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Isozyme variation in the Canarian endemic *Neochamaelea pulverulenta* (*Cneoraceae*): implications for population differentiation in the Canaries and first molecular insights on the floristic link with the Mediterranean.

Abstract

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We examined levels of population genetic variation at 9 isozyme loci in the Canarian monotypic endemic *Neochamaelea pulverulenta* and in a stand of the Mediterranean *Cneorum tricoccon*. Basic descriptors of genetic variation indicate that this Canarian endemic fits the emerging picture of a higher genetic diversity in Canary Island endemics than in those from more remote oceanic archipelagos. Genetic variation is highly subdivided in *N. pulverulenta*, as indicated by the high average values of Gst and Fst, which hint at a very low level of gene flow. Patterns of isolation by distance manifest only moderately in the inter-island comparisons, with the population from La Sorrueda (Gran Canaria) unexpectedly closer to Teno (Tenerife) than to the other two Gran Canarian populations in the UPGMA cluster, probably as an effect of drift. The low number of isozyme alleles shared by *N. pulverulenta* and *C. tricoccon* and the low average genetic identity between them (I = 0.245) bolsters the hypothesis of an antique evolutionary divergence, as suggested by previous systematic and pallynologic studies.

Introduction

The three species of the family Cneoraceae, with a distribution in the Mediterranean (*Cneorum tricoccon*), the Canary Islands (*Neochamaelea pulverulenta*) and Cuba (*Cneorum trimeron*), make up one of the most intriguing geographical disjunctions among vascular plants and furnish evidence of the close floristic connection between the Mediterranean and the Canaries. *Neochamaelea pulverulenta* (2n = 36; Borgen 1974) is noteworthy in that it has not diversified in the Canary Islands in spite of being a presumably antique endemic. The taxonomic position of *N. pulverulenta* as related to *Cneorum* (2n = 36; Goldblatt 1981) had long been debated until the morphological and palynological studies carried out by Erdtman (1952) established their current separation into two different genera.

Reproductive biology surveys for this family provide compelling evidence that *C. tricoccon* is andromonoecious (Traveset 1995). Tébar and Llorens (1997) refine this

Table 1. Gel/electrode buffer systems used for the enzymes which could be scored unambiguously.

Buffer system	Enzyme	EC CODE
Lithium borate pH 8,3	*GOT*	2.6.1.1
Tris-citrate pH 8,2	*PRX*	1.11.1.7
	ACP	3.1.3.2
Histidine pH 7	*PGI*	2.6.1.1
	PGM	5.4.2.2
	IDH	1.1.1.42
	MDH	1.1.1.37

conclusion and extend it to the Canarian *N. pulverulenta* based on observations of a small sample of specimens from Tenerife. Although they state that the reproductive system of *C. tricoccon* and *N. pulverulenta* is identical, preliminary reproductive data (Pérez de Paz 2002) hint that, unlike *C. tricoccon*, individuals of *N. pulverulenta* can (1) have both morphologically hermaphroditic (but functionally female) flowers and masculine flowers; (2) have only masculine flowers; or (3) exhibit a strong dichogamy, protandry o protogyny (depending on the year and the individual) [Pérez de Paz and Febles, unpublished data). Further, the anthers of the hermaphroditic flower have very scarce pollen and do not dehisce in any of the specimens observed, which entails that they are functionally feminine. These observations suggest that *N. pulverulenta* is an example of androdioecy and is probably in transit towards dioecy, which likely entails a high percentage of functional dioecy (and, therefore, of xenogamy).

The paucity of molecular data for this family has stood in the way of assessing the extent of genetic differentiation between *Cneorum* and *Neochamaelea*, thereby preventing an integrated understanding of how and why genetic diversity is maintained in these taxa. In addition, the use of molecular markers would likely allow us to assess the extent to which genetic differentiation reflects the presently accepted taxonomic subdivisions within the Cneoraceae.

