Variation at five polymorphic microsatellite loci was used to investigate genetic diversity and differentiation of two tetraploid Canarian endemics, *Bencomia exstipulata* and *B. caudata*. Data were analysed and are discussed in terms of tetrasomic (autotetraploid) and disomic (allohexaploid) inheritance. In both cases, genetic diversity values were similar to those described in other tetraploid plant species. High genetic differentiation between the only two described natural populations of *B. exstipulata* was detected ($F_{ST} = 0.411$). Bayesian cluster analysis revealed a geographical structure with distinct genetic groups from each island. High genetic differentiation and low genetic diversity of the *B. exstipulata* population from Tenerife suggest a recent population bottleneck, perhaps caused by the most recent major volcanic eruption, for this natural locality. This may be heightened by possible inbreeding depression and the monoecy of these species. Polymorphic microsatellite loci were also tested across all species in the *Bencomia* alliance. These reliably amplified the target sequence, suggesting a high degree of conservation of the sequences flanking the microsatellites. © 2009 The Linnean Society of London, Botanical Journal of the Linnean Society, 2009, 160, 429–441. ADDITIONAL KEYWORDS: Allotetraploid – autotetraploid – *Bencomia caudata* – conservation genetics – endangered species – tetraploidy.

INTRODUCTION

Polyploidy is a major process in plant speciation, providing opportunities for rapid diversification. The success of polyploids can be explained by several factors, including increased heterozygosity and genetic diversity, multiple copies of genes (and therefore enzymes) and increased biochemical diversity (Levin, 1983; Soltis & Soltis, 2000). Their establishment in nature may be favoured by vegetative (asexual) multiplication and perennial habitat (Soltis & Soltis, 2000). Moreover, polyploids often have a broader ecological amplitude than the parental diploids and a better colonizing ability, resulting in availability of new ecological niches (Soltis & Soltis, 2000). Recent genetic studies suggest that some lineages may undergo one or more episodes of polyploidization followed by extensive diploidization (Soltis, Soltis & Tate, 2003).

Polyploids are commonly categorized as either autopolyploids or allopolyploids, depending on their presumed origin (Stebbins, 1950; Soltis & Soltis, 2000). Allopolyploids result from an increase of chromosome
number through hybridization and subsequent chromosome doubling and they are characterized by bivalent formation and disomic inheritance. In contrast, autopolyploids result from chromosome doubling of genetically similar genomes by fusion of unreduced gametes (Stebbins, 1950; Soltis & Soltis, 2000) and are expected to form multivalents at meiosis and to exhibit polysomic inheritance (Stebbins, 1947; Jackson & Casey, 1982; Levin, 1983). Most documented cases involve allopolyploidy or segmental allopolyploidy (Stebbins, 1947, 1950; Grant, 1981) and few examples of well-documented autopolyploidy are known from nature. Grant (1981) noted that ‘old polyploids tend to be more diploid-like than newly formed polyploids’. This process, which is known as diploidization, affects both the cytological behaviour and genic constitution of polyploids (Soltis et al., 2003).

Models of population structure based on codominant markers have been mainly developed for diploids. Alterations to the most common estimates of gene flow and genetic diversity have also been developed for autotetraploids (Ronfort et al., 1998), but studies of genetic structure in polyploid plant species are still rare and they focus mainly on cases in which the events that gave rise to polyploids are recent. Population studies of palaepolyploid species are even scarcer because of the lack of information concerning the origin of such polyploids and the lack of analytical tools to interpret codominant markers (Thrall & Young, 2000).

The Bencomia alliance forms part of tribe Sanguisorbeae in subfamily Rosoideae of Rosaceae. In addition to the three genera in the alliance (Bencomia Webb & Berthel., Dendriopoterium Svent. and Marcetella Svent.), the tribe includes 14 other genera (Hutchinson, 1964; Pérez de Paz, 2004). The genera in the Bencomia alliance are all endemic to the Macaronesian region. Bencomia is a Canarian endemic genus with four species (B. extispulata Svent., B. caudata Webb & Berthel., B. brachystachya Svent. and B. sphaerocarpa Svent.) that occupy different and non-overlapping ecological zones (Sventenius, 1948; Helfgott et al., 2000) and show both monoecy and dioecy. Both species of Dendriopoterium (D. mendozii Svent. and D. pulidoi Svent. ex Bramwell) are dioecious and also restricted to the Canary Islands. Marcetella consists of two species M. moquiniana (Webb & Berthel.) Svent. and M. maderensis (Bornm.) Svent. that are endemic to the Canary Islands and Madeira, respectively; both species are monoecious. The group is monophyletic. Welsh (1997), based on a morphological cladistic analysis, recovered a putatively monophyletic group, broadly in agreement with the current generic delimitation of the endemic genera of the Bencomia alliance, albeit requiring the transfer of Marcetella maderensis to Bencomia. However, neither Welsh (1997), using internal transcribed spacer 1 (ITS1) sequences, nor Helfgott et al. (2000), using nuclear ribosomal DNA (nrDNA) ITS1 and ITS2 sequences, fully clarified the molecular phylogenetic relationships among the members of the alliance. The Bencomia alliance has been hypothesized to be a relic of the European subtropical flora of the Tertiary period or, alternatively, a recently derived group that has acquired woodiness on the islands (Helfgott et al., 2000; Carine et al., 2004). All members are wind-pollinated, long-lived shrubs. Like most members of Sanguisorbeae, all species in the alliance are tetraploids with \(2n = 28\) and a base chromosome number of \(x = 7\). The Bencomia alliance has been considered one of the most important groups in the Macaronesian flora of a relicual nature and the study of the reproductive ecology and biogeography of the group may help to clarify evolutionary events in these island ecosystems.

