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A new vanilla species from Costa Rica closely related to *V. planifolia* (Orchidaceae)

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Abstract. We describe a new vanilla species growing in sympatry with *Vanilla planifolia* Jacks. ex Andrews (Orchidaceae) in the province of Limón, Caribbean coast of Costa Rica. The morphology of the reproductive and vegetative organs observed on vines cultivated under shade-house, the nuclear (Internal Transcribed Spacer) and plastid (*mat*K) nucleotide sequences, as well as the contents of aromatic compounds measured in ripe fruits, show that this species is close to but distinct from *V. planifolia.* The name *V. sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. is proposed for this new *Vanilla* species endemic in Costa Rica. It is especially distinguished from *V. planifolia* by a reduction of about 30% of the size of the fruits and flowers, by a divergence of ITS sequences for at least two species-conserved nucleotides compared to seven other species of the *V. planifolia* group, and by the presence of anisic compounds and low content of phenolic compounds (including vanillin) in the fruits. These results confirmed the extension of the area of distribution of *V. planifolia* southward to Costa Rica, where a recent speciation process occurred. Because of its particular agronomic and aromatic properties, *V. sotoarenasii* sp. nov. could represent a valuable biological resource for the vanilla industry.

Keywords. Barcoding, Costa Rica, Limón, radiation, *Vanilla planifolia*, *V. sotoarenasii*.

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Introduction

The *Vanilla* genus, *Vanilla* Plum. ex Miller (Miller 1754), belongs to the subfamily Vanilloideae, tribe Vanillinae (Cameron 2010). It is an ancient group of tropical orchids that originated in America about 70 million years ago and differentiated in America, Africa and Asia (Ramírez *et al.* 2007; Bouétard *et al.* 2010). The classification of the species of *Vanilla* was recently reviewed by Soto Arenas & Cribb (2010), who divided the genus into two subgenera, *Vanilla* and *Xanata* Soto Arenas & P.J.Cribb*,* and further split the subgenus *Xanata* into two sections *Xanata* and *Tethya* Soto Arenas & P.J.Cribb. Currently, about 110 *Vanilla* species are reported and clustered into 20 groups (Portères 1954; Soto Arenas & Cribb 2010; Cameron 2011; Pansarin *et al.* 2012). The *V. planifolia* group is the most important by the number of species (16) and, economically, because it contains the commercial species *V. planifolia* Jacks. ex Andrews (Andrews 1808) and *V.* × *tahitensis* J.W.Moore (Moore 1933), the aromatic fruits of which provide the vanilla flavor used by the food and perfumes industries. The taxonomy of the species from Mexico and Central America was reviewed by Soto Arenas & Dressler (2010) on the basis of their morphology and Internal Transcribed Spacer (ITS) DNA sequences. Recently, two new species have been described in the region, *V. esquipulensis* Archila & Chiron (Archila & Chiron 2012) in Guatemala and *V. rivasii* Molineros, R.T.González, Flanagan & J.T.Otero (Molineros-Hurtado *et al.* 2014) in Chocó, northern Colombia, plus two more in French Guiana, *V. inornata* Sambin & Chiron (Sambin & Chiron 2015) and *V. aspericaulis* Sambin & Chiron (Sambin & Chiron 2015).

Ten out of the 17 species recorded in Mexico and Central America have been reported in Costa Rica (Soto Arenas & Dressler 2010), namely: *V. costaricensis* Soto Arenas (Soto Arenas & Dressler 2010), *V. dressleri* Soto Arenas (Soto Arenas & Dressler 2010), *V. hartii* Rolfe (Rolfe 1899), *V. helleri* A.D.Hawkes (Heller & Hawkes 1966), *V. inodora* Schiede (Schiede 1829), *V. odorata* C.Presl (Presl

1827), *V. planifolia*, *V. pompona* Schiede (Schiede 1829), *V. sarapiquensis* Soto Arenas (Soto Arenas & Dressler 2010) and *V. trigonocarpa* Hoehne (Hoehne 1944).

During surveys carried out in Costa Rica since 2012 (Azofeifa-Bolaños *et al*. 2014) abundant populations of vanilla which did not fit any species previously described in the country were observed in Limón Province (Caribbean coast of Costa Rica). This vanilla, called "vanilla Limón" (VanL), had strong morphological similarities with a vanilla accession collected in Cahuita (Limón, Costa Rica) in 1993, referenced *Pignal 396b* (P00075132) in the herbarium of the Muséum national d'Histoire naturelle in Paris, P (herbarium acronym following Thiers continuously updated). Living specimens of this accession were preserved in the Emmanuel Liais park (Cherbourg-en-Cotentin, France) under accession number CH554, and subsequently in the Biological Resources Centre (BRC) Vatel (Saint Pierre, La Réunion) under accession number CR0068, and were tentatively classified as *V.* aff. *planifolia*.

Molecular approaches have enhanced plant taxonomy by allowing reliable genealogy-based classifications (Besse 2014). In the last decade, nuclear, mitochondrial and chloroplastic DNA sequencing has been used to study plant diversity and resolve taxonomic positions in all plant families, including the puzzling group of vanilloid orchids (Cameron 2009, 2010; Soto Arenas & Dressler 2010).

The aim of this study was thus to investigate the taxonomic status of VanL by using complementary approaches involving morphology of reproductive and vegetative organs, molecular barcoding and the accumulation of secondary metabolites in fruits.

Material and methods

Plant and DNA samples

The plant samples used in this study were obtained from field surveys carried out in Costa Rica, Mexico and Guatemala, supplemented with lyophilized plant materials preserved in the Mexican National Collection (Reyes-López *et al*. 2014), the BRC Vatel (Roux-Cuvelier & Grisoni 2010), and DNA from the herbarium of the Instituto Chinoin, AMO (Thiers continuously updated).

The survey in Costa Rica was conducted between November 2012 and March 2016 in the entire country. Prospections were targeted on areas where vanilla were recorded based on herbarium specimen information and consulting orchid experts, local guides, and earlier project experiences, among others. The material was collected according to permit number 061-2013 issued on 12 Jun. 2013 by National System of Conservation Areas (SINAC) of the Ministry of Environment and Energy (MINAE).

All plant and DNA samples analyzed in this study are listed in Table 1.

Morphological traits

To compare the morphology of vegetative and reproductive organs of VanL to those of *V. planifolia*, the following traits were measured on plants cultivated in controlled conditions in BRC Vatel in La Réunion: length and greater width of the third and fourth leaves, diameter and length of the third inter-node, length and width of the tube, length of the ovary, length and width of the dorsal sepal. Measurements were carried out on two to six plants per accession grown under shade-house in November, which is the optimum vegetative growth and flowering period in La Réunion. From 14 to 30 fresh organs were measured per accession according to the availability of material. Length and weight of fruits were also measured at maturity (in July). We used the experimental tool "collaboratoire" of the national French infrastructure e-ReColNat (ANR-11-INBS-0004) for specimen comparisons.

Table 1. Identification and origin of the accessions of species of *Vanilla* Plum. ex Miller used in this study. [continued on next 3 pages]

Code	Species	Country of origin	Depository	Locus	Code in collection / voucher
bahi0071	*V. bahiana*	Brazil	VATEL	*mat*K	CR0071
bahi0086	*V. bahiana*	Brazil	VATEL	ITS & *mat*K	CR0086
bahi0098	*V. bahiana*	Brazil	VATEL	ITS	CR0098
bahi0099	*V. bahiana*	Brazil	VATEL	ITS & *mat*K	CR0099
bahi0668	*V. bahiana*	Brazil	VATEL	*mat*K	CR0668
caly-01	*V. calyculata*	Honduras	UNAM	ITS	Linares_3386
cham0666	*V. chamissonis*	Brazil	VATEL	ITS & *mat*K	CR0666
crib0109	*V. cribbiana*	French Guyana	VATEL	ITS & *mat*K	CR0109
crib0122	*V. cribbiana*	French Guyana	VATEL	*mat*K	CR0122
cribb-01	*V. cribbiana*	Mexico	UNAM	ITS	Soto_7940
cribb-02	*V. cribbiana*	Mexico	UNAM	ITS	Soto8439(nueva)
cribb-03	*V. cribbiana*	Mexico	UNAM	ITS	Soto_8370
dres-01	*V. dressleri*	Costa Rica	UNAM	ITS	ByrdIA6(azul)
dres-02	*V. dressleri*	Costa Rica	UNAM	ITS	Byrd_IIC3
dres-03	*V. dressleri*	Costa Rica	UNAM	ITS	Byrd_ID3
dres-04	*V. dressleri*	Costa Rica	UNAM	ITS	Byrd_IIC2
hart-01	*V. hartii*	Costa Rica	UNAM	ITS	Byrd_IIF1
hart-03	*V. hartii*	?	UNAM	ITS	MAS7956
hart-04	*V. hartii*	Costa Rica	UNAM	ITS	Salas_1
hell-0	*V. helleri*	Mexico	UNAM	ITS	Soto8818
hell-01	*V. helleri*	Costa Rica	UNAM	ITS	Byrd_IID5
hell-02	*V. helleri*	Costa Rica	UNAM	ITS	Byrd_IIF4
insi-02	*V. insignis*	Mexico	UNAM	ITS	Soto_7668
insi-03	*V. insignis*	Honduras	UNAM	ITS	Linares7
insi-04	*V. insignis*	Guatemala	UNAM	ITS	Soto_8611
insi2507	*V.* cf. *insignis*	Mexico	ITT	*mat*K	muestra-13
insi2519	*V.* cf. *insignis*	Mexico	ITT	ITS	muestra-25
insi2520	*V.* cf. *insignis*	Mexico	ITT	ITS	muestra-26
insi2521	*V.* cf. *insignis*	Mexico	ITT	ITS & *mat*K	muestra-27
insi2542	*V.* cf. *insignis*	Mexico	ITT	ITS & *mat*K	muestra-20
insi2545	*V.* cf. *insignis*	Mexico	ITT	ITS	muestra-23
insi2583	*V. insignis*	Mexico	ITT	ITS	muestra-33
insi2584	*V.* cf. *insignis*	Mexico	ITT	ITS	muestra-34
insi2594	*V.* cf. *insignis*	Mexico	BUAP	ITS	#005
insi2618	*V. insignis*	Mexico	BUAP	*mat*K	CR2618
insi2672	*V. insignis*	Guatemala	Orquideario_Archila	ITS	VG-002
insig2548	*V.* cf. *insignis*	Mexico	ITT	*mat*K	muestra-26
lind0682	*V. lindmaniana*	French Guyana	VATEL	ITS & *mat*K	CR0682
odor-01	*V. odorata*	?	UNAM	ITS	Soto_8822
odor0116	*V. odorata*	French Guyana	VATEL	ITS & *mat*K	CR0116
odor0117	*V. odorata*	French Guyana	VATEL	*mat*K	CR0117

Code	**Species**	**Country of origin**	**Depository**	**Locus**	**Code in collection / voucher**
odor-02	*V. odorata*	?	UNAM	ITS	Soto_8797
odor-04	*V. odorata*	?	UNAM	ITS	Soto_8365
odor-05	*V. odorata*	?	UNAM	ITS	Soto_8356
odor-06	*V. odorata*	?	UNAM	ITS	Soto_7955
odor2154	*V. odorata*	Mexico	CITRO	ITS	V. odorata
odor2671	*V. planifolia*	Guatemala	OrquidearioArchila	ITS	VG-001
odor686	*V. odorata*	?	VATEL	*mat*K	CR0686
odor-sn	*V. odorata*	Surinam	UNAM	ITS	Hagsater11881
phae-01	*V. phaeantha*	Mexico	UNAM	ITS	Carnevali_4825
phae-02	*V. phaeantha*	?	UNAM	ITS	Kew
phae-03	*V. phaeantha*	Panama	UNAM	ITS	Soto_9920
phae1522	*V. phaeantha*	Madagascar	VATEL	*mat*K	CR1522
phae1524	*V. phaeantha*	Madagascar	VATEL	*mat*K	CR1524
phae1525	*V. phaeantha*	Madagascar	VATEL	*mat*K	CR1525
phae1526	*V. phaeantha*	Madagascar	VATEL	*mat*K	CR1526
pla2506	*V. planifolia*	Mexico	ITT	*mat*K	CR2506
plan_KJ566306	*V. planifolia*	?	Genbank	*mat*K	KJ566306
plan0001	*V. planifolia*	La Réunion	VATEL	ITS & *mat*K	CR0001
plan0020	*V. planifolia*	La Réunion	VATEL	ITS & *mat*K	CR0020
plan0024	*V. planifolia*	La Réunion	VATEL	ITS & *mat*K	CR0024
plan0027	*V. planifolia*	La Réunion	VATEL	ITS	CR0027
plan0038	*V. planifolia*	La Réunion	VATEL	ITS & *mat*K	CR0038
plan0043	*V. planifolia*	La Réunion	VATEL	ITS	CR0043
plan0196	*V. planifolia*	La Réunion	VATEL	ITS & *mat*K	CR0196
plan-02	*V.* cf. *planifolia*	Costa Rica	UNAM	ITS	Byrd_IIA1
plan-03	*V.* cf. *planifolia*	Honduras	UNAM	ITS	Linares8BF
plan-05	*V. planifolia*	Mexico	UNAM	ITS	Soto8355(nueva)
plan-06	*V. planifolia*	Costa Rica	UNAM	ITS	Pupulin_1966
plan0628	*V. planifolia*	La Réunion	VATEL	ITS	CR0628
plan-08	*V. planifolia*	Mexico	UNAM	ITS	MAS_8526
plan0802	*V. planifolia*	La Réunion	VATEL	ITS & *mat*K	CR0802
plan0831	*V. planifolia*	Mayotte	VATEL	ITS	CR0831
plan0836	*V. planifolia*	Mayotte	VATEL	ITS	CR0836
plan0852	*V. planifolia*	Comores	VATEL	ITS	CR0852
plan0862	*V. planifolia*	Comores	VATEL	ITS	CR0862
plan0876	*V. planifolia*	Comores	VATEL	ITS	CR0876
plan0883	*V. planifolia*	Comores	VATEL	ITS	CR0883
plan-14	*V. planifolia*	?	UNAM	ITS	KewPWC
plan1563	*V. planifolia*	Madagascar	VATEL	ITS	CR1563
plan1570	*V. planifolia*	Madagascar	VATEL	ITS	CR1570
plan-18	*V. planifolia*	Mexico	UNAM	ITS	Soto8808
plan-22	*V. planifolia*	?	UNAM	ITS	clon1324
plan2497	*V. planifolia*	Mexico	ITT	*mat*K	muestra-03
plan2499	*V.* cf. *planifolia*	Mexico	ITT	*mat*K	muestra-05

Code	Species	Country of origin	Depository	Locus	Code in collection / voucher
plan2500	*V.* cf. *planifolia*	Mexico	ITT	*mat*K	muestra-06
plan2518	*V. planifolia*	Mexico	ITT	ITS & *mat*K	muestra-24
plan2523	*V. planifolia*	Mexico	ITT	ITS	muestra-29
plan2530	*V. planifolia*	Mexico	ITT	ITS	muestra-01
plan2531	*V. planifolia*	Mexico	ITT	ITS	muestra-02
plan2533	*V. planifolia*	Mexico	ITT	ITS & *mat*K	muestra-09
plan2534	*V. planifolia*	Mexico	ITT	ITS	muestra-10
plan2535	*V. planifolia*	Mexico	ITT	ITS	muestra-11
plan2540	*V. planifolia*	Mexico	ITT	ITS	muestra-18
plan2541	*V. planifolia*	Mexico	ITT	ITS	muestra-19
plan2544	*V. planifolia*	Mexico	ITT	ITS	muestra-22
plan2549	*V. planifolia*	Mexico	ITT	ITS	muestra-28
plan2551	*V. planifolia*	Mexico	ITT	ITS	muestra-30
plan2552	*V. planifolia*	Costa Rica	UNA	ITS	UNA-VAN-0126
plan2555	*V. planifolia*	Costa Rica	UNA	ITS	UNA-VAN-0198
plan2559	*V. planifolia*	Costa Rica	UNA	ITS	none
plan2582	*V. planifolia*	Mexico	ITT	ITS	muestra-32
plan2586	*V. planifolia*	Mexico	ITT	ITS	muestra-36
plan2587	*V. planifolia*	Mexico	ITT	ITS	muestra-37
plan2588	*V. planifolia*	Mexico	ITT	ITS & *mat*K	muestra-38
plan2589	*V. planifolia*	Mexico	ITT	*mat*K	muestra-39
plan2590	*V. planifolia*	Mexico	ITT	ITS	muestra-40
plan2591	*V. planifolia*	Mexico	ITT	ITS	muestra-41
plan2592	*V. planifolia*	Mexico	BUAP	ITS	#001
plan2599	*V. planifolia*	Mexico	BUAP	ITS	#027
plan2605	*V. planifolia*	Mexico	BUAP	ITS	#044
plan2608	*V. planifolia*	Mexico	BUAP	ITS	#056
plan2616	*V. planifolia*	Mexico	BUAP	ITS	#081
plan2625	*V. planifolia*	Mexico	BUAP	ITS	#111
plan2632	*V. planifolia*	Mexico	BUAP	ITS	#128
plan2645	*V. planifolia*	Mexico	BUAP	ITS	#180
plan2650	*V. planifolia*	Mexico	BUAP	ITS	#mut
plan2678	*V. planifolia*	Guatemala	Orquideario_Archila	ITS	VG-008
plan2679	*V. planifolia*	Guatemala	Orquideario_Archila	ITS	VG-009
plan2680	*V. planifolia*	Guatemala	Orquideario_Archila	ITS	VG-010
plan2681	*V. planifolia*	Guatemala	Orquideario_Archila	ITS	VG-011
plan2682	*V. planifolia*	Guatemala	Orquideario_Archila	ITS	VG-012
pomp0018	*V. pompona*	French Polynesia	VATEL	*mat*K	CR0018
pomp0052	*V. pompona*	La Réunion	VATEL	*mat*K	CR0052
pomp0064	*V. pompona*	?	VATEL	*mat*K	CR0064
pomp0070	*V. pompona*	Brazil	VATEL	*mat*K	CR0070
pomp0079	*V. pompona*	Guadelupe	VATEL	*mat*K	CR0079
pomp0096	*V. pompona*	French Guyana	VATEL	*mat*K	CR0096
pomp-02	*V. pompona*	?	UNAM	ITS	Soto_7632

Code	Species	Country of origin	Depository	Locus	Code in collection / voucher
pomp0691	*V. pompona*	?	VATEL	*mat*K	CR0691
pomp1529	*V.* cf. *pompona*	Madagascar	VATEL	*mat*K	CR1529
pomp1923	*V. pompona*	Mexico	VATEL	*mat*K	CR1923
pomp2581	*V. pompona*	Mexico	VATEL	ITS	CR2581
sp.0068	*V.* sp.	Costa Rica	VATEL	ITS & *mat*K	CR0068
sp.2180	*V.* sp.	Costa Rica	VATEL	ITS & *mat*K	CR2180
sp.2543	*V.* cf. *planifolia*	Mexico	ITT	ITS	muestra-21
sp.2552	*V.* sp.	Costa Rica	VATEL	*mat*K	CR2552
sp.2553	*V.* sp.	Costa Rica	UNA	ITS & *mat*K	UNA-VAN-0002
sp.2554	*V.* sp.	Costa Rica	UNA	ITS & *mat*K	UNA-VAN-0047
sp.2557	*V.* sp.	Costa Rica	UNA	ITS & *mat*K	UNA-VAN-0002
sp.2719	*V.* sp.	Costa Rica	VATEL	ITS & *mat*K	CR2719
sp.2720	*V.* sp.	Costa Rica	VATEL	ITS	CR2720
sp.2721	*V.* sp.	Costa Rica	VATEL	ITS	CR2721
sp.2722	*V.* sp.	Costa Rica	VATEL	ITS & *mat*K	CR2722
sp.UNA022	*V.* sp.	Costa Rica	UNA	ITS	UNA-VAN-0022
sp.UNA049	*V.* sp.	Costa Rica	UNA	ITS	UNA-VAN-0049
sp.UNA059	*V.* sp.	Costa Rica	UNA	ITS	UNA-VAN-0059
sp.UNA228	*V.* sp.	Costa Rica	UNA	ITS	UNA-VAN-0228
sp.UNA229	*V.* sp.	Costa Rica	UNA	ITS	UNA-VAN-0229
sp.UNA230	*V.* sp.	Costa Rica	UNA	ITS	UNA-VAN-0230
xtah0017	*V.* × *tahitensis*	French Polynesia	VATEL	ITS & *mat*K	CR0017
xtah0163	*V.* × *tahitensis*	French polynesia	VATEL	*mat*K	CR0163
xtah0164	*V.* × *tahitensis*	French Polynesia	VATEL	ITS & *mat*K	CR0164
xtahi-02	*V.* × *tahitensis*	French Polynesia	UNAM	ITS	Colin_sn

DNA extraction and sequencing

Total DNA was extracted from lyophilized leaf samples using the Qiagen DNA plant minikit (Dusseldorf, Germany) according to manufacturer protocols. Quantity and quality of the DNA were estimated using a Nanodrop spectrophotometer (Wilmington, USA), and DNA extracts were adjusted to 20 ng μl^{-1} for polymerase chain reaction (PCR) amplification.

The Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA and part of the plastid maturase K (*mat*K*)* gene were chosen for molecular characterization because they are among the most discriminant loci for orchids (Cameron 2009; Hollingsworth *et al.* 2009; Xu *et al.* 2015) and have distinct inheritances, biparental for ITS and maternal for *mat*K. The DNA samples were PCR amplified using GoTaq kit (Promega, USA) with the two primer pairs AB101/AB102 (Sun *et al.* 1994) for the ITS sequence, and *mat*K-743F (5’-CTTCTGGAGTCTTTCTTGAGC-3’) / *mat*K-1520R (5’-CGGATAATGTCCAAATACCAAATA-3’) for *mat*K. The 25 µl PCR reaction mixture contained: PCR reaction buffer, 50 nmol $MgCl_2$, 1 U *Taq* polymerase (GoTaq, Promega, USA), 5 nmol dNTPs, 10 nmol of each forward and reverse primer and 40 ng of genomic DNA. Amplification reactions were performed using a 96-well GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). The annealing temperatures for primers were 60°C for ITS and 56°C for *mat*K. Samples from Costa Rica were also amplified using the Multiplex PCR kit (Qiagen) with the same primers, and the PCR Mix and cycling conditions defined by the provider. Amplicons were sequenced in both directions as part of the

Bibliothèque du Vivant project (Paris, France), and by Genwiz (Takeley, UK) and Genoscreen (Lille, France). Nucleotide sequences were aligned using the ClustalW package included in Bioedit software (Hall 1999) and cleaned manually to generate consensus sequences. The data set was complemented with reference sequences obtained previously (Soto Arenas & Dressler 2010).

Identification and quantification of aromatic precursors in mature fruits

Hand-pollinated fruits of VanL (CR0068) and three *V. planifolia* accessions (CR0196, CR0040 and CR0038), cultivated under shade-house in La Réunion, were harvested at 8 months after pollination, in 2013 and 2014, then freeze-dried to minimize possible enzyme degradation. The fruits were ground to a fine powder with a mortar and pestle and stored at minus 20°C until extraction.

Extraction of volatile compounds was performed using a protocol adapted from Palama *et al*. (2009) and Pérez Silva *et al*. (2011). Fifty milligrams of the ground material was suspended in 10 ml of phosphate buffer (0.1 M; pH 5). The mixture was ultra-sonicated (frequency 35 kHz; AXTOR Model CD-4820) for 10 min at ambient temperature. The mixture was rapidly heated to 80°C for 10 min to inactivate endogenous enzymes and, after cooling at 25°C, was centrifuged at 5000 rpm for 3 min and then filtrated on a Whatman no.1 paper (Sigma-Aldrich, USA). The filtrate was complemented with 0.4 ml of a glycosidase rich enzyme preparation (AR2000®, Sigma–Aldrich, Mexico; 70 mg·ml^{-1} in phosphate buffer mentioned above) and adjusted to a volume of 10 ml with the pH 5 buffer solution. The extract was vortexed and incubated for 4 h at 40°C for enzymatic hydrolysis of the glycosylated precursors. One milliliter of hydrolyzed extract was filtered at 0.45 µm prior to HPLC analysis.

The samples were analyzed on an Agilent 1100 series equipped with a UV–VIS detector (G1314A), degasser (G1379A), column oven (G1316A), quaternary pump unit (G1311A) and autosampler (G1313A) controlled by OpenLAB CDS EZChrom Edition (Agilent Technologies, Inc. 2013). The column used was Zorbax Eclipse plus XDB C18 (150 mm long and 4.6 mm in diameter; 5 µm, Agilent Technologies, Mexico) and the mobile phase was a mixture of two solvents: A (0.1 M KH_2PO_4, pH 3.2) and B (MeOH, HPLC grade). Elution was achieved at 30°C with a gradient of 3−7% B for 2 min (0.8 ml·min^{-1}), 7% B for 10 min (0.8 ml·min^{-1}), 7% B for 11 min (1.5 ml·min^{-1}), followed by isocratic elution in 19% B for 25 min (1.5 ml·min^{-1}) and 19% B for 40 min (2.0 ml·min^{-1}). The compounds were monitored at 230 nm, and the injection volume was 10 µl. The compounds were quantified using the external standard technique. Solutions at concentrations ranging from 10 to 100 mg·ml^{-1} in the mobile phase were injected into the HPLC system to build the calibration curve.

All chemicals used were of analytical grade. The standard compounds for HPLC analyses, i.e., vanillin, vanillic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol, vanillyl alcohol, anisyl alcohol and anisic acid, were from Sigma–Aldrich (Saint Quentin Fallavier, France). Anisaldehyde was obtained from Chromadex (Irvine, CA, USA) and methanol (HPLC-grade) from J.T. Baker® (Saint Quentin Fallavier, France). The water used was Milli-Q-purified.

For each sample, extraction and HPLC were run in duplicate.

Data analysis

All statistical analyses were performed with the R statistical software (R Core Team 2013). Multiple comparisons of means of morphological and aromatic traits were performed using the Multcomp package in R (Hothorn *et al*. 2008).

Phylogenetic trees were inferred using the maximum likelihood method implemented in MEGA7 (Kumar *et al.* 2016) after determining the best substitution model using the online version of JModelTest2 (Guindon *et al.* 2003; Darriba *et al.* 2012). Branch robustness was assessed by bootstrapping 1000 datasets and branches with less than 65% bootstrap support were collapsed.

Results

Field observations

A total of 131 vanilla plants were collected in the survey carried out throughout Costa Rica in the time period 2013−2016. Among them, 17 were tentatively classified as *V. planifolia* (12) or VanL (5) according to the morphology of the leaves, stem or flower. The geographic origin and code numbers of these plants are provided in Fig. 1 and Table 2.

Morphologically, the VanL plants showed most similarity with *V. planifolia* and *V. ×tahitensis*, a species having a hybrid origin involving *V. planifolia* and *V. odorata* (Lubinsky *et al*. 2008). However, compared to these two species VanL plants had much smaller leaves, flowers and fruits (Fig. 2) and a distinct shape of leaves; elliptic-obovate in the case of VanL, oblong for *V. planifolia* and lanceolate for *V.* × *tahitensis*. On the other hand, VanL plants differed markedly from the 12 other species of the *V. planifolia* group (namely *V. appendiculata* Rolfe (Rolfe 1895), *V. bahiana* Hoehne (Hoehne 1950), *V. cristagalli* Hoehne (Hoehne 1944), *V. dubia* Hoehne (Hoehne 1944), *V. dungsii* Pabst (Pabst 1975), *V. fimbriata* Rolfe (Rolfe 1899), *V. helleri, V. insignis* Ames (Ames 1934), *V. odorata*, *V. phaeantha* Rchb.f. (Reichenbach

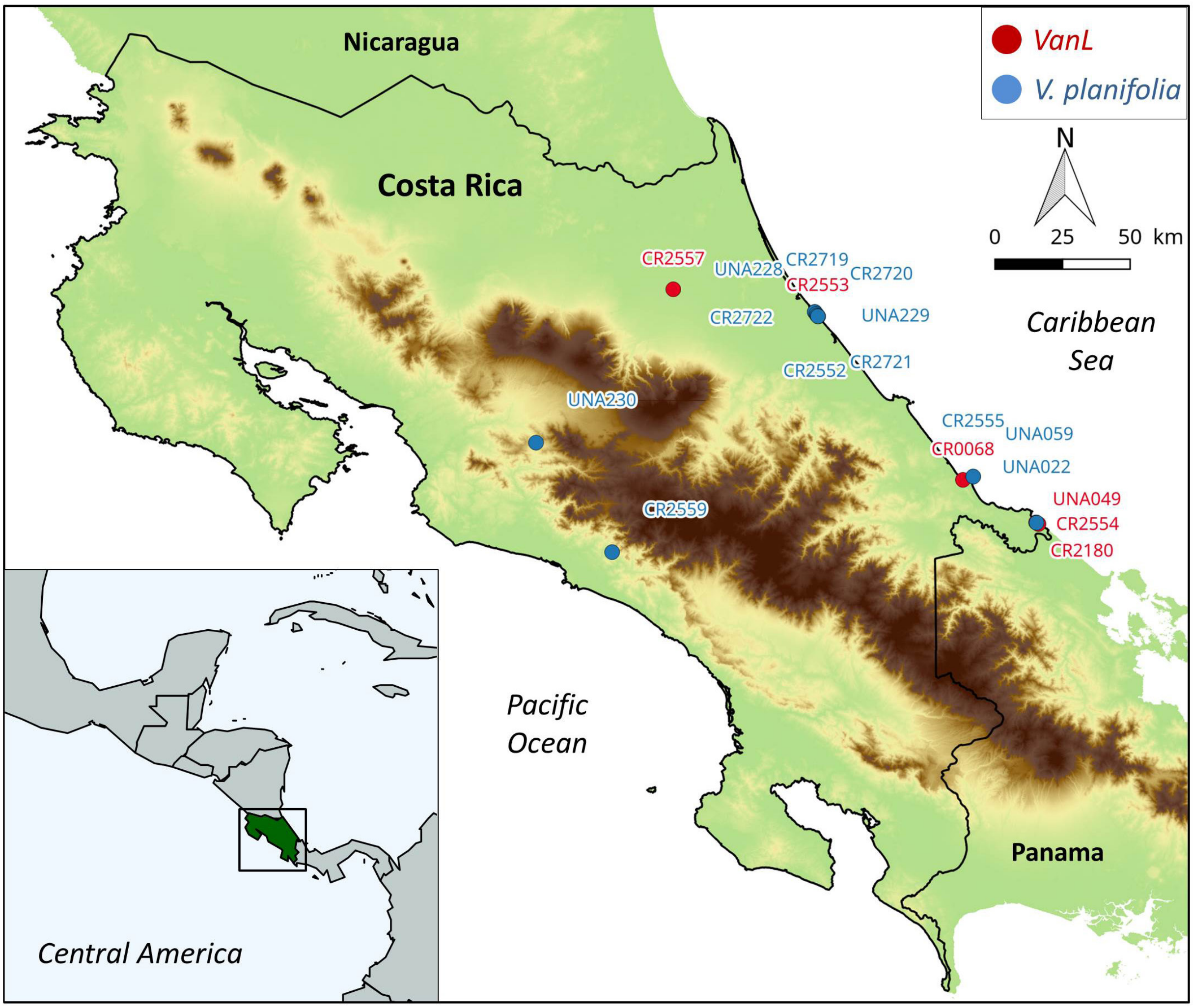

Fig. 1. Localization of *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. (VanL) and *V. planifolia* Jacks. ex Andrews samples collected in Costa Rica.

Table 2. Geographic origin of the samples collected in Costa Rica.

Collection date	Municipality (Province)	Site	Latitude (°N)	Longitude (°W)	Elevation (m)	Collectors	*Ex situ* conservation	Code in collection	DNA code	Genotype	Status
21 Jan. 1993	Cahuita (Limón)	–	–	–	–	*B. Gosselin*	MNHN	CH588	CR0068	VanL	?
8 Feb. 2013	Siquirres (Limón)	Barra de Parismina	10.294	83.338	14	*B. Azofeifa, JA Garcia, A. Paniagua*	UNA	UNA-VAN-00126	CR2552	*planifolia*	wild
8 Feb. 2013	Siquirres (Limón)	Barra de Parismina	10.281	83.329	18	*B. Azofeifa, JA Garcia, A. Paniagua*	UNA	UNA-VAN-00002	CR2553	VanL	wild
14 Nov. 2013	Talamanca (Limón)	Refugio Nacional Mixto de Vida Silvestre Gandoca-Manzanillo	9.594	82.602	14	*M. Grisoni, B. Azofeifa*	BRC VATEL	CR2180	CR2180	VanL	wild
14 Nov. 2013	Talamanca (Limón)	Refugio Nacional Mixto de Vida Silvestre Gandoca-Manzanillo	9.594	82.601	17	*B. Azofeifa, JA Garcia*	UNA	UNA-VAN-00022	FSC-22	*planifolia*	wild
14 Nov. 2013	Talamanca (Limón)	Refugio Nacional Mixto de Vida Silvestre Gandoca-Manzanillo	9.595	82.603	16	*B. Azofeifa, JA Garcia*	UNA	UNA-VAN-00047	CR2554	VanL	wild
14 Nov. 2013	Talamanca (Limón)	Refugio Nacional Mixto de Vida Silvestre Gandoca-Manzanillo	9.5895	82.595	19	*B. Azofeifa, JA Garcia*	UNA	UNA-VAN-00049	FSC-08	VanL	wild
10 Sep. 2014	Talamanca (Limón)	Parque Nacional Cahuita-Puerto Vargas	9.747	82.812	27	*B. Azofeifa, JA Garcia*	UNA	UNA-VAN-00059	FSC-07	*planifolia*	wild
10 Sep. 2014	Talamanca (Limón)	Parque Nacional Cahuita-Puerto Vargas	9.747	82.812	27	*B. Azofeifa, J.A. Garcia*	UNA	UNA-VAN-00198	CR2555	*planifolia*	wild
11 Nov. 2014	San Isidro (Dota)	–	9.496	84.012	847	*A. Barquero, B. Azofeifa*	Finca Santiago Parra	–	CR2559	*planifolia*	cultivated
11 Nov. 2014	Guápiles (Limón)	–	10.353	83.804	79	*A. Barquero, B. Azofeifa*	UNA	UNA-VAN-00216	CR2557	VanL	cultivated
18 Nov. 2014	Siquirres (Limón)	Barra de Parismina	10.295	83.338	15	*B. Azofeifa, JA Garcia*	UNA	UNA-VAN-00228	FSC-01	*planifolia*	wild
No data	Mora (San José)	Guayabo, Colón	9.859	84.265	962	*A. Paniagua, JA Garcia*	UNA	UNA-VAN-00230	FSC-02	*planifolia*	cultivated
23 May 2015	Siquirres (Limón)	Barra de Parismina	10.287	83.333	6	*B. Azofeifa*	UNA	UNA-VAN-00229	FSC-06	*planifolia*	wild
12 Mar. 2016	Siquirres (Limón)	Barra de Parismina	10.279	83.327	6	*M. Grisoni, B. Azofeifa, A. Paniagua, M. Vieira Nascimiento*	BRC VATEL	CR2719	CR2719	*planifolia*	wild
12 Mar. 2016	Siquirres (Limón)	Barra de Parismina	10.279	83.327	8	*M. Grisoni, B. Azofeifa, A. Paniagua, M. Vieira Nascimiento*	BRC VATEL	CR2720	CR2720	*planifolia*	wild
12 Mar. 2016	Siquirres (Limón)	Barra de Parismina	10.279	83.327	8	*M. Grisoni, B. Azofeifa, A. Paniagua, M. Vieira Nascimiento*	BRC VATEL	CR2721	CR2721	*planifolia*	wild
12 Mar. 2016	Siquirres (Limón)	Barra de Parismina	10.279	83.327	8	*M. Grisoni, B. Azofeifa, A. Paniagua, M. Vieira Nascimiento*	BRC VATEL	CR2722	CR2722	*planifolia*	wild

1865), *V. ribeiroi* Hoehne (Hoehne 1910) and *V. schwackeana* Hoehne (Hoehne 1944)) by color of sepal, color of petal, size and shape of tube, label ornamentation, and shape, thickness and texture of leaves.

Five out of the six VanL samples, CR0068, CR2180, CR2553, CR2554 and UNA-049, were collected in natural forests at three localities of the Caribbean province of Limón (Costa Rica), indicated in Table 2. The sixth VanL accession (CR2557) was cultivated in a farm with no information on its origin. During the survey, ten *V. planifolia* plants were found growing wild at two localities of the Limón province. Five of these accessions were collected in Barra Parismina, a human settlement established about 50 years ago that is still not connected by road to the rest of the country and where, according to one of the first settlers, vanilla was present prior to human occupation and no vanilla was ever introduced by man (Gabriel Taylor, Parismina, Costa Rica, pers. comm.). They are therefore considered to be occurring

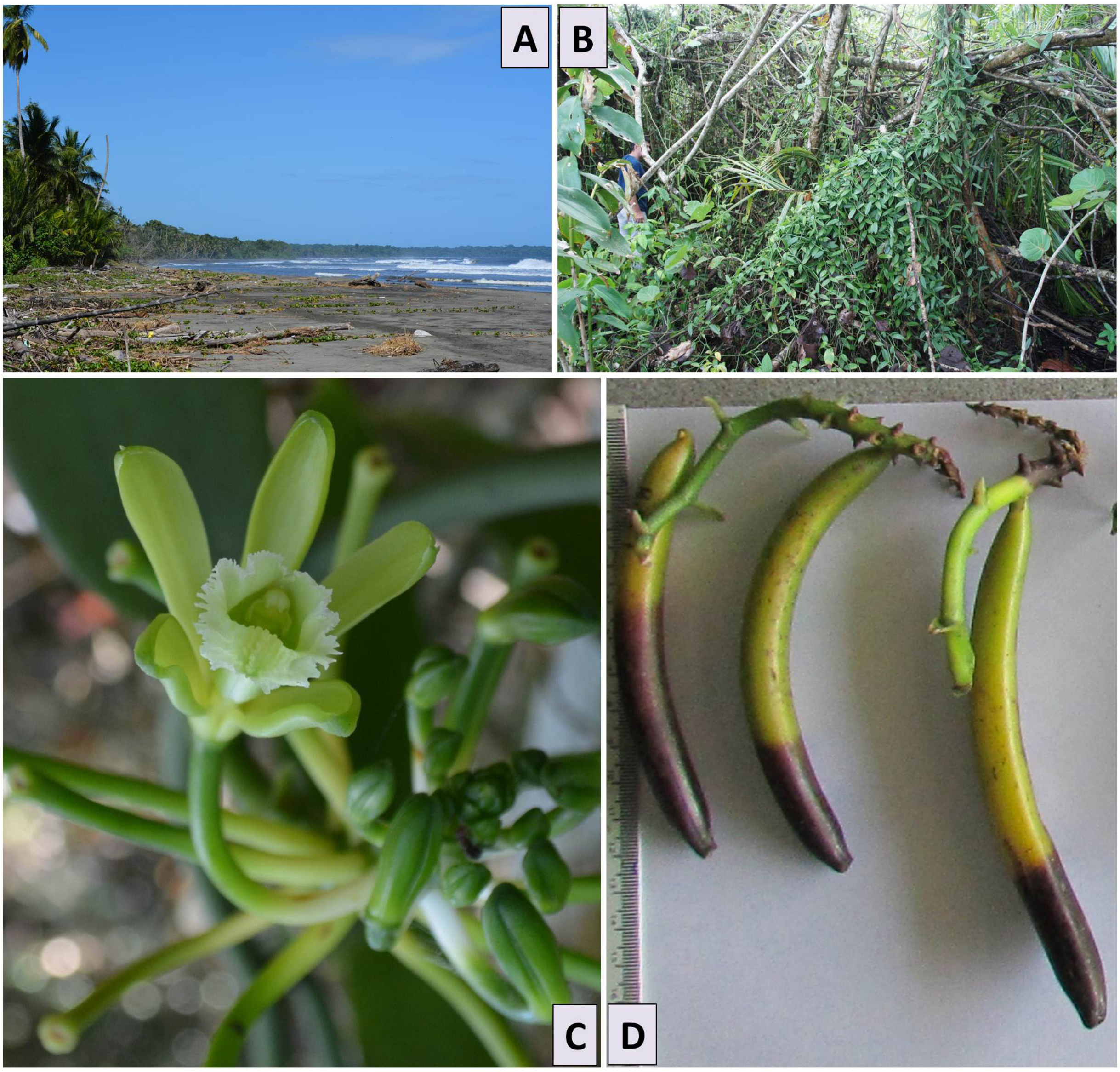

Fig. 2. Natural biotope of *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. (VanL) at Refugio Nacional Mixto de Vida Silvestre, Gandonca Manzanillo, Costa Rica. **A**. View of the littoral region of Limón Province harboring VanL populations. **B**. Important development of VanL in the humid littoral forests of Limón. **C**. Flower of VanL. **D**. Naturally pollinated fruits of VanL at maturity.

naturally in this area and not having escaped from local cultivation. They were morphologically indistinguishable from cultivated *V. planifolia* collected at farms in San Isidro (CR2559) and Mora (UNA-0230) or the three *V. planifolia* from Talamanca (UNA-0022, UNA-0059, CR2555).

