Allopolyploid Origin of the Mediterranean Endemic, *Centaurium bianoris* (Gentianaceae), Inferred by Molecular Markers

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**ABSTRACT.** *Centaurium bianoris* (Gentianaceae) is restricted to Majorca, the main island of the Balearic Archipelago. This tetraploid species is characterised by salmon-coloured corollas (var. *bianoris*), but pink (var. *roseum*) and yellow (var. *sulfureum*) varieties have also been described. An allopolyploid origin has been proposed between the diploids *C. maritimum* (yellow flowers) and *C. tenuiflorum* var. *acutiflorum* (pink flowers), both occurring on Majorca and in other places of the Mediterranean basin. In this study, we tested the proposed hybrid origin of *C. bianoris* by using RAPD fingerprinting, and both direct and cloned sequences of the nuclear ribosomal ITS, and the chloroplast *trnLF* regions. Our molecular data confirmed the hypothesis of an allotetraploid origin of *C. bianoris* via hybridisation between *C. tenuiflorum* and *C. maritimum*, the latter being the maternal parent. The so-called varieties *roseum* and *sulfureum* appeared to be only floral morphs that may have arisen via genomic processes such as gene silencing. Hybridisation is probably the cause of the ITS sequence polymorphism observed in *C. bianoris*, whereas backcrosses with either parent may be responsible for the apparent bidirectional homogenisation observed in ITS clones. Finally, the polyphyletic behaviour of *C. bianoris* on the ITS cladogram, combined with the differential rates of homogenisation observed in ITS sequences, may denote a recurrent origin for that taxon. This result contrasts with the narrow distribution of *C. bianoris*, compared to that of its diploid parents, suggesting instead a single origin for this hybrid.

**KEYWORDS:** allopolyploidy, Balearic Islands, *Centaurium*, endemism, Gentianaceae, RAPD.

Since the beginning of the 20th century, polyploidy (i.e. evolution by genome duplication) has been recognised as a major evolutionary force leading to plant speciation (Stebbins 1971; Grant 1981; Levin 2002). Until the 1980’s, the mechanisms generating polyploids and the evolutionary processes acting in their genomes were poorly documented. However, in the last twenty years, molecular biology and genetics have greatly enhanced our knowledge of the different processes characterising polyploid occurrence and evolution (Wendel 2000; Liu and Wendel 2003; Soltis et al. 2003).

Natural polyploids have been classified into different categories, depending on the degree of homology among coexisting genomes. Allopolyploidy is a polyploidisation process involving both interspecific (and intergeneric) hybridisation followed by chromosome doubling. Hence, allopolyploids contain two (or more) homeologous genomes that have diverged before hybridisation and polyploidisation. Allopolyploids are thought to be the predominant form of polyploidy in flowering plants although this hypothesis has not been fully demonstrated (Soltis et al. 2003). Many questions concerning the emergence and the evolution of allopolyploids have been recurrently addressed (Ramsey and Schemske 1998, 2002; Soltis et al. 2003), such as the mode and frequency of polyploid emergence in natural populations, and more recently the processes leading to the genome evolution of newly arisen allopolyploids (Wendel 2000; Liu and Wendel 2003). An abundant literature has been devoted to the mode of polyploid formation (e.g., Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Although in some taxa somatic doubling may be responsible, fertilisation between unreduced gametes is thought to be the predominant mode of polyploid emergence. Furthermore, recent analysis of polyploid genomes has considerably strengthened the idea that multiple origins are common in generating polyploids (Soltis and Soltis 1993; Leitch and Bennett 1997; Soltis and Soltis 1999; Soltis et al. 2003). Few examples of polyploid taxa that seem to have originated once have been documented (Ainouche et al. 2004).

The recent analysis of genome evolution after allopolyploidisation has revealed an unsuspected dynamism within allopolyploid genomes (Soltis and Soltis 1993; Leitch and Bennett 1997; Wendel 2000; Soltis et al. 2003), including intragenomic rearrangement, intergenomic movement of genetic material, and concerted evolution. Extensive intragenomic reorganisation, involving chromosomal translocations or inversions, and intergenic recombination following homeologous chromosome pairing at meiosis (Leitch and Bennett 1997; Soltis and Soltis 1999; Levin 2002; Soltis et al. 2003), may result in the loss of chromosome- or genome-specific sequences or may generate new polymorphism in neopolyploids (Song et al. 1995; Kovarik et al. 2004; Lim et al. 2004). On the other hand, concerted evolution leads to both intra- and interlocus
gene conversion, and subsequent sequence homogenisation (Elder and Turner 1995; Wendel et al. 1995; Kovarik et al. 2004). Furthermore, concerted evolution may either act in the direction of one diploid parental species or the other in different populations (Wendel et al. 1995).

