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Morphological and biochemical genetic evidence for hybridization in the genus *Centella* (Apiaceae), with notes on phylogenetic and taxonomic implications

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ABSTRACT

The main aim of this paper is to explore the occurrence of hybridization in the genus *Centella*. Morphological as well as genetic characters are investigated to confirm the identity of a putative hybrid between *C. triloba* and *C. macrocarpa*. These two independent data sets, one from enzyme electrophoresis and one from morphology, are compared and interpreted by means of cladistic analysis. *Centella glauca* and *C. virgata* were included in the analysis and the effect of hybridization on cladistics is demonstrated. Hybridization gives a new perspective on infrageneric relationships within the genus *Centella*, as it may have obscured discontinuities between previously discrete infrageneric groups.

INTRODUCTION

The subject of hybrids and hybridization has been covered extensively by a number of authors (e.g. Stace 1989; Nason *et al.* 1992), and the influence of hybridization on cladistics has also previously been the subject of detailed analyses (McDade 1990, 1992, 1995). These authors indicated that hybridization significantly affects infrageneric classification because the limits between taxa may become obscured by reticulate evolution. The following facts pointed to hybridization in the genus *Centella*: 1, lack of correlation between characters of species; 2, reported occurrence of putative hybrids in the herbarium record; and 3, the discovery of a putative hybrid initially thought to be a new species. Apart from the two populations of parent species (*C. triloba* and *C. macrocarpa*) and their putative hybrid, one population each of *C. virgata* and *C. glauca* (and an additional population of *C. macrocarpa*) were included in a cladistic study of morphological characters and enzyme data. A second *C. macrocarpa* population was included to compare relationships at the population and species level. *Centella virgata* and *C. glauca* were chosen because of their obvious close relationship to *C. macrocarpa*. *Centella macrocarpa* from the Swartberg Pass and *C. virgata* are reseeders, i.e. plants that are killed by fire and which can only regenerate from seed after fire, whereas all the other populations and species are resprouters, i.e. plants that survive fire by coppicing, and have a slow rate of seed germination. As a result, only a relatively small number of seedlings are added to the populations after each fire. The inclusion of reseeders and resprouters gives the results broader applications in terms of the effects of fire-survival strategy on genetic variation, and also on the circumscription of species in the *C. virgata* group.

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MATERIALS AND METHODS

Herbarium specimens and material preserved in FAA of *Centella triloba*, *C. macrocarpa* and the putative hybrid, as well as *C. glauca* and *C. virgata* were examined and the leaves, fruits and inflorescences were drawn under camera lucida. For microtome sectioning, leaves and mature fruits preserved in FAA were used. Material was embedded in glycol methacrylate (GMA) according to a modification of the method of Feder & O'Brien (1968) as used by Van Wyk & Tilney (1994), who also give the procedures for ultramicrotome sectioning, staining and photography.

Voucher specimens

C. triloba (7): 3418 Simonstown: Kogel Bay, (–BD), *Schubert & Van Wyk 15.*

C. macrocarpa (4): 3322 Oudtshoorn: Swartberg Pass, (–AC), *Schubert & Van Wyk 90.*

C. macrocarpa (7): 3418 Simonstown: Kogelberg, (–BD), *Schubert & Van Wyk 53.*

C. virgata (5): 3320 Montague: Tradouws Pass, nr Barrydale, (–DC), *Schubert & Van Wyk 67.*

C. glauca (5): 3219 Wuppertal: Groot Winterhoek plateau, (–CC), *Schubert & Van Wyk 101.*

Putative hybrid (2): 3418 Simonstown: Kogelbaai, *Schubert & Van Wyk 98.*

Extraction, sample preparation and gel loading methods are described in Van der Bank *et al.* (1995); a Tris-HCl extraction buffer (pH = 7.5) was used. The supernatant was absorbed directly onto paper wicks, and twelve percent starch (Sigma: S-4501) gels were used. Genetic interpretation of enzyme banding patterns was based on the subunit structure and subcellular compartmentalization of the enzymes (Gottlieb 1981, 1982). Locus nomenclature followed Harris & Hopkinson (1976), Soltis & Soltis (1989), Hillis & Moritz (1990) and Shakalee *et al.* (1990). Locus abbreviations, monomorphic loci, enzyme commis-

TABLE 1.—Locus abbreviations, enzyme commission numbers (E.C.No.), optimal buffer systems and pH