To date, the wild populations of VanL and *V. planifolia* were both sampled in tropical humid forests of the southern coastal lowlands of Limón Province (Fig. 3). This biotope is characterized by plant species adapted to sandy soils such as *Terminalia catappa* L. (Combretaceae), *Coccoloba uvifera* (L.) L. (Polygonaceae), *Cocos nucifera* L. (Arecaceae), *Costus spicatus* (Jacq.) Sw. (Costaceae), *Hibiscus pernambucensis* Arruda (Malvaceae), *Acrostichum aureum* L. (Pteridaceae), *Chrysobalanus icaco* L. (Chrysobalanaceae), *Amphitecna latifolia* (Mill.) A.H.Gentry (Bignoniaceae), *Rhizophora mangle* L. (Rhizophoraceae) and *Pterocarpus officinalis* Jacq. (Fabaceae).

The VanL plant from Guápiles (CR2557) was sampled in a vanilla plot where vanilla was associated with pepper and cinnamon.

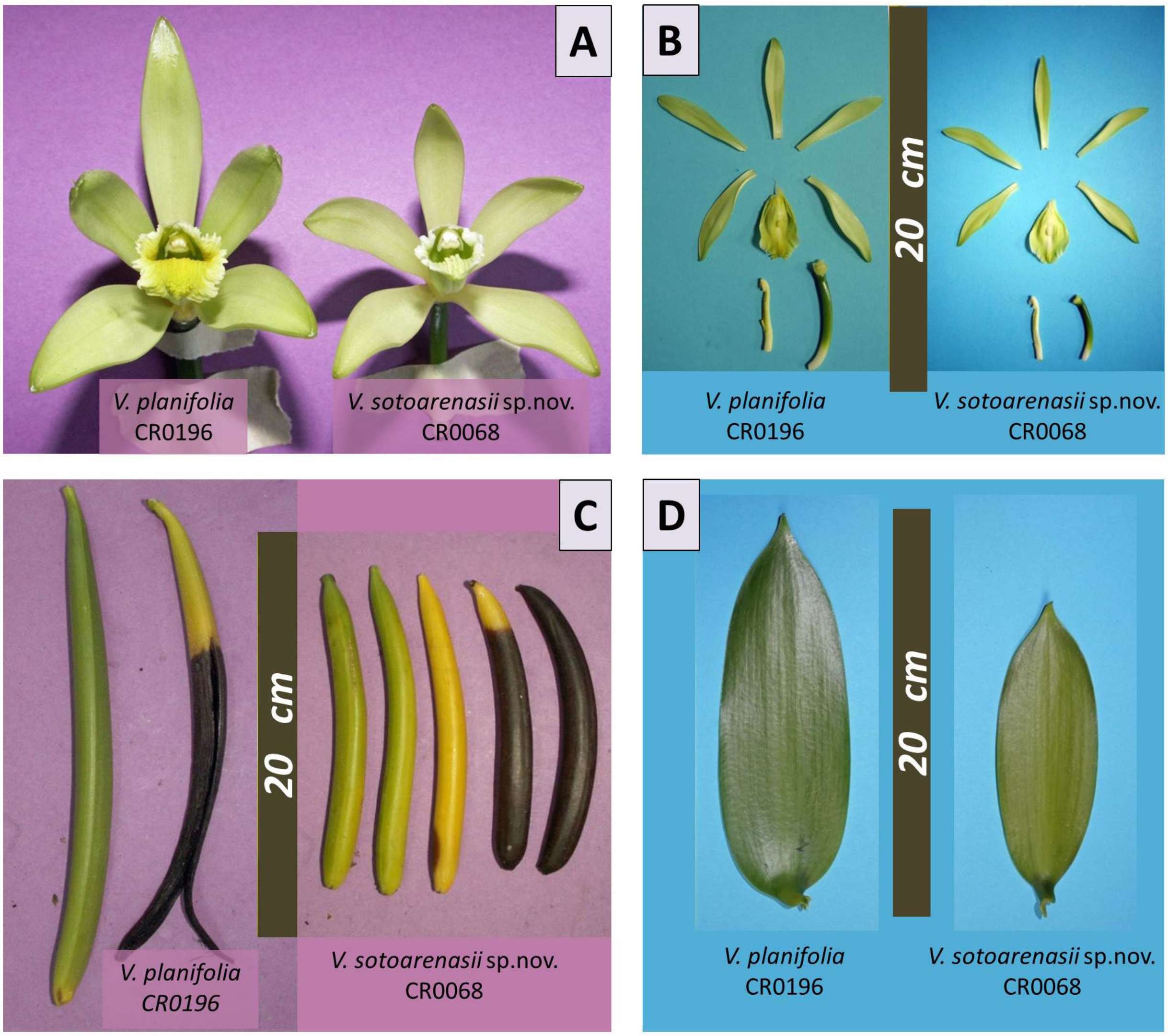

Fig. 3. Comparison of morphological traits between *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. (accession CR0068) and *V. planifolia* Jacks. ex Andrews (CR0196) cultivated under shade house in La Réunion. **A**. Front view of entire flowers. **B**. Separated flower parts. **C**. Mature fruits. **D**. Leaves.

In Gandoca-Manzanillo, Parismina and Cahuita the flowering happened from October to January, as deduced from the many racemes found in mid-November bearing flowers and young fruits. Mature vanilla beans were harvested in December 2015 from the VanL population of Guápiles (CR2557), which indicated a flowering time probably ending in March.

Phylogenetic analysis

To elucidate the taxonomic position of VanL plants, the nuclear ITS DNA and the *mat*K plastid gene were sequenced for 125 and 55 vanilla accessions, respectively. The sample set included VanL specimens, accession representatives of the diversity of the *V. planifolia* group, as well as outgroup species (Table 1).

The phylogenetic tree inferred from the 506 positions of the 125 aligned ITS sequences (465 to 500 nt) revealed a clade separated with 93% bootstrap support, containing the five VanL accessions (CR2180, CR2553, CR2554, CR2557 and UNA049) along with CR0068 from BRC Vatel and two accessions from the AMO, collected in Costa Rica and Honduras respectively, identified as *V. planifolia* cf. plan-02 and plan-03 (Fig. 4). This clade was within the *V. planifolia* accessions and close to the *V. bahiana* / *V. phaeantha* clade. It was more distantly related to the *V. insignis* and the *V. helleri* – *V. odorata* – *V.* × *tahitensis* clades.

The eight partial ITS sequences in the VanL clade differed from all the accessions of the *V. planifolia* clade by two conserved nucleotides at positions 390 and 479 of the alignment (Table 3). Similarly, *V. bahiana* differed from *V. phaeantha* by only two conserved nucleotides (nt 172 and 420). In contrast, the *V. planifolia* clade differed from the *V. bahiana* and *V. phaeantha* group by 13 conserved nucleotides, and from *V. insignis* by 15 conserved nucleotides.

The phylogenetic tree inferred from the 741 positions of the 55 aligned *mat*K sequences (699 to 734 nt) separated *V. planifolia* with high bootstrap support from its most closely related species, including *V. odorata, V. insignis, V. bahiana* and *V. phaeantha* (Fig. 5). The eight VanL accessions sequenced fell within the *V. planifolia* clade, confirming their very close relationship with this species.

Phylogenetic trees inferred using the Neighbor Joining, Parsimony and Bayesian methods were congruent with the ML trees (data not shown).

Morphology of vegetative and reproductive organs of VanL

The morphology of the leaf, stem, and flower of VanL is very similar to that of *V. planifolia* (Fig. 2). However, the former differs from the latter by a highly significant reduction in size of the vegetative and reproductive organs (Table 4). In average, leaf and stem dimensions are 29 to 41% smaller in VanL compared to *V. planifolia*.

The size reduction in VanL is less important in the flower, with tube length only 7% and sepal length 12% lesser than for *V. planifolia*. However, in VanL, the dorsal sepal is 29% narrower and the ovary 19% shorter than in *V. planifolia*. The blooming period of VanL cultivated in La Réunion was approximately two weeks earlier than that of *V. planifolia* accessions, but they overlapped from October to November.

Mature fruits of VanL were 45% shorter and 55% lighter than those of *V. planifolia* (Fig. 2; Table 4). They were also indehiscent and more cylindrical, contrary to most *V. planifolia* fruits, which dehisce at maturity and have a more triangular section.

