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Interspecific differentiation and hybridization in *Vanilla* species (Orchidaceae)

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Vanilla claviculata, *V. barbellata* and *V. dilloniana* are distributed throughout the Caribbean islands and are all found in Puerto Rico. The vegetative parts of the species are similar; however, their conspicuous flowers easily distinguish them. Electrophoresis of seven polymorphic enzymes revealed that the genetic composition of the three species is also very similar: they deviate mainly from each other in allele frequencies rather than by specific alleles. A hierarchical analysis of genetic differentiation showed that the between-species component is slightly higher ($F_{SG} = 0.237$) than the component between populations within species ($F_{PS} = 0.141$). Nevertheless, they are efficiently recognized by their genotypic compositions. In *V. barbellata* and *V. claviculata* 97–99% of all individuals were assigned to the correct species. Assignment to a wrong species occurred only with individuals at localities where species coexist. This suggests that the species may hybridize.

Keywords: differentiation, hybridization, population structure, *Vanilla*.

Introduction

Only few cases of genetic incompatibility between closely related species have been discovered in Orchidaceae (Sanford, 1964, 1967; Johansen, 1990; Dressler, 1993). Instead, prepollination mechanisms such as pollinator specificity, separate flowering phenologies and mechanical barriers in flowers seem to be the principal factors preventing hybridization in the family (van der Pijl & Dodson, 1966; Dressler, 1981; Gill, 1989; Paulus & Gack, 1990; Grant, 1994).

In Puerto Rico three native species of *Vanilla*, *V. claviculata* (W. Wright) Swartz, *V. barbellata* Reichenbach f. and *V. dilloniana* Correll (Orchidaceae) are found in the western part of the island. It is difficult to distinguish the species by the vegetative parts only, but characteristics of their labella separate the *Vanilla*'s. There seem to be no prepollination barriers (L. R. Nielsen & J. D. Ackerman, unpubl. obs.), leaving open the possibility of hybridization in areas where more than one species is found. This is particularly expected if interspecific differentiation is small compared to intraspecific differentiation, indicating a relatively high level of genetic similarity. If hybridization occurs, the genetic identity of species may become blurred in sympatric populations.

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In this paper we compare the differentiation among populations with the level of interspecific differentiation. We ask whether it is possible to separate the species from one another by their genetic composition and discuss the possibility of natural hybridization in sympatric populations.

Materials and methods

Plant species

In Puerto Rico, *V. claviculata* grows in several districts ranging from Guajataca State Forest in the north to the Susua Forest Reserve in the south (Fig. 1). *Vanilla barbellata* was found in the south-western to southern part of the island where the Susua Forest Reserve is the northernmost locality. In addition, a population of *V. barbellata* was rediscovered on the small island, Isla de Vieques, east of Puerto Rico. *Vanilla dilloniana* is uncommon and only found at three western/south-western localities all shared with *V. claviculata*. *Vanilla claviculata* and *V. dilloniana* were found in moist serpentine shrub forest and *V. barbellata* in drier areas.

The species are lianas with long, succulent stems that cover the ground and attach to the surrounding vegetation. The small, scaly leaves are shed early, particularly in *V. claviculata* and *V. barbellata*, and aerial roots are produced at each stem node. The spirally arranged resupinate and nectarless flowers open sequentially and last for one day only. The petals and

