-like with parallel inconspicuous rugose keels above the penicillate callus; penicillate callus 0.5–0.6 cm long. made up by 6-7 rows of flattened and whitish fimbriae; column 4.5–4.9 cm long, semi-cylindrical, slender, sinuous, creamy-white, apex with two small membranous wings; rostellar flap prominent; anther versatile, saddle-shaped; pollen in monads. **Fruits** 8.0–9.9 cm long, cylindrical, obscurely canaliculate, green, base shortly attenuate, basipetal dehiscence.

Material examined: BRAZIL. **Amazonas:** São Gabriel da Cachoeira, Rio Negro, Iauaretê, levantamento Projeto RADAM-58, trepadeira muito comum na Campina, 12/XII/1975, sterile, *L. Coelho & Francisco 222* (INPA!); Santa Isabel do Rio Negro, Along rio Marie, at Marauná, campina, 00°40'S, 66°45'W, 6/VII/1979, fl. (buds), *L. Alencar 474* (NY!, photograph!).); Juruá, Campo Petrolífero de Urucu – Locação RNI, epífita em Mata de Terra Firme, solo argilo-arenoso, 12/VII/1995, fr., *L. Chaves et al. 134* (HUAM!); Manaus, Ramal Vale do Novo Amanhecer, 2°51"22.02"'S, 60°13'18.96"'W, on *Mauritia flexuosa*, 13/VII/2019, fr., *A. H. Krahl & D. R. P. Krahl 1283* (HUAM!); *idem*, 12/XII/2020, fl. (buds), *A. H. Krahl et al. 1526* (HUAM!). COLOMBIA. **Caquetá:** Sierra de Chiribiquete, cerca del campamento base, 0°56'15" N, 72°42'06"W, 600 m a.s.l., 20/XI/1992, sterile, *P. Palacios et al. 2753* (COL!); idem, 27/XI/1992, fl., *P. Palacios et al. 2776* (COL!); **Guainía**: riberas del Río Inírida, Raudal Guacamayo, margen izquierda, 69°45'W, 180 m a.s.l., 4/II/1992, fr., *A. Fernández 2146* (US!); **Vaupés:** Rio Apaporis, Cachivera de Jirijirimo y alrededores, 250 m a.s.l., 10/VI/1951, fl. (buds), *R. E. Schultes & I. Cabrera 12497* (A, AMES and COL!); GUYANA. **Cuyuni-Mazaruni:** Pakaraima Mts., Mazaruni River, NW of Chi-Chi Falls, 5°35'48.6"N, 60°12'48.8"W, 762 m a.s.l., 01/II/2004, fr., *K. M. Redden et al. 2518* (NY and US, photograph!); Mazaruni River, 0.25 miles S of Base Camp 6, 6°2'26.6" N, 60°39'09.6" W, 547 m a.s.l., 11/II/2004, fl., *K. M. Redden et al. 1843* (US, photograph!); VENEZUELA. **Amazonas:** Casiquiare, R. Casiquiare, 162 Kms. de la boca dela Casaquiare, 3/II/1991, fr., *M. Colella et al. 1766* (NY, photograph!).

Distribution: Occurs in Brazil (based on this study), Colombia, Guyana and Venezuela (Rolfe 1896; Soto-Arenas and Cribb 2010; Damián-Parizaca and Mitidieri-Rivera 2023). From the consultation of herbarium material, we conclude *V. sprucei* also occurs in the municipality of Santa Isabel do Rio Negro, São Gabriel da Cachoeira, Juruá and Manaus, in the State of Amazonas, Brazil. From the new data collated here, the species distribution appears to be limited to the Rio Negro watershed and the Guiana shield area, where white sand forest ecosystems commonly occur as islands of habitats, and host specialized species (Adeney *et al.* 2016; Daly *et al.* 2016). These ecosystems are particularly vulnerable to anthropogenic pressures, being a priority area for conservation (Fine and Bruna 2016).

Habitat and ecology: In Brazil *V. sprucei* was found in a white sand vegetation ("campinarana") in the north of the municipality of Manaus, state of Amazonas, northern Brazil (Figure 5). The species was found in flower from July to December. In July, unripe and ripe fruits were also observed. The opening of the flowers takes place in the early hours of the morning and lasts up to six hours (the flowers close before midday). *Vanilla sprucei* was more commonly recorded as hemiepiphyte on the buriti palm (*Mauritia flexuosa* L.), although the species can also use Eudicotiledons as a phorophyte.

Conservation status: Based on the studied specimens, at least 30-35 sub-populations of *Vanilla sprucei,* with ca. 300-350 mature individuals are estimated to occur along their distribution to Brazil, Colombia, Guiana and Venezuela. The Extent of occurrence (EOO) is over 1,100,000 km² (falling into the IUCN Least concerned [LC] category), whereas the Area of Occupancy (AOO) is so far estimated as 116 km². Under the IUCN Red list criterion B2, this very limited AOO corresponds to the Endangered (EN) category. Additionally, the species presents a greatly fragmented geographic range (criterion B2a), and the majority of the known populations are located outside conservation units. Habitat degradation in the Amazon region continues, including the localities of this species, with likely loss of subpopulations (criterion B2b). Therefore, we suggest that the species be preliminary assigned the conservation status in the EN category (B2ab).

In Brazil the conservation status of the taxon is of more acute concern. Only four small populations (ca 30-40 mature individuals, IUCN criterion C) are hitherto known (B2a), with an AOO of 16 km² (B2) and a reasonable EOO (191,173 km² - LC category). These subpopulations are located in "campina", biotopes on white sand, where the illegal extraction of sand leads to habitat destruction. Thus, we may

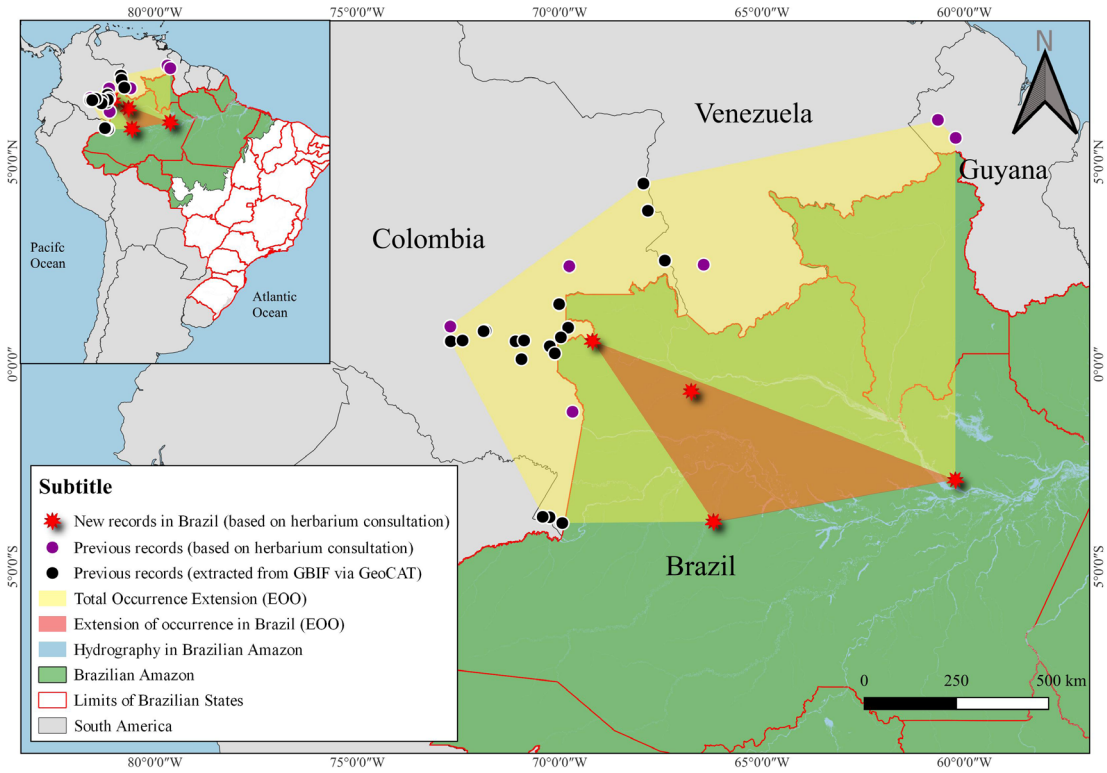

Figure 5. Geographic distribution of ***Vanilla sprucei*** in South America. New records from Brazil are presented in red.

expect a continued decline of AOO (B2b) as well as a severe and continuing decline of the total number of mature plants (C1) if this land-use pattern is maintained. In conclusion, we suggest a preliminary conservation status for the Brazilian population of *V. sprucei* in the CR category (B2abC1).

