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Genetic polymorphism detected with RAPD analysis and morphological variability in some microspecies of apomictic *Alchemilla*

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Alchemilla L. (Rosaceae) contains numerous agamospermous microspecies, which are often treated as species. However, many of them are not clearly morphologically distinct, and their genetic variability is practically not investigated. In the present study, we used RAPD analysis to assess the genetic relatedness between *Alchemilla* microspecies. In all, 51 plants from 12 *Alchemilla* microspecies were analysed, and 116 characters were considered (68 RAPD bands over three primers and 48 morphological characters). Phylogenetic trees were constructed by the unweighted pair-group method, neighbour-joining and maximum parsimony methods. The genetic data supported most Fröhner's system of sections. Despite the use of a limited set of data in the investigation and weak support values, some tentative conclusions could be based on congruence of the RAPD analysis and morphological data. *Alchemilla acutiