

Genetic relatedness among Asian *Cotoneaster* species investigated with DNA marker analysis

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Summary: The widespread genus *Cotoneaster* has its centre of diversity in the Himalayas and surrounding areas. Most taxa appear to be polyploid and apomictic, and many of them have become popular ornamentals due to their attractive foliage and berries. One of the taxonomically most critical groups is Section *Alpigeni* which contains several important ornamental plants. The number of species belonging to this section varies widely between different taxonomic treatises, depending on whether 'splitting' or 'lumping' of species is preferred. Using a rather narrow species definition, we have investigated 13 different species using RAPD analysis. A simple matching (SM) coefficient-based principle coordinate analysis (PCO) was calculated from the RAPD data. Some species were clearly more similar to each other than to other species in the analysis. The levels of similarity did, however, not correspond very well to the lumping together of several taxa under the same species name as performed e.g. in the recent Flora of China. Obviously, the complex hybridogenous origination and, in some cases, still ongoing recombination with sexual species or among the apomictic taxa themselves, produces a genetic variability structure that cannot be properly reflected in a hierarchical taxonomy.

Key words: *Cotoneaster*, Section *Alpigeni*, DNA-marker, RAPD, systematics, taxonomy

Introduction

The genus *Cotoneaster* belongs to Rosaceae, and consists of woody plants, varying in size and habitus from 0.2 m prostrate shrubs to 15–20 m high trees. Many *Cotoneaster* species have become popular ornamentals due to their attractive foliage, abundant flowers and, especially, for their bright red (sometimes black or, more rarely, pink or yellowish) fruits.

The genus *Cotoneaster* is widespread throughout the Northern hemisphere, including all of Europe, North Africa, and the temperate areas of Asia. The western limits are the Atlas Mountains of Morocco, the Granada and Pyrenees in Spain, and N. Wales in Britain. In the east, *Cotoneaster* taxa are found in Taiwan, on the island of Ullung between Korea and Japan, and in Korea (but not in Japan). The southern limits are the Sinai in Egypt, Jabaleth Shan of Oman, the Nilgiri Hills of southern India, and Mount Victoria in Burma. In the north, the genus occupies the Kola Peninsula and the area around Lake Baikal and Yatutsk in Siberia. The epicentre of distribution for the genus is the Himalayas and surrounding mountains of China in the provinces of Yunnan and Sichuan.

The botanical exploration of the Himalayas began around

1800 when the Scottish medical man Francis Buchanan-Hamilton collected in, and also the area east of, Nepal. Around this time the interest in the genus *Cotoneaster* arose in several other countries as well. Soon after, the very first species of *Cotoneaster* from China was described (*Cotoneaster acutifolius* Turcz. Bull. Soc. Nat. Mosc. 4:190, 1832), from what was to become a centre of future *Cotoneaster* exploration. With the immense interest in gardening and in new species around the beginning of the twentieth century, the era of the professional plant-hunter emerged. Throughout the next 40 years, around 50 new species of *Cotoneaster* were discovered in China alone, and the total number of taxa recognized in the genus rose accordingly. Thus, Rehder (1927) mentions a total of only 80 species but this number increased to 176 in Flinck & Hylmö (1966) and to 261 species in Phipps et al. (1990). At present, a total of approximately 300 *Cotoneaster* species are recognized. Lumping of species has, however, been undertaken by Lu & Brach (2003) who recognize only about 90 'broad sense' species, 59 of which occur in China where 37 are endemic.

Most *Cotoneaster* taxa appear to be polyploid and apomictic, i.e. capable of producing offspring without prior fertilization (Hylmö & Fryer, 1999; Bartish et al., 2001).

Taxonomy is usually controversial in apomictic genera, due to the allopolyploid origination of large numbers of identifiable but often not clearly differentiated units (Asker & Jerling 1992). In the genus *Cotoneaster*, groups of related taxa sometimes contain one (or a few) diploid ($2n = 34$) species together with several polyploid (triploid, tetraploid or, rarely, pentaploid) taxa (J. Bailey & H. McAllister, respectively, pers. comm.). Infrageneric taxonomy is especially critical in such groups since the polyploid taxa appear to have originated by hybridization events involving the diploid group member. One of the most difficult groups is found in the Section *Alpigeni* (Koch) Hurusawa, which contains several taxa with small leathery leaves (Klotz, 1963). Several of these have become economically important ornamentals, like the prostrate *C. procumbens* 'Queen of Carpets' and the somewhat more upright *C. dammeri* 'Coral Beauty'.