Phylogenetic analyses

Phylogenetic inferences obtained by analysis of the ITS (nrDNA) region using different methods (BI, ML and MP) resulted in trees with similar topologies (ML is shown; Figure 6). The Neotropical *Vanilla* with membranaceous leaves (PP 1, BS 99%;) was basally placed to a clade (PP .87, BS 71%) with two subclades: a predominantly Old-World/ Caribbean clade (PP .99, BS 92%), and a clade including the remaining Neotropical taxa (PP .96, BS 90%). *Vanilla sprucei*, in addition to the members of the *V. palmarum* group, i.e. *V. palmarum* (Salzm. ex Lindl.) Lindl. and *V. bicolor* Lindl., emerged in a polytomy along with a larger clade of Neotropical taxa. The latter clade is well-supported (PP .93, BS 67%) and it included *V. trigonocarpa*, which emerged as sister to the remaining (Figure 6).

DISCUSSION

Vanilla sprucei has been originally described from the Vaupés River (upper Amazon) in, Colombia, as indicated in the protologue of Rolfe (1896). The most of the materials collected and deposited in herbaria show that its occurrence almost restricted to Colombia and considered by Soto-Arenas and Cribb (2010) to be endemic to that country. The early suggestion that this species occurs in Brazil (in the State of Amazonas) as pointed out by Cogniaux (1904-1906), Hoehne (1945), Flora e Funga do Brasil (2023) and Govaerts *et al.* (2023) is arbitrary as they do not indicate any voucher other than the holotype and, no material has been recorded for the Brazilian territory to date. We believe that this mistake by Cogniaux (1904-1906) and subsequent authors is due to the fact that the Vaupés River arises in Colombia, but flows into Brazilian territory. Furthermore, the term "Upper Amazon" contained in the protologue may have mistakenly led these authors to indicate the occurrence of this species in the Brazilian Amazon (State of Amazonas in Brazil).

Flowers of *V. sprucei* are fragile and ephemeral, lasting for a few hours. Perhaps for this reason, this species is poorly represented in floristic inventories and herbaria collections, and has been considered an obscure taxon. In fact, although our data show this species is distributed in northern South America, only three specimens of *V. sprucei* are available in Brazilian scientific collections prior this study (Flora e Funga do Brasil 2023). This species has sometimes been confused with several other Amazonian *Vanilla*, such as *V. trigonocarpa* and *V. appendiculata*. However, the morphology of *V. sprucei* strongly differs from that of *V. appendiculata*. In *V. sprucei*

