Plant Syst Evol (2009) 279:1–10
DOI 10.1007/s00606-008-0064-5

ORIGINAL ARTICLE

Low levels of genetic differentiation between *Opuntia echios* varieties on Santa Cruz (Galapagos)

P. Helsen · P. Verdyck · A. Tye · S. Van Dongen

Received: 29 January 2008 / Accepted: 12 June 2008 / Published online: 24 April 2009
© Springer-Verlag 2009

Abstract Three processes play an important role in plant speciation: isolation, hybridization and polyploidization. Galapagos endemic *Opuntia* display putatively all of these processes. On this archipelago most islands are inhabited by a single *Opuntia* taxon. Santa Cruz, however, houses two morphologically distinct *O. echios* varieties (*echios* and *gigantea*). Morphological intermediates are found where these two geographically isolated varieties meet. Here we used ten microsatellite loci to reveal the population genetic structure of this system. In contrast to earlier studies, we found high genetic variability within localities. Genetic structuring was weak and no evidence for the existence of hybrids was found. The reasons for this weak genetic structure may include: the species' hexaploid nature, high levels of gene flow, recent colonization, and the lack of geographic barriers. This first detailed genetic study on these threatened species will be important for further conservation planning.

Keywords *Opuntia echios* · Prickly pear · Population genetics · Hybridization · Microsatellite · Galapagos · Polyploidy · Flow cytometry

P. Helsen (✉) · P. Verdyck · S. Van Dongen
Evolutionary Ecology Group, University of Antwerp,
Groenenborgerlaan 171, 2020 Antwerp, Belgium
e-mail: philippe.helsen@ua.ac.be

A. Tye
Charles Darwin Research Station (CDRS),
Isla Santa Cruz, Galapagos, Ecuador

Present Address:
A. Tye
Secretariat of the Pacific Regional Environment Programme,
PO Box 240, Apia, Samoa

Introduction

For many decades the enormous radiation and evolutionary divergence of the Galapagos fauna and flora have fascinated evolutionary biologists such as Darwin (1858) and Mayr (1942). Many of these organisms have been studied profoundly. However, scientists have paid little attention to one of the most characteristic and dominant elements of the arid ecosystem of these islands, the cacti. Three cactus genera inhabit Galapagos, but only the *Opuntia* (prickly pear) genus is both widespread and highly diverse (Stewart 1911; Howell 1933; Dawson 1965; Wiggins and Porter 1971; Browne et al. 2003). The morphology of the Galapagos *Opuntia* ranges from 12 m high treelike plants with hard dimorphic spines and long pendant branches to 2 m high scrubby plants without branches and with soft bristly spines. Providing food, water, shelter, and nesting places for many of Galapagos native animal species, *Opuntia* cacti play a keystone role in the arid ecosystem (Hicks and Mauchamp 1996). Due to the introduction of goats, donkeys, and other mammalian herbivores, some *Opuntia* populations nowadays are under serious threat (Jackson 1993; Mauchamp 1997; Desender et al. 1999; Tye 2005). Plants are eaten and/or their bark and root system is damaged. Although successful efforts have been made to remove these animals from many of the islands, on Santa Cruz, the main human population centre on the archipelago, removal programs are still in their infancy. Natural replacement of *Opuntia* may currently be insufficient to counteract the severe effects of this unnatural herbivory. Therefore, *Opuntia* restoration efforts have been begun on some islands (Tye 2005). On the IUCN red list (IUCN 2007) one species is considered critically endangered, all other species are vulnerable or endangered.

The current classification of Galapagos *Opuntia* species is based on perceived discontinuities in vegetative and

reproductive morphological characters and recognizes six endemic species further divided into 14 varieties (Wiggins and Porter 1971; Hicks and Mauchamp 1996), and suggested to comprise two clades stemming from separate colonizations of the archipelago (Porter 1983). However, as most cactus species are highly polymorphic (Gibson and Nobel 1986) and environmental factors influences cactus morphology (Racine and Downhower 1974; Nobel 1981; Hicks and Mauchamp 1996, 2000), a classification on morphology alone may be misleading. But experiments indicate that not all morphological variation in Galapagos *Opuntia* is explained by their polymorphic nature (Racine and Downhower 1974). We therefore conclude that genetic studies are needed to disentangle the role of genome and environment in determining morphology and species boundaries.

Browne et al. (2003) evaluated the diversity in and the phylogenetic relationships between eight of the 14 Galapagos *Opuntia* taxa (240 individuals), using eight allozyme markers, but found no variation at all, probably due to conservatism in the allozyme markers they used. We used a larger set of neutral and highly variable microsatellite markers (Goldstein and Schlötterer 1999) to re-evaluate the genetic variability of two *Opuntia echios* varieties. The ample genetic diversity uncovered by these markers is postulated to allow population structures to be revealed more accurately than by other markers (Liu et al. 2003). To score these markers correctly in suspected polyploid species (Gibson and Nobel 1986; Pinkava et al. 1998; Rebman and Pinkava 2004) it is crucial to know ploidy levels (Esselink et al. 2004; Nybom et al. 2006).

In Galapagos, most islands are inhabited by a single morphologically defined *Opuntia* taxon. On Santa Cruz, however, an evolutionarily interesting system seems to occur which may provide insight into the genetic variability and population genetic structure of these plants. Two geographically separated *O. echios* varieties co-occur in the arid lowlands which encircle the more humid highlands of the island. On the southern part *O. echios* var. *gigantea*, a high treelike cactus with a tall trunk and short spines, dominates this vegetation zone. On the northern side of the island *O. echios* var. *echios*, a scrub-like cactus with long spines and a short trunk can be found. Identification in the field is difficult because many overlapping morphological characteristics were used in the currently accepted classification (Wiggins and Porter 1971). For individuals of intermediate size, one rule of thumb was developed to distinguish between the two varieties in the field: if it is possible to touch the cactus pad surface between the spines (without being pricked) you are probably dealing with a *gigantea* specimen. Frequently, however, locality of an individual is the only parameter used for identification. In the relatively small area where these two described varieties meet, morphological intermediates are found that are characterized most strikingly by their intermediate size (Verdyck and Tye, personal observation). Yet, evidence for a genetic foundation of this variation is lacking.