Although taxonomy in *Cotoneaster* is traditionally based on morphological characters determined in field studies and on herbarium material, DNA-based data have the potential to provide a valuable addition. Since apomictic taxa in general have evolved recently and are closely related, standard DNA sequencing may not produce data that are sufficiently variable for phylogenetic interpretations (Stace et al., 1997). By contrast, various DNA marker methods have frequently proved successful for analyses of apomictic species complexes (reviewed in Nybom, 1996; Weising et al., 2005). One of the most efficient molecular marker methods in terms of the ability to produce polymorphic markers within a comparatively short time and with a limited budget is RAPD (random amplified polymorphic DNA). RAPD has been shown to be a useful method for analyses of genetic variability within and among many apomictic taxa (Stace et al., 1997; Sepp et al., 2000; Bartish et al., 2001; Storchova et al., 2001; Rossello et al., 2002; Persson & al. 2004; Reisch, 2004).

The present investigation was undertaken with RAPD analysis to study interspecific relationships in the taxonomically critical Section *Alpigeni* with species from China, Nepal, Tibet and India.

Material and method

Plant material

A total of 14 species were analysed, one of which is not yet identified (Table 1). Plants were sent in winter to Balsgård from England where they had been grown in the plant nursery belonging to Jeanette Fryer. Newly developed leaves were harvested and kept in $-80\text{ }^{\circ}\text{C}$ until processing. Vouchers are kept at Balsgård, SLU.

Extraction of DNA and PCR amplifications

One or two leaves (30–60 mg of fresh weight leaf tissue) were crushed in an Eppendorf tube with a glass rod in 400 μl of extraction buffer, provided with DNeasy Plant Mini Kit (Qiagen). All further steps of the extraction procedure followed the recommendations of the manufacturer of the kit.

PCR amplifications were performed in volumes of 20 μl . Each reaction contained 10–20 ng of DNA, reaction buffer IV with 1.5 mM of MgCl_2 (Advanced Biotechnologies), 0.1% of Triton X-100 (Sigma), 20 μM Nucleotide Mix (Roche) and 1.0 unit *Taq* DNA Polymerase (Advanced Biotechnologies). Nine primers from Operon Technologies (OPE-01, OPE-05, OPE-14, OPF-03, OPF-04, OPF-07, OPF-08, OPF-09, OPF-13) were selected for the present study and used in a concentration of 0.6 μM per reaction. These primers were chosen from a set of twelve primers, which had been selected in a previous RAPD analysis of genetic diversity in *Cotoneaster* based on their ability to produce clear and polymorphic bands (Bartish et al., 2001). Amplifications were carried out in a thermal cycler (MJ Research) under the following conditions: 3 min at $94\text{ }^{\circ}\text{C}$, followed by 40 cycles at $94\text{ }^{\circ}\text{C}$ for 30 s, $42\text{ }^{\circ}\text{C}$ for 45 s, and $72\text{ }^{\circ}\text{C}$ for 1 min. An additional cycle of 6 min at $72\text{ }^{\circ}\text{C}$ was added at the end of each PCR run. The amplification products were separated by electrophoresis in 1.8 % agarose gels in 1 x TAE buffer, containing 0.5 $\mu\text{g/ml}$ ethidium bromide. The reproducibility of PCR reactions was controlled by

Table 1. Plant material used in RAPD analysis of *Cotoneaster*.

Species	Origin of sample
<i>C. cashmiriensis</i> G. Klotz	W Himalaya, border Kashmir–Himachal Pradesh, India
<i>C. cochleatus</i> (Franch.) G. Klotz	Dali Cangshan track–Lonquan peak, Yunnan, China
<i>C. congestus</i> Baker	Langtang, Nepal
<i>C. elatus</i> G. Klotz	Dali Cangshan track–Lonquan peak, Yunnan, China
<i>C. glacialis</i> (J.D. Hooker ex Wenzig) G. Panigrahi & A. Kumar	Chu at lake station no. 17, Pasum Tso, Tibet
<i>C. integrifolius</i> (Roxburgh) G. Klotz	Nilgiri, India
<i>C. marginatus</i> (Loudon) D.F.L. von Schlechtendal	Gosainkund, Sing Gompa, Langtang, Nepal
<i>C. melanotrichus</i> (Franch.) G. Klotz	Lijiang Yulongshan Gan Hai Zi, Yunnan, China
<i>C. morrisonensis</i> Hayata	Mt. Morrison, Taiwan
<i>C. procumbens</i> G. Klotz	SW Kangting Erlan Shan, Sichuan, China
<i>C. prostratus</i> Baker	Marsyandi Gorge NE of Annapurna, Nepal
<i>C. rockii</i> G. Klotz	SE Kongbo, Tibet
<i>C. rotundifolius</i> Wall. ex Lindl.	Unknown
<i>C. sp.</i>	Pso Tangpo, Tibet

amplification of identical DNA (two samples from each of three plants, each representing a different species) in separate runs.