Bencomia extispulata has been catalogued as ‘critically vulnerable’ in the National Endangered Species Catalogue (BOE, 1998) and it is also listed in the Spanish Red List of Vascular Flora (VV.AA., 2000) and in Annex I of the Bern Convention. It is known from only two natural populations located within the boundaries of National Parks: Tajodeque (Caldera de Taburiente National Park, La Palma) with 21 individuals and Tiro del Guanche (Teide National Park, Tenerife) with 52 plants. These natural populations have suffered a severe reduction in size because of human activity and pressure from introduced herbivores (e.g. goats and rabbits) and the remaining plants are located on steep slopes prone to landslides at the edge of the lava flow. Bencomia extispulata has been reported to be dioecious, with occasional hermaphrodite flowers on female plants (Nordborg, 1966), although recent studies have shown it to be monoecious with mixed female and male inflorescences or exclusively female plants and occasional hermaphrodite flowers (Pérez de Paz, 2004).

Since the 1990s, the National Park of Caldera de Taburiente (La Palma) and the National Park of Teide (Tenerife) have carried out conservation activities by implementing a Recovery Plan for B. extispulata to increase the size of the populations, area of occupancy and mitigate the threatening factors. Seeds and vegetative material from natural sources were used to establish new populations. As a result, the IUCN category was re-evaluated and changed from endangered to vulnerable (Bañares et al., 2003). Although only material from each island was used for reintroduction, the lack of knowledge about genetic diversity and structure of the natural populations and the genetic composition of the material used for planting has made conservation management difficult and
specific genetic conservation strategies are needed to avoid stochastic events (Sosa et al., 2002). The populations and the individuals involved in such reintroductions are of primary importance for developing and implementing successful programmes.

*Bencomia caudata*, known from four islands in the Canarian Archipelago (El Hierro, La Palma, Tenerife and Gran Canaria; Izquierdo et al., 2004) has been classified as vulnerable in the Spanish Red List of Vascular Flora (VV.AA., 2000). The inflorescences in *B. caudata* are mainly unisexual, but small numbers of flowers of the opposite sex can be found at the base of the inflorescence (Nordborg, 1966; Pérez de Paz, 2004).

Microsatellite loci have been characterized for *B. exstipulata* and *B. caudata* (González-Pérez et al., 2004b). Their usefulness in other members of the *Bencomia* alliance could also prove to be important as a conservation tool for other endangered species (e.g. *B. brachystachya*, listed in the Red Data Book as critically endangered by Bañares et al., 2003).

The aims of this study were: (1) to investigate the level of genetic diversity, differentiation and spatial genetic structure in natural and reintroduced populations of *B. exstipulata*, in order to contribute to the implementation of the Recovery Plan for the species; (2) to compare the genetic structure of two congeneric species with different sexual systems and different population sizes; (3) to compare genetic parameters under different models of inheritance (auto- vs. allotetraploid) in *B. exstipulata*; and (4) to evaluate the cross-amplification ability of the microsatellite loci in other species of the *Bencomia* alliance.

**MATERIAL AND METHODS**

**PLANT MATERIAL**

Sampling for *B. exstipulata* included 64 known individuals from both natural populations, 21 from La Palma (LPN) and 43 from Tenerife (TFN) (Fig. 1, Table 1). In addition, 128 samples from reintroduced plants from different localities on each island were

![Figure 1](https://academic.oup.com/botlinnean/article-abstract/160/4/429/2418383)

**Figure 1.** Locations of natural (●) and reintroduced (○) populations of *B. exstipulata* and *B. caudata* (▲). Population codes are given in Table 1.

analysed; 76 from La Palma (LPR) and 52 from Tenerife (TFR). For *B. caudata*, 57 individuals were collected outside the boundaries of the National Parks: Bco. Seco (BSE) and Bco. Suitos (BAUI) in La Palma and Bco. Añavingo (BAV) in Tenerife (Fig. 1, Table 1). Whereas, the BSE and BAUI populations were made up of only 15–30 individuals, the BAV population included around 300 specimens.

In order to test cross amplification of the microsatellite loci isolated for *B. exstipulata* and *B. caudata* (González-Pérez et al., 2004b) in the remaining *Bencomia* alliance species, samples of *B. brachystachya* (9 individuals), *B. sphaerocarpa* (9), *D. menedezii* (19), *D. pulidoi* (24), *M. maderensis* (2) and *M. moquiniana* (42) were also analysed.