Enzyme (loci)	E.C.No.	Buffer	pH
Aspartate aminotransferase AAT-1, -2*	2.6.1.1	Lithium-hydroxide-borate Tris-citrate (Cooke & Buckley 1987)	electrode : 8.1 gel : 8.4
Acid phosphatase ACP-1* ACP-2	3.1.3.2	 Histidine-citrate (Kephart 1990) Morpholine-citrate (Clayton & Tretiak 1972)	 6.5 6.1
Cytosol aminopeptidase CAP-1	3.4.11.1	Tris-EDTA-borate (Goncharenko *et al.* 1992) Lithium-hydroxide-borate Tris-citrate	8.6 electrode : 8.1 gel : 8.4
CAP-2*		Morpholine-citrate	6.1
Glucose-6-phosphate isomerase GPI	3.5.1.9	Tris-EDTA-borate (Markert & Faulhaber 1965) Lithium-borate Tris-citrate (Ridgway *et al.* 1970)	8.6 electrode : 8.0 gel : 8.7
Isocitrate dehydrogenase IDH*	1.1.1.42	Histidine-citrate	6.5
Malate dehydrogenase MDH*	1.1.1.37	Tris-EDTA-borate (Markert & Faulhaber 1965)	8.6
Peptidase (substrate : leucil-tyrosine) PEP-S*	3.4._._	Tris-EDTA-borate (Markert & Faulhaber 1965)	8.6
Peroxidase PER	1.11.1.7	Lithium-hydroxide-borate Tris-citrate	electrode : 8.1 gel : 8.4
Phosphoglucomutase PGM-1, -2	5.4.2.2	Tris-EDTA-borate (Goncharenko *et al.* 1992)	8.6
6-phosphogluconate dehydrogenase PGD*	1.1.1.44	Lithium-borate Tris-citrate	electrode : 8.0 gel : 8.7
Superoxide dismutase SOD*	1.15.1.1	Lithium-borate Tris-citrate	electrode : 8.0 gel : 8.7

* monomorphic loci

TABLE 2.—Allele frequencies of polymorphic loci for six *Centella* populations: *C. triloba* (P1); a putative hybrid between *C. triloba* and *C. macrocarpa* (H); *C. macrocarpa* (resprouter, population from Kogel Bay) (P2); and *C. macrocarpa* (reseeder, population from Swartberg Pass) (P3); *C. virgata* (V); and *C. glauca* (G). Allele frequencies with significant differences ($P<0.5$) among populations are marked with an asterisk

		Populations					
Locus	Allele	P1	H	P2	P3	V	G
AAT-1	A	0.214	0.500	0.600	0.250	0.300	0.875
	B	0.786	0.500	0.400	0.750	0.700	0.125
ACP-2	A	0.143	0.000	0.143	0.250	0.800	0.000
	B	0.857	1.000	0.857	0.750	0.200	1.000
CAP-1	A	0.167	0.000	0.000	0.000	0.000	0.000*
	B	0.833	0.000	0.200	0.000	1.000	0.800*
	C	0.000	1.000	0.800	1.000	0.000	0.000*
	D	0.000	0.000	0.000	0.000	0.000	0.200
GPI	A	0.143	0.500	0.286	0.000	0.000	0.900*
	B	0.000	0.000	0.000	1.000	0.000	0.000
	C	0.857	0.500	0.714	0.000	1.000	0.100*
PER	A	0.000	0.000	0.000	0.000	1.000	0.000
	B	0.000	0.000	0.417	1.000	0.000	0.000*
	C	1.000	1.000	0.583	0.000	0.000	0.000*
	D	0.000	0.000	0.000	0.000	0.000	1.000
PGM-1	A	0.167	0.000	0.000	0.000	0.000	0.167*
	B	0.250	1.000	0.750	1.000	0.000	0.667*
	C	0.583	0.000	0.250	0.000	1.000	0.167*
PGM-2	A	0.357	1.000	1.000	1.000	1.000	1.000*
	B	0.643	0.000	0.000	0.000	0.000	0.000*

sion numbers and buffer system combinations yielding the best results are listed in Table 1.

Data analysis

We used DISPAN (Ota 1993) for phylogenetic analysis of allozyme data by using neighbour-joining and bootstrap methods (1000 replications) and Nei's (1978) genetic distance values. The analysis of allozyme data was executed using BIOSYS-1 (Swofford & Selander 1981). The morphological data as well as the allele frequencies in the different populations were polarized using *C. triloba* as outgroup. It is probably the least derived of all the species included in the study, judged by the shrubby habit and the broad, dentate, dorsiventral leaves, which we consider to be plesiomorphic within the genus. Of these, the dorsiventral leaves are perhaps the most convincing plesiomorphy, as this character state occurs in related genera and in all the basal species of *Centella* (*C. asiatica* and the *C. eriantha* group). All character states of the outgroup were polarised as plesiomorphic. Alleles that were absent from one or more populations, while exhibiting both a low and a high frequency of occurrence in other populations, were treated as binary or multistate characters. Allele frequencies without any obvious discontinuities (such as AAT-1 and ACP-2) were not included in the analysis. Only those frequencies of which the values were obviously low or high, with no intermediate values (low taken as less than 0.3, high taken as 0.5 or more) were polarised. Again, *C. triloba* was used as outgroup. The intermediate states of the GPI A and PGM-1 B alleles were present in the outgroup, so we were unable to polarize these characters (only the one polarity is shown in Table 3). However, reversing this polarity had no effect, neither on the topology nor on the tree lengths or consistency indices, even when the genetic data were analysed separately (Figure 3D, E). Table 2 lists the allele frequencies used to polarize the enzyme characters, as shown in Table 3. These data were analysed using HENNIG86 (Farris 1988). Five to ten individuals were studied in each population except for the hybrid where only two individuals were present at the locality sampled. Despite the small sample size on which allele frequencies for the hybrid were based, we believed that useful results could be obtained because the observed allele frequencies in the other populations were generally either very high or very low.

TABLE 3.—Characters and polarization of character states in *C. triloba* (P1); a putative hybrid between *C. triloba* and *C. macrocarpa* (H); *C. macrocarpa* (resprouter, population from Kogel Bay) (P2); *C. macrocarpa* (reseeder, population from Swartberg Pass) (P3); *C. virgata* (V); and *C. glauca* (G)

Characters and character states	Species and populations	P1	H	P2	P3	V	G
	morphological data						
1		0	1	2	2	2	2
2		0	1	2	2	2	2
3		0	0	1	1	1	1
4		0	1	1	1	1	1
5		0	1	2	2	0	2
6		0	0	2	2	1	2
7		0	0	1	1	1	1
	genetic data						
8		0	1	1	1	1	1
9		0	2	1	2	0	0
10		0	1	1	1	0	0
11		1	2	1	0	0	2
12		0	0	0	2	0	1
13		0	0	1	1	0	0
14		0	0	0	1	1	1
15		0	1	1	1	1	0
16		1	2	2	2	0	2
17		0	2	1	2	0	1
18		0	1	1	1	1	1
19		0	1	1	1	1	1

Characters and polarization of character states using *C. triloba* as outgroup:

1. Leaf type: broad (6–36 mm wide) = 0; narrow (3–5 mm wide) = 1; acicular (approximately 1 mm wide) = 2.
2. Number of teeth on leaf margin: 3(5–11) = 0; 1(–3) = 1; 1 = 2.
3. Presence of petiole: distinguishable from lamina = 0; not distinguishable from lamina = 1.
4. Tissue arrangement in leaf: dorsiventral = 0; isobilateral = 1.
5. Surface sculpturing in fruit: smooth = 0; ribbed = 1; prominently ribbed = 2.
6. Size of fruit: 3–4 × 3 mm = 0; 4.0–5.5 × 4.0 mm = 1.
7. Indumentum of petals: glabrous or villous = 0; glabrous = 1.
8. CAP-1 A: allele present = 0; allele absent = 1.
9. CAP-1 B: allele absent = 2; allele present at low frequencies = 1; allele present at high frequencies = 0.
10. CAP-1 C: allele absent = 0; allele present = 1.
11. GPI A: allele absent = 0; allele present at low frequencies = 1; allele present at high frequencies = 2.
12. GPI C: allele present at high frequencies = 0; allele present at low frequencies = 1; allele absent = 2.
13. PER B: allele absent = 0; allele present = 1.
14. PER C: allele present at high frequencies = 0; allele absent = 1; allele present at high frequencies = 2.
15. PGM-1 A: allele present = 0; allele absent = 1.
16. PGM-1 B: allele absent = 0; allele present at low frequencies = 1; allele present at high frequencies = 2.
17. PGM-1 C: allele present at high frequencies = 0; allele present at low frequencies = 1; allele absent = 2.
18. PGM-2 A: allele present at low frequencies = 0; allele present at high frequencies = 1.
19. PGM-2 B: allele absent = 1; allele present = 0.

RESULTS

Occurrence of hybrids in the herbarium record

Some established species of *Centella* may in actual fact be of hybrid origin, but this is difficult to prove. Only a few herbarium specimens are possible hybrids. *Esterhuysen 16840,* for example, was identified as a hybrid on the label, and the presence of both parents at the same locality [Uniondale Dist., Zitzikamma Mountains near Joubertina (3323 DD)] was noted. Representative specimens of both parents were collected: *C. eriantha, Esterhuysen 16838* (BOL); *C. montana, Esterhuysen 16939* (BOL). Another specimen of a possible hybrid between *C. triloba* and *C. eriantha* is *Pillans 5894* (BOL), from the Noordhoek Mountains (3418 AB).

Hybridization between C. triloba *and* C. macrocarpa

At Kogel Bay we discovered two morphologically intermediate plants in the transitional zone between a population of *C. macrocarpa* and *C. triloba*. Since the plants were morphologically intermediate between the only two *Centella* species present at this site, we concluded that they must be hybrids. Supporting evidence for their hybrid origin is presented below. Different data sets were analysed to obtain a better understanding of character state distributions in the study group.

FIGURE 1.—Comparison of leaves, fruit and inflorescences of *C. triloba*, *C.* hybrid and *C. macrocarpa*. *C. triloba*: A1–A5, leaves; D1, D2, inflorescence; G, fruit. *C.* hybrid: B1–B5, leaves; E, inflorescence; H, fruit. *C. macrocarpa*: C1–C4, leaves; F1, F2, inflorescence; I, fruit. A1, D2, G, *Barker 6091*; A2, *Adamson 4175*; A3, *Ecklon & Zeyher s.n.*; A4, *Compton 13512*; A5, *Stokoe s.n.*; B1, B2, E, H, *Boucher 564*; B3–B5, *Schubert & Van Wyk 98*; C1, *Esterhuysen 32366*; C2, C4, *Zeyher 4901*; C3, *Parker 4262*; D1, *De Vos 1035*; F1, I, *Compton 17587*; F2, *Adamson 4262*. Scale bars: 10 mm.