Analysis of RAPD data

A data matrix was created from evaluation of photographs of the gels by assigning 1 to present and 0 to absent bands. Only bands that were reproducible in all three pairs of parallel amplifications were included in numerical analyses, providing altogether 50 phenotype characters = polymorphic bands. In addition, 41 monomorphic characters were scored but these were not used in the analyses.

The resulting binary data matrix was analysed statistically using the NTSYS-pc software version 1.8 (Rohlf 1996). Similarity matrices were calculated for pairwise comparisons among samples using two different methods, Jaccard's coefficient of similarity and the Simple Matching coefficient of similarity (SM). A three-dimensional principal coordinate analysis was carried out on each of the Jaccard's and SM similarity matrices (procedures Double Center and Eigenvectors in NTSYS-pc) to provide a representation of the phenetic relationships among accessions.

Results and discussion

Section *Alpigeni* is generally considered to be a taxonomically critical group (Klotz 1963). The number of species belonging to this section varies widely between different taxonomic treatments, depending on whether

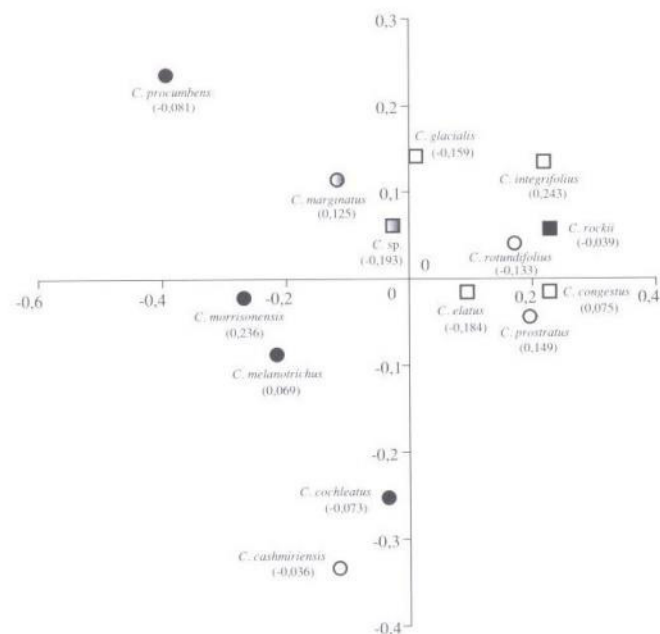


Fig. 1. Three-dimensional representation of a principal coordinate analysis (PCO) of the phenetic relatedness among species of *Cotoneaster*. Two axes of the plot represent the first two principal coordinates (PC1 and PC2), and the third value (PC3) is indicated in parentheses near the symbol and species name. Symbols were marked according to taxonomic affinity sensu Klotz (1963): filled squares, series *Buxifolii*; shaded circle, series *Marginati*; open circles, series *Rotundifolii*; open squares, series *Alpigeni*; filled circles, series *Procumbentes*, shaded square, *C. sp.*

'splitting' or 'lumping' of species is preferred. Using a relatively narrow species definition, we analysed 14 species in the present study. Klotz (1963) treated these species as *C. rockii* in series *Buxifolii*, *C. marginatus* in series *Marginati*, *C. cashmiriensis*, *C. prostratus* and *C. rotundifolius* in series *Rotundifolii*, *C. congestus*, *C. elatus*, *C. integrifolius* and *C. microphyllus* var. *glacialis* in series *Alpigeni*, and *C. cochleatus*, *C. melanotrichus* (as *C. buxifolia* f. *melanotricha* under *C. cochleatus* but later treated as a distinct species), *C. morrisonensis* and *C. procumbens* in series *Procumbentes*. Several of these species have later been lumped together in a recent treatise on the flora of China (Lu & Brach, 2003).

Results of our RAPD analysis revealed considerable diversity among the sampled species. This diversity was interpreted by the application of PCO. The first three principal coordinates accounted for 23.4 %, 13.5 %, and 13 % of the total variation, respectively (cumulative value 49.9 %) when based on the SM similarity matrix. This means that nearly half of the genetic variation is unveiled by our numerical analysis. For Jaccard's coefficients the cumulative value was somewhat lower, 41.7 %. Therefore, only the matrix based on SM was used in further evaluation of the data.