**Microsatellite analysis and genotyping**

DNA was extracted from silica-gel dried young leaves using a modified 2 × cetyl trimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). Forward and reverse primers described by González-Pérez et al. (2004b) were used to amplify five polymorphic microsatellite loci. PCR amplifications were carried out following the protocols in González-Pérez et al. (2004b). The products were detected using an ABI 3100 Genetic Analyzer and fragment sizes were determined using GeneScan version 2.02 and GENOTYPER version 1.1 (Applied Biosystems Inc.). In order to allow for uncertainty about the model of inheritance, both auto- and allotetraploidy were assumed. Under the autotetraploid (tetrasomic inheritance) model, we identified allele peak profiles at each locus and assigned a genotype to each individual. In those individuals with two or three different alleles, genotypes were characterized considering values for peak area provided by the software. Peak area ratio was compared with the relationship between each pair of alleles expected in a tetraploid individual (Esselink, Nybom & Vosman, 2004). For those individuals in which allele dosage did not correspond to the expected ratio, a ‘null’ allele was considered. For the allotetraploid model, as each allele cannot be assigned to a separate chromosomal locus we performed a binary coding of the simple sequence repeat (SSR) data with the presence or absence of each fragment recorded, similar to the method often used with dominant markers (e.g. AFLPs).

**Data analysis**

Under the autotetraploid model, genetic diversity indices; allelic richness (*A*), allelic richness within individuals (*Ai*), genotypic richness (*G*), observed (*Ho*) and expected (*He*) heterozygosity and fixation index (*F*) were calculated from genotype data per population using AUTOTET software (Thrall & Young, 2000). In autotetraploids, two types of *He*, and therefore *F*-value, can be estimated: the expected heterozygosity and fixation index assuming (1) random chromosomal segregation or (2) some level of chromatid segregation (Bever & Felber, 1992; Ronfort et al., 1998). Chromatid segregation occurs if double reduction takes place. We do not know if double reduction takes places in *Bencomia* and therefore we estimated *He* and *F* assuming chromosome and chromatid segregation.

In order to clarify genetic differentiation among populations, overall genetic divergence within species and pairwise divergence among populations were analysed using the *F*<sub>ST</sub> approach (Weir & Cockerham, 1984). Genetic differentiation measures (1000 permutations) were calculated using SPAGeDi 1.0 (Hardy & Vekemans, 2002). A multivariate representation of populations analysed was carried out by subjecting pairwise estimates of *F*<sub>ST</sub> to a multidimensional scaling analysis (MSD) in SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

Population structure was also inferred using a Bayesian clustering procedure (implemented in STRUCTURE; Pritchard, Stephens & Donnelly, 2000) to identify the *K* (unknown) populations of origin of the sampled individuals and to assign the individuals
GENETIC DIVERSITY IN BENCOMIA EXSTIPULATA 433

simultaneously to the populations. The most likely value of $K$ is assessed by comparing the likelihood of the data for different values of $K$. Populations and individuals were assigned to one cluster if their proportion of membership ($q_i$) to that cluster was equal to or larger than 0.05. We assumed the model to be of population admixture and that the allele frequencies are independent. We conducted a series of independent runs for each value of $K$ (the number of subpopulations) between 1 and 7. Analyses consisted of $10^5$ burn-in period replicates and a run length of $10^6$ replicates.

For the autotetraploid model, genetic diversity indices such as gene diversity ($H$), Shannon diversity ($I$) and the proportion of polymorphic loci ($P$) and genetic distance among populations (Nei, 1972) were calculated using POPGENE 3.2 software (Yeh et al., 1997). The resulting tree was visualized using TreeView software (Page, 1996). The genetic differentiation among populations was studied using the genetic differentiation coefficient ($F_{ST}$) calculated using the genetic analysis software ARLEQUIN version 3.1 (Excoffier, Laval & Schneider, 2005). This method was also used to estimate genetic differentiation among natural and cultivated populations.

RESULTS

GENETIC DIVERSITY AND STRUCTURE

Autotetraploid assumption

Forty-nine alleles were detected at five polymorphic microsatellite loci. Allelic richness ranged from 1.6 alleles per locus in TFR to 6.2 alleles per locus in BAV (Table 2). Observed heterozygosity ($Ho$) varied from 0.32 (TFN) to 0.55 (BAV).

Although the natural population of $B$. exstipulata from Tenerife (TFN) is twice the size of the natural population from La Palma (LPN), the genetic diversity detected in TFN ($A = 2.40; G = 2.00$) is approximately half that observed in LPN ($A = 5.20; G = 6.20$). For all analysed loci, a higher number of alleles was found in La Caldera de Taburiente National Park (21 alleles) than in El Teide National Park (12). Seventeen alleles were exclusive to La Palma whereas two alleles (MS4-182 and MS6-187) were exclusive to the natural population of Tenerife (TFN). In addition, 14 different genotypes were identified in the natural population from La Palma (LPN), but only seven were recorded on Tenerife (TFN). The diversity recorded in both natural and reintroduced populations from Tenerife is as a result exclusively of the locus MS-2. For the other four loci, all individuals showed the same genotype.