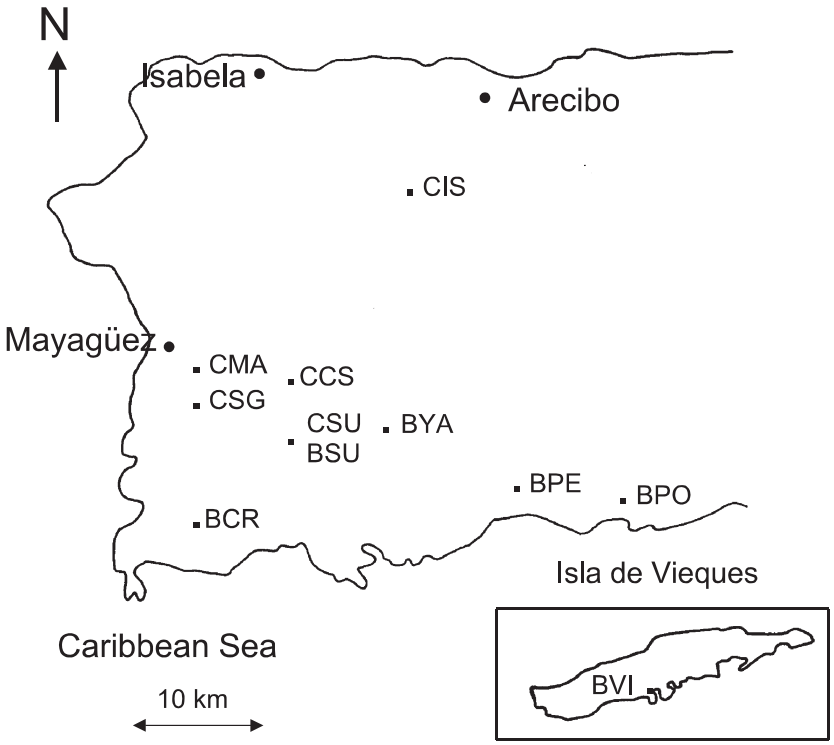

Fig. 1 Localities of *Vanilla claviculata*, *V. barbellata* and *V. dilloniana* in Puerto Rico. Abbreviations beginning with C refer to *V. claviculata*, and with B to *V. barbellata*. *Vanilla dilloniana* was collected at CCS, CSG and CSU/BSU. The smaller island, Isla de Vieques, is approximately 20 km east of the main island of Puerto Rico. See Table 1 for the population abbreviations.

sepals are green to olive-green and only the labellum varies significantly between the three species (Ackerman, 1995). The number of flowers setting fruits is low in all three species, ranging from 0.5% (*V. dilloniana*) to 5.3% (*V. claviculata*) (L. R. Nielsen & J. D. Ackerman, unpubl. obs.).

Sampling

A total of 237 samples was collected from 14 populations of *V. claviculata*, *V. barbellata* and *V. dilloniana* at 10 different localities (Table 1) in May 1997. Because of the similarity of the three species, only material from

Table 1 Sample size of each species of *Vanilla* before and after pooling identical, closely sampled individuals (defined individuals)

Species	Locality	Abbreviation of locality	Sample size	Number of defined individuals
V. claviculata	Campamento-Santana	CCS	6	5
	Isabela	CIS	20	18
	Maricao	CMA	30	29
	San German	CSG	16	16
	Susua	CSU	23	21
	Total		95	89
V. barbellata	Cabo Rojo	BCR	6	6
	Penuelas	BPE	16	12
	Ponce	BPO	29	24
	Susua	BSU	35	25
	Vieques	BVI	27	17
	Yauco	BYA	3	3
	Total		116	87
V. dilloniana	Campamento-Santana	DCS	5	3
	San German	DSG	17	5
	Susua	DSU	4	2
	Total		26	10

flowering individuals was collected at sites where more than one species were known to grow (based on Ackerman, 1995 and on herbarium material of *Vanilla* specimens kept at the herbarium of the University of Puerto Rico, Río Piedras). In the field, only one sample was taken from each patch to avoid unintentional mixing of individuals. Moreover, to prevent resampling individuals the samples were separated by approximately 10 m. Of each sample a piece of stem (~40 cm) was collected and grown at the Botanical Garden of Copenhagen.

Enzyme electrophoresis

Fresh pieces of stem tissue (about 0.125 cm^3) were homogenized in an extraction buffer of Corrias *et al.* (1991) consisting of 2 mM EDTA, 200 mM ascorbic acid, 14 mM 2-mercaptoethanol and 0.2 M Tris-HCl (pH 7). The plant extracts were absorbed onto wicks of Whatman no. 4 chromatography paper.

Seven interpretable systems, polymorphic in at least one population, were routinely surveyed on 12% starch gels with two buffer systems.