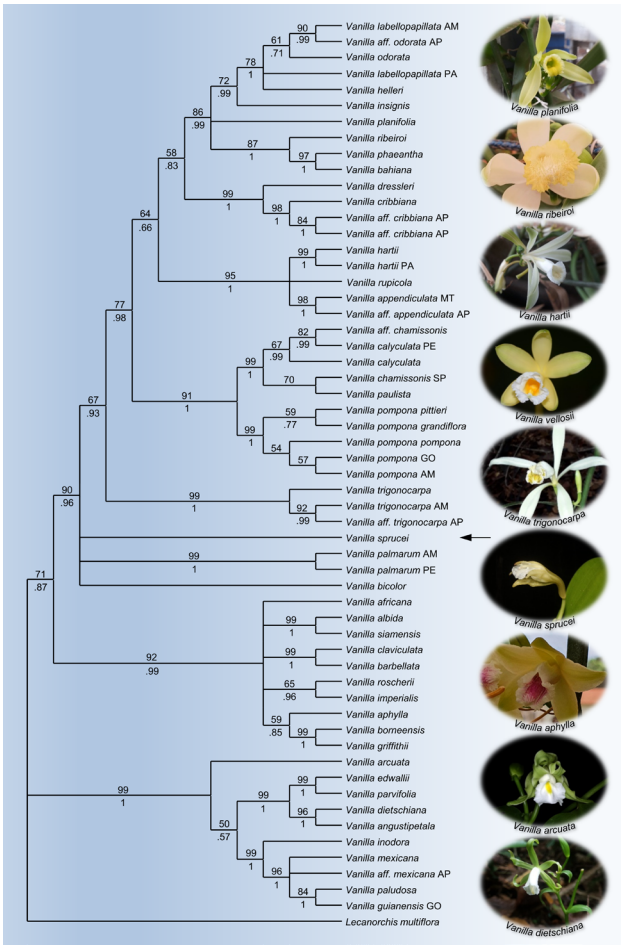

Figure 6. Bootstrap consensus tree from maximum likelihood analysis of *Vanilla* (Orchidaceae) based on the region ITS1, 5.8S, ITS2 (nrDNA) showing the position of *V. sprucei* among the Neotropical congeners (arrow). Bootstrap values >50 obtained by maximum parsimony analysis (MP %) are given above the branches, while posterior probabilities values > 0.5 (BI) are given below branches. Acronym after species names = Brazilian states: AM = Amazonas, AP = Amapá, GO = Goiás, MT = Mato Grosso, PA = Pará, PE = Pernambuco, SP = São Paulo.

the leaves are elliptic to oblanceolate with an acuminate apex *vs.* obovoid to spathulate with a cuspidate to mucronate apex in *V. appendiculata* (Engels and Rocha 2016; Barona-Colmenares 2018). The inflorescence is apical or axillary and produces 8 flowers on average in *V. sprucei vs.* apical with 8–30 flowers in *V. appendiculata*. The floral bracts of both species differ in shape (ovate *vs.* triangular) and the pedicel + ovary is shorter in *V. sprucei* (2–2.5 cm *vs.* 2.5–4 cm). The petals and sepals are usually ≤ 6.5 × 0.6 cm in *V. sprucei vs.* ≥ 6.5 × 0.6 cm in *V. appendiculata*. In addition, the petals are narrowly linear in *V. sprucei*, and narrowly lanceolate in *V. appendiculata*. The labellum of *V. appendiculata* is yellowish, barbellate, foliaceous appendages, absent in *V. sprucei*. The fruits of *V. sprucei* are also smaller (8–9.9 cm long) than *V. appendiculata* (10–17.4 cm long) and have a cylindrical shape over their entire length with a slightly attenuated base, whereas the fruits of *V. appendiculata* are cylindrical and swollen towards the apex with a curved base (see Engels and Rocha 2016; Barona-Colmenares 2018). Although Soto-Arenas and Cribb (2010) includes *V. appendiculata* in the *V. planifolia* group recent findings have shown that *V. appendiculata* emerges in a clade comprising *V. hartii* and *V. rupicola*. Thus, the *Vanilla planifolia* group is polyphyletic (Pansarin and Menezes 2023).

According to Soto-Arenas and Cribb (2010), *V. sprucei* is part of the subgen. *Xanata* sect. *Xanata* and is included in the *V. trigonocarpa* group. The *V. trigonocarpa* group is characterized by species with few-flowered racemes, fragrant flowers, which can be small to huge, very inflated lip with very crenulate margins and by the trigonal fruit, very long and fleshy. Currently the group consists of four species, distributed throughout tropical America (from Costa Rica to the Amazon in South America): *V. espondae* Soto-Arenas, *V. hartii*, *V. sprucei* and *V. trigonocarpa* (Soto-Arenas 2010; Soto-Arenas and Cribb 2010). However, according to Pansarin and Menezes (2023), *V. hartii* is closely related to *V. appendiculata* and to a recently described species from Brazilian campos rupestres, *V. rupicola*. In fact, the three species emerge in a well-supported clade sister to the remaining species in sect. *Xanata* (Pansarin and Menezes 2023), suggesting that further realignments of the groups created by Soto-Arenas and Cribb (2010) will be necessary, as the *V. trigonocarpa* group is polyphyletic (Pansarin and Menezes 2023). The inclusion of *V. sprucei* in the analyses reinforces the polyphyly of the *V. trigonocarpa* group, as this Amazonian species emerges in a basal polytomy among the non-membranaceous *Vanilla*, while *V. trigonocarpa* is nested as sister to the remaining hemiepiphytes or nomadic vines thick-leafed congeners (Pansarin and Menezes 2023).