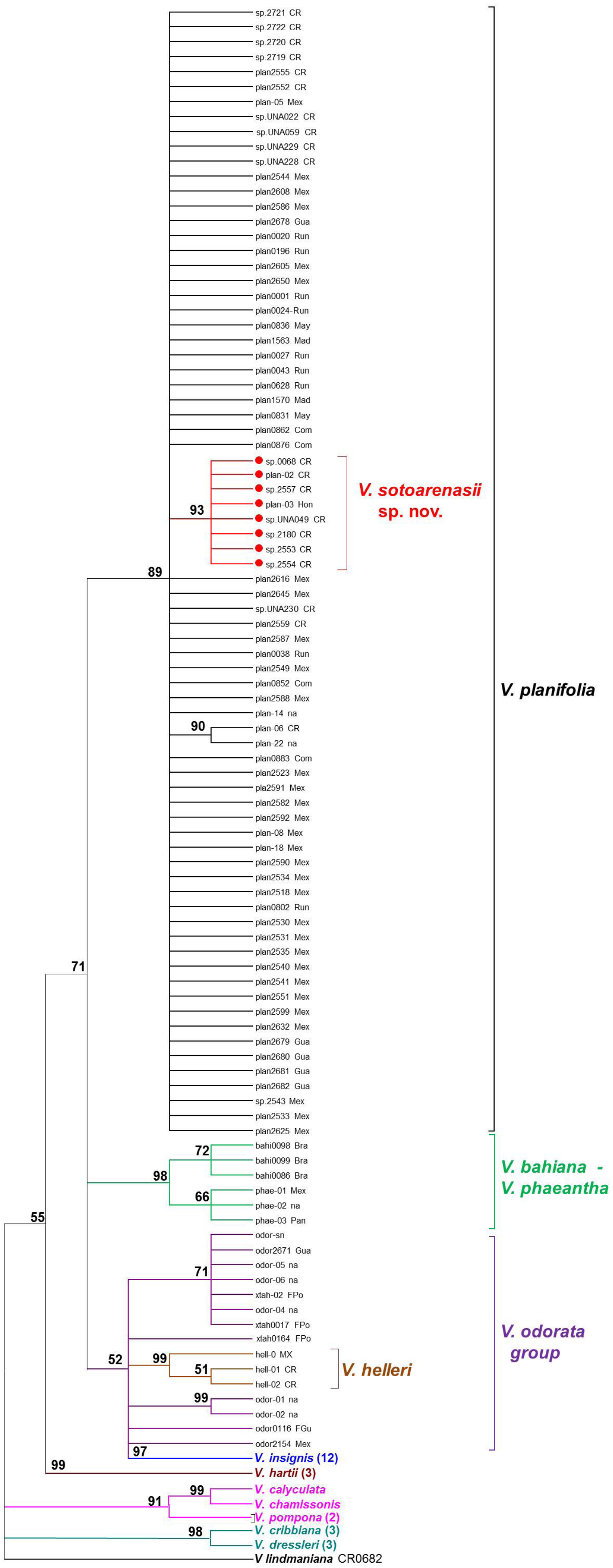

Fig. 4. Phylogenetic tree derived from the partial ITS sequences (506 positions) of the 125 accessions listed in Table 1, showing the differentiation of the *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. clade from *V. planifolia* Jacks. ex Andrews and all other related species. The tree was inferred using the Maximum Likelihood method based on the Tamura-Nei model with invariant sites and Gamma distribution of evolutionary rates. The figures indicate the percentage of bootstrap support. Branches with less than 65% support were collapsed. Countries of origin: Bra = Brazil; Com = Comoros: CR = Costa Rica; FGu = French Guiana; FPo = French Polynesia; Gua = Guatemala; Hon = Honduras; Mad = Madagascar; May = Mayotte; Mex = Mexico; Run = La Réunion; na = geographic origin not available. Numbers in brackets indicate the number of similar accessions merged in one branch for outgroup species.

Table 3. Inter-clade polymorphisms of within-clade-conserved nucleotides along the partial ITS sequence of *Vanilla planifolia* Jacks. ex Andrews, *V. sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. and their most closely related species. The triangular matrix on the right indicates the number of polymorphic sites between species. Nucleotides diverging from the *V. planifolia* sequence are in bold. Y = C or T, K = G or T.

Position	*12*	*25*	*33*	*34*	*54*	*58*	*80*	*93*	*112*	*127*	*172*	*178*	*188*	*192*	*204*	*390*	*399*	*420*	*422*	*423*	*424*	*444*	*473*	*479*	*480*	*493*	*494*	***V. planifolia***	***V. sotoarenasii***	***V. bahiana***	***V. phaeantha***	***V. insignis***
V. planifolia	C	T	G	C	T	T	Y	A	-	A	T	A	C	Y	C	C	C	T	C	G	G	C	G	A	Y	C	K	■				
V. sotoarenasii	C	T	G	C	T	T	T	A	-	A	T	A	C	C	C	**G**	C	T	C	G	G	C	G	**G**	C	C	T	2	■			
V. bahiana	C	T	**A**	**T**	**C**	**C**	**C**	**C**	**T**	**T**	**C**	A	C	T	C	C	**T**	T	**T**	G	G	**T**	**A**	A	T	**T**	G	13	19	■		
V. phaeantha	C	T	**A**	**T**	**C**	**C**	**C**	**C**	**T**	**T**	T	A	C	T	C	C	**T**	**C**	**T**	G	G	**T**	**A**	A	T	**T**	G	13	18	2	■	
V. insignis	**T**	**C**	G	C	T	C	T	A	-	A	T	**G**	**T**	T	**A**	C	**T**	Y	**T**	**T**	**C**	C	**A**	A	T	**T**	G	11	17	16	15	■

Table 4. Means of different measurements on vegetative and reproductive organs of two accessions of *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. (CR0068 and CR2180) and two accessions of *V. planifolia* Jacks. ex Andrews (CR0040 and CR0196). Measurements are in mm unless otherwise indicated. In each line, means with different letters are significantly different (p < 0.005, Tukey's test). StE = standard error.

	***V. sotoarenasii* sp. nov.**						***V. planifolia***					
	CR0068			**CR2180**			**CR0040**			**CR0196**		
Characters	**mean**	**StE**		**mean**	**StE**		**mean**	**StE**		**mean**	**StE**	
length of leaf	76.0	2.28	*a*	105.8	3.85	*b*	149.4	2.58	*c*	146.5	3.65	*c*
width of leaf	32.7	0.58	*a*	27.4	1.29	*a*	50.0	0.96	*b*	47.9	1.08	*b*
diameter of stem	5.8	0.09	*a*	6.1	0.12	*a*	10.3	0.25	*b*	9.9	0.26	*b*
length of internode	71.4	3.00	*a*	91.0	3.03	*a*	117.7	3.65	*b*	122.2	4.66	*b*
Number of items	30			14			24			30		
length of sepal sup	50.3	0.45	*a*	50.6	0.64	*a*	57.9	0.43	*b*	56.8	0.53	*b*
width of sepal sup	9.5	0.22	*a*	8.9	0.33	*a*	13.5	0.29	*b*	12.6	0.33	*b*
length of tube	42.6	0.32	*a*	43.2	0.67	*a*	47.0	0.40	*b*	45.7	0.48	*b*
width of tube	10.8	0.12	*a*	11.3	0.18	*a*	13.6	0.19	*b*	13.0	0.22	*b*
length of ovary	37.8	0.53	*a*	45.9	0.93	*b*	53.1	0.61	*c*	49.9	0.72	*c*
Number of items	30			19			22			30		
weight of mature fruit (g)	6.6	0.17	*a*	–	–		18.1	0.84	*b*	18.3	0.65	*b*
length of mature fruit	114	2.20	*a*	–	–		201	3.66	*b*	203	2.26	*b*
Number of items	16			–			17			22		

Aromatic content of mature fruits

Strongly contrasted aromatic profiles were observed between VanL and *V. planifolia* by comparing the contents of nine volatile precursors detected in mature fruits by HPLC (Table 5). The fruits of VanL had a much lower content of vanillyl compounds, particularly vanillin which did not exceed 0.26% of dry matter, while it was over 2.28% in *V. planifolia* beans. Conversely, VanL fruits had significant amounts of anisyl compounds which were not detected by HPLC in *V. planifolia* fruits, and *p*-hydroxybenzyl (PHB) alcohol was present at a much higher titer in VanL compared to *V. planifolia.* The contents of the two other *p*-hydroxyl compounds were not significantly different between VanL and *V. planifolia*.

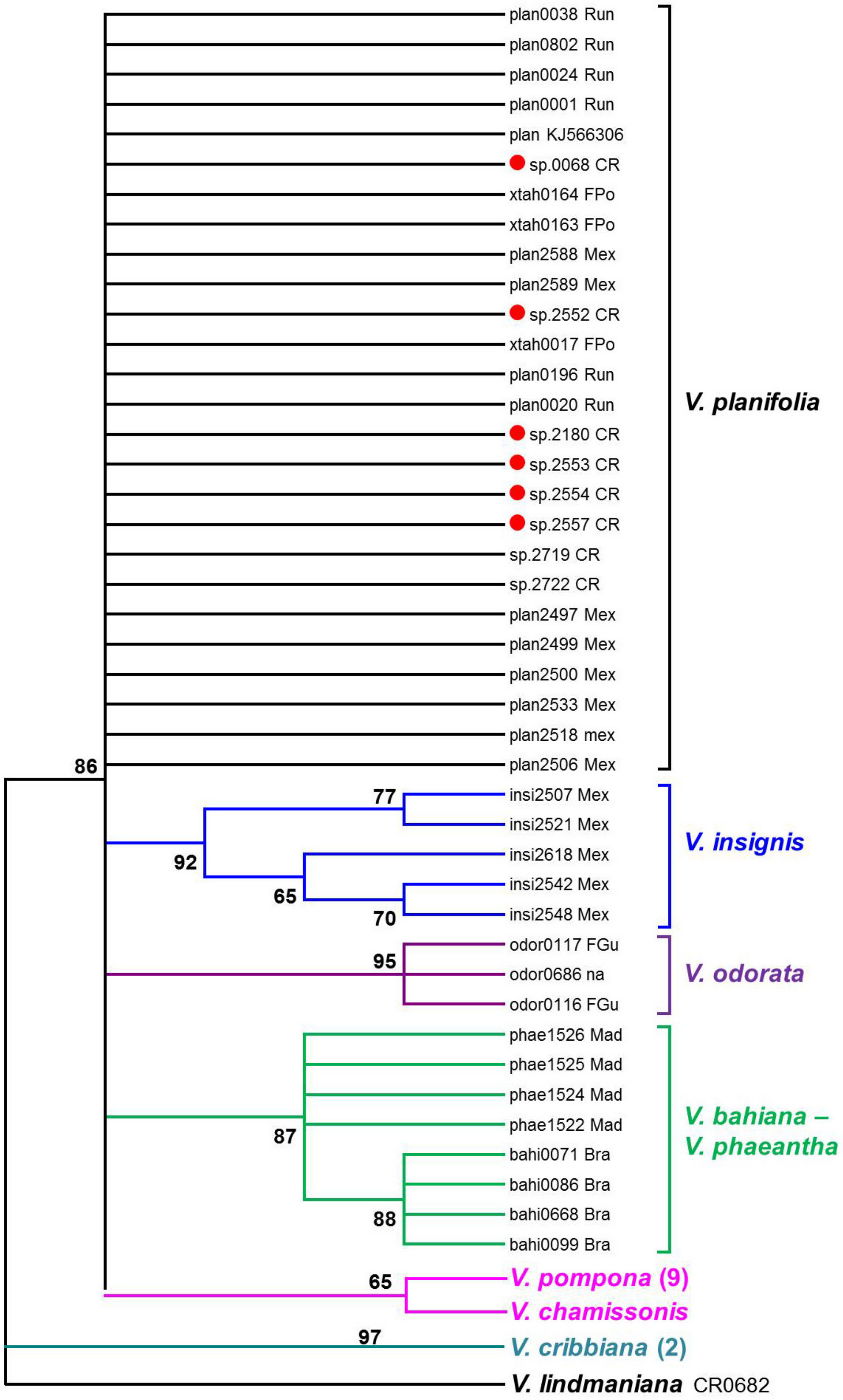

Fig. 5. Phylogenetic tree derived from the partial *mat*K sequences (725 positions) of the 55 accessions listed in Table 1, showing the *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. accessions (red dots) within the *V. planifolia* clade but distinct from other related species. The tree was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model and Gamma distribution of evolutionary rates. The figures indicate the percentage of bootstrap support. Countries of origin: Bra = Brazil; CR = Costa Rica; FGu = French Guiana; FPo = French Polynesia; Mad = Madagascar; Mex = Mexico; Run = La Réunion; na = geographic origin not available. Numbers in brackets indicate the number of similar accessions merged in one branch for outgroup species.