The combination of current conservation problems and the poor knowledge of the genetic variability and phylogenetic relationships of these keystone species make this study important in the conservation of these species. Ten microsatellite loci were used to investigate: (1) the genetic status of these two earlier described taxa, (2) to what extent these two varieties interact and form morphological intermediates, (3) levels of gene flow among localities within the island and between morphological varieties along a North–South gradient, and (4) the genetic variability at the studied locations.

Materials and methods

Field samples

Galapagos, an Ecuadorian archipelago located in the eastern Pacific Ocean, is separated from the continent by approximately 1,000 km of open sea. The archipelago consists of 13 islands greater than 10 km^2 and more than 130 smaller islands and rocks. Santa Cruz, the second largest and central island, currently carries the largest human population and the two *Opuntia* varieties under study (Wiggins and Porter 1971). Twelve sites (Fig. 1) were sampled along the western coast, including typical *echios* varieties (110 individuals) in the north and northwest, typical *gigantea* varieties (100) in the south and southwest and morphological intermediates (25) in the west. Because *Opuntia* species may reproduce vegetatively (Rebman and Pinkava 2004) individuals selected at a single locality were at least 100 m from each other (Browne et al. 2003). One fresh mature cladode was chopped off at the joint with a machete, a process comparable to natural damage by wind, and transported in a paper bag to the Charles Darwin Research Station (CDRS) where it was stored at room temperature. Close to shipping day pads were cut into pieces of approximately 10 × 10 cm and transported in paper bags at ambient temperature to both the Royal Belgian Institute of Natural Sciences (RBINS) and the University of Antwerp (UA) where they were put in plastic bags and stored at −20°C.

DNA extraction and microsatellite genotyping

We isolated genomic DNA from all samples using the DNAeasy Plant Mini Kit protocol (Qiagen). In addition to the standard procedures, we conducted an extra centrifugation step to the lysate of 5 min at 13,000 RPM to remove

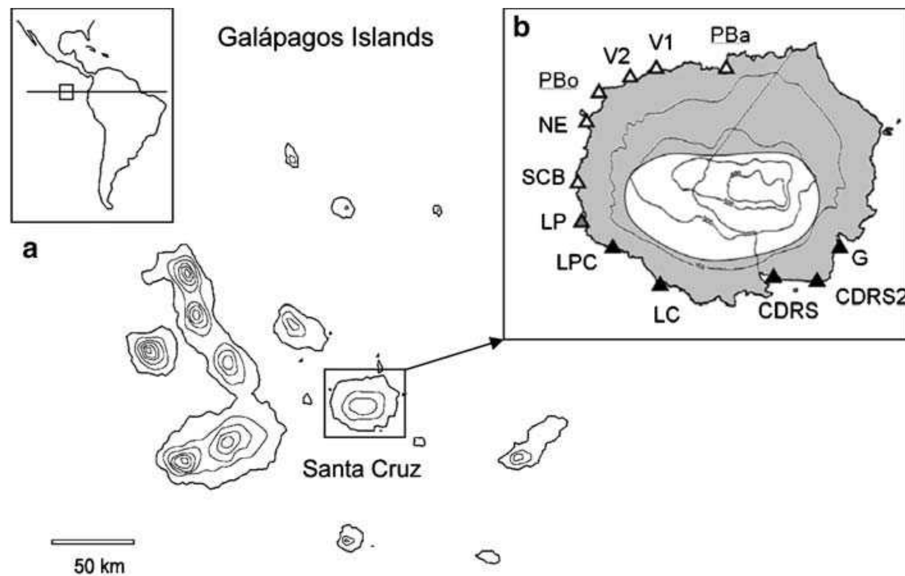

Fig. 1 **a** Galapagos and its location, **b** geographic distribution (*shaded*) and sampling location of *O. echios* varieties on Santa Cruz: *gigantea* (*black triangles*), *echios* (*white triangles*) and morphological intermediates (*grey triangles*). Location abbreviations with numbers of samples between brackets: *PB* Playa Bachas (39), *V1* Venecia1 (11), *V2* Venecia2 (30), *PBo* Punta Bowditch (10), *NE* North of Eden (10), *SCB* South of Cerro Ballena (10), *LP* Las Palmas (25), *LPC* Las Palmas Chica (10), *LC* Los Corales (10), *CDRS1* Charles Darwin Research Station 1 (33), *CDRS2* Charles Darwin Research Station 2 (7), and *G* Garrapatera (40) with number of samples between brackets