1 Histidine-citrate consisted of a stock solution of 0.13 M histidine titrated to pH 6.0 with citric acid (monohydrate; approximately 0.03 M). The electrode buffer was a 1:1 dilution of the stock buffer, and the gel buffer was obtained from a 1:12 aqueous dilution of the stock (Ellstrand, 1984). This buffer system was used to resolve phosphoglucomutase (PGM, EC 5.4.2.2), malate dehydrogenase (MDH, EC 1.1.1.37), isocitrate dehydrogenase (IDH, EC 1.1.1.42), shikimate dehydrogenase (SKDH, EC 1.1.1.25), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44) and UTP-glucose-1-phosphate uridyltransferase (UGUT, EC 2.7.7.9).
2 Sodium borate pH 8.5/Tris-citrate pH 7.8 (Torres & Bergh, 1978) was utilized to resolve diaphorase (DIA, EC 1.6.99.1). The electrode buffer consisted of a 1:1 aqueous dilution of 0.6 M boric acid, adjusted to pH 8.5 with NaOH and the gel buffer of a 1:9 dilution of a buffer containing 0.165 M Tris base, titrated to pH 7.8 with citric acid.

Staining methods for enzyme assays, excluding agar overlay, for PGM, MDH, SKDH, PGD, DIA and IDH followed Weeden & Wendel (1989) with minor modifications for IDH and SKDH. When staining for IDH only half the concentration of D,L-isocitric acid was used and for SKDH the following modified recipe was applied: 50 mL Tris-HCl pH 8.0, 90 mg shikimic acid, 5 mg NADP, 15 mg MTT and 1.2 mg PMS. UGUT was stained according to Harris & Hopkinson (1976). The staining for MDH revealed three stained zones of which two loci were variable (*Mdh1* and *Mdh3*), where *Mdh1* codes for the most anodally moving enzymes. The most common allele at each locus was given the number 100. The remaining alleles were labelled according to their mobilities relative to the most common allele.

Data analysis

Although precautions were taken when collecting the samples, some re-sampling could have occurred because of the scandent growth of the plants. Therefore, before analysing the data, multilocus genotypes of the samples were examined. Individuals collected close to each other (approximately 10 m apart) with the same multilocus genotype were pooled and treated as one.

Deviations of genotypic distributions from Hardy–Weinberg proportions were tested by Guo & Thompson's (1992) Monte Carlo permutation test. In each test 1000 permutations were used where the probability of the permutated genotypic distribution was estimated. The probabilities of significance were calculated as the frequency of the permutated values equal to or exceeding the probability of the observed genotypic distribution. The Bonferroni procedure was applied to calculate tablewide levels of significance (Holm, 1979). For each population, potential associations between alleles at different loci were tested for gametic phase equilibria (Weir, 1979).

The following analyses were only conducted for *V. claviculata* and *V. barbellata*, because the total sample size of *V. dilloniana* was low. Differentiation between populations was quantified using Wright's *F*-statistics as implemented by Weir & Cockerham (1984). To compare the amount of differentiation found between populations within species with the amount of differentiation found between species, the total genetic differentiation, F_{IG}, was partitioned into three hierarchies: F_{IP}, F_{PS} and F_{SG}, within populations, between populations within species and between species within the genus, respectively. The components are related by the equation:

$$1 \quad F_{IG} = (1 \quad F_{IP})(1 \quad F_{PS})(1 \quad F_{SG}).$$

The analysis was carried out with POPGENE (Yeh & Boyle, 1997).

As the same alleles were found in both species, but with different frequencies, genetic drift is presumably the main evolutionary force responsible for the differences among populations. Therefore, the programme CONTML from the PHYLIP package of Felsenstein (1993) was used to describe the genetic relationship among populations (the input order of the populations was permutated 100 times with the 'global rearrangement' option).

To examine whether allele frequencies are sufficiently different to assign individuals to their populations (and species) the procedure of Paetkau *et al.* (1995) was

applied. Based on Hardy–Weinberg proportions and gametic phase equilibria the likelihood of a genotype belonging to a population was determined by multiplying the expected genotype frequencies across all loci based on the allele frequencies in the given population. Each individual was assigned to the population with the highest likelihood of containing a member with the observed multilocus genotype (see Paetkau *et al*., 1995).

Results

Genotypic proportions and genetic population size

The multilocus genotypes revealed that several samples collected close to each other had identical genotypes, and these were pooled which reduced the sample sizes (Table 1). In *V. barbellata* the sample size was reduced by 25%. Further, at one locality (Yauco) only three individuals were discovered, and therefore this population was excluded from the analyses. In *V. claviculata* only a few samples were pooled (Table 1). In *V. dilloniana* a total sample size of 26 collected at three sites corresponded to only 10 presumed individuals (Table 1). The populations were therefore pooled and treated as one population (DIL). This population was not considered further in interspecific comparisons.

Tests for Hardy–Weinberg proportions resulted in six significant deviations at the 5% level: one in *V. barbellata* (*Pgm*, BVI) and five in *V. claviculata* (Table 2). Three of the deviations in *V. claviculata* were found in the CMA population (*Dia*, *Idh*, *Skdh*), the other two in CIS (*Pgm*) and CSU (*Dia*). However, when adjusted with the sequential Bonferroni technique for each species over all populations and loci, none of the deviations was significant at the 5% tablewide level. This suggests that the genotypic proportions accord to Hardy–Weinberg proportions in both species. The average differentiation within populations, F_{IP}, was −0.062 (Table 3). In *V. dilloniana* the pooled sample agreed with Hardy–Weinberg proportions at all loci at the 5% level.

In only one population, CMA, two alleles, Idh^{119} and $Mdh3^{75}$, were in gametic phase disequilibrium ($P = 0.043$). Thus, of approximately 130 tests only one was significant. If adjusted with a sequential Bonferroni procedure, none of the tests was significant at the 5% tablewide level. This suggests that all populations were in gametic phase equilibria.

Differentiation within species

The overall differentiation between populations within species, F_{PS}, was 15.8% and 12.3% in *V. claviculata* and *V. barbellata*, respectively, with an average of 14.1% (Table 3).

The population that deviated most from others in *V. barbellata* was found on the Isla de Vieques (BVI). This was the only population monomorphic at *Skdh*. At *Pgm*, the allele Pgm^{108} was frequent (0.38) and only found in one other population, BSU, at a low frequency. In *V. claviculata*, the population from Isabela (CIS) was most differentiated. At *Idh*, Idh^{119} was the most frequent allele; an allele that was only found in one individual in one other population (CMA). Further, the rare allele $Mdh1^{109}$ was observed in several individuals.

The genetic relationships among populations are illustrated in Fig. 2. The most geographically isolated population in each species (CIS and BVI) has the longest branches representing the two most differentiated populations. Furthermore, the smallest population in each species (CCS and BCR) also has relatively long branches. The populations of *V. barbellata* are clearly separated from the populations of *V. claviculata*. It is noteworthy that the two populations occurring at the same locality (BSU and CSU) represent the pair of populations from each species with the shortest distance to each other in the tree.

As the assumption of gametic phase equilibria appeared fulfilled and the populations showed Hardy–Weinberg proportions, the assignment test was applied. In both species assignment to the original population was high (Table 4). The populations in both *V. barbellata* and *V. claviculata* which appeared most differentiated in the tree (BCR, BVI, CCS, CIS) also had a high likelihood of being assigned to the correct population (83, 100, 80 and 78%, respectively).

Differentiation between species

At two loci, *Skdh* and *Idh*, alleles were found in several populations in one species only (Table 2). The remaining differences were found alone in allele frequencies and the overall differentiation between species (F_{SG}) amounted to 0.24 (Table 3). Nevertheless, the differences were sufficient to place almost all individuals in the correct species with the assignment test. In *V. barbellata*, 99% of all individuals were assigned to the correct species. Only one population (BSU) contained individuals that were assigned to an incorrect species (Table 4). Likewise, in *V. claviculata* the overall assignment to the correct species was high (97%) and only in the population (CSU) coexisting with the two other species did some individuals (15%) have a higher likelihood of assignment to *V. barbellata*. These findings are in agreement with the above-described differentiation of populations, where the two closest populations of each species were BSU and CSU (Fig. 2).

Table 2 Allele frequencies in the studied populations of *Vanilla* (*V. dilloniana* pooled as one population; DIL). *F*, Wright's fixation index; *P*, significance level for deviation from Hardy–Weinberg proportions; *N*, sample size. See Table 1 for the abbreviations of localities