Assuming chromosome segregation, values of expected heterozygosity ($He$) ranged from 0.27 (TFR) to 0.68 (BAV). Values of observed heterozygosity were lower than those of expected heterozygosity in all populations except TFN and TFR (Table 2). The value of the fixation index ($F_{CE}$) estimated over all loci was significantly positive ($P < 0.01$) in all populations analysed, except TFN and TFR ($F = -0.10, F = -0.21$, respectively), suggesting a deficit of heterozygotes in most populations analysed. Considering chromatid segregation (maximum double reduction, $\alpha = 1/7$), $He$

Table 2. Genetic variation in the analysed populations of Bencomia exstipulata and B. caudata under the assumption of autotetraploidy and under the assumption of autotetraploidy

<table>
<thead>
<tr>
<th>Population</th>
<th>Autotetraploid model</th>
<th>Allotetraploid model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A$</td>
<td>$Ai$</td>
</tr>
<tr>
<td>LPN</td>
<td>5.20</td>
<td>1.98</td>
</tr>
<tr>
<td>LPR</td>
<td>4.80</td>
<td>1.72</td>
</tr>
<tr>
<td>TFN</td>
<td>2.40</td>
<td>1.50</td>
</tr>
<tr>
<td>TFR</td>
<td>1.60</td>
<td>1.50</td>
</tr>
<tr>
<td>Average for $B$. exstipulata</td>
<td>3.50</td>
<td>1.67</td>
</tr>
<tr>
<td>BSE</td>
<td>5.00</td>
<td>2.04</td>
</tr>
<tr>
<td>BSUI</td>
<td>4.20</td>
<td>1.99</td>
</tr>
<tr>
<td>BAV</td>
<td>6.20</td>
<td>2.21</td>
</tr>
<tr>
<td>Average for $B$. caudata</td>
<td>5.13</td>
<td>2.08</td>
</tr>
</tbody>
</table>

$A$, allelic richness (average allele per locus); $Ai$, allelic richness within individuals (average alleles per individual at a locus); $G$, genotypic richness (the number of four allele genotypes at a locus); $Ho$, observed heterozygosity; $He$, expected heterozygosity; $F$, fixation index; ($Ce$), $He$ and $F$ assuming chromosome segregation; ($Cd$), $He$ and $F$ assuming chromatid segregation; $H$, Nei's (1972) genetic diversity; $I$, Shannon's information index; $P$, percentage of polymorphic loci.

Population codes as in Table 1.
ranged from 0.25 to 0.63 (BAV), whereas F-values were significantly positive in all populations, except TFN and TFR ($F = -0.17$ and $F = -0.30$, respectively), indicating an excess of heterozygotes in these two populations. Expected heterozygosity values were higher than those of observed heterozygosity in all populations except for natural and cultivated populations from Tenerife (TFN and TFR). On the whole, estimations assuming chromosome segregation did not differ significantly from those calculated assuming chromatic segregation. Taking this into account, and considering that there is no evidence of multivalent formation in Bencomia, we assumed for the remaining data analysis that only chromosomal segregation occurs. This allows a more conservative data processing method to be used when attempting to determine the type of polyploid origin for this species (auto- or allopolyploid).

**Allotetraploid assumption**

Fixed heterozygous diallelic profiles were observed for locus MS-4 and MS-7 in all individuals from natural and cultivated populations of Tenerife. In addition, fixed homozygous profiles were observed for locus MS-6 and MS-9 in these populations.

Under the allotetraploid assumption genetic diversity ($I$) range from 0.037 (TFN) to 0.227 (LPN). Genetic diversity detected in LPN (0.227) was four times greater than those observed in the natural population from Tenerife (0.056; Table 2). Similarly to the results obtained under the tetrasomic inheritance model, the cultivated population from La Palma (LPR) maintained 89% of the total genetic diversity ($P$) detected in the natural population (LPN). The cultivated population from Tenerife (TFR) only included 33% of the genetic variation ($P$) estimate in the natural one (TFN).

**GENETIC DIFFERENTIATION AMONG POPULATIONS AND SPECIES**

**Autotetraploid assumption**

Pairwise F$_{ST}$ values ranged from 0.030 (between LPN and LPR) to 0.569 (between TFR and BSE) (Table 3). Pairwise comparisons of F$_{ST}$ among populations suggest a high genetic differentiation between the two natural populations of *B. exstipulata* (0.411). In addition, genetic differentiation among *B. exstipulata* samples from La Palma and Tenerife was greater than that observed between the former and any *B. caudata* population. In contrast, average genetic differentiation among *B. caudata* populations was 0.087 (Table 3).

The first two dimensions of the multidimensional scaling analysis (MSD) together accounted for 98.7% of the total variance (Fig. 2). The first dimension separated TFN and TFR populations from the rest, whereas LPN and LPR from *B. caudata* populations (BSUI, BSE and BAV) was resolved by the second dimension. In the multidimensional space defined by MSD, *B. exstipulata* populations from La Palma (LPN and LPR) were closer to *B. caudata* than *B. exstipulata* populations from Tenerife (TFN and TFR).

In the Bayesian analysis, using the total data set (249 individuals, five microsatellite loci, seven sampled populations) and $K = 1–7$, the probability of the data was maximum with $K = 4$, suggesting that the individuals analysed can be split into four distinct genetic clusters (Table 4). *Bencomia exstipulata* natural individuals from La Palma were assigned to three different clusters; clusters I (LPN$_{q1} = 0.092$), III (LPN$_{q3} = 0.377$) and IV (LPN$_{q4} = 0.525$), whereas LPR individuals were assigned to only clusters III (LPR$_{q3} = 0.731$) and IV (LPR$_{q4} = 0.252$). However, natural and reintroduced populations from Tenerife showed a higher proportion of mean individual membership in cluster II ($q_{II} = 0.977$ and 0.976, respectively).