The primarily Mediterranean genus Centaurium Hill (Gentianaceae) is an ideal group to investigate abrupt speciation via hybridisation and polyploidy. This group, commonly known as ‘‘centauries,’’ mainly comprises annual or biennial species that form polyploid complexes in the Mediterranean basin and surrounding areas (Zeltner 1970; Mansion 2004; Mansion et al. 2005). Hybridisation has previously been described in the genus (Melderis 1931) and episodes of allopolyploidy have been well documented in the British Isles (Ubsdell 1979). Centaurium bianoris, a tetraploid species (2n = 4x = 40) endemic to Majorca (Balearic Islands, Spain), has been hypothesised to have arisen via a polyploidy event (Contandriopoulos and Cardona 1984). This species generally shows salmon-coloured corollas (var. bianoris), but yellow (var. sulfureum) and pink (var. roseum) forms have also been described and treated as separate varieties (Zeltner 1978). Owing to the additive patterns observed in flower colour and chromosome number, a hybrid origin of C. bianoris has been postulated involving two diploid Majorcan species: C. maritimum (yellow corollas, 2n = 2x = 20) and C. tenuiflorum subsp. acutiflorum (pink corollas, 2n = 2x = 20) (Zeltner 1978). Centaurium maritimum has a disjunct and possibly relict distribution mostly in the western part of the Mediterranean basin (Zeltner 1970; Mansion et al. 2005), while C. tenuiflorum is more widespread, with a circum-Mediterranean distribution (Fig. 1). Both species sometimes occur in sympatric populations in the Mediterranean (A. Guggisberg & G. Mansion, pers. obs.), but so far, C. bianoris has been encountered only on Majorca.

The goals of the present study are: (1) to test the hypothesis of a hybrid origin of C. bianoris; (2) if of hybrid origin, to explore the patterns of genome evolution (e.g., concerted evolution of ITS sequences) following allopolyploidisation; and (3) to investigate alternative hypotheses concerning the formation (i.e., multiple vs. single origin coupled with introgression) of this polyploid species.

**Materials and Methods**

**Plant Sampling.** Plants were collected during spring and summer 2000–2001, on the mainland (southern France and Italy).
and on several Mediterranean islands (Majorca, Corsica, and Sar- 
dinia; Fig. 1). The sampling strategy was designed to cover the
geographical range where both hypothetical parents occur. Thir-
teen populations of C. binorius were collected on Majorca, and were
mainly represented by the salmon variety (over 95% of the indi-
viduals sampled; Appendix 1). Eighteen populations of C. mariti-
mum and 16 populations of C. tenuiflorum were also collected. As
no Majorcan populations of C. maritimum were found, none were
available for RAPD analyses, and one herbarium specimen was
used for DNA sequencing. Vouchers have been deposited at the
herbarium of Neuchâtel (NEU).

Flow Cytometry and Chromosome Counts. We determined the
ploidy level of each accession using either flow cytometry analyses
or chromosome counts.

In the first case, seeds collected in the field were sown in the
greenhouse to obtain fresh leaf material. The ploidy level of each
seedling was then estimated by quantifying the relative DNA con-
tent of interphase nuclei by flow cytometry as described in Bre-
tagnolle (2001). Leaves were chopped in FACScan flow buffer (Becton
Dickinson, USA), in order to extract cell nuclei. The resulting so-
lution was stained with propidium iodide, and analysed using a
FACStrak flow cytometer (Becton Dickinson, USA). The accuracy
of the analyses was checked by comparison with an internal stan-
dard (chicken red blood cells, CRBC, BioSure, USA) and specimen
controls (individuals with known chromosome number).

For chromosome counts, floral buds or root tips were fixed in
Carnoy’s solution (absolute ethanol and glacial acetic acid in a final
3:1 volume) for at least 24 h, then stained with acetic carmin for 1 h, gently heated for 2 min, and squashed in acetic carmin. Chro-
mosome numbers were then obtained from observation of either
mitosis in ovary tissues, or meiosis in pollen mother cells (tetrads).

DNA Isolation and PCR Amplification. Total DNA was ex-
tracted from leaves dried in silica gel by using the DNeasy Plant
Mini Kit (Qiagen, Switzerland), according to the manufacturer’s in-
nstructions.

RAPD FINGERPRINTING. In this study, we developed random
amplified polymorphic DNA (RAPD) markers (Williams et al. 1990),
a method that has been extensively used to investigate hy-
bridisation processes in plants, most notably in polyploid com-
plexes (Smith et al. 1996; Brochmann et al. 1998; Neuffer et al.
1999; Steen et al. 2000). RAPD amplifications were carried out in
25 µl reaction volumes containing 1X buffer, 1 mM MgCl₂, 0.4X
Q solution, 0.1 mM dNTPs, 0.4 µM primer, and one unit Taq poly-
merase (Qiagen). A DNA-free mix was run as negative control, to
take into account no contamination occurred. Among the 60 RAPD prim-
ers surveyed (kits OPB, OPP, and OPI from Operon Technologies
Inc.), we chose those producing readable, reproducible, and poly-
morphic bands. For each selected primer, optimal annealing tem-
perature was tested on a gradient thermocycler (Biometra). The
selected amplification conditions were as follows: a first cycle at 94
°C for 4 min; 40 cycles at 93°C for 1 min, 41°C or 48°C for 1 min
(depending on the primer) and 72°C for 1 min; and a final cycle of
15 min at 72°C. Amplified products were then separated on 1.5%agarose gels stained with ethidium bromide; in the presence of a
molecular weight standard (100 bp ladder, BioLabs, USA). Each
PCR was repeated twice in order to assess the reproducibility of
the method.