Many *Vanilla* species has been synonymized in a recent treatment for Neotropical *Vanilla* based on examination of herbarium materials (Karremans *et al.* 2020). This is the case with *V. sprucei* and *V. trigonocarpa*. (Karremans *et al.* 2020, page 490). According to our findings, *V. sprucei* is not a previous name for *V. trigonocarpa* as suggested (Karremans *et al.* 2020). Both taxa show enough differences to deserve being treated as separate species. The leaves of *V. sprucei* are smaller them those *V. trigonocarpa* (11.5–17.5 × 3.7–5.2 *vs.* 15–30 × 5.5–7 cm); the inflorescence produces more flowers (on average 8 *vs.* up to 4); the pedicellate ovary is shorter (2.0–2.5 *vs.* 4–4.5 cm); the perianth parts are also smaller: sepals (5.9–6.4 × 0.5–0.6 *vs.* 9.3–11.5 × 1.4–2.0 cm), petals (5.9–6 × 0.1–0.2 *vs.* 9.4–11.0 × 1.4–1.8 cm), and labellum (3.8–4.7 × 2.1–2.4 *vs.* 8.9–11.5 × 4.1–5.5 cm.) (see Soto-Arenas and Dressler 2010; Krahl 2020). Strong differences can also be observed in the floral parts. Sepals in *V. sprucei* are narrowly oblanceolate (*vs.* lanceolate, elliptical or oblanceolate in *V. trigonocarpa*); petals are linear to narrowly oblong (*vs.* oblanceolate to narrowly oblanceolate); the labellum is entire to subtrilobed (*vs.* trilobed); the midlobe is sub-obtuse (*vs.*

emarginate); and the column is smaller (4.5–4.9 *vs.* 8–8.5 cm long). Finally, the fruit shows a cylindrical transverse section (*vs*. triangular) (see Soto-Arenas and Dressler 2010; Krahl 2020).

Importantly, our molecular data strongly support *V. sprucei* and *V. trigonocarpa* as distinct taxa. According to our phylogenetic analyses (Figure 6), the hemiepiphyte *V. sprucei,* in addition to the epiphytes *V. bicolor* and *V. palmarum,* occupy a basal position among the non-membranaceous Neotropical *Vanilla. Vanilla bicolor* and *V. palmarum* are species widely known by their relation with palms (see Barberena *et al.* 2019; Pessoa *et al.* 2015; Klein and Piedade 2019; Krahl 2020; Cantuária *et al.* 2021; Ferreira-Filho *et al.* 2021), while *V. sprucei* shows a non-obligatory association with palms. These three species are widely distributed throughout the Amazon, suggesting that the origin of Neotropical non-membranous *Vanilla* (section *Xanata*) may be this region. In fact, the ancient Amazon Forest and Brazilian Atlantic Forest were spatially interconnected and continuous during the Paleogene (Sobral-Souza and Lima-Ribeiro 2017). The Cerrado vegetation evolved between the two Biomes because of extreme environmental conditions and climatic oscillations during the Tertiary and Quaternary (Morley 2000) allowing the diversification of taxa adapted to more xeric conditions, including *Vanilla* (Pansarin and Menezes 2023).

CONCLUSION

Despite the importance of alpha-taxonomy over the centuries, recent studies have shown that integrative taxonomy is extremely important in the understanding of species boundaries in Vanilloideae. It is also the case with *V. sprucei*, a taxon recently proposed as conspecific with *V. trigonocarpa.* Here, our data on living specimens, detailed studies on morphology and molecular phylogenetics reveal these species to be distinct. In addition, our studies on Neotropical *Vanilla* have updated the geographical distribution of several taxa.

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