most of the precipitates. To maximize overall DNA yield, extraction products were dissolved in 100 µl H_2O. Twelve microsatellite loci (Opuntia1, Opuntia2, Opuntia5, Opuntia6, Opuntia8, Opuntia9, Opuntia10, Opuntia11, Opuntia12, Opuntia13, Opuntia15, and Opuntia17), specifically developed for these taxa were used to genotype the collected samples (Helsen et al. 2007). Primers, primer labeling, and PCR conditions were identical to those described by Helsen et al. (2007). Labeled PCR products were mixed with M13mp8 DNA standards and blue dextran formamide loading dye and heated to 95°C. This mixture was electrophoresed using an automated ALFexpress DNA Sequencer (Amersham Biosciences). Since we did not sequence individual microsatellite PCR products because of possible polyploidy issues, exact allele lengths could not be defined. We therefore assigned allele lengths to two reference individuals using M13 size standards as guidance. By adding these two individuals to all runs, we ensured scoring consistency between gels (Berckmoes et al. 2005). Allele sizes were determined automatically using the program AlleleLocator 1.03 (Amersham Biosciences) and checked manually afterwards. Due to poor quality of some of the material not all individuals could be genotyped for all loci, even after repeated amplifications. These loci were treated as missing data for those specimens. Microsatellite amplification and peak patterns proved to be reproducible as indicated by the reprocessing of 25 individuals. Checking these peak patterns exposed problems in that most individuals had more than two alleles per locus. In order to be able to score peak patterns properly we had to find an explanation for these multiple peaks.

Ploidy analysis

Although polyploidization has played a major role in the evolution of the Cactaceae (Gibson and Nobel 1986), and in the subfamily Opuntioidea 64.3% of the species are polyploid (Pinkava et al. 1998), nothing is known about the ploidy level of Galapagos' Opuntias. Within the Opuntioidea ploidy levels range from di- to octaploid (Pinkava et al. 1998). This makes it crucial to study the chromosome number of the species for an accurate interpretation of the microsatellite peak patterns (Esselink et al. 2004; Nybom et al. 2006).

We used DNA flow cytometry, a method recently reviewed by Dolezel and Bartos (2005), to estimate DNA quantity in a cell nucleus. Because microsatellite analyses were run prior to ploidy analysis, knowledge on the number of alleles was used to make a decision on which samples to analyze. For each of the two varieties and the morphological intermediates the five allele-richest individuals (displaying more than eight bands for some loci) were analyzed together with the five least variable individuals.

To minimize the chance of obstructions in the flow cytometry equipment due to the slimy secretions of the cacti parenchymal layer (Mauseth 1995), only the outermost layer of mature cladodes was used. A 2 cm^2 piece of cuticle was pulled off the plant and most of the remaining parenchymal

cells were removed with a scalpel. The resulting samples were further treated as described by Otto (1990) with some minor adjustments proposed by Nybom et al. (2006). Ploidy analyses were performed on PAS III flow cytometry equipment with mercury lamps (Partec, Münster, Germany). Perennial grass (*Lolium perenne*) was used as an internal standard to calibrate this system (Nybom et al. 2006). *Opuntia quimilo*, *O. sulphurea* var. *hildmannii* and *O. engelmannii*, collected at the National Botanical Garden of Belgium, were used respectively as a diploid (Yuasa et al. 1974) and two hexaploid (Yuasa et al. 1974; Pinkava et al. 1998; Powell and Weedin 2001) references. For each plant two to four independent histograms, plotting the frequency of nuclei versus the fluorescence channel, were produced. Peak positions of our *O. echios* varieties were compared to the reference plants. As discussed further on, all plants appeared to be hexaploid.

Analysis of genetic variation

Due to the polyploid nature of the species studied, partial heterozygosity (Bruvo et al. 2004) makes it impossible to score genotypes exactly. Several methods have been developed to counter this problem, of which two are used here. The most often cited technique scores alleles as presence-absence data (Rodzen et al. 2004); therefore, the resulting data matrix hereafter will be called the P/A dataset. This method scores co-dominant microsatellites as dominant markers thereby reducing information content, and consequently the power of the analysis (Bjorklund 2005). To overcome this problem a relatively novel approach, termed Microsatellite DNA Allele Counting using Peak Ratios (MAC-PR), was used to quantify allelic configuration (Nybom 2004; Esselink et al. 2004; Nybom et al. 2006). This method takes into account that during PCR reactions, abundant alleles within a locus should amplify more often than less abundant ones. The relative peak areas found in peak diagrams are therefore thought to be correlated with the relative number of copies of that allele within the genome (Jenneckens et al. 2001). Using this technique one should correct for differences in amplification success of alleles. Because not all alleles amplify equally, peak area ratios (peak area allele A:peak area allele B) in heterozygotic diploid individuals may differ from 1. One should therefore first evaluate these ratios to be able to correct for this effect (see Esselink et al. 2004 for detailed description of this method). Because of high genetic variability and the hexaploid nature of the study species we sometimes could not define clear correction factors (Esselink et al. 2004). Therefore, we used a more robust method comparable to the one described by Jenneckens et al. (2001).

Genetic diversity of the P/A dataset was estimated for each locality and putative variety using Nei's unbiased diversity estimator for each marker. Total genetic diversity was estimated for localities and varieties by pooling all localities with the same variety. We compared genetic diversity between varieties with a mixed-model ANOVA with variety as fixed effect and locality (nested within group) and allele as random effects. We also tested for inter-locality differences. All statistical analyses were conducted in SAS release 9.1 (SAS Institute Inc.).

Genetic differentiation at different hierarchical levels was calculated on the P/A dataset using analysis of molecular variance (AMOVA) in ARLEQUIN 3.01 (Excoffier et al. 2005). Because AMOVA analysis assumes Hardy–Weinberg equilibrium, the P/A dataset was tested for deviations from this equilibrium using a Bayesian-based deviance information criterion (DIC) selection statistics model implemented in Hickory v1.0.4 (Holsinger et al. 2002; Holsinger and Wallace 2004). The DIC values are comparable to Akaike's information criterion (AIC) in that they take into account both how well a particular model fits the data and the number of parameters that are incorporated in that model.