DNA SEQUENCING. Both nuclear and chloroplast genomes
were used as source of sequence data to reconstruct molecular
phylogenies. The internal transcribed spacer region of nrDNA (ITS),
as well as amplified primers ITS4 and ITS5 (Baldwin et al. 1995).
The invariable 5.8S intron was excluded from the analyses. ITS
sequences have been successfully used to reconstruct molecu-
lar phylogenies within the Gentiana family (e.g., Mansion and
Struwe 2004), or to detect parental additivity in putative hybrids (e.g.,
Sang et al. 1995; O’Kane et al. 1996; Campbell et al. 1997;
Fuertes Aguilar et al. 1999; Widmer and Baltisberger 1999; An-
dreasen and Baldwin 2003). The trnL intron plus trnL-trnF spacer
(hereafter called trnLF) of cpDNA were amplified using primers
trnl-c, trnL-d, trnl-e and trnL-f, respectively (Taberlet et al. 1991).
The tools (individuals of known nrDNA and cpDNA markers enables
the detection of reticulate patterns (Wendel and Doyle 1998). In
this aspect, an allopolyploid will share the biparental inherited
nrDNA with both progenitors and the maternal cpDNA with the
seed parent (Corriveau and Coleman 1988).

Polymerase chain reactions (PCR) were performed in a final
volume of 25 µl containing 1X buffer, 2 mM MgCl₂, 0.1 mM dNTPs,
0.2 µM of each primer, and one unit Taq polymerase (Promega,
USA). Amplifications were carried out on a Biometra thermocycler
using the following conditions: a first cycle at 94°C for 3 min; 30
cycles at 94°C for 10 s, 50°C for 20 s and 72°C for 1.5 min; and a
final cycle of 4 min at 72°C. PCR products were purified using QIAquick PCR columns (Qiagen), and sequenced using the
BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied
Biosystems) according to the manufacturer’s protocols, but using a
5 µl excess. Reaction of dye terminators and primers were re-
moved by a sodium-acetate precipitation, and purified products
were sequenced on an Applied Biosystems 310 DNA automated
sequencer (Applied Biosystems, USA). The DNA sequences ob-
tained were carefully checked for accuracy and the presence of
polymorphic sites such as Sequence Navigator (Applied Biosystems,
USA).

CLONING. Direct sequencing of ITS repeats produced hetero-
geneous electropherograms in accessions of C. binorius. Therefore,
PCR products from four individuals of C. binorius (including the
respective salmon, pink, and yellow varieties) were cloned by li-
gation into a pCR2.1-TOPO v5.0 vector (Invitrogen, Basel). The
clones were verified and then transformed according to the manu-
facturer’s instructions (TOPO TA Cloning® kit, Invitrogen, Basel).
After growing the col-
oneies at 37°C overnight on agar plates with Luria-Bertani (LB)
medium and ampicillin, white colonies were randomly selected
and their ITS inserts amplified using universal primers M13F and
M13R. The PCR amplifications were performed in a final volume
of 25 µl containing 1X buffer (including 1.5 mM MgCl₂), 0.625 mM
MgCl₂, 0.2 mM dNTPs, 1.25 µl DMSO, 0.325 µM of each primer,
and one unit Taq Polymerase using the following cycling condi-
tions: a first cycle at 95°C for 5 min; 35 cycles at 95°C for 45 s,
55°C for 1 min and 72°C for 1.5 min; and a final cycle of 10 min
at 72°C. Sequencing reactions were prepared as described above,
using the same ITS primers. Sequencing products were then pu-
rified on 96-well multiscreen filter plates (Millipore) to remove
eccess excess dye terminators, and run on an ABI Prism 3100 automated
sequencer (Applied Biosystems). Sequencer 4.2 (Gene Codes
Corp.) was used to compile contiguous sequences (contigs) of each
accession and check for accuracy.

Molecular Analyses. For RAPD analyses, each selected frag-
ment was coded for absence (0) or presence (1) in a data matrix
(available upon request from the main author), and transformed in
a distance matrix by using the Dice coefficient (Dice 1945),
which takes into account shared presence of RAPD bands but not
shared absence and assigns a higher weight to shared presence
than to dissimilarity. The Dice distance matrix was then used to
produce a phylogram via the Neighbour Joining clustering method
implemented in the Clustering Calculator program (http://
phylogram was visualised with the TreeView software (Page
1996).

Sequence alignment was done by eye because it was straight-
forward; only a few gaps had to be included to align the ingroup
with the outgroup. For the parental repeats that did not vary in
length, the polymorphic sites found in the direct accessions of C.
binorius were assumed to result from mutations between the pa-
rental accessions, and were coded with IUPAC ambiguity codes.