Morphology

Morphological evidence for hybridization was based on characters of the leaves, petioles and mature fruit of the parent species and the hybrid. Figure 1A–C shows that the leaves of the putative hybrid are intermediate between the parent species in width and in the number of marginal teeth. The leaves are laminar in *C. triloba* and acicular in *C. macrocarpa*. Based on the outgroup method, broad leaves as well as the presence of three or more teeth are polarized as plesiomorphic while narrow and acicular leaves with few or no teeth respectively are considered as the apomorphic character states of these multistate characters (Table 3). Furthermore, in the hybrid and in *C. triloba*, a lamina can be distinguished from the petiole (as in most other Hydrocotyloideae), whereas in *C. macrocarpa*, no differentiation between lamina and petiole is evident. The differentiation of lamina and petiole is considered plesiomorphic whereas the phyllodinous state of the leaves of *C. macrocarpa* is polarized as apomorphic in Table 3. Transverse sections through the lamina and the petiole of the two species and the hybrid (Figure 2) show differences in shape and in the presence of a layer of palisade cells around the entire circumference of the leaf (dorsiventral in *C. triloba*, isobilateral in *C. macrocarpa* and the hybrid). In the petiole this palisade layer is absent. The continuous layer of palisade cells is not as evident in the lamina of the putative hybrid (Figure 2E) as in *C. macrocarpa* (Figure 2F). The leaves of *C. triloba* are clearly dorsiventral (Figure 2D). In Table 3 dorsiventral leaves are thus considered plesiomorphic whereas isobilateral leaves are considered apomorphic (see note under Data analysis). The leaves of *C. virgata* and *C. glauca* are acicular and isobilateral with no differentiation of lamina and petiole. Transverse sections of the petioles and the lamina of *C. triloba*, the hybrid and *C. macrocarpa*, (Figure 2D, G; E, H; F, I) confirm the intermediacy of the hybrid because of the intermediate shape of both the lamina and the petiole. The petiole of *C. macrocarpa* is winged, that of the hybrid is slightly winged and the petiole of *C. triloba* is not winged at all.

Centella macrocarpa and the hybrid have ribbed fruits, but the ribs on the fruits of *C. macrocarpa* are more distinct than those of the hybrid (Figure 2B, C). The fruits of the putative hybrid are intermediate in shape and are polarized as intermediate between the plesiomorphic character state (smooth fruits) of *C. triloba* and the apomorphic character state (distinctly ribbed fruits) of *C. macrocarpa* (Table 3). Ribbed fruits are exceptionally rare, not only within *Centella*, but within the subfamily Hydrocotyloideae as a whole. As a result, virtually any choice of outgroup would lead to the same polarity decision. Furthermore, the fruits of *C. macrocarpa* are much larger than those of the other species and the hybrid (Figure 1G–I) and the relatively large size is considered apomorphic. Smaller fruits are universal in *Centella* and the polarity decision will therefore not be different for any other outgroup. The fruits of *C. virgata* are smaller than those of *C. macrocarpa* and less distinctly ribbed, whereas the fruits of *C. glauca* are particularly large with well pronounced ribs. *Centella triloba* may be andromonoecious