To overcome potential problems when analyzing dominant P/A datasets, the same Bayesian program (Hickory v1.0.4) was used to estimate parameters related to genetic structure (θ_B: an estimate of F_{ST} under random-effects model population sampling). This Bayesian method does not assume that genotypes are in Hardy–Weinberg proportions within localities, and it does not treat presence–absence phenotypes as haplotypes. It takes full advantage of the information provided by dominant markers, allowing us to incorporate uncertainty about the magnitude of the within-population inbreeding coefficient into estimates of θ_B (Holsinger et al. 2002; Holsinger and Wallace 2004; Ge et al. 2005; Liu et al. 2006). Default settings were used for burn-in (50,000), sampling (250,000), and thinning (50). The Bayesian estimator of genetic diversity is calculated for each of the four models: (1) a full model which includes priors for f, π_i [the mean of the beta distribution (Holsinger et al. 2002)], and θ; (2) f, a F_{IS} analogue, =0 assuming no inbreeding; (3) $\theta_B = 0$ assuming no population structure; and (4) f-free allowing the incorporation of uncertainty about f into the analysis. The four models were applied to the data and evaluated using measures of deviance information criterion (DIC). Pairwise F_{ST} values were also calculated for the MAC-PR dataset using SPAGeDi 1.2 (Hardy and Vekemans 2002). Two-sided P values were calculated with a 20,000 permutation test of individuals and genes. Calculations were made between *echios*, *gigantea*, and morphological intermediates.

Although we are aware of the potential pitfalls when using F_{ST} to calculate gene flow in an indirect way especially in polyploid organisms (Whitlock and McCauley 1999), we used the island model [$F_{ST} = 1/(4Nm + 1)$] to

estimate the number of migrants per generation. Slatkin's private allele method (1985) was used as an alternative.

Population genetic structure

Since the distributions of the two morphological varieties do not overlap, the observed pattern could also be an effect of current or past isolation by distance. We therefore first used a partial Mantel test to verify this. Because of the current debate on the varietal status of *O. echios* var. *echios* and var. *gigantea*, a principal coordinate analysis (PCoA) on the P/A dataset was performed in GenAlEx 6 (Peakall and Smouse 2006) to reveal aggregations of individuals. PCoA differs from PCA in that it uses a distance matrix instead of the raw data. It therefore treats situations with some missing data in a more satisfactory manner than ordinary PCA (Rohlf 1972).

Since differences between the two varieties could be small and due to differences in allele frequencies rather than the presence or absence of an allele, a model-based Bayesian clustering method was used on the MAC-PR dataset to define the most likely number of clusters (STRUCTURE v. 1.0, Pritchard et al. 2000). Here we are not interested in fine scale population structuring within or between locations but want to test for potential cryptic speciation. The clustering method assumes the existence of K subpopulations (cfr. clusters), where each is characterized by a set of allele frequencies at each locus. The model accounts for the presence of Hardy–Weinberg or linkage disequilibrium by introducing population structure and attempts to find population groupings that (as far as possible) are not in disequilibrium. Based on preliminary analysis we evaluated the likelihood for $K = 1$–12. Four independent runs of the Gibbs sampler for each K were evaluated to check for mixing. An admixture model was assumed and the analysis was run with no prior population information. Because of the close relatedness, possible migration, and shared recent ancestor a correlated allele frequency model was used (Pritchard et al. 2000). Based on stationary in several statistics burning length was set to 5×10^4 and 10^6 MCMC replicates. Possible admixture of the morphological intermediates was evaluated to test the hybridization scenario.

Results

Ploidy analysis

Flow cytometry histograms of the 30 tested individuals, representing allele rich and poor individuals of both varieties and their morphological intermediates, all showed peaks comparable to those of *O. sulphurea* var. *hildmannii* and *O. engelmannii*. All taxa under study are therefore considered hexaploid; 37 and 29% of all individuals, however, showed more alleles than this ploidy level for microsatellite markers *Opuntia12* and *Opuntia17*, respectively. Because primers were specifically developed for these varieties, and all bands/alleles are present in a small range of length, we believe that this is not a generic primer problem but more likely a result of them being multicopy genes. Therefore, we excluded microsatellites *Opuntia 12* and *Opuntia17* from all further analyses.

Genetic variability

Genetic variation within all *Opuntia echios* varieties (including morphological intermediates) was high, as for the 10 usable loci 223 bands were scored, of which 99% (221 bands) were polymorphic. All individual plants had unique microsatellite patterns, suggesting that none of them is a vegetative clone of any other sampled cactus. The total number of bands per locus within populations ranged from 6 to 44. When intermediates were excluded, 41 and 26 alleles were exclusively found within respectively *gigantea* and *echios* varieties. The frequency of these private alleles was never above 6%. Four of the private alleles of each variety were also found within the morphological intermediate group which itself had two unique alleles that were not found within any variety.

Genetic diversity indices (calculated using Nei's unbiased diversity estimator) for morphological intermediates, *echios* and *gigantea* varieties were 0.1455, 0.1399, and 0.1597, respectively. Differences between the two named varieties were significant (DF $= 9$, T value $= 3.41$, $P = 0.0077$), but morphological intermediates displayed an intermediate level which was not significantly different from the two varieties (DF $= 9$, T value $= 1.36$ and -0.54, $P = 0.21$ and 0.60 for *echios* and *gigantea* varieties, respectively). There were no significant inter-locality differences, but plants at Los Corales (var. *gigantea*) displayed the highest diversity. The mean frequency of private alleles was 0.0151 and 0.0167 for respectively *echios* and *gigantea* varieties, respectively.