Table 2. Basic indicators of population genetic variability at the nine loci surveyed in the populations of *N. pulverulenta* and in the specimens of *Cneorum tricoccon*. N: number of individuals per population; A: average number of alleles per locus; P: proportion of polymorphic loci; Ho and He: observed and expected heterozygosities; Fis: Wright's (1951) fixation index. Numbers in brackets are standard errors. GC: Gran Canaria; TF: Tenerife.

				Average heterozygosities		
Population	N	A	P	observed (H_o)	expected (H_e)	Fixation index F_{is}
Sorrueda (GC)	22	1,4 (0,2)	33,3	0,217 (0,132)	0,137 (0,080)	-0,499
Vicentillos (GC)	20	1,6 (0,2)	44,4	0,367 (0,156)	0,243 (0,097)	-0,556
Tasartico (GC)	20	1,8 (0,3)	33,3	0,169 (0,109)	0,129 (0,067)	-0,120
Teno (TF)	20	1,6 (0,2)	44,4	0,267 (0,134)	0,199 (0,085)	-0,285
Averages *N. pulverulenta*		1,6	38,85	0,255	0,177	-0,365
C. triccocon	10	1,3 (0,2)	33,3	0,333 (0,167)	0,175 (0,088)	-1,000

In this survey, we couple isozyme variation assessments with reproductive data for several populations of *N. pulverulenta* from the Canaries to (1) provide the first estimates of the levels of genetic variation in this Canarian monotypic endemic genus; and (2) explore the impact of the reproductive system on the generation and maintenance of genetic variation.

Material and Methods

PLANT MATERIAL - We selected four populations of *Neochamaelea pulverulenta*: three from Gran Canaria and one from Tenerife (Table 2). For comparative purposes, we included 10 individuals of *Cneorum triccoccon* from the Mediterranean that correspond to an undetermined population from the island of Mallorca collected by D. Bramwell in 1973. These specimens are in culture at the Jardín Botánico Canario "Viera y Clavijo" and consist of both seeds from the original collection from 1973 and their offspring. Our sampling design in the Canarian stands followed transects along the areas of population occurrence.

ELECTROPHORETIC ANALYSIS - Enzyme extracts were obtained from leaf tissue of adult plants using an extraction buffer that followed Wendel & Weeden (1989). Electrophoretic and staining protocolos followed Murphy & al. (1996). We examined 18 enzyme systems, out of which only seven (Table 1) could be unambiguously scored. Putative enzyme loci and their alleles were labelled according to their relative migration towards the anode following the numerical (loci) and alphabetic (alleles) sequences. The number of bands of heterozygotes and their relative intensities conformed to the quaternary structures expected (Acquaah 1992) and to the hypothesis of Mendelian codominant inheritance in all cases.

The average number of alleles per locus (A), percentage of polymorphic loci (P), observed and expected heterozygosities (Ho and He) and Wright's 1951 fixation indices (Fis) and Nei's 1978 genetic identities were calculated from genotype data using BIOSYS-1 version 1.7 (Swofford & Selander 1989). The estimates of the apportionment of genetic variation among populations (Gst; Nei 1973) were obtained through Genestat (Lewis 1993). Ewens-Watterson tests of neutrality and the estimates of Wright's (1951) F-ststistics were obtained using the computer program Popgene version 32 (Yeh & al. 1997). The matrix of allele frequencies was implemented in the computer program NTSYS-pc (Rohlf 1988) to obtain an UPGMA cluster based on Nei's (1978) genetic distance.

Results and Discussion

The Canarian populations of *N. pulverulenta* exhibit levels of isozyme variation (average He = 0,177, Table 2) which are higher than those reported for other self-incompatible Canarian endemics like *Cistus* (average He = 0,126; Batista & al. 2001), and rank only slightly lower than the average published for Canarian endemics (He = 0,186; Francisco-Ortega & al. 2000). Thus, *N. pulverulenta* fits the emerging picture of a higher genetic diversity in Canary Island endemics than in those from more remote oceanic archipelagoes (average He = 0,064; DeJoode and Wendel 1992), although factors related to lineage ascription and reproductive traits may have influenced its levels of variation even more than its geographic distribution, as hypothesized by recent works (Karron 1987, 1991; Gitzendanner & Soltis 2000; Elgar & Clode 2001).