The resulting matrices were analysed using the maximum par-
simony (MP) method implemented within PAUP® 4.0, version beta 10 (Swofford 2002). Heuristic searches were performed with char-
acters states equally weighted, gaps/polymorphic sites treated as
missing data/uncertainties, respectively, and the following param-
eters: TBR branch swapping, steepest algorithm on, MULPAR on,
and collapse branches on for branches with a minimum length of
zero. Two hundred replicates with random addition sequence were
performed, saving no more than five trees per replicate. The
trees obtained were used as starting trees for a new search, running
as many trees as possible (Manson and Zeltner 2004). Branch sup-
port was determined using the bootstrap method (Felsenstein 1985). Bootstrap values (BS) were obtained from 100 bootstrap replicates using heuristic searches with simple stepwise addition and TBR branch swapping. To identify the phylogenetic position of C. bianoris within the genus, sequences of representative species (C. erythraea, C. pulchellum, C. scilloides, C. glypisola, C. fariniger) occurring in the same geographical range, and including the type species C. latifolium, were added to the matrix. All the analyses were rooted with the sister genera Chromia and Orphium (Mansion and Struve 2004). Accession numbers of sequences submitted to GenBank are listed in Appendix 1. The respective data sets were submitted to TreeBASE (study accession number S1366, matrix accession numbers M2420–M2422).

Results

Flow Cytometry and Chromosome Counts. Diploid and tetraploid individuals were easily distinguished by flow cytometry analyses, with no overlap of the respective fluorescence peaks. The particular valences were generally congruent with the chromosome numbers established for individuals fixed in the field and with the literature (Zeltner 1978). Our results confirmed that all salmon-coloured specimens of C. bianoris were tetraploid (no triploids were found). Moreover, all the C. maritimum accessions were diploid, whereas both diploid and tetraploid cytotypes were detected for C. tenuiflorum.

Yellow and pink varieties of C. bianoris rarely germinated; most of them had to be extracted from herbarium vouchers. Their identification was thus partly done a posteriori, by direct comparison with Louis Zeltner’s herbarium specimens (including the holotypes of the respective varieties).

RAPD Fingerprinting. A total of 138 accessions (65 C. bianoris, 46 C. maritimum, 27 C. tenuiflorum) was analysed, corresponding to a range of 1–9 samples per population. Of the 60 primers surveyed, seven were chosen for the final analysis (OPT14, OPT15, OPB11, OPB14, OPF7, OPP11, and OPF14). The number of bands scored per primer ranged from three (OPT14) to ten (OPT15), for a total of 38 polymorphic RAPD markers (Fig. 2A).

Twelve RAPD bands were effective molecular markers for the various species (one to C. bianoris, seven to C. maritimum, four of which were limited to populations of Baillaury Valley, France, and four to C. tenuiflorum). Twenty-two bands were shared between two taxa, among the 15 bands found in C. maritimum but absent in C. tenuiflorum, eight were shared with C. bianoris (53.3% band additivity). When excluding the atypical populations of Baillaury Valley, band additivity reached 72.7% (8/11). Sixteen bands were present in C. tenuiflorum but absent in C. maritimum, 12 of which were shared with C. bianoris (75.0% band additivity). Further, two bands were shared between C. maritimum and C. tenuiflorum, and four markers were present in the three species investigated. Finally, nineteen bands occurred with a high overall frequency (more than 70%) in either the parental taxa or in C. bianoris. Sixteen such high-frequency bands (84.2%) agreed with the hypothesis of an allopolyploid origin because they occurred in the presumed hybrid and in at least one of the putative progenitors (Allan et al. 1997; Neuffer et al. 1999; Nieto Feliner et al. 2002).

The NJ phylogram depicted three clusters (Fig. 2B), each containing all the accessions of a single species, whereas the C. bianoris group has an intermediate placement between C. maritimum and C. tenuiflorum. No geographical structure could be detected within each group and the varietal ranks hitherto proposed for the yellow and pink forms of C. bianoris (Zeltner 1978) were also not supported. However, two populations of C. maritimum (Baillaury Valley, France) formed a well-differentiated subgroup.

Phylogenetic Analyses. Parsimony analyses performed on the ITS matrix (28 accessions, 459 aligned characters) produced six most parsimonious trees (L=130, CI=0.87, RI=0.96, RC=0.83). The strict consensus tree depicted three major clades with high bootstrap support (Fig. 3). One well-supported clade comprised C. maritimum and C. pulchellum (BS=98%), along with seven accessions of C. bianoris (three of var. bianoris, three of var. roseum, and one of var. sulfureum). All accessions of C. maritimum and C. bianoris further formed a subclade “M” (BS=97%), in which one sample of C. maritimum (C.maritimum3, Baillaury Valley, France) strongly differed from all the remaining ones. A second major clade “T” (BS=98%) comprised all the accessions of C. tenuiflorum, along with two accessions of C. bianoris (one of var. sulfureum and one of var. bianoris). Finally, the five remaining species of Centaurea formed a third major clade (BS=100%).