Genetic differentiation

There was a non-significant correlation between geographical and genetic distances ($r = -0.069$, $P \leq 0.35$ under 10^5 permutations). Separate examination of the varieties indicated that this correlation was due to var. *echios* ($r = -0.33$, $P > 0.2$ under 10^5 permutations) rather than to var. *gigantea* ($r = 0.25$, $P > 0.19$ under 10^5 permutations). But overall we can conclude that the observed pattern is not an effect of isolation by distance.

Genetic variation within localities accounted for over 95% of the total genetic variation whether or not

Table 1 Analysis of molecular variance (AMOVA) of the P/A data for *Opuntia echios* varieties on Santa Cruz

Source of variation	Percentage of total variance			
	var. *gigantea*, *echios* and morph. interm.	var. *gigantea* and *echios*	var. *gigantea* and morph. interm.	var. *echios* and morph. interm.
(a)				
Between varieties	1.11	0.73	2.01	1.76
Between localities within varieties	3.4	3.36	1.94	4.86
Within localities	95.49	95.91	96.05	93.39
(b)				
O. echios var. *gigantea*				
Between localities	1.8			
Within localities	98.15			
O. echios var. *echios*				
Between localities	4.94			
Within localities	95.06			

P values estimated with a permutation test (10,000 permutations) were all highly significant ($P < 0.005$)

morphological intermediate individuals were included (Table 1). When analyzing the two varieties separately, differentiation between localities was highest for var. *echios* (Table 1). Differentiation between the earlier proposed varieties was 2% or lower yet statistically significant (Table 1).

Looking at alternative population genetic structures, the lowest DIC value (6,020) was found for the "no inbreeding" model ($f = 0$), followed by the "Full model" (DIC score 6,025). But with the difference in DIC scores higher than 2 (Spiegelhalter et al. 2002) only the "no inbreeding" model deserves consideration. The DIC score for the "no population" structure model (6,773) indicated that there are genetic differences among varieties. According to the f free model, Θ^B an F_{ST} analogue was 0.0367 ± 0.0038.

According to the MAC-PR dataset, F_{ST} values (Table 2) showed a low but significant genetic differentiation between the two varieties. Gene flow estimated on pairwise F_{ST} values was: $Nm = 35.5$. The private allele method (Slatkin 1985) suggest an estimate of $Nm = 32.3$ and 26.51 for gene flow from *gigantea* to *echios* and *echios* to *gigantea*, respectively.

The results from the PCoA are presented in Fig. 2. The first, second, and third PC accounted for respectively 20.14, 17.79, and 16.80% of the total variation detected among individuals. There were no clear separations between the two named varieties (Fig. 2). However, there is a slight tendency for the intermediates to be clustered with *gigantea* rather than with *echios* varieties (Fig. 2a, b). Overall this provides little evidence for genetic structuring within the dataset.

Because we might be dealing with cryptic speciation, and only 54% of the variation in the present markers is included in the first three principal components, we used a second method to reveal potential genetic structuring. The Bayesian clustering approach implemented in STRUCTURE v1.0 (Pritchard et al. 2000) yielded estimated Ln probabilities for $1 \leq K \leq 12$ that ranged from −17,703 to −15,730. LnP(D) values decreased with increasing K values, coming to a plateau. As mentioned by the designers of the program, $\log(P(X|K))$ gives a reliable idea of the correct number of clusters (Pritchard et al. 2000; Falush et al. 2003). However, the differences in $\log(P(X|K))$ for $K = 1$, $K = 2$, and $K = 3$ were too small to make any decisions. Also the alternative and more precise method to define the optimal number of clusters, ΔK (Evanno et al. 2005), is not applicable for $K = 1$. Therefore, we checked the biological relevance of the two ($K = 2$) and three cluster ($K = 3$) scenarios, by calculating the mean percentage of ancestry of the two varieties and the intermediates to these newly constructed clusters (Table 3). Just like the intermediates, *gigantea* and *echios* varieties were evenly distributed over the two or three inferred clusters, giving evidence for no population genetic structuring ($K = 1$). For $K = 2$ (Table 3a), intermediates were more likely assigned to the second cluster. Because this second cluster is not representative of the *echios* variety but rather a newly constructed subpopulation, this does not contradict the earlier described position of the intermediates.

Table 2 Pairwise F_{ST} values based on MAC-PR dataset according to SPAGeDi and corresponding two sided *P* values (in italic)

	var. *gigantea*	var. *echios*
var. *echios*	0.0070	
	<0.001	
intermediates	0.0150	0.0175
	0.0014	*0.0008*

F_{ST} between all localities 0.0194 ($P < 0.001$)