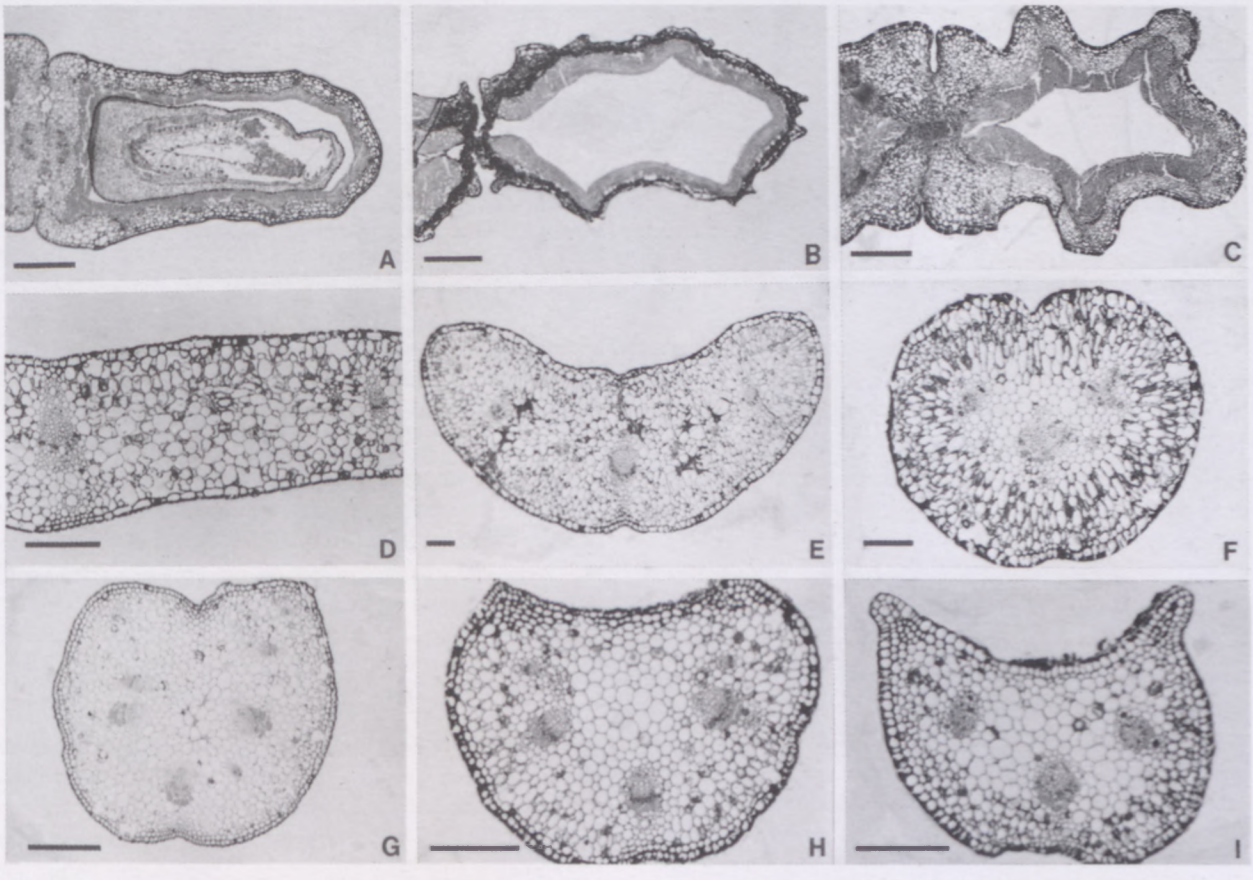

FIGURE 2.—Ultramicrotome sections showing anatomy of leaf lamina, petiole and fruit of *C. triloba*, *C.* hybrid and *C. macrocarpa*. *C. triloba*: A, fruit; D, leaf lamina; G, petiole. *C.* hybrid: B, fruit; E, leaf lamina; H, petiole. *C. macrocarpa*: C, fruit; F, leaf lamina; I, petiole. A, D, G, *Schubert & Van Wyk 15*; B, *Boucher 564*; E, H, *Schubert & Van Wyk 98*; C, F, I, *Schubert & Van Wyk 53*. Scale bars: 0.5 mm.