Table 3. F$_{ST}$ values for all pairwise populations analysed under the autotetraploid model (above diagonal) and under the allotetraploid model (below diagonal)

<table>
<thead>
<tr>
<th>Population</th>
<th>LPN</th>
<th>LPR</th>
<th>TFN</th>
<th>TFR</th>
<th>BSE</th>
<th>BSUI</th>
<th>BAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPN</td>
<td>0.030**</td>
<td>0.411***</td>
<td>0.433***</td>
<td>0.118***</td>
<td>0.067*</td>
<td>0.112***</td>
<td></td>
</tr>
<tr>
<td>LPR</td>
<td>0.161***</td>
<td>0.396***</td>
<td>0.417***</td>
<td>0.194***</td>
<td>0.141*</td>
<td>0.202***</td>
<td></td>
</tr>
<tr>
<td>TFN</td>
<td>0.697***</td>
<td>0.614***</td>
<td>0.083***</td>
<td>0.550***</td>
<td>0.476***</td>
<td>0.429***</td>
<td></td>
</tr>
<tr>
<td>TFR</td>
<td>0.721***</td>
<td>0.632***</td>
<td>−0.020NS</td>
<td>0.569***</td>
<td>0.479***</td>
<td>0.453***</td>
<td></td>
</tr>
<tr>
<td>BSE</td>
<td>0.188**</td>
<td>0.115*</td>
<td>0.730***</td>
<td>0.755***</td>
<td>0.081**</td>
<td>0.116***</td>
<td></td>
</tr>
<tr>
<td>BSUI</td>
<td>0.139**</td>
<td>0.173***</td>
<td>0.711***</td>
<td>0.730***</td>
<td>0.053NS</td>
<td>0.063**</td>
<td></td>
</tr>
<tr>
<td>BAV</td>
<td>0.245***</td>
<td>0.387***</td>
<td>0.401***</td>
<td>0.424***</td>
<td>0.277***</td>
<td>0.134***</td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

Population codes as in Table 1.
respectively). Thus, the STRUCTURE analysis revealed a geographical structure related to islands and it provides further evidence of a close genetic relationship among *B. exstipulata* from La Palma and *B. caudata*.

In *B. caudata*, individuals from BAV (Tenerife) were located in clusters I (BAVqI = 0.888) and IV (BAVqIV = 0.094), BSE individuals can be attributed to clusters I (BSEqI = 0.711), III (BSEqIII = 0.191) and IV (BSEqIV = 0.056), whereas BSU individuals could be assigned to all clusters considered (BSUqI = 0.642, BSUqII = 0.181, BSUqIII = 0.115 and BSUqIV = 0.0061) (Table 4).

### Allotetraploid assumption

Pairwise *F*\textsubscript{ST} values ranged from 0.000 (between TFN and TFR) to 0.753 (between TFR and BSE). As with the autotetraploid assumption, genetic differentiation between both natural populations of *B. exstipulata* was high and significant (0.697, *P* < 0.001; Table 3), whereas, average *F*\textsubscript{ST} values among *B. caudata* populations was 0.155. The neighbor joining tree based on Nei’s (1972) genetic distance clustered together *B. exstipulata* populations from La Palma and *B. caudata*, whereas *B. exstipulata* from Tenerife was separated from these groups (Fig. 3).

AMOVA analysis (Table 5) revealed that the percentage of variation was maintained among islands (60%) but, within each island, variation was contained within populations (36%) at the expense of variation among populations within islands (4%). Low variation among natural and reintroduced populations (4%) indicates that both National Parks have picked up most genetic diversity of the natural populations of *B. exstipulata* in the reintroduced ones.

### Genetic conservation

There is no substantial loss of genetic diversity in the reintroduced populations of *B. exstipulata* from La Palma (LPR) in comparison with the natural one (LPN). In fact, of the 24 alleles recorded in the natural population from La Palma (LPN), 23 (96%) were also detected in the reintroduced localities (LPR). Allele MS7-126, recorded in the reintroduced population (LPR), was not observed in any individual from the natural one. On Tenerife, only 11 alleles (64.7%) of the 17 recorded in the natural populations...
were detected in the reintroduced population (TFR).

**Cross-species amplification**

For all five loci, primers amplified the target sequence across all species tested, except in *M. moquiniana*, where no products were amplified for locus MS-9. In addition, 15 new alleles not detected in *B. exstipulata* and *B. caudata* were recorded, six of these at locus MS-4 (Table 6).