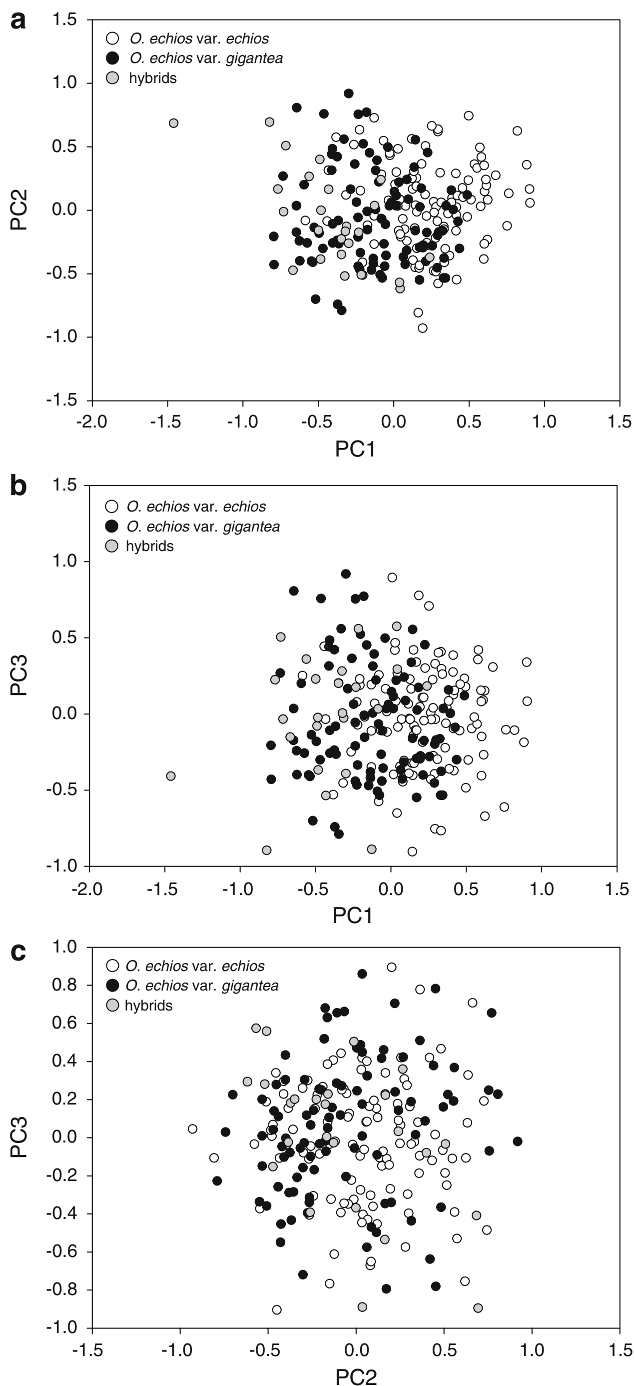

Fig. 2 Two-dimensional representation of the Principal Coordinate Analysis (PCoA) of the P/A dataset for all 235 individuals with **a** PC1 versus PC2 2, **b** PC versus PC3 and 2, **c** PC2 versus PC3

Discussion

High levels of genetic variability were found within all studied *Opuntia echios* localities on Santa Cruz. These contrast with the low levels of genetic (allozyme) variability within Galapagos *Opuntia* species described earlier (Browne et al. 2003) and will have consequences for conservation. Using standard population genetic approaches, the global fixation index was low ($F_{ST} = 0.007$) but statistically significant. We should however be cautious interpreting this value because highly variable markers, such as microsatellites, may yield statistically significant measures of differentiation (F_{ST} values) that have no biological relevance (Hedrick 1999). When we verified the biological significance of potential genetic substructuring, using PCoA and Bayesian clustering, no pattern was exposed, implying we should not restrict our view to this significance. Our analysis suggests high levels of gene flow between the earlier described varieties, which could be attributed to insect-mediated pollen flow, and/or seed dispersal by birds and reptiles (Grant and Grant 1981; Loveless and Hamrick 1984; Guerrero and Tye, unpublished data). Carpenter bees *Xylocopa darwinii* and Cactus finches *Geospiza scandens* act as the major and most mobile pollinators (Jackson 1993 and personal observation) and Oedemerinae beetles visit flowers at night, clearly distributing pollen (P. Verdyck, personal observation). Mocking birds *Nesomimus* spp. and Darwin's finches Geospizinae have been reported as intra-island seed dispersers (Arbogast et al. 2006, Guerrero and Tye, unpublished data). Short-eared owls *Asio flammeus* who prey upon these finches are therefore potential long-distance dispersers (Grant et al. 1975). But when it comes to germination success, tortoises and land iguanas are the most effective dispersers. Seeds deposited in their excrements have the highest germination rates (Estupiñan and Mauchamp 1995).

On the other hand low F_{ST} may reflect recent divergence. Although Galapagos is a relatively young archipelago (<9 Myr), time of the first *Opuntia* colonization may even be more recent. During the last Myr climate at Galapagos shifted from hot and wet to dry (Seltzer et al. 2002), making it less likely for cactus species to colonize or survive on these islands earlier than during the last Myr. If colonization was very recent, the high genetic variability would imply a large number of colonizers.

Low F_{ST} values come together with no clear population genetic structure. Earlier findings on these plants such as: relatively dense and continuous population structure, presumed outcrossing, a relatively long lifespan, insect pollination, and seed dispersal by birds and reptiles (Loveless and Hamrick 1984), suggested Hicks and Mauchamp (1996) that population genetic structuring would be unlikely, except where strong barriers such as significant bodies of sea water or lava flows separate two populations. However, at present no such barriers are to be found within Santa Cruz. With the two varieties sharing the same ploidy level, there is also no barrier in genome structure permitting differentiation. All these factors can contribute to the very weak population genetic structure observed. We were

Table 3 Proportion of membership of the two predefined varieties and the morphological intermediates in each of the (a) two (b) three constructed clusters

Variety	Inferred cluster (*K*)			Number of individuals
	1	2	3	
(a)				
Gigantea	54.4 ± 32.4	45.6 ± 33.1		96
Echios	40.9 ± 36.4	59.1 ± 35.9		110
Intermediates	36.7 ± 36.5	63.3 ± 36.5		25
(b)				
Gigantea	33.8 ± 9.6	35.9 ± 9.8	30.3 ± 8.5	96
Echios	28.4 ± 7.9	30.8 ± 8.7	40.7 ± 10.2	110
Intermediates	29.5 ± 10.4	44.8 ± 10.9	25.7 ± 7.8	25

unable to tell the two varieties apart on a molecular genetic basis, and likewise could not genetically define morphological intermediates. These results suggest that there are no genetic discontinuities between *Opuntia* cacti living at different localities on Santa Cruz.