As a whole, the VanL fruit had a significantly lower (about 30%) level of total aromatic content (all nine molecules) and a very distinct aromatic profile compared to *V. planifolia*. Indeed, the volatiles of *V. planifolia* fruits were strongly dominated by vanillyl compounds (83.1% of all volatiles, particularly vanillin which represented 68%). By contrast, the VanL fruits contained a more equilibrated profile with a slight dominance of vanillyl compounds (41.9% of all volatiles, in which vanillyl-alcohol represented 31%), *p*-hydroxybenzyl and anisyl compounds (35.9% and 22.2%, respectively).

The above results indicate than VanL and *V. planifolia* are very closely related but significantly distinct in morphological and biochemical traits, as well as in nuclear nucleotide sequences. We therefore propose to place VanL in a specific taxon distinct from *V. planifolia*, and to name it *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov.

Taxonomy

Class Equisetopsida C.Agardh (Agardh *et al.* 1825)
Subclass Magnoliidae Novák ex Takht. (Takhtajan 1967)
Superorder Lilianae Takht. (Takhtajan 1967)
Order: Asparagales Link (Link 1829)
Family Orchidaceae Juss. (de Jussieu 1789)
Subfamily Vanilloideae (Lindl.) Szlach. (Szlachetko 1995)
Tribe Vanilleae (Blume 1835)

Genus ***Vanilla*** Plum. ex Mill. (Miller 1754)
Subgenus ***Xanata*** Soto Arenas & Cribb (Soto Arenas & Cribb 2010)
Section ***Xanata*** Soto Arenas & Cribb (Soto Arenas & Cribb 2010)

Vanilla sotoarenasii M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov.
urn:lsid:ipni.org:names:77160155-1
Figs 2, 3, 6

Diagnosis

A Vanilla planifolia similis, sed folia caulesque breviores (folium: 7.6–10.6 × 2.7–3.3 cm versus 14.7–14.9 × 4.8–5 cm), laminae breviores, ellipticae-oblongaeque, flores albiores, tubus floris, sepala petalaque breviores, sepalum dorsale angustius, labellum angustiius cum papillis salientioribus, ovarium brevitius (3.8–4.6 cm versus 5–5.3 cm), fructus brevitior (11.4 cm versus 20.1–20.3 cm) indehiscensque, sectio fructi cylindrica (versus trigona). Moleculae aromaticae fructi absimilis.

Etymology

This species is dedicated to Dr. Miguel Angel Soto Arenas (1963–2009), authority in orchid floristics and ecology, particularly in the *Vanilla* genus.

Type material

Holotype

COSTA RICA: Cahuita (ex hort. parc E. Liais, from plants collected by B. Gosselin in 1993), 8 Oct. 1996, *Pignal 396 b* (holo-: P: P00075132).

Paratypes

COSTA RICA: Limón Province: Talamanca, Refugio Nacional Mixto de Vida Silvestre Gandoca-Manzanillo, 9.594° N, 82.602° W, altitude 2 m, 14 Nov. 2013 (originally collected), cultivated in BRC Vatel, Saint Pierre, La Réunion, France CRV2180, 28 Feb. 2016, *M. Grisoni & J.B. Azofeifa-Bolaños*

Table 5. Means of volatile compounds quantified by HPLC in mature beans of *V. sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. (CR0068; 3 samples) and *V. planifolia* Jacks. ex Andrews (CR0196, CR0038, CR0040; 9 samples). Contents are expressed as grams of compound per 100 g dry matter. nd = compound not detected. Averages with different letters within rows are significantly different ($p < 0.05$).

	***V. sotoarenasii* sp. nov.**		***V. planifolia***	
Compounds	**g / 100g dw**	**%**	**g / 100g dw**	**%**
Vanillin	0.26 a	10.9	2.28 b	68.0
Vanillic acid	nd a	0.0	0.19 b	5.5
Vanillyl alcohol	0.73 a	31.0	0.32 b	9.6
Total vanillyl compounds	0.99 a	41.9	2.79 b	83.1
p-hydroxybenzaldehyde	0.20 a	8.5	0.18 a	5.4
p-hydroxybenzoic acid	0.37 a	15.8	0.37 a	11.2
p-hydroxybenzyl alcohol	0.27 a	11.6	0.01 b	0.3
Total *p*-hydroxybenzyl compounds	0.85 a	35.9	0.56 b	16.9
Anisyl alcohol	0.24 a	10.2	nd b	0.0
Anisaldehyde	0.03 b	1.4	nd b	0.0
Anisic acid	0.25 a	10.6	nd b	0.0
Total anisyl compounds	0.52 a	22.2	nd b	0.0
Total	**2.36 a**	**100**	**3.35 b**	**100**

JJMM01 (REU: REU13363); Canton of Talamanca, Refugio de Vida Silvestre Gandoca-Manzanillo (originally collected), cultivated in INISEFOR, Heredia, Costa Rica, 14 Nov. 2013, *M. Grisoni, B. Azofeifa and J. García 2180* (CR 281507), cultivated at the same locality, *B. Azofeifa and J. García 0047* (CR 281508); Canton of Siquirres, Barra de Parismina (originally collected), cultivated in INISEFOR, Heredia, Costa Rica, 8 Feb. 2013, *B. Azofeifa, J. García and A. Paniagua 0002* (CR 281509).

Description

Hemiepiphytic vine up to 15 m high. Stems flexuous, terete, smooth, green, 5−6 mm thick; internodes, sometimes slightly curved apically, ca 10 cm long. Terrestrial roots pubescent, ramified, ca 2 mm thick; both attaching and free aerial roots terete, pubescent, ca 2 mm thick at base, ca 3.5 mm at middle. Leaves regularly alternate. Blade elliptic to obovate, slightly fleshy (ca 27 veins visible on dry specimens); base rounded to cuneate, shortly pseudopetiolate; apex shortly acuminate (acumen sharp, 12 mm long, 5 mm at base), slightly recurved; margin thinned; pseudopetiole canaliculate, 16 × 5 mm. Inflorescence: raceme, ca 10−20-flowered, 7−13 cm, sometimes located on short axillary branches, sometimes lying on leafy stems. 3−4 inflorescence bracts, foliaceous, basal bract pseudopetiolate (40 × 20 mm), upper bract sessile and shorter (20 × 8 mm and 12 × 3 mm). Flowers at anthesis successively, ephemeral, 1−2 simultaneously, white green, sepals forming an angle of approximately 45° with axis of column, petals more or less parallel to this axis. Ovary terete, smooth, slightly arcuate, white at base and green on upper ⅔, 30 mm long and 4 mm in diameter. Parts of perianth with whitish inclusions (visible on dry specimens), longitudinally oriented, ca 0.1−2 mm long, more numerous on petals, dorsal sepal narrow elliptic to oblanceolate, ca 11-veined, 40 × 10 mm, apex acute, rounded, base attenuate-clawed, slightly concave, canaliculate. Lateral sepals, elliptic asymmetric, slightly falciform, ca 11-veined, 38 × 10.5 mm, apex acute, slightly cupuliform, base attenuate, canaliculate, 4 mm wide. Petals elliptic asymmetric, slightly falciform, ca 12-veined, carinate dorsally (carena 1.2 mm wide), 38 × 9 mm, apex rounded, base attenuate, 3 mm wide (Fig. 6). Labellum attached to column along margins of 5/7 (ca 20 mm), funnel-shaped, spread apically (opening about 10 mm), trilobed, ca 30-veined ramified in distal third, margin crenulate. With 5−6 fimbriated scales, ca 1.5 × 1.5 mm, at about middle of labellum. Lateral lobes, obliquely triangular, ca 11-veined, 10 mm high and 7 mm wide, margins widely undulate. Midlobe quadrate, 20 mm wide and 6 mm high, about 12-veined, converging at apex. Papillae on four central veins, on apical half. Column trigonous-semicylindrical, 28 mm long, 2.5 mm wide, apically with 2 lateral auricles, crenulate, 2.5 mm high and 4 mm wide. Rostellum quadrangular, ca 3 × 3 mm. Stigma bilobed. Anther articulated, connective keel-shape, 2 × 2 mm, bicarinate on top. Operculum helmet-shaped, 2 × 3 mm. Pollinarium, 2. Fruit arcuate, banana-shaped, green, turning yellowish and then brown, 11−16 × 1.5 cm, with cylindrical section.

Phenology

The flowering period occurs from October to March in Costa Rica.