The trnLF data set (28 accessions, 799 aligned characters) produced two most parsimonious trees (L=31, CI=0.97, RI=0.99, RC=0.96). The strict consensus tree depicted a clade “M” comprising all the accessions of C. bianoris and C. maritimum (BS=98%; Fig. 4). Another clade included all the remaining species of Centaurea (BS=63%), of which C. tenuiflorum and C. pulchellum (excluding C.pulchellum1) formed a subclade “T” (BS=70%).

ITS Polymorphism and Clones. Direct sequencing of C. bianoris accessions produced ambiguous sites, suggesting the occurrence of heterogeneous ITS arrays (Baldwin et al. 1995). Polymorphism was especially concentrated at so-called “parental diagnostic sites” (27%; i.e., sites distinguishing the putative parents of C. bianoris, namely C. maritimum and C. tenuiflorum subsp. acutiflorum, see Discussion), and relatively rare (<2%) at so-called “parental constant sites” (i.e. sites where C. maritimum and C. tenuiflorum subsp. acutiflorum do not differ, data not shown). Moreover, all polymorphisms found at parental diagnostic sites showed additivity of both parental repeat types (Table 1).

Four accessions of C. bianoris representing different
Fig. 2. RAPD analyses highlighting the intermediate position of C. bianoris between diploids C. maritimum and C. tenuiflorum. A. Schematic representation of the RAPD matrix. Each column corresponds to one RAPD marker, and each row to one individual plant. The upper block represents samples of C. maritimum, the intermediate block individuals of C. bianoris, and the lower block specimens of C. tenuiflorum. A filled cell indicates marker (band) presence. Acronym: BV (bands specific to populations of C. maritimum from Baillaury Valley, France). B. Unrooted NJ phylogram of 138 samples based on a Dice similarity matrix of 38 RAPD bands. Main clusters are encircled with dotted lines. Acronym: BV (populations of C. maritimum from Baillaury Valley, France).
**FIG. 3.** Strict consensus of six most parsimonious trees based on direct ITS nrDNA sequences for *Centaurium* species, using *Chironia* and *Orphium* as outgroups. Bootstrap supports (BS) are indicated above branches (BS > 50). Abbreviations for terminal taxa are as in Appendix 1. Accessions of *C. bianoris* are in bold. "M" and "T" indicate clades containing all accessions of *C. maritimum* and *C. tenuiflorum*, respectively.

**FIG. 4.** Strict consensus of two most parsimonious trees based on trnLF cpDNA sequences for *Centaurium* species, using *Chironia* and *Orphium* as outgroups. Bootstrap supports (BS) are indicated above branches (BS > 50). Abbreviations for terminal taxa are as in Appendix 1. Accessions of *C. bianoris* are in bold. "M" and "T" indicate clades containing all accessions of *C. maritimum* and *C. tenuiflorum*, respectively.

**TABLE 1.** Nucleotide variation in accessions of *C. bianoris* at "parental diagnostic sites", i.e. sites that differ between *C. maritimum* and *C. tenuiflorum* subsp. *acuti¯orum*. Site numbering follows ITS DNA sequence alignment. Sites identical to the first sequence are represented by dots. Polymorphic sites are underlined, whereas autapomorphies are in italic. Direct sequences are included for comparison with the clones (designated by nos. 1±15) and are shown in bold. Possible recombinant clones are followed by an asterisk. IUPAC ambiguity codes were used to code polymorphic regions. Accession abbreviations are given in Appendix 1.
levels of polymorphism (Table 1) were cloned for ITS sequences: one of var. bianoris (C. bianoris3), two of var. sulfureum falling either in clade M or T (Fig. 3; C. bianoris sulfureum1, C. bianoris sulfureum2), and one of var. roseum (C. roseum2). Among the 42 clones obtained, five were suspected to be recombinants because they were composed of both parental repeat types (cf. Table 1). Regardless of their origin (natural vs. experimental), they were excluded from the phylogenetic analyses. Figure 5 shows the strict consensus of four trees (L = 187, CI = 0.86, RI = 0.98, RC = 0.84) obtained from heuristic searches including the cloned ITS sequences, but excluding the direct sequences of C. bianoris (56 taxa and 459 aligned characters). All the ITS clones of “C. bianoris” (nos. 1–15) fell in clade M (BS = 96%). All accessions actually displayed the repeat type of C. maritimum at parental diagnostic sites (Table 1). Likewise, all clones of “C. sulfureum1” grouped in clade T (BS = 98%); they presented the repeat type of C. tenuiflorum at parental diagnostic sites (Table 1). Seven of the nine clones obtained for “C. sulfureum2” (nos. 1–6, 10) are placed in clade T, whereas two (nos. 7, 9) appear in clade M. Among the four ITS clones of “C. roseum2” obtained in this study, three (nos. 1, 3–4) grouped in clade M, whereas one (No. 2) was placed in clade T.

**DISCUSSION**

*Origin of Centaurium bianoris.* Our study, using different sets of molecular markers, strongly supports the hypothesis of an allopolyploid origin for C. bianoris, and determines with confidence the diploid progenitors. In addition, our results provided enough information to formulate hypotheses related to the origin of varieties sulfureum and roseum.