Although rather well dispersed in the SM-based PCO analysis, variation among taxa is not continuous (Fig. 1). Instead some taxa appear to be considerably more related to each other than to other taxa in the analysis. The largest group consists of nine samples collected at high elevations in China, India, Nepal and Tibet. In the Flora of China, Lu & Brach (2003) treated *C. congestus* and *C. glacialis* as synonyms, with a status as var. *glacialis* under *C. microphyllus*. Under *C. microphyllus* var. *microphyllus*, they placed *C. elatus*, and under *C. microphyllus* var. *thymifolius* they placed *C. integrifolius*. In the same treatise, *C. prostratus* was treated as a synonym of *C. rotundifolius*, whereas both *C. rockii* and *C. marginatus* were treated as varieties of *C. buxifolius*.

Another four species appear to be quite distinct from the large group, and belong to two smaller groups: *C. cochleatus* from Yunnan forms a pair with *C. cashmiriensis* from India, whereas *C. melanotrichus* from Yunnan forms another pair with *C. morrisonensis* from Taiwan. *Cotoneaster cochleatus* was treated as a variety of *C. microphyllus* by Lu & Brach (2003) and *C. cashmiriensis* was not mentioned. In the other pair, *C. morrisonensis* was accorded species status by Lu & Brach (2003) whereas *C. melanotrichus* was treated as a synonym of *C. microphyllus* var. *microphyllus*.

Finally, one more Chinese species, *C. procumbens* from Sichuan, was strongly differentiated from all other taxa. This species was not treated by Lu & Brach (2003). There are several closely related taxa, including the well-known cultivar 'Queen of Carpets', which are often included under this species name.

In some cases, genetic distances among the samples studied here with RAPD conformed rather well with the taxonomic treatises presented by Klotz (1963) and by Lu & Brach (2003). There are, however, several major discrepancies. Samples belonging to different species according to Lu & Brach (2003) were sometimes closer together in the PCO analysis than were samples of the same species. Thus the

largest group in our PCO analysis contained representatives of three different species (*C. buxifolius*, *C. microphyllus* and *C. rotundifolius*) according to Lu & Brach (2003). The delineation of taxa into series according to Klotz (1963) fits somewhat better with our RAPD data. Species in three series (*Buxifolii*, *Alpigeni* and *Rotundifolii*) are clustered in the upper right corner (high values on both PCO axes) except for the outlying *C. cashmiriensis*. Series *Marginati* (represented by only *C. marginatus*) has a low value on PC1 and a higher on PC2. The remaining four species, *C. procumbens*, *C. morrisonensis*, *C. melanotrichus* and *C. cochleatus* form a continuum from the upper left corner (low values on PC1, high on PC2) to the low central part, i.e. medium value for PC1, low for PC2.

In a previous RAPD-based survey of the genus *Cotoneaster*, 12 European species in Section *Cotoneaster*, series *Cotoneaster* and *Melanocarpi*, were investigated together with the Chinese species *C. albokermesinus* in Section *Chaenopetalum*, series *Tomentelli* (Bartish et al., 2001). As expected, the Chinese species was found to differ very strongly from the European species. These, in their turn, clustered mostly as expected from morphology-based interpretations of their taxonomic relationships.

One reason for the higher degree of inconsistency between RAPD-derived data and presumed levels of relatedness among the investigated taxa in the present study, is probably the fact that this study was carried out in a taxonomically very difficult group including both sexual diploids and apomictic taxa. Consequently, speciation could still be an ongoing process, resulting in allopolyploid taxa that may even be polyphyletic in some cases.

In the present study, only one accession of each species was available for analysis. In the previous RAPD-based *Cotoneaster* study (Bartish et al., 2001), intraspecific variability was however found to be very low; plants from different accessions were usually much more similar to each other than to accessions from any of the other species. In general, apomictic species have been found to harbour considerably lower levels of genetic variation compared to sexual species (Stace et al., 1997; Storchova et al., 2002).

However, even if a different set of samples from the same *Cotoneaster* species would produce different estimations of their genetic relatedness, the overall pattern is likely to remain; i.e. some presumably closely related taxa are quite similar also with RAPD data but the overall taxonomic structure created by lumping some taxa as synonyms or according them varietal status is not supported by RAPD. A similar lack of correspondence between morphology-based taxonomy and RAPD data has been found in other apomictic species as well (Stace et al., 1997; Rossello et al., 2002; Storchova et al., 2002). The complex (and sometimes polyphyletic) hybridogenous origination and, in some cases, still ongoing recombination with sexual species or among the apomictic taxa themselves, produces a genetic variability structure that cannot be properly reflected in a hierarchical taxonomy.

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