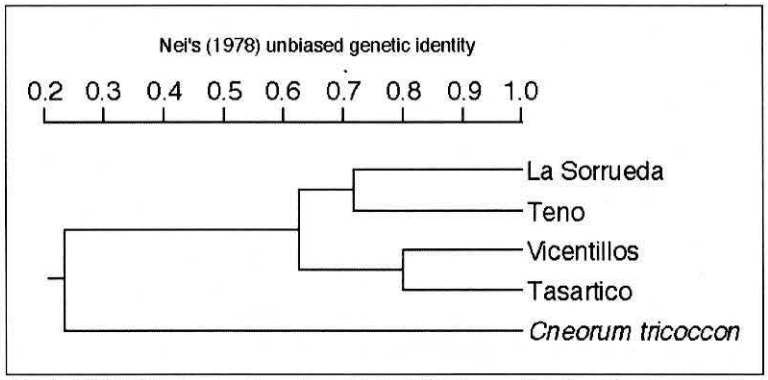

Fig. 1. UPGMA cluster based on Nei's (1978) genetic identity showing the relationships among the five populations sampled.

The moderate levels of variation detected in *N. pulverulenta* (Table 2) are probably a consequence of the relict condition of this endemic, the uniformity of the arid environments where it occurs and its preferentially outcrossing breeding system. In the absence of evidence to argue for selection against homozygotes (all Ewens-Watterson tests were non-significant), our favoured hypothesis to explain heterozygote excess in this species is a major effect of obligate outcrossing through functional dioecy and perhaps positive assortive mating. If some kind of polyploidy has played a role in generating almost-fixed

Table 3. Population structure statistics following Nei (1973) and Wright (1951) and gene flow (Nm) for the nine loci surveyed in *N. pulverulenta*. Gene flow estimated from Nm = 0,25 (1 - Fst)/Fst.

	Nei's (1973) population structure statistics								Wright's F-statistics			
LOCUS	Hs	Js	Ht	Jt	Dst	CDst	Gst	CGst	Fis	Fit	Fst	Nm
All *N. pulverulenta*												
Pgm	0,0773	0,9227	0,4302	0,5698	0,3529	0,4820	0,8203	0,8569	-0,153	0,746	0,779	0, 1
Pgi	--	--	--	--	--	--	--	--	--	--	0,000	--
Idh	0,0120	0,9880	0,4880	0,5120	0,4760	0,6574	0,9754	0,9820	--	--	0,000	--
Mdh-1	0,2549	0,7451	0,4000	0,6000	0,1451	0,2166	0,3627	0,4240	-0,910	-0,312	0,313	0,5
Mdh-2	0,6190	0,3810	0,6204	0,3796	0,0014	0,0037	0,0023	0,0038	-0,540	-0,509	0,020	12,3
Prx	0,0000	1,0000	0,6667	0,3333	0,6667	1,0986	1,0000	1,0000	--	1,000	1,000	0,0
Acp	0,0537	0,9463	0,0595	0,9405	0,0058	0,0061	0,0972	0,0999	0,319	0,382	0,092	2,5
Got-1	0,3551	0,6449	0,4583	0,5417	0,1032	0,1744	0,2252	0,2844	-0,600	-0,280	0,200	1,0
Got-2	0,2221	0,7779	0,3386	0,6614	0,1165	0,1622	0,3441	0,3925	0,052	0,333	0,297	0,6
Averages	0,1993	0,8007	0,4327	0,5673	0,2334	0,3447	0,5395	0,6080	-0,481	0,127	0,410	0,4
Gran Canarian *N. pulverulenta*												
Pgm	0,0160	0,9840	0,0160	0,9840	0,0000	0,0000	0,0002	0,0002	-0,024	-0,008	0,016	15,4
Pgi	--	--	--	--	--	--	--	--	--	--	0,000	--
Idh	0,0160	0,9840	0,6507	0,3493	0,6347	1,0356	0,9754	0,9847	--	--	0,000	--
Mdh-1	0,1707	0,8293	0,3333	0,6667	0,1626	0,2182	0,4878	0,5383	-1,000	-0,200	0,400	0,4
Mdh-2	0,6134	0,3866	0,6121	0,3879	0,0000	0,0033	0,0000	0,0034	-0,514	-0,492	0,015	16,8
Prx	0,0000	1,0000	0,6667	0,3333	0,6667	1,0986	1,0000	1,0000	--	1,000	1,000	0,0
Acp	0,0716	0,9284	0,0793	0,9207	0,0077	0,0084	0,0975	0,1012	0,319	0,375	0,083	2,8
Got-1	0,3452	0,6548	0,5000	0,5000	0,1548	0,2697	0,3096	0,3891	-1,000	-0,500	0,250	0,8
Got-2	0,2960	0,7040	0,4235	0,5765	0,1275	0,1998	0,3010	0,3627	0,052	0,276	0,237	0,8
Averages	0,1911	0,8089	0,4102	0,5898	0,2191	0,3159	0,5341	0,5983	-0,523	0,005	0,347	0,5