		BCR	BPE	BPO	BSU	BVI	CCS	CIS	CMA	CSG	CSU	DIL
Dia	104	0	0.05	0.15	0.44	0.29	1.00	0.53	0.81	0.53	0.47	0.45
	100	1.00	0.95	0.85	0.56	0.71	0	0.47	0.19	0.47	0.52	0.55
	F	—	−0.05	0.16	0.03	−0.42	—	−0.42	0.21	−0.07	−0.50	−0.01
	P	—	1.000	0.398	1.000	0.221	—	0.169	0.043	1.000	0.027	1.000
	N	6	11	24	25	17	4	17	29	15	20	10
Idh	119	0	0	0	0	0	0	0.67	0.03	0	0	0
	100	1.00	1.00	0.85	0.96	1.00	1.00	0.33	0.97	1.00	1.00	1.00
	60	0	0	0.15	0.04	0	0	0	0	0	0	0
	F	—	—	−0.17	−0.04	—	—	0.25	1.00	—	—	—
	P	—	—	1.000	1.000	—	—	0.104	0.016	—	—	—
	N	6	12	24	24	17	5	18	29	16	21	10
Mdh 1	109	0	0	0	0	0.03	0.10	0.19	0	0	0	0
	100	1.00	1.00	1.00	1.00	0.97	0.90	0.81	1.00	1.00	1.00	1.00
	F	—	—	—	—	−0.03	−0.11	−0.24	—	—	—	—
	P	—	—	—	—	1.000	1.000	1.000	—	—	—	—
	N	6	12	24	25	17	5	18	29	16	21	10
Mdh 3	129	0	0	0	0.02	0	0	0	0	0	0	0
	121	0.17	0.27	0.02	0.32	0	0	0	0.02	0.06	0.02	0.15
	114	0.58	0.36	0.77	0.40	0.62	0	0	0	0	0.05	0
	100	0.25	0.36	0.21	0.24	0.38	0.90	0.44	0.90	0.63	0.50	0.35
	75	0	0	0	0.02	0	0.10	0.56	0.09	0.31	0.43	0.50
	F	−0.46	−0.10	−0.15	−0.06	−0.37	−0.11	0.33	0.27	−0.35	−0.10	−0.49
	P	0.375	0.549	0.131	0.982	0.301	1.000	0.194	0.279	0.336	0.583	0.312
	N	6	11	24	25	17	5	18	29	16	21	10
Pgd	107	0	0	0	0	0	0	0	0	0	0	0.50
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50
	F	—	—	—	—	—	—	—	—	—	—	−0.60
	P	—	—	—	—	—	—	—	—	—	—	0.207
	N	6	12	24	25	17	5	15	29	16	21	10
Pgm	113	0	0	0	0	0	0	0.12	0	0	0	0
	108	0	0	0	0.02	0.38	0.40	0.24	0.19	0.27	0.07	0.15
	100	0.83	0.33	0.42	0.57	0.06	0.40	0.26	0.34	0.43	0.36	0.60
	91	0	0.25	0.42	0.17	0.56	0.20	0.24	0.40	0.23	0.36	0.15
	80	0.17	0.42	0.17	0.21	0	0	0.15	0.07	0.07	0.21	0.10
	74	0	0	0	0.02	0	0	0	0	0	0	0
	F	−1.00	−0.02	0.07	−0.03	−0.64	−0.25	−0.05	−0.16	−0.27	−0.03	−0.03
	P	0.020	0.588	0.201	0.589	0.003	0.479	0.018	0.293	0.467	0.589	0.132
	N	6	12	24	21	17	5	17	29	15	21	10
Skdh	114	0	0	0	0	0	0	0	0.04	0	0	0
	108	0	0	0	0	0	0.38	0.07	0.17	0.07	0.06	0.11
	100	0.67	0.60	0.56	0.77	1.00	0.50	0.93	0.56	0.86	0.94	0.72
	88	0.33	0.20	0.17	0.15	0	0.13	0	0.24	0.07	0	0.11
	73	0	0.20	0.27	0.08	0	0	0	0	0	0	0.06
	F	−0.50	0.11	−0.22	−0.21	—	0.16	−0.07	0.14	−0.12	−0.6	−0.23
	P	1.000	0.331	0.319	1.000	—	0.664	1.000	0.019	1.000	1.000	1.000
	N	6	10	24	24	17	4	15	27	14	18	9

Table 2 (*Continued*)