**DISCUSSION**

**Genetic diversity and structure**

The proportion of species with unisexual flowers (both monoecious and dioecious) on oceanic islands is higher than that found in continental floras (Bawa, 1980; Schultz & Ganders, 1996), but the factors that could facilitate the change from hermaphroditism to unisexuality are still unknown. In general, it is accepted that dioecious species would have higher levels of genetic diversity among individuals than monoecious species and, in hermaphroditic systems, predominantly selfing populations would have lower diversity than outcrossing ones (Hamrick & Godt, 1990). As well as low population numbers in *B. exstipulata*, its monoecious condition could therefore also result in lower genetic diversity compared with its dioecious congener *B. caudata* (Table 2). In addition, this result is in agreement with the wider distribution and greater population size of *B. caudata*. Genetic diversity parameters estimated for *B. exstipulata* using microsatellites were similar to those detected in other endemic and/or endangered species (Fréville et al., 2000; Bottin et al., 2005; Ueno et al., 2005). Despite this, 60% loci analysed showed heterozygosity deficiency. This effect could be because of a bottleneck effect followed by genetic drift, although polyploid inheritance could reduce the effects of genetic drift. In tetraploids, each locus can accommodate up to four different alleles, instead of two alleles for a diploid organism. Thus, tetraploid inheritance should theoretically lead to the maintenance of greater heterozygosity in natural populations of polyploids (Soltis & Soltis, 2000). Average genetic variability values found in natural populations of *B. exstipulata* (*Ho = 0.45*) are similar to those described in other tetraploid species such as *Arracacia xantrhorrhiza* Banc. (Apiaceae: *Ho = 0.44*; Morillo et al., 2004), *Shorea ovalis* (Korth.) Blume ssp. *sericea* (Dyer) P.S.Ashton (Dipterocarpaceae: *Ho = 0.50*; Kevin, Lee & Koh, 2004), but higher than in the endangered tetraploid *Borderea chouardii* Gaussen & Heslot (Dioscoreaceae: *Ho = 0.14*; Segarra-Moraguez et al., 2005) and lower than those recorded in other tetraploid species, such as the pioneer *Betula pubescens* Ehrh. ssp. *tortuosa* (Ledeb.) Nyman (Betulaceae: *Ho = 0.67*; Truong et al., 2005), the invasive weed *Bryophyllum delagoense* (Eckl. & Zeyh.) Schinz (Crassulaceae: *Ho = 0.72*; Hannan-Jones et al., 2005) and the self-pollinating *Echium vulgare* L. (Boraginaceae: *Ho = 0.95*; Korbecka et al., 2003).

Both natural and reintroduced populations from Tenerife showed the lowest heterozygosity and a reduced number of alleles (Table 2). Moreover, these populations showed signatures of bottlenecks (lower allelic richness and observed heterozygosity than any other population) and a high population differentiation with natural (average *Fst* = 0.422) and reintroduced (average *Fst* = 0.406) populations of *B. exstipulata* from La Palma. The natural population from Tenerife is situated inside the Teide National Park. The 52 individuals that comprise this population survive on the edge of a lava flow. The last eruption from the volcano El Teide in 1798 produced a lava flow that covers an area of 4.5 km² (Araña & Carracedo, 1978; Martinez-Pison & Quirantes, 1981; Carracedo & Day, 2002), which is inside the bound-
aries of the National Park. Most of the individuals forming the original population were probably exterminated by the lava flow and the few individuals left on the edges were the founders of the current smaller population. In this scenario, the bottleneck followed by genetic drift suffered in this site was so severe that polyploid inheritance could not mask it. The depauperate genetic diversity in Tenerife is also reflected in its reintroduced population (TFR), as all individuals used for propagation were originally from Tenerife.

In populations of autotetraploid species, equilibrium frequencies under random mating are reached after several generations, not in a single generation as for randomly mating diploid species (Bever & Felber, 1992). This can theoretically result in deviations from Hardy–Weinberg equilibrium (Mahy et al., 2000). Positive fixation index values were found in most of the analysed populations, indicating a deficiency of heterozygosity compared with Hardy–Weinberg equilibrium. A heterozygosity deficiency could be explained as a result of different factors: (1) unrecognized genetic structure within populations; (2) inbreeding as a result of reduced population size; or (3) presence of null alleles (Pemberton et al., 1995). The presence of null alleles is unlikely, however, as the sets of primers used to amplify these loci also worked in other genera of the alliance (see below). Thus, inbreeding, as a result of the small size of both natural populations of B. exstipulata is likely to be the cause of the heterozygosity deficiency observed.

**GENETIC DIVERSITY AMONG POPULATIONS AND SPECIES**

On the one hand, the high genetic differentiation among natural Bencomia populations (Table 3, Fig. 2) could be explained because they are located in different islands. High differentiation among populations is a common situation in oceanic islands in general (Francisco-Ortega et al., 2000) and in the Canary Islands in particular (Francisco-Ortega et al., 2000; Batista et al., 2001, 2004; González-Pérez, 2001; Bouza et al., 2002; Sosa et al., 2002; González-Pérez, Caujapé-Castells & Sosa, 2004a). Nevertheless, the differentiation between populations from each island differs between the species studied here, as $F_{ST}$ values among B. caudata populations from Tenerife (BAV)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Variant components</th>
<th>Percentage of variation</th>
<th>Fixation indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among islands</td>
<td>1</td>
<td>2.335</td>
<td>59.67</td>
<td>$F_{CT} = 0.597^{**}$</td>
</tr>
<tr>
<td>Among populations within islands</td>
<td>2</td>
<td>0.158</td>
<td>4.04</td>
<td>$F_{SC} = 0.100^{**}$</td>
</tr>
<tr>
<td>Within populations</td>
<td>187</td>
<td>1.420</td>
<td>36.28</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>190</td>
<td>3.914</td>
<td></td>
<td>$F_{ST} = 0.637^{***}$</td>
</tr>
</tbody>
</table>