It has been suggested that wind and competition for light may explain phenotypic variability within Galapagos *Opuntias* (Arp 1973; Racine and Downhower 1974; Nobel 1981). Moreover, relative humidity is known to be an important factor in plant growth rate (Malda et al. 1999a, b). Precipitation data from 1995 until 2004 (unpublished data) indicated that rainfall on the southern part of the island, where the tree-like *gigantea* variety is to be found, is ten times higher than on the northern side. This is a first clear indication of a variable environmental setting correlated with phenotypic variability within these species. The current study conforms to others mentioning high morphological but low genetic differentiation ($Q_{ST} > F_{ST}$), which is commonly interpreted as evidence for divergent selection and adaptation to local environments (e.g. Latta and Mitton 1997; Storz 2002; Volis et al. 2005). The question remains however whether the observed phenotypic differences are caused by an adaptive genetic response to this environmental difference (a result of changes in genes we did not sample), phenotypic plasticity, or an epigenetic basis (e.g. Rapp and Wendel 2005) among others. Not knowing which genes influence this phenotypic variability we strongly believe common garden or transplanting experiments could help resolve this question, but the slow growing nature of these plants makes such experiments difficult. As long as there is uncertainty on the exact reason for this phenotypical divergence, conservation planning should aim to preserve both morphological varieties.

The lack of genetic differentiation between the two named varieties makes us question the taxonomic validity of these and other *Opuntia* taxa. We therefore plan to study gene flow between neighbouring islands and the genetic differentiation between all Galapagos' *Opuntia* taxa using microsatellites and other molecular tools.

Acknowledgments This work is financially supported by a BOF-NOI project (FAO70400/4) at the Evolutionary Biology Group of the University of Antwerp. We thank Willem De Smet for his practical support, Peter Galbusera and Kurt Jordaens for their useful comments on the first draft, and the Entomology Department of the Royal Belgian Institute of Natural Sciences for their financial support. This is Contribution 1064 of the Charles Darwin Research Station, the institute that provided cooperation and field logistics support. We thank the National Botanical Garden of Belgium, and especially Viviane Leyman, for letting us collect reference *Opuntia* species. We are grateful to the Institute for Agricultural and Fisheries Research (ILVO) where flow cytometry analysis took place, and Natalie Van Houtte who supported us in the lab.

References

Arbogast BS, Drovetski SV, Curry RL et al (2006) The origin and diversification of Galapagos mockingbirds. Evolution 60:370–382

Arp GK (1973) The Galápagos *Opuntias*: another interpretation. Noticias de Galápagos 21:33–37

Berckmoes V, Scheirs J, Jordaens K et al (2005) Effects of environmental pollution on microsatellite DNA diversity in wood mouse (*Apodemus sylvaticus*) populations. Environ Toxicol Chem 24:2898–2907

Bjorklund M (2005) A method for adjusting allele frequencies in the case of microsatellite allele drop-out. Mol Ecol Notes 5:676–679

Browne RA, Anderson DJ, White MD, Johnson MA (2003) Evidence for low genetic divergence among Galápagos' *Opuntia* cactus species. Noticias de Galápagos 62:11–13

Bruvo R, Michiels NK, D'Souza TG, Schulenburg H (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. Mol Ecol 13:2101–2106

Darwin C (1858) On the origin of species by means of natural selection. Murray, London

Dawson EY (1965) Further studies of *Opuntia* in the Galápagos archipelago. Cact Succ J 37:135–148

Desender K, Baert L, Maelfait JP, Verdyck P (1999) Conservation on Volcan Alcedo (Galápagos): terrestrial invertebrates and the impact of introduced feral goats. Biol Conserv 87:303–310

Dolezel J, Bartos J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot 95:99–110

Esselink GD, Nybom H, Vosman B (2004) Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting-peak ratios) method. Theor Appl Genet 109:402–408

Estupiñan B, Mauchamp A (1995) Interacción planta–animal en la dispersión de *Opuntia* de Galápagos. Conference Proceeding. II Congreso Nacional de Botánica. Quito, Ecuador

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Ge XJ, Zhou XL, Li ZC et al (2005) Low genetic diversity and significant population structuring in the relict *Amentotaxus argotaenia* complex (Taxaceae) based on ISSR fingerprinting. J Pl Res 118:415–422

Gibson AC, Nobel PS (1986) The cactus primer. Harvard University Press, Cambridge

Goldstein DB, Schlötterer C (1999) Microsatellites: evolution and applications, 3 edn. Oxford University Press, New York

Grant BR, Grant PR (1981) Exploitation of *Opuntia* cactus by birds on the Galápagos. Oecol 49:179–187

Grant PR, Smith JNM, Grant BR, Abbott IJ, Abbott LK (1975) Finch numbers, owl predation and plant dispersal on Isla Daphne Major. Galápagos Oecol 19:239–257

Hardy OJ, Vekemans X (2002) SPAGeDI: a versatile computer program to analyse spatial genetic structure at the individual or population level. Mol Ecol Notes 2:618–620

Hedrick PW (1999) Perspective: highly variable loci and their interpretation in evolution and conservation. Evolution 53:313–318

Helsen P, Verdyck P, Tye A et al (2007) Isolation and characterization of polymorphic microsatellite markers in Galapagos prickly pear (*Opuntia*) cactus species. Mol Ecol Notes 7:454–456

Hicks DJ, Mauchamp A (1996) Evolution and conservation biology of the Galápagos Opuntias (Cactaceae). Haseltonia 4:89–102

Hicks DJ, Mauchamp A (2000) Population structure and growth patterns of *Opuntia echios* var. *gigantea* along an elevational gradient in the Galápagos Islands. Biotropica 32:235–243