**Allopolyploid Origin.** The high percentage of RAPD markers shared between C. bianoris and its putative progenitors, C. maritimum and C. tenuiflorum (72.7% and 75.0%, respectively; Fig. 2A), unambiguously supports an allopolyploid origin of C. bianoris. Among the 19 RAPD bands occurring at a high frequency in the three species investigated (more than 70%), only three bands (15.8%) reject the hybrid origin of C. bianoris. Similar patterns were found in other groups of plants such as *Cyrtandra* (Gesneriaceae; Smith et al. 1996), *Encelia* (Asteraceae; Allan et al. 1997), *Viola* (Violaceae; Neuffer et al. 1999), and *Armorina* (Plumbaginaceae; Nieto Feliner et al. 2002). Since RAPD markers are biparentally inherited, one would expect additive banding in allopolyploids (Williams et al. 1990; Harris 1999). However, a deviation from complete additivity and the existence of unique polyploid bands often occurs. First, although present at high rates within the parental species, diagnostic bands usually show inter- and intrapopulational frequency variation. A hybrid may thus lack some RAPD bands of either one or both parental species. Second, recessive alleles of a heterozygous progenitor may have remained undetected in C. bianoris, due to the dominant nature of RAPD markers (Williams et al. 1990; Harris 1999). Third, it is also possible that some parental populations sharing additional polymorphism have not been sampled. Finally, departure from strict additive banding pattern in polyploids and the existence of specific C. bianoris bands may be due to the dynamic nature of polyploid genomes (Soltis and Soltis 1993; Leitch and Bennett 1997; Wendel 2000; Soltis et al. 2003). Rapid genome changes have been recently documented in neopolyploids (Song et al. 1995; Lim et al. 2004). As a conclusion, the alternative topological positions of C. bianoris on the ITS tree, clustering either with C. tenuiflorum or C. maritimum (Figs. 3, 5), along with its intermediate placement on the RAPD phylogram (Fig. 2B), strengthen the hypothesis of an allopolyploid origin (cf. McDade 1992).

**Parental Species.** Because a hybrid generally inherits the nrDNA of both progenitors and the cpDNA of only one—commonly maternal—parent, incongruence between nrDNA and cpDNA phylogenetic reconstructions including this hybrid is expected (Wendel and Doyle 1998). In our analyses, all the trnLF accessions of C. bianoris cluster with C. maritimum (Fig. 4). This result leads us to propose C. maritimum as the chloroplast donor of C. bianoris, and consequently, the diploid C. tenuiflorum subsp. acutiflorum, as the paternal progenitor. We can also rule out the tetraploid C. tenuiflorum subsp. tenuiflorum as a potential parent of C. bianoris, due to its topological position in the ITS cladogram (Figs. 3, 5). Further, no additive polymorphic sites (APS) were apparently shared between these two species (Mansion et al. 2005).

**Origin of the Floral Forms.** We did not find any hierarchical structure within C. bianoris consistent with the existing taxonomy (Zeltner 1978). Actually, accessions of var. sulfureum and var. roseum formed mixed clusters with those of the salmon form (Figs. 2B, 3–5). If these two varieties had an autoployploid origin or were the result of backcrosses with one of the putative parents, the yellow form would have grouped with C. maritimum, or the pink with C. tenuiflorum, on both nuclear- and chloroplast-DNA based trees. These varieties are more likely “local morphs” of C. bianoris, for they only differ from the type by the colour of their corollas (pink or yellow vs. salmon). Allopolyploidy is frequently associated with variation and instability in phenotypes (Comai et al. 2000). One of the main reasons for this phenotypic variability is gene silencing, a consequence of genome doubling that leads to polyploid stabilisation and evolution via pseudogenisation, genomic deletion, or epigenetic modifications (Wendel 2000; Liu and Wendel 2003). The occurrence of pink and yellow variants in the allopolyploid C. bianoris...
FIG. 5. Strict consensus of three most parsimonious trees obtained from phylogenetic analysis of the ITS sequence data for *C. bianoris* clones (and representatives of other major lineages), using *Chironia* and *Orphium* as outgroups. Bootstrap supports (BS) are indicated above branches (BS > 50). Abbreviations for terminal taxa are as in Appendix 1. Cloned sequences of *C. bianoris* are designated by nos. 1–15 and highlighted in bold. “M” and “T” indicate clades containing all accessions of *C. maritimum* and *C. tenuiflorum*, respectively.
therefore could be explained by mechanisms of gene silencing. Such phenotypic variation was indeed observed in synthetic allotetraploid F₁ progenies of *Arabidopsis thaliana* x *A. arenosa* (Comai et al. 2000). In this study, three of the four F₁ plants had flowers that were intermediate in size and colour to those of the parents, whereas the inflorescence of the fourth hybrid resembled that of *A. thaliana*. Phenotypic instability was also detected among F₂, F₃, and F₄ generations (e.g., morphologically intermediate F₁ individuals reversed to an *A. thaliana*-like phenotype in the F₂ generation).