**$P < 0.01; \; ***P < 0.001.$**

---

**Table 6. Cross-species amplification of five microsatellite loci in eight Bencomia alliance species, showing allele size range (bp) detected in B. exstipulata and B. caudata, and new alleles recorded in the other species from the alliance**

<table>
<thead>
<tr>
<th>Species</th>
<th>MS-2</th>
<th>MS-4</th>
<th>MS-6</th>
<th>MS-7</th>
<th>MS-9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(GA)$_{10}$</td>
<td>(GA)$_{10}$</td>
<td>(GT)$_{10}$</td>
<td>(GAA)$_{6}$</td>
<td>(CAA)$<em>{5}$ ... (GCA)$</em>{6}$</td>
</tr>
<tr>
<td>Bencomia caudata Webb &amp; Berthel.</td>
<td>183-200</td>
<td>162-196</td>
<td>188-212</td>
<td>105-123</td>
<td>282-307</td>
</tr>
<tr>
<td>Bencomia brachystachya Svent. &amp; Nordborg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bencomia sphaerocarpa Svent.</td>
<td>+</td>
<td>177</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dendriopoterium menendezii Svent.</td>
<td>+</td>
<td>185</td>
<td>208</td>
<td>+</td>
<td>279, 285</td>
</tr>
<tr>
<td>Dendriopoterium pulidoi Svent. ex Bramwell</td>
<td>182</td>
<td>167, 175, 208</td>
<td>+</td>
<td>279, 285, 317</td>
<td></td>
</tr>
<tr>
<td>Marcetella maderensis (Bornm.) Svent.</td>
<td>+</td>
<td>173</td>
<td>+</td>
<td>99, 109</td>
<td>294</td>
</tr>
<tr>
<td>Marcetella moquiniana (Webb &amp; Berthel.) Svent.</td>
<td>190</td>
<td>165, 173</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+, successful amplification; −, no amplification.
and La Palma (BSE and BSUI) were three and four times lower than among those for *B. exstipulata*. As populations of both species are influenced by the same geographic barriers, gene flow alone could not explain the high genetic differentiation detected between populations of *B. exstipulata* from each island. However, different breeding strategies could explain the different \( F_{ST} \) values detected in both species. On the other hand, the volcanic eruption, which is assumed to have drastically reduced the population size of *B. exstipulata*, did not affect the extant populations of *B. caudata* in Tenerife, as these two species occupy different ecological zones, and this historical event alone could have been sufficiently catastrophic to produce this result. Reduced or no gene flow between islands could explain the genetic differentiation among all natural populations, whereas genetic drift after a massive reduction in population size may have increased the genetic differentiation observed in *B. exstipulata* in Tenerife. Nevertheless, natural populations of *B. exstipulata* from both islands show lower genetic diversity than *B. caudata*. Our results, together with the fact that putative hybrids have been recorded in cultivation (A. Palomares, pers. comm.), suggest that these *Bencomia* spp. are genetically closely related, possibly products of a recent radiation. Thus, a combination of a strong extinction event on the natural population from Tenerife, along with putative inbreeding depression, as a result of the monoecious condition of *B. exstipulata*, could explain the results obtained.

### Genetic Conservation of *B. exstipulata*

In general, genetic variability indices and genotype analysis indicate that most genetic diversity detected in the natural populations of *B. exstipulata* from La Palma is present in the reintroduced populations (Table 2). In addition, a missing allele (MS7-126) from the natural population of La Palma (LPN) has been rescued in the reintroduced populations (LPR) at a low frequency. Although this allele may result from a recent mutation in the reintroduced population, it could also come from one of the three individuals from the natural populations that were successfully cultivated before they died in 1993 (A. Palomares, pers. comm.). In addition, the genetic structure detected within the natural population in La Palma (LPN: clusters III and IV) was also reflected in the cultivated one (LPR), indicating that the LPR population is representative of the natural one (LPN). However, the fact that 73.1% individuals from LPR (Table 4) were assigned to cluster III, suggests that most of the reintroduced individuals in La Palma came from individuals belonging to cluster III of the natural population. In 1993, seeds from three random individuals of the natural population from La Palma were planted in a reintroduced population. Of these individuals, only one (B-503) could be attributed to cluster IV. Over 2 years these three plants produced 12 new cultivated individuals which have been used to produce most of the current reintroduced plants. Of these 12 individuals, only two were assigned to cluster IV. Thus, conservation efforts in La Palma should be focused on those individuals assigned to cluster IV in the natural population.

In contrast, conservation efforts in Tenerife should be focused on the reinforcement of the natural population in order to mitigate endogamy. To minimize the risks derived from catastrophic phenomena (volcanic activity, landslides) or climatic variability (droughts), new populations should be established in suitable habitats.