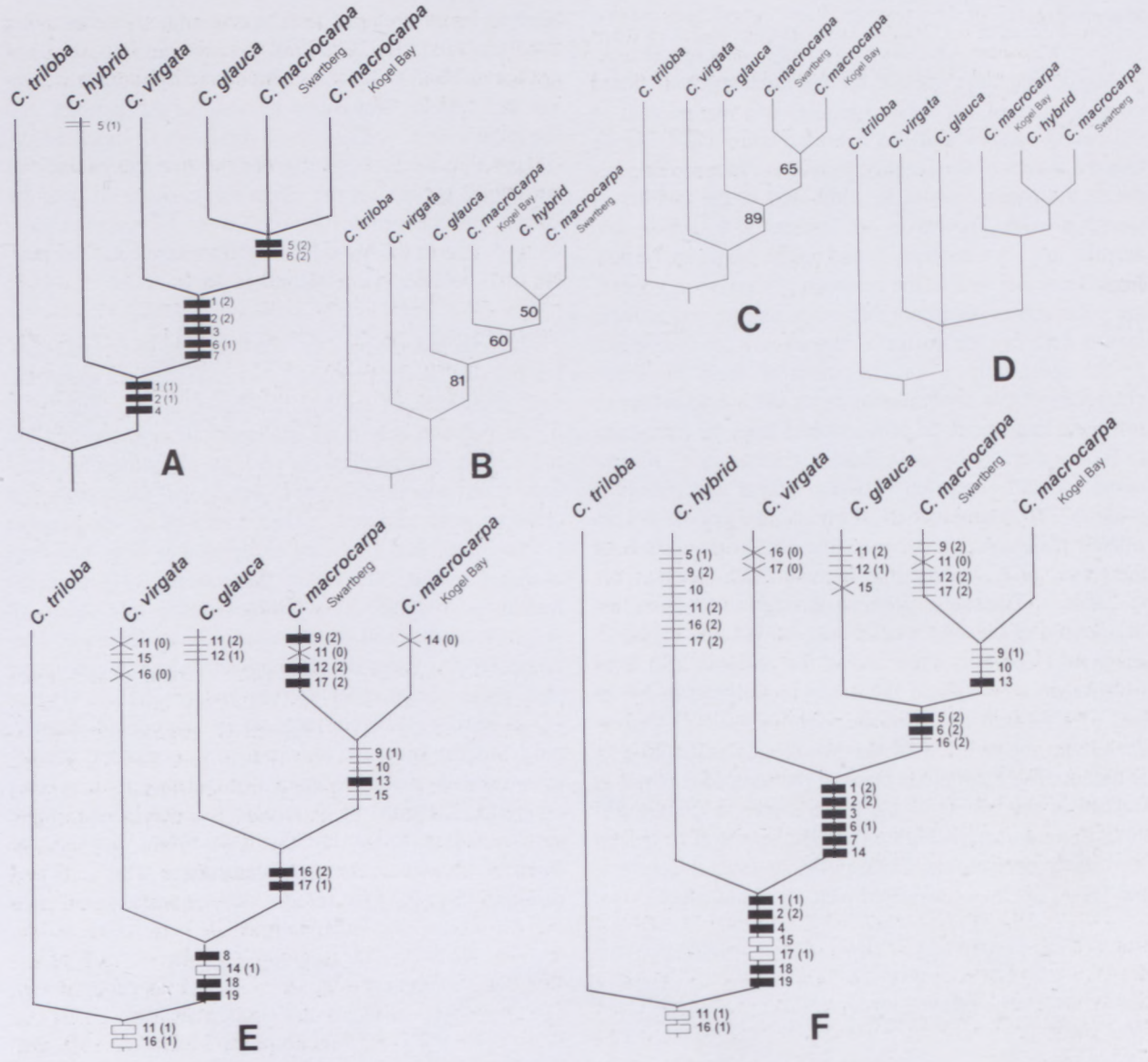

FIGURE 3.—Cladograms and phenograms showing relationships between some species of *Centella*. A, cladogram based on morphological data. B, phenogram, including hybrid, constructed with DISPAN (Ota 1993). C, phylogenetic tree, excluding hybrid, constructed with DISPAN (Ota 1993). D, cladogram, including hybrid, based on genetic data. E, cladogram, excluding hybrid, based on genetic data. F, cladogram based on morphological and genetic data. Characters and polarization of characters used in construction of cladograms listed in Table 3. Solid square, apomorphy without homoplasy; open square, apomorphy with reversal higher up; =, convergence; x, reversal.

(male and bisexual flowers on the same plant but no female flowers) or androdioecious (male and bisexual flowers on different plants and no female flowers) (Figure 1D1, 1D2). The bisexual, functionally female umbellules are made up of a single fruit while the male umbellules have three to five flowers of which the petals occasionally are villous. *Centella macrocarpa* may be andromonoecious or androdioecious as well, but instead of the inflorescence of functionally female plants having many functionally female umbellules as in *C. triloba*, it is made up of a single umbellule (Figure 1F1). Furthermore, when male and bisexual flowers are present, the inflorescence will bear only a single functionally female umbellule (Figure 1F2). The male umbellules have three to five male flowers with glabrous petals. The hybrid is andromono-ecious (Figure 1E), but only a limited number of specimens is available. The inflorescence is otherwise compar- able to that of *C. macrocarpa* with villous male flowers (Figures 1F1, 1F2). In Table 3 the glabrous petals of *C. virgata*, *C. glauca* and the two *C. macrocarpa* populations are polarized as apomorphic, because of the presence of villous petals in the outgroup. *Centella virgata* is andromonoecious and *C. glauca* may be androdioecious or andromonoecious.

The morphological data analysis resulted in a partially resolved cladogram with a length of 11 steps and a consistency index of 0.90 (Figure 3A). The cladogram is not fully resolved as a polytomy occurs between the two *C. macrocarpa* populations and *C. glauca*.

Allozyme data

Genetic variation within species was observed at seven of the 15 enzyme-coding loci. The choice of five of these loci, i.e., CAP-1, GPI, PER, PGM-1 and PGM-2, for further data analysis was confirmed by their relatively high fixation index (F-statistic) values (Table 4, the values are explained in the caption). The alleles that contributed most to population differences at these loci are represented by characters 8 to 19 in Table 3 (their F_{ST} values are all

TABLE 4.—Summary of F-statistics at all loci. F_{IS} and F_{IT} are the fixation indices of individuals relative to the total population and its subpopulations, respectively. F_{ST} measures the amount of differentiation among subpopulations relative to the limiting amount under complete fixation

Locus	F_{IS}	F_{IT}	F_{ST}
AAT-1	0.188	0.364	0.217
ACP-2	1.000	1.000	0.429
CAP-1	0.201	0.781	0.726
GPI	0.850	0.944	0.630
PER	0.657	0.961	0.885
PGM-1	0.077	0.566	0.530
PGM-2	0.067	0.627	0.600
Mean	0.417	0.767	0.601

above 0.500). Figure 3B shows the dendrogram produced with DISPAN and the complete set of allozyme data whereas Figure 3C shows the dendrogram without the hybrid. Four cladograms were produced from the characters (8–19) in Table 3, of which the consensus tree is shown in Figure 3D. The length of the consensus tree is 23 steps with a consistency index of 0.73. When the hybrid was excluded from the data set, the resulting cladogram was fully resolved, with a length of 21 steps and a consistency index of 0.80 (Figure 3E). According to McDade (1995), a higher consistency value is to be expected, since the removal of a hybrid from the analysis should lead to a significant reduction in homoplasy.