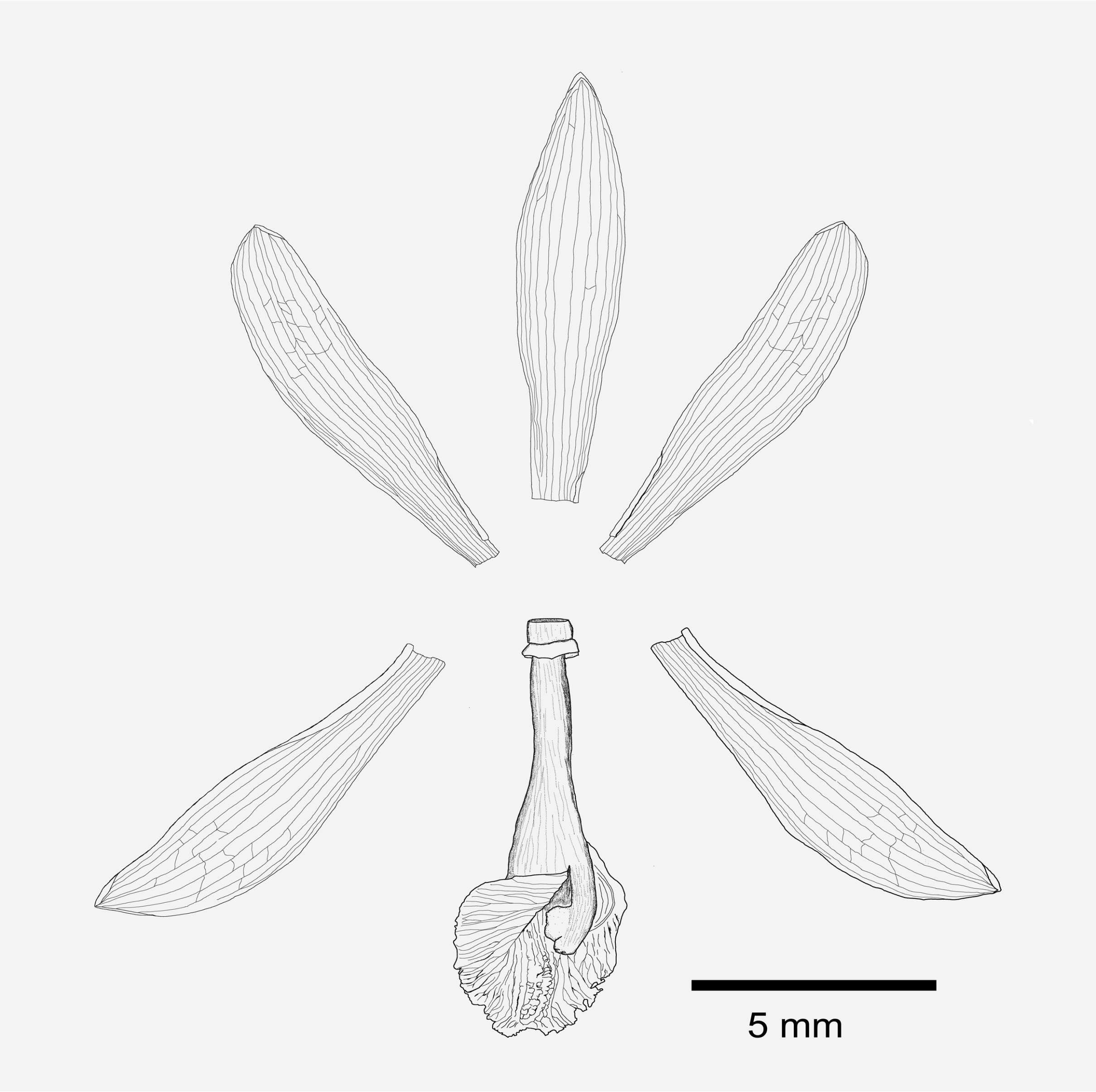

Fig. 6. Croquis drawing of a flower of *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov.

IUCN status –Vulnerable

The populations of *V. sotoarenasii* sp. nov. observed in the province of Limón showed strong vegetative development of vines and natural seed set was frequently observed. However, a small number of populations have so far been observed and only over a very limited area of Limón Province (10 populations over a coastal strip of about 50 km²), and its habitat is periodically submerged by the ocean. This makes us inclined to tentatively classify *V. sotoarenasii* sp. nov. as vulnerable D2 (IUCN 2012) until further data on distribution and population dynamics have been obtained.

Discussion

Using complementary approaches involving morphology of the reproductive and vegetative systems, molecular barcoding and the accumulation of secondary metabolites in fruits, we highlighted specific traits for the vanilla populations sampled on the Caribbean coast of Costa Rica which revealed a new taxon, *V. sotoarenasii* sp. nov. The morphology of the flowers and leaves clearly assigned *V. sotoarenasii* sp. nov. within the *V. planifolia* group (Soto Arenas & Dressler 2010). Based on flower morphology, *V. sotoarenasii* sp. nov. was more similar to *V. planifolia* and *V.* × *tahitensis* than to any other vanilla species of this group. However, *V. sotoarenasii* sp. nov. differs from the other two species by several characteristics (Table 6).

Firstly, our data and previous data by Costantin & Bois (1915) and Portères (1953) showed that *V. sotoarenasii* sp. nov. has a significantly smaller size for all organs measured (stem, leaf, flower and fruit) compared to *V. planifolia* and *V.* × *tahitensis*, and had a distinct shape of the leaves: elliptic to obovate in the case of *V. sotoarenasii* sp. nov., elliptic to oblong for *V. planifolia* and narrowly oblong to lanceolate for *V.* × *tahitensis* (Table 6). In addition, the flowers of *V. sotoarenasii* sp. nov. are more whitish, with a narrower label showing marked papillae, compared to those of *V. planifolia*, which are more greenish, with a wider label and smooth papillae.

On the basis of HPLC quantification of hydrolyzed volatile compounds in mature fruits, the aromatic precursors of *V. sotoarenasii* sp. nov. are very distinct from those of *V. planifolia*. In particular, fruits of *V. sotoarenasii* sp. nov. were characterized by less predominant vanillin content and the presence of anisyl compounds. They should develop, after over-maturation or curing, flavors extremely different from those of *V. planifolia* and more similar to those of *V. pompona* or *V.* × *tahitensis* (Pérez-Silva *et al.* 2006; Brunschwig *et al.* 2009; Maruenda *et al.* 2013).

On the other hand, molecular analysis of nuclear DNA sequences (ITS) unambiguously separated the *V. sotoarenasii* sp. nov. group of plants from the *V. planifolia* group including 68 accessions originating from seven countries (Comoros, Costa Rica, Guatemala, Madagascar, Mayotte, Mexico, and La Réunion). This result is corroborated by amplified fragment length polymorphism (AFLP) analyses by Bory *et al*. (2008) that clearly separated *V. sotoarenasii* sp. nov. CR0068 from 303 *V. planifolia* genotypes of diverse origins with an average distance comparable to the distance between *V. planifolia* and *V.* × *tahitensis.* The facts that i) at the plastid DNA level (partial *mat*K sequence) *V. sotoarenasii* sp. nov. and *V. planifolia* share the same clade, and ii) in the ITS phylogeny *V. sotoarenasii* sp. nov. is in an internal position within the *V. planifolia* clade (like *V. helleri* within the *V. odorata* cluster), suggest the recent radiation of *V. sotoarenasii* sp. nov. from *V. planifolia* populations.

So far, *V. sotoarenasii* sp. nov. has only been observed in the Limón Province of Costa Rica where it is sympatric with *V. planifolia*, which has been reported as native to Costa Rica (Soto Arenas & Dressler 2010). However, recent introductions in the country of *V. planifolia* cultivars and hybrids have also been documented (Soto Arenas & Dressler 2010; Belanger & Havkin-Frenkel 2011; Varela Quirós 2011). Historical, genetic and phylogenetic data are insufficient to decide whether *V. sotoarenasii* sp. nov. derived from natural or introduced populations of *V. planifolia*, nor how and when the radiation occurred.

Table 6. Characters differentiating *Vanilla sotoarenasii* M.Pignal, Azofeifa-Bolaños & Grisoni sp. nov. from related species.

	V. planifolia	***V. × tahitensis***	***V. sotoarenasii* sp. nov.**
Leaf shape	elliptic to oblong	narrowly oblong to lanceolate	elliptic to obovate
Leaf length	more than 14 cm long	more than 14 cm long	less than 12 cm long
Leaf length/width ratio	less than 4 times as long as wide	more than 4 times as long as wide	about 3 times as long as wide
Sepal size (length × width)	more than 55 × 10 mm	more than 55 × 10 mm	no more than 53 × 8 mm
Fruit section	triangular, generally dehiscent	triangular, generally indehiscent	rounded, indehiscent

Orchid species are often interfertile, which allows them to create interspecific and even intergeneric fertile hybrids. Many interspecific hybrids between species of *Vanilla* have been produced in the last decades by botanists and agronomists (Knudson 1950; Theis & Jiménez 1957; Divakaran *et al.* 2006). In nature, however, interspecific hybrids are prevented by reproductive barriers that result primarily from the inability of pollinators to transfer pollen from one species to another. Within the limits of our sampling, we have never observed intermediary types, at the genetic or morphological level, in the Limón area, which suggests that a reproductive barrier may exist between *V. planifolia* and *V. sotoarenasii* sp. nov. Indeed, in the case of sympatric populations of the two very closely related species *V. barbellata* Rchb.f. and *V. dilloniana* Correll, having synchronous flowering and the same pollinator in western Puerto Rico, intermediary types were observed, demonstrating natural interspecific hybridizations (Nielsen 2000). We hypothesize that the differences in flower morphology observed between *V. planifolia* and *V. sotoarenasii* sp. nov. impede gene flow from one species to another, which enhanced the radiation of the population in Limón. Vanilla pollination is bee-dependent (Ackerman 1986; Gigant *et al.* 2011) and flower size difference is likely to constitute a reproductive barrier between species by selecting compatible pollinators. This has been observed for instance in Peru where, due to their small size, the *Mellipona* bees did not remove pollen when visiting *V. grandiflora* Lindl. flowers, and therefore did not contribute to its pollination (Lubinsky *et al.* 2006). Given the significant size reduction of the flowers of *V. sotoarenasii* sp. nov. compared to those of *V. planifolia*, it is unlikely that a bee capable of pollinating one of the two species would be able to pollinate the other. In addition to size compatibility, the probability of pollinator visits is frequently increased by the aromatic metabolites emitted by orchid flowers, which act as bee attractants (Ackerman 1986; Pansarin & Pansarin 2014). This is particularly important for vanilla flowers that are open a single day, which reduces the chances of pollen-pollinator encounter. Analysis of the volatile compounds emitted by flowers from various *Vanilla* species (unpublished data) has shown in particular that in *V. planifolia* (n = 4) the major compounds are a hydrocarbon monoterpene, (E)-β-ocimene (representing 17.10 ± 14.08% of all detected compounds), and an aromatic compound, 2,6-bis (1,1-dimethylethyl)-4-(1-oxopropyl) phenol (11.59 ± 8, 04%), while in *V. sotoarenasii* sp. nov. (n = 1), only (E)-β-ocimene was predominantly found (63.26%). This difference in the nature of volatile emissions between the two species could also contribute to the specificity of the pollinators visiting the flowers. Furthermore, the earlier flowering of *V. sotoarenasii* sp. nov. compared to *V. planifolia* may also contribute to favor self or geitonogamous pollination as well as provide greater reproductive success to *V. sotoarenasii* sp. nov., as pointed out for non-rewarding orchids (Jersáková *et al.* 2006; Sun *et al.* 2009).

All these factors hindering gene flow between populations of *V. sotoarenasii* sp. nov. and *V. planifolia* may account for the radiation that led to the emergence of *V. sotoarenasii* sp. nov., a species that is genetically, morphologically, and biochemically distinct from *V. planifolia*.

Finally, given its original aromatic content combining vanilyl and anisic notes, its high level of resistance (at least for CR0068) to the *Fusarium* root rot of vanilla (Koyyappurath *et al.* 2015), and its early and abundant flowering in culture, *V. sotoarenasii* sp. nov. could be a promising genitor for breeding programs aiming to produce new vanilla varieties for the agroindustry.

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