		BCR	BPE	BPO	BSU	BVI	CCS	CIS	CMA	CSG	CSU	DIL
Ugut	100	1.00	0.79	0.98	0.74	0.88	0.70	0.11	0.05	0.19	0.19	0.50
	90	0	0.17	0.02	0.04	0	0.30	0.89	0.71	0.78	0.64	0.45
	79	0	0.04	0	0.22	0.12	0	0	0.24	0.03	0.17	0.05
	F	—	−0.21	−0.02	−0.29	−0.13	0.52	−0.13	−0.10	−0.24	0.09	−0.10
	P	—	1.000	1.000	0.431	1.000	0.317	1.000	1.000	1.000	0.073	1.000
	N	6	12	24	25	17	5	18	29	16	21	10

Table 3 Hierarchical *F*-statistics combined across loci for *Vanilla claviculata* and *V. barbellata*

X	Y	F_{XY}
Individual (I)	Population (P)	−0.062
Population (P)	Species (S)	0.141
Species (S)	Genus (G)	0.237
Individual (I)	Genus (G)	0.304

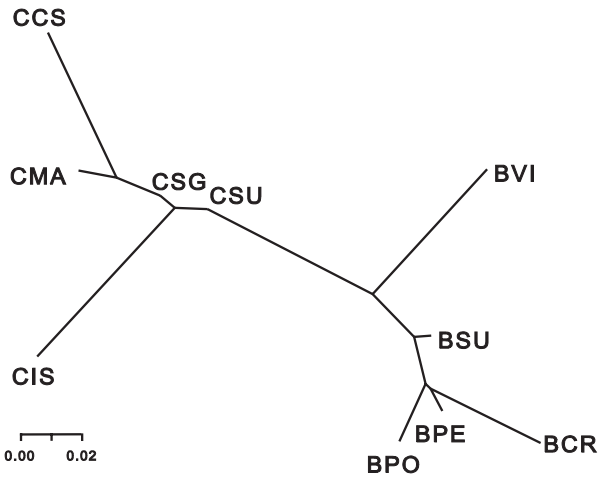

Fig. 2 Genetic relationships among the *Vanilla* populations based on evolution by genetic drift (Felsenstein, 1981, 1993). The angles between branches are arbitrary. The scale indicates the expected accumulated variance of allele frequencies.

Discussion

Differentiation within species

Compared to other studies on orchid species, the differentiation among populations in *V. claviculata* and *V. barbellata* (F_{PS}) is generally high (see Fig. 3). Out of 23 species, only five were more differentiated than observed in the present study. This may be a consequence of the small population sizes discovered in all the present localities. The total number of genetic individuals was generally low (3–29) and an estimation of the effective population size showed that it was a quarter of the census size (L. R. Nielsen & J. D. Ackerman, unpubl. obs.). Genetic drift has a high impact on populations with persistently small effective population sizes, resulting in both loss and reorganization of variation (Ellstrand & Elam, 1993). This was especially reflected in the smallest populations of both *V. claviculata* and *V. barbellata*,

Table 4 Assignment of individuals from populations of *Vanilla claviculata* and *V. barbellata*. The populations listed in the left-hand column represent the population from which the assigned individual originated. The other columns indicate the percentage of individuals from the population in question that are assigned to a given population

	BCR	BPE	BPO	BSU	BVI	CCS	CIS	CMA	CSG	CSU
BCR	**83**	17	0	0	0	0	0	0	0	0
BPE	42	**42**	8	8	0	0	0	0	0	0
BPO	33	4	**54**	0	8	0	0	0	0	0
BSU	20	12	12	**40**	12	4	0	0	0	0
BVI	0	0	0	0	**100**	0	0	0	0	0
CCS	0	0	0	0	0	**80**	0	0	20	0
CIS	0	0	0	0	0	6	**78**	0	0	17
CMA	0	0	0	0	0	21	3	**52**	17	7
CSG	0	0	0	0	0	31	0	6	**25**	38
CSU	10	0	0	5	0	5	0	0	24	**57**