Table 5 lists the genetic distances between the species and populations studied. From this information it is evident that the shortest genetic distance was found between the hybrid and the *C. macrocarpa* population from Kogel Bay. The genetic distance between the two *C. macrocarpa* populations is relatively small (0.091) when compared to the average distance between species (0.218). The species with the shortest genetic distance are *C. macrocarpa* and *C. triloba* (0.108) and the species with the longest distance between them are *C. macrocarpa* and *C. virgata* (0.350). The shortest genetic distances are those between *C. triloba*, the hybrid, and the two *C. macrocarpa* populations. This is what one would expect, given the proposed hybrid origin. *Centella glauca* and *C. virgata* are only linked into this group at a much greater genetic distance.

Table 5 lists results of the BIOSYS analysis used to establish the genetic diversity within populations. The genetic diversity within the *C. triloba* and the *C. macrocarpa* (Kogel Bay) populations is by far the greatest (mean heterozygosity per locus 14.2%, 15.4%; percentage of loci polymorphic 40, 40; mean number of alleles per locus 1.4, 1.13 respectively) while that of the *C. virgata* and the *C. macrocarpa* (Swartberg) populations is the smallest (mean heterozygosity per locus 4.9, 5.0; percentage of loci polymorphic 13.3, 13.3; mean number of alleles per locus 1.13, 1.13 respectively).

When the morphological and genetic data sets were combined, the result was a fully resolved cladogram with a length of 40 steps and a consistency index of 0.70 (Figure 3F). When the hybrid was removed from this data set, the tree length reduced to 32 steps and the consistency index improved to 0.87. This reduction in homoplasy is again consistent with our assumption of hybridization, following McDade (1995).

Geographical distribution of the putative hybrid and the parent species

Both *C. triloba* and *C. macrocarpa* occur in the Western Cape but *C. triloba* is limited to the Cape Peninsula and coastal areas of the Caledon District, whereas *C. macrocarpa* is more widespread in the region. The hybrid is very localized in occurrence. Only two plants were found at Kogel Bay and both parent species occur at the same locality (Figure 4). The *C. triloba* population occurs on a relatively flat area closer to the sea than the *C. macrocarpa* population, which grows on a steep scree slope. The putative hybrids occurred between the two populations at the foot of the scree slope.

DISCUSSION

Centella triloba, the hybrid and *C. virgata* formed morphologically discrete clades with only one overlapping morphological character between *C. virgata* and the hybrid, namely the presence of ribbed fruit. *Centella glauca* and *C. macrocarpa* are mainly distinguished by their habit differences and the distinctly glaucous leaves of the former species (Schubert & Van Wyk 1996). A polytomy results as these autapomorphic characters were not used in the cladogram (Figure 3A). The morphological data showed that the hybrid is intermediate between *C. triloba* and the rest of the study group. Characters, such as the presence of isobilateral leaves in the hybrid, group it with *C. macrocarpa*, whereas the similarities with *C. triloba* all appear to be symplesiomorphies. McDade (1990) also found that hybrids do not lead to unresolved cladograms with high levels of homoplasy, but that they emerge as

TABLE 5.—Genetic variation and differentiation within and between: *C. triloba* (P1); a putative hybrid between *C. triloba* and *C. macrocarpa* (H); *C. macrocarpa* (resprouter, population from Kogel Bay) (P2); and *C. macrocarpa* (reseeder, population from Swartberg Pass) (P3); *C. virgata* (V); and *C. glauca* (G)

Population	mean heterozygosity per locus	% of loci polymorphic	mean # of alleles per locus
P1	14.2%	40.0	1.4
H	6.7%	13.3	1.13
P2	4.9%	13.3	1.13
P3	8.1%	26.7	1.13
V	5.0%	13.3	1.13
G	15.4%	40.0	1.13

Nei's (1978) unbiased genetic distance between: *C. triloba* (P1); a putative hybrid between *C. triloba* and *C. macrocarpa* (H); *C. macrocarpa* (resprouter, population from Kogel Bay) (P2); and *C. macrocarpa* (reseeder, population from Swartberg Pass) (P3); *C. virgata* (V); and *C. glauca* (G)

Population	H	P2	P3	V	G
P1	0.139	0.108	0.297	0.151	0.208
H		0.005	0.124	0.311	0.156
P2			0.091	0.198	0.127
P3				0.350	0.254

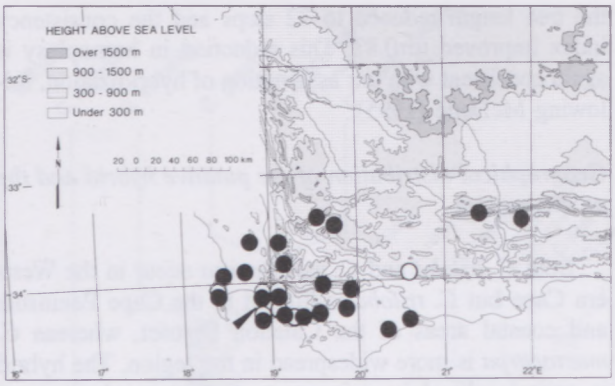

FIGURE 4.—The known geographical distribution of *C. triloba*, ○; *C.* hybrid (indicated by arrow); and *C. macrocarpa*, ●.

basal to the clade which includes the most derived of the two parents (Figure 3A, F).