Table 4. Nei's (1978) genetic identities (above diagonal) and distances (Below diagonal) between pairwise combinations of the five populations sampled.

population	1	2	3	4	5
1 La Sorrueda	****	0,8648	0,7971	0,0654	0,8378
2 Teno	0,1453	****	0,7040	0,0983	0,7360
3 Vicentillos	0,2268	0,3510	****	0,1741	0,9078
4 Tasartico	0,1770	0,3066	0,0968	****	0,1360
5 *C. tricoccon*	2,7276	2,3199	1,7479	1,9952	****

heterozygosis at several loci, it must have been early in the history of the genus, since the chromosome number in the Cneoraceae (2n = 36) is frequent in the Rutaceae (Federov 1974). The observed heterozygote excess might have been enhanced by recent bottlenecks (in general, *N. pulverulenta* inhabits areas under a high anthropic pressure in the West of Gran Canaria), although the low number of polymorphic loci per population prevented us from carrying out a bottleneck test as described in Cornuet and Luikart (1996). The case for heterozygote excess in *C. triccoccon* is more uncertain because of the scarce representativity of the samples used, and will not be discussed in this paper.

The degree of population differentiation in *N. pulverulenta* is high (Table 3) either including the population from Teno (average Gst = 0,540, Fst = 0,410, Nm = 0,360) or excluding it (average Gst = 0,534, Fst = 0,347, Nm = 0,470), which hints at a poor level of gene flow among the populations sampled. Quite unexpectedly, the cluster (Fig. 1) groups the population from La Sorrueda (Gran Canaria) more tightly to Teno (Tenerife) than to the other populations surveyed in Gran Canaria. At the present state of phylogenetic ignorance for this genus, the more feasible explanation for this topology is the effect of drift on the allele frequencies.

Quantitatively, the average indicators of genetic variation in *N. pulverulenta* and *C. tricoccon* are similar (Table 2). Qualitatively, these two species share only 3 alleles out of the total 25 scored, and their degree of relatedness as measured by Nei's (1978) genetic distance and identity (Table 4, average D = 1,408, I = 0,245) is low as compared with published surveys with congeneric species (I = 0,67 ± 0,07, Gottlieb 1977). The average value cited in Gottlieb's (1977) review might change if updated; however, our molecular data do support the present taxonomic position of C. tricoccon and N. pulverulenta in different genera, and suggest that they may derive from a considerably antique diversification event, in accordance with palynological data (Erdtman 1952; Lobreau-Callen & al. 1978).

Although still much prospective, our results suggest two future avenues of research in the Cneoraceae. First, an enhanced data analysis and population sampling of these taxa are necessary to assess whether the levels of genetic variation reported in this work are representative of their areas of occurrence in the Canaries and the Mediterranean. This is of special relevance to explore the relative impact of historical factors and reproductive systems on the levels and maintenance of genetic variation of *N. pulverulenta* and *C. tricoccon* And second, in the face of the probable bias resulting from the application of only one molecular technique, our assessment should benefit from the confrontation with evidence from other sources of molecular and biological information to streamline the present conclusions.

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