The low genetic diversity found in the reintroduced population from Tenerife (TFR) is a reflection of the low genetic variability in the natural source (TFN). In order to obtain populations with an adequate distribution of genotypes, further reintroductions in Teide National Park are actually focused on the rare alleles that have not been recovered yet in the reintroduced sites. However, several factors linked to the reproductive biology and ecology of the species should be considered, as some rare genotypes correspond to male or hermaphroditic individuals that produce few seeds. Demographic and reproductive studies (age classes and recruitment) have been promoted from Teide National Park prior to defining more specific conservation strategies for this population in Tenerife. Also, for both islands, ex-situ conservation by asexual propagation and controlled pollinations are conducted to preserve rare or unique genotypes and to generate progenies.

The relationship between the diversity of natural vs. reintroduced populations is different in each island. Reintroduced populations from La Caldera de Taburiente (LPR) show similar genetic diversity to that found in the natural population. In contrast, in the island of Tenerife reintroduced populations (TFR) display lower genetic variability indices than those for the natural population (TFN) (Table 2).

Populations from both islands are genetically isolated. The lack of gene flow between them is highlighted by the presence of exclusive alleles for La Palma and Tenerife and high \( F_{ST} \) values (Table 3). Compared with the diversity found in La Palma, the population from Tenerife is genetically impoverished, which is reflected in the cultivated population as well. Nevertheless, any attempt to rescue this population by means of genetic influx of new genetic diversity from La Palma should be avoided. It is dangerous to draw conclusions about the viability of populations.
exclusively based on observed levels of molecular variation (Ouborg, Vergeer & Mix, 2006). In addition, adaptive differentiation as a result of geographic isolation and local adaptations might lead to outbreeding depression (Montalvo & Ellstrand, 2001). The magnitude of this outbreeding depression increases linearly with genetic distance (Sosa et al., 2002; Edmans & Timmerman, 2003). Levels of contemporary gene flow between the islands are unknown and common garden experiments to test for adaptive differentiation are necessary to evaluate outbreeding depression risks.

**AUTOTETRAPLOID VS. ALLOTETRAPLOID INHERITANCE MODELS**

In order to confirm the inheritance model in *B. exstipulata* with certainty, a more detailed study which includes cytogenetic analysis or genetic analysis of controlled crosses is needed. Nevertheless, comparing the results obtained with our different analyses, we can draw some preliminary conclusions. Although no fixed heterozyosity was observed, both unbalanced and balanced heterozygotes were recorded in all possible combinations. If allotetraploids are derived from close relatives (e.g. hybridization between sibling species), or if autoploids have recently acquired disomic inheritance, even under an infinite allele model, variation and unbalanced heterozygotes will be seen. Moreover, in the case of microsatellites, stepwise mutations may easily lead to apparent polysomic inheritance in a disomic lineage, in an effect sometimes called ‘cryptic disomy’ (De Silva et al., 2005).

We have acquired three pieces of evidence that suggest disomic inheritance: (1) all the alliance members are tetraploid, suggesting that tetrasomic inheritance must also have been the case for their common ancestor. Autopolyploids diploidize over time and often evolve disomic inheritance rapidly after formation. Genomic rearrangements, gene silencing and other genomic processes apparently diploidized genomes of species over time to such an extent that they now appear to be functionally diploid (Gaut et al., 2000; Vision, Brown & Tanksley, 2000); (2) multivalent formation has not been seen; (3) in two of the five microsatellite loci analysed (MS-4 and MS-7), fixed heterozygous diallelic profiles were observed in all individuals analysed in the natural population of Tenerife, which complies better with the assumption of allotetraploidy. We note that populations of *B. exstipulata* from La Palma do not show fixed heterozygosity. We know that differences in the rate at which disomy is restored can lead to heterogeneity in the mode of inheritance within a species (Sears, 1969).

**CROSS-SPECIES AMPLIFICATION**

Crucial to the use of microsatellites for assessing the genetic variation within and among species is the ability of primers from one species to amplify homologous loci in related species (Kijas, Fowler & Thomas, 1995). One aim of the present work was to determine to what extent the primer pairs designed for the amplification of microsatellite loci in *B. exstipulata* and *B. caudata* (González-Pérez et al., 2004b) could be used for assessing genetic diversity in other members of the *Bencomia* alliance. Although only few individuals of each taxon were tested, 15 new alleles were recorded for the loci analysed. The allele size difference between *B. exstipulata* and species such as *D. meneedzii*, *D. pulidoi*, *M. maderensis* and *M. moquiniana* range from ± 2 to ± 10 bp (Table 6). The utility of the primers to amplify products across the group was demonstrated, indicating a high degree of conservation of the primer sequences. This in turn implies a close genetic relationship between all the species of the *Bencomia* alliance and suggests a relatively recent radiation. The microsatellite loci presented here could be used to evaluate the genetic variability of other endemic species within the group. Four out of eight species in the alliance are endangered with different levels of risk, from vulnerable to critical (Bañares et al., 2003). Studies of this type could help in the effective management of populations. In addition, phylogenetic relationships within the alliance are not fully resolved by molecular means (Helfgott et al., 2000). Highly variable loci, such as the flanking regions of microsatellites, could potentially be used to evaluate relationships among closely related species (Rossetto, McNally & Henry, 2002).

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**REFERENCES**


Kijas JM, Fowler CS, Thomas MR. 1995. An evaluation of sequenced tagged microsatellite site markers for genetic