In choosing *C. triloba* as outgroup, we expected the topology A in Figure 3. Obviously, the similarities between the hybrid and *C. macrocarpa* would be interpreted as synapomorphies. If we had chosen *C. virgata* or *C. glauca* as outgroup, the reverse would have been true, i.e. the hybrid would have grouped with *C. triloba*. According to McDade (1995), 'Hybrids may express the derived states (apomorphies) of both parents, the apomorphies of one parent, partially derived conditions that are intermediate between the parents, or states that are more extreme than either (autapomorphies)'. If our putative hybrid was unrelated to the two species with which it was found growing, we would not have expected any intermediate characters. The data in Table 3 show the following pattern of character expression: four characters shared with *C. triloba*, seven characters shared with *C. macrocarpa*, three intermediate characters and three unique characters. It thus seems reasonable to conclude that the relatively high number of intermediate characters support our assumption of hybrid origin based on field observation. Even if the two plants found at Kogel Bay eventually turned out to be a new species (we do not exclude this possibility) we are arguing that it is of hybrid origin, being derived from *C. triloba* and *C. macrocarpa*.

Allele frequencies calculated at CAP-1 (A, B and C alleles), PGM-1 (A and B alleles) and PGM-2 (B and C alleles) grouped the hybrid together with *C. macrocarpa*, whereas the allele frequencies recorded for PER (B and C alleles) coincided for the hybrid and *C. triloba*. The larger number of allele frequencies shared with *C. macrocarpa* resulted in the hybrid being nested in the polytomy in Figure 3D. This is again in agreement with the proposals of McDade (1995), who suggested that the hybrid would be placed near to the parent that has the most derived characters. The combined morphological and genetic data also present supporting evidence for the hypothesized hybrid origin. When the hybrid was omitted, a fully resolved topology resulted, with a substantial improvement in both the tree length and consistency index (Figure 3E). The morphology gives only a partially resolved cladogram (Figure 3A) but the *C. glauca*/*C. macrocarpa* polytomy becomes fully resolved in the combined analysis, with the genetic data responsible for the improvement. These analyses are consistent with two generalizations: 1, removal of the hybrid improves the resolution and 2, both data sets (morphological and genetic) contribute to resolving different parts of the topology, as is evident in Figure 3A & F. *C. virgata* is clearly separated from *C. macrocarpa* in all the analyses, which gives useful support for considering it as a distinct species despite the close similarity to some forms of *C. macrocarpa* (particularly the reseeding forms). Fire-survival appears to be a homoplasious character which has evolved convergently in different species.

The *C. macrocarpa* populations were grouped together on the final cladogram (Figure 3F). This is interesting since the Swartberg population is a reseeder and the Kogel Bay population is a resprouter, and the genetic distance between them (Table 5) was less (0.091) than between the *C. macrocarpa* reseeder population and another closely similar reseeder species, *C. virgata* (0.350). Likewise, the distance of 0.091 is less than the distance between the resprouting *C. glauca* and the resprouting population of *C. macrocarpa* (0.127). Thus the phenomena of reseeding and resprouting (Schutte *et al.* 1995) may occur in a single species.

There is a possible correlation in the genetic variation of the reseeder populations and also of the resprouter populations, i.e. that resprouters are genetically more variable than the reseeder populations. The latter are more prone to genetic bottlenecks, as was proposed by Schutte *et al.* (1995), because presumably only one generation is present at any given time, while the survival of the resprouters leads to a mixture of numerous generations within the same population. Further studies, including larger sample sizes, are needed to confirm the relationship between genetic variation and fire-survival strategy in *Centella*.

The demonstration that hybridization occurs between two species of *Centella* that are not sister taxa indicates that a strictly hierarchical infrageneric classification may be an unobtainable goal. Even the cladistic method may become problematic, particularly when nodes are weakly supported [see McDade (1992, 1995) for a detailed discussion of the impact of hybrids on cladistic analysis]. The assumption of divergent evolution needed for the cladistic method may thus not be an accurate reflection of the actual mechanisms of evolution within the genus. If evolution is indeed reticulate, then the identification of lineages of ancient hybrid origin becomes problematic (McDade 1990). Nevertheless, the use of different data sets in determining phylogenetic relationships between *Centella* species, resulted in a closer proximation to the true phylogeny than would have been possible with either of the data sets (Shakalee & Whitt 1981; McDade 1995).

The new insight into evolutionary relationships in *Centella* provides a possible explanation for the reticulate pattern of character expression in the genus. Adamson (1951) was unable to create mutually exclusive infrageneric taxa and coped with the problem by including some species in more than one series. Roux *et al.* (1978), Winter & Van Wyk (1995) and Allison (1995) have used cladistic methods to study Apiaceae, marking the beginning of a

rigorous, empirical approach to classification in this family. However, if a hybrid disrupts the pattern of character expression by inheriting the defining apomorphies of unrelated parents from different sections or series, then it will not be possible to use cladistics in the normal way because the method relies on divergence. McDade (1995) highlighted several new attempts at resolving this problem.

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