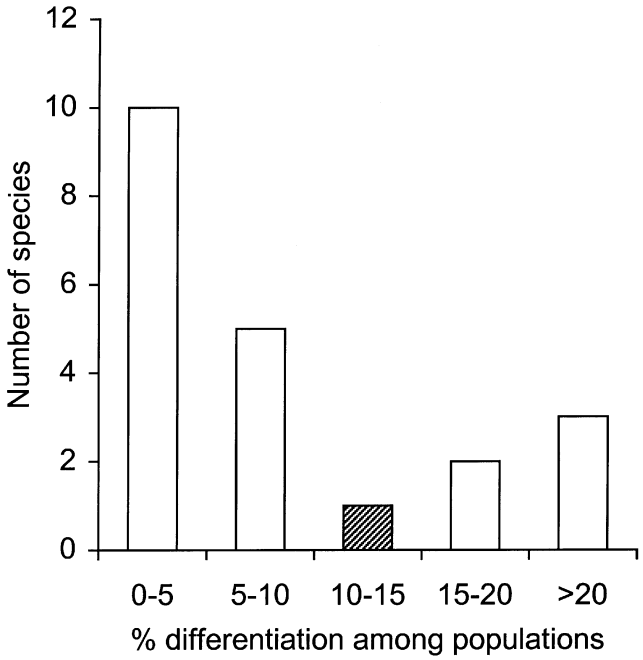

Fig. 3 Differentiation among populations of orchid species. Values taken from Scacchi *et al.* (1990, 1991), Corrias *et al.* (1991), Rossi *et al.* (1992), Case (1994), Arduino *et al.* (1995), Hollingsworth & Dickson (1997) and Sun (1997). The grey bar represents the location of *Vanilla barbellata* and *V. claviculata*.

which particularly differed from the remaining populations. The higher differentiation of *V. claviculata* and *V. barbellata* compared to other orchid species may also reflect the dispersal of fruit and seeds. Most orchids have dry capsular fruits and disperse many thousands of dust-like seeds by the wind (Dressler, 1981). In *Vanilla*, however, the seeds are associated with a moist pulp and may be dispersed by bees (Madison, 1981) or other animals. Hence, the dispersal distances may be shorter in *Vanilla* than in wind-dispersed orchid species and, consequently, genetic drift is likely to be more powerful. The BVI population, which is located on a smaller island, may thus be more differentiated from the remaining populations of *V. barbellata* because of a low level of gene flow. The sea between the two islands is presumably a strong barrier for genetic exchange between this population and others.

The differentiation of the smallest populations (BCR and CCS) and of the distant populations (BVI and CIS) is reflected in their genotypic composition. Individuals from these populations have a high likelihood of being assigned to the correct population. As suggested by Paetkau *et al.* (1995) wrongly assigned individuals may be immigrants and therefore have a higher likelihood of assignment to the population from which they originated. Thus, the high level of correct assignment to the most distant populations in both *V. claviculata* and *V. barbellata* (CIS and BVI, respectively), may be a result of reduced gene flow to and from the two populations. The perfect assignment of all individuals from the Vieques population therefore supports the hypothesis that gene flow into this population is very low.

Differentiation between species

The genetic differences found between the two species are mostly differences in allele frequencies; only few alleles appear in only one species. This is reflected in the analysis of hierarchical differentiation which shows that the differentiation between species ($F_{SG} = 0.237$) is only a little higher than the differentiation between populations within species ($F_{PS} = 0.141$) (Table 3). Even so, the differences are strong enough to separate *V. claviculata* and *V. barbellata* into two groups by both the genetic relationship among populations and by the assignment procedure. Interestingly, only the two populations in the Susua Forest Reserve (BSU and CSU) contain individuals which are assigned to the wrong species. As this was the only locality where both species were found growing together, an explanation for the incorrect assignment may be that the species interbreed and exchange genes, which blurs the genetic differences. This may also be seen by the close location of these two populations in the tree. Several points suggest that hybridization may indeed take place at this locality: two individuals were discovered where some characters appeared morphologically intermediate to the two hypothetically parental species (*V. claviculata* and *V. barbellata*). A study on genotypic composition revealed a high level of heterozygosity in these individuals and in eight other specimens collected near the Susua Forest Reserve. This, together with a multivariate analysis on floral characters, suggested that the individuals were hybrids between *V. barbellata* and *V. claviculata* (L. R. Nielsen, unpubl. obs.). The third species of *Vanilla*, *V. dilloniana*, growing in the Susua Forest Reserve flowers later than *V. barbellata* and *V. claviculata* (L. R. Nielsen & J. D. Ackerman, unpubl. obs.) and therefore the possibility for gene exchange between this species and the two other species is smaller.

The low differentiation between species and the evidence of hybridization in sympatric populations make it reasonable to believe that the species are closely related, with a relatively recent common ancestor.

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