**Genome Evolution Following Allopolyploidisation in *C. bianoris*.** The complete additivity encountered at diagnostic polymorphic ITS sites supports an allopolyploid origin of *C. bianoris* (Table 1). Homogenisation of ITS sequences is generally less efficient in lineages of hybrid and/or polyploid origin, and may proceed independently in each of the parental genomic contributions, allowing two different nrDNA types to persist (Sang et al. 1995; O’Kane et al. 1996; Campbell et al. 1997). Nonetheless, concerted evolution is expected to progressively reduce and eventually eliminate genetic divergence among repeats of multigene families (Elder and Turner 1995). In the allopolyploid *C. bianoris*, 27% of nucleotides at parental diagnostic sites are polymorphic, the other 73% are identical to one parental repeat type (Table 1). Moreover, both direct and cloned accessions of the hybrid appears in two divergent parental clades on the ITS cladogram (clades M and T; Figs. 3, 5). This suggests that polymorphic nucleotide sites in *C. bianoris* are bidirectionally homogenised (Wendel et al. 1995). Such homogenisation generally occurs directionally with the introgressant parent (Fuertes Aguilar et al. 1999; Andreasen and Baldwin 2003). Therefore, accessions with most clones falling in the clade of *C. maritimum* (clade M) or *C. tenuiflorum* (clade T), might be viewed as the products of backcrosses with the relative parents (Fig. 5). Further, ITS introgression with the maternal progenitor appears to be more frequent for a majority of the direct and cloned accessions of *C. bianoris* are found in clade M (Fig. 5).

Possible alternatives to reticulation as an explanation for the occurrence of ITS polymorphism concern lineage sorting (i.e. stochastic accumulation of ancestral polymorphism within lineages; Wendel and Doyle 1998), the occurrence of paralogous sequences, or the presence of pseudogenes (Alvarez and Wendel 2003). In the first case (lineage sorting), the expectation would be random positions of some polymorphic ITS accessions in the respective cladograms, which is not the case in this study. Most of the polymorphic ITS sequences, and their corresponding clones, belong either to clade M or clade T (Fig. 5). The second possibility (paralogy) would require a random distribution of APS within the respective taxa. Yet, in our study, APS mainly occur at the sites that differ between the two proposed parental species (data not shown), and therefore providing better support for reticulation processes. Finally, possible pseudogenes or recombinant sequences (C. bianoro sulfureum1 nos. 8–10/13 and C. bianor sulfureum2–8), which could have influenced phylogenetic reconstruction, were excluded from the analyses.

**Multiple vs. Single Origin of the Allopolyploid C. bianoris.** The different kinds of markers used in this study do not allow us to unambiguously discriminate between a recurrent and a single origin for *C. bianoris*. On one hand, the high number of subdivisions encountered within the *C. bianoris* cluster on the RAPD tree (Fig. 2B), coupled with the different rates of ITS homogenisation (direct accessions, Table 1), and the polyphyletic position of the hybrid on the ITS cladogram (cloned accessions, Fig. 5) support a multiple origin. On the other hand, only one cpDNA haplotype was detected in our study, a fact corroborated by more extensive sampling (Manson et al. 2005), suggesting a single origin. Despite the large distributional overlap of the parental species (Fig. 1), *C. bianoris* was only encountered on Majorca. The most parsimonious explanation for such a restricted distribution favors a single origin of the hybrid (Brochmann et al. 1998; Steen et al. 2000), along with habitat requirements specific to the island or a recent origin, which limited its expansion until now (Arft and Ranker 1998; Widmer and Baltisberger 1999; Sästad et al. 2001).

The development of additional chloroplast markers (e.g., chloroplast microsatellites) would help to confirm a single vs. polytopic origin of *C. bianoris*. Further, new populations of *C. bianoris* recently discovered on Ibiza (Balearic Islands; J. Rossello, pers. comm.), may be seen as either the result of a recent introduction/expansion by man, or the consequence of a second origin on this island.

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**LITERATURE CITED**


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APPENDIX I

Plant material sampled for the present study. Data are presented in the following sequence: Taxon, OTU abbreviation of sequenced accessions (in bold), population location, voucher information, GenBank accession number for ITS1, ITS2, trnL intron and trnL-F spacer. Accessions followed by an asterisk were used for RAPD analysis. Sequences directly retrieved from GenBank are in italic. Accession numbers of ITS clones are indicated in parentheses. All vouchers were deposited in Neuchâtel (NEU). Name of collectors: AG (A. Guggisberg), FB (F. Bretagnolle), GM (G. Mollard), LD (L. D’Andrea), LZ (L. Zeltner), MC (M. Callmander), PK (P. Küpper), SW (S. Wohlhäuser).

Centaurium biansi var. biansi (Senn.) Sennen, Castille de Bel-ver, Majorca, Spain, AG & GM CB010608-4-(1/2/3/4)*, between S’Arraco and Sant Elm, Majorca, Spain, AG & GM CB010609-4-(1/2/3/4)*, Canonges harbour, Majorca, Spain, AG & GM CB010609-4-(1/2/3/4)*, between Orient and Bunyola, Majorca, Spain, AG & GM CB010609-5-(1/2/3/4)*, between Orient and Bunyola, Majorca, Spain, AG & GM CB010609-5-(1/2/3/4)*, between Orient and Bunyola, Majorca, Spain, AG & GM CB010609-5-(1/2/3/4)*.