Holsinger KE, Wallace LE (2004) Bayesian approaches for the analysis of population genetic structure: an example from *Platanthera leucophaea* (Orchidaceae). Mol Ecol 13:887–894

Holsinger KE, Lewis PO, Dey DK (2002) A Bayesian approach to inferring population structure from dominant markers. Mol Ecol 11:1157–1164

Howell JT (1933) The Cactaceae of the Galápagos Islands. Proc Calif Acad Sci 21:41–54

IUCN (2007) IUCN Red list of threatened species (2007). http://www.iucnredlist.org

Jackson MH (1993) Galápagos: a Natural History guide. University of Calgary Press, Calgary

Jenneckens I, Meyer JN, Horstgen-Schwark G et al (2001) A fixed allele at microsatellite locus LS-39 exhibiting species-specificity for the black caviar producer *Acipenser stellatus*. J Appl Ichthyol 17:39–42

Latta RG, Mitton JB (1997) A comparison of population differentiation across four classes of gene marker in limber pine (*Pinus flexilis* James). Genetics 146:1153–1163

Liu K, Goodman M, Muse S et al (2003) Genetic structure and diversity among Maize inbred lines as inferred from DNA microsatellites. Genetics 165:2117–2128

Liu YF, Wang Y, Huang HW (2006) High interpopulation genetic differentiation and unidirectional linear migration patterns in *Myricaria laxiflora* (Tamaricaceae), an endemic riparian plant in the Three Gorges valley of the Yangtze River. Am J Bot 93:206–215

Loveless MD, Hamrick JL (1984) Ecological determinants of genetic structure in plant populations. Ann Rev Ecol Syst 15:65–95

Malda G, Backhaus RA, Martin C (1999a) Alterations in growth and crassulacean acid metabolism (CAM) activity of in vitro cultured cactus. Pl Cell Tiss Org 58:1–9

Malda G, Suzan H, Backhaus R (1999b) In vitro culture as a potential method for the conservation of endangered plants possessing crassulacean acid metabolism. Sci Hortic (Amsterdam) 81:71–87

Mauchamp A (1997) Threats from alien plant species in the Galápagos islands. Biol Conserv 11:260–263

Mauseth JD (1995) Collapsible water-storage cells in cacti. Bull Torrey Bot Club 122:145–151

Mayr E (1942) Systematics and the origin of species. Columbia University Press, New York

Nobel PS (1981) Influences of photosynthetically active radiation on cladode orientation stem tilting, and height of cacti. Ecology 62:982–990

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 13:1143–1155

Nybom H, Esselink GD, Werlemark G, Leus L, Vosman B (2006) Unique genomic configuration revealed by microsatellite DNA in polyploid dogroses, *Rosa* sect. *Caninae* J Evol Biol 19:635–648

Otto F (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewicz Z, Crissman HA (eds) Methods in cell biology. Academic Press, New York, pp 105–110

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Pinkava DJ, Rebman JP, Baker MA (1998) Chromosome numbers in some cacti of western North America-VII. Haseltonia 6:32–41

Porter DM (1983) Vascular plants of the Galápagos: origin and dispersal. In: Bowman RI, Benson M, Leviton AE (eds) Patterns of evolution in Galápagos organisms. AAAS Pacific Division, San Fransisco, pp 33–96

Powell AM, Weedin JF (2001) Chromosome numbers in Chihuahuan Desert Cactaceae. III. Trans-Pecos Texas. Am J Bot 88:481–485

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Racine CH, Downhower JF (1974) Vegetative and reproductive strategies of *Opuntia* in the Galápagos Islands. Biotropica 6:175–186

Rapp RA, Wendel JF (2005) Epigenetics and plant evolution. New Phytol 168:81–91

Rebman JP, Pinkava DJ (2004) *Opuntia* cacti of North America—an overview. Fla Entomol 84:474–483

Rodzen JA, Famula TR, May B (2004) Estimation of parentage and relatedness in the polyploid white sturgeon (*Acipenser transmontanus*) using a dominant marker approach for duplicated microsatellite loci. Aquaculture 232:165–182

Rohlf FJ (1972) Empirical comparison of 3 ordination techniques in numerical taxonomy. Syst Zool 21:271–280

Seltzer GO, Rodbell DT, Baker PA et al (2002) Early warming of tropical South America at the Last Glacial–interglacial transition. Science 296:1685–1686

Slatkin M (1985) Rare alleles as indicators of gene flow. Evolution 39:53–65

Spiegelhalter DJ, Best NG, Carlin BR, van der Linde A (2002) Bayesian measures of model complexity and fit. J R Stat Soc B 64:583–616

Stewart A (1911) A botanical survey of the Galapagos islands. Proc Calif Acad Sci IV 1:7–288

Storz JF (2002) Contrasting patterns of divergence in quantitative traits and neutral DNA markers: analysis of clinal variation. Mol Ecol 11:2537–2551

Tye A (2005) Restoration of the vegetation of the dry zone in Galápagos. Lyonia 1–24

Volis S, Yakubov B, Shulgina I, Ward D, Mendlinger S (2005) Distinguishing adaptive from nonadaptive genetic differentiation: comparison of Q(ST) and F-ST at two spatial scales. Heredity 95:466–475

Whitlock MC, McCauley DE (1999) Indirect measures of gene flow and migration: FST not equal $1/(4Nm + 1)$. Heredity 82:117–125

Wiggins IL, Porter DM (1971) Flora of the Galápagos Islands. Stanford University Press, Stanford

Yuasa H, Shimizu H, Kashiwai S, Kondo N (1974) Chromosome numbers and their bearing on the geographic distribution in the subfamily Opuntioideae (Cactaceae). Rep Inst Breed 4:1–10