Centaurium biansi var. roseum (Zeltner, AG&GM) sulfus010612±8*; between Titulcia and Chinchon, Lago di Fondi, Italy, AG & GM MG001012, A Y251672, A Y251700, A Y251730, A Y251754.

Centaurium gypsicoloud (Boiss & Reut.) Ronninger, AG & GM MG001012, A Y251672, A Y251730, A Y251754.

France, AG & GM CM010628±4±2*; Ostricorni, near Ogliastro, Corsica, France, AG & GM CM010630±1±23*; between Pont de Castirla and Popolasca, Corsica, France, AG & GM CM010630±2-(8/910//11)*; C.maritimum1, Serra di Pigno, Corsica, France, AG & G M CM010701±3±9*, DQ166324, DQ166255, DQ166306, DQ166315; Serra di Pigno, Corsica, France, AG & GM CM010701±3±11*; between Morsiglia and Port Centuri, Corsica, France, AG & GM CM010702±1-(6/10/12/13)*; C.maritimum2, Lac des Rêves, near Lattes, France, FB CM010719±1±6*, DQ166325, DQ166256, DQ166307, DQ166316; Lac des Rêves, near Lattes, France, FB CM010719±1-(8/9)*; C.maritimum3, Baillaury Valley, Banuyls-sur-Mer, France, GM MG98703, A Y251673, A Y251703, A Y251733, A Y251756; Lago di Fondi, Italy, PK CM0006±1-(1/9)*; C.maritimum4, Ile du Levant, Hyères Islands, France, LZ MG030102, A Y879833, A Y879884, A Y879934, A Y879992; C.maritimum5, Majorca, Spain, LZ MG040509, DQ166327, DQ166308, DQ166317

Centaurium pulchellum (Sw.) Druce, C.pulchellum1, Gap, Hautes-Alpes, France, MG MG98505, AY047787, AY047872, AY251734, AY251758; C.pulchellum2, Houston airport, Texas, USA, GM & LZ MG97701, AY879835, AY879886, AY879936, AY879994; C.pulchellum3, church of San Michele de Plaianu, Sardinia, Italy, AG, GM & LD LT-1, AY879836, AY879887, AY879937, AY879995

Centaurium scilloides (L. fil.) Samp., C.scilloides, Playa de Baldaio, Spain, LZ MG981111, AY251675, AY251705, AY251737, AY251761

Centaurium tenuiflorum subsp. acutiflorum (Schott) Zeltner, between Andratx and Estellencs, Majorca, Spain, AG, GM & LD CTX010608±5±1*; between Orient and Bunyola, Majorca, Spain, AG, GM & LD CTX010609±5-(1/2/5)*; C.tenuiflorum1, Maratsinet, Majorca, Spain, AG, GM & LD CTX010610±1±10*; Col de Soller, Majorca, Spain, AG, GM & LD CTX010610±2-(4/5)*; C.tenuiflorum2, Torrent de Pareis, Majorca, Spain, AG, GM & LD LD-D2-1, AY879847, AY879897, AY879948, AY880006; Torrent de Pareis, Majorca, Spain, AG, GM & LD CTX010612±8-(4/6/7)*; Ostricorni, near Ogliastro, Corsica, France, AG, GM & LD CTX010629±1±3*; C.tenuiflorum3, Ile du Levant, Hyères Islands, France, AG, GM & LD LD-D2±1, AY879847, AY879897, AY879948, AY880006; Torrent de Pareis, Majorca, Spain, AG, GM & LD CTX010612±8-(4/6/7)*; Ostricorni, near Ogliastro, Corsica, France, AG, GM & LD CTX010629±1±3*; Cap Corse, between Barcaggio and Tollare, Corsica, France, AG & GM GM010702±1-(2/3/8)*; Lac des Rêves, near Lattes, France, FB CTX010719±1-(3/5/9)*; Aresquiers, France, FB CTX010719±1-(1/7/9)*; Centaurium tenuiflorum subsp. tenuiflorum (Hoffmgg, & Link) Fritsch, between Orient and Bunyola, Majorca, Spain, AG, GM & LD CTX010609±4±11*; C.tenuiflorum1, Jimena de la Frontera, Spain, AG, GM & LD LD-F3-9, AY879848, AY879899, AY879949, AY880007; C.tenuiflorum2, between Puerto de Galis and Ubrique, Spain, AG, GM & LD LD-G1-3, AY879849, AY879990, AY880008; Chiromia baccifera, South Africa, MC & SW A005, AY251690, AY251720, AY251746, AY251783

Chironia baccifera, South Africa, MC & SW A005, AY251693, AY251723, AY251748, AY251785

Orphium frutescens E. Meyer, Orphium frutescens, South Africa, MC & SW A005, AY251693, AY251723, AY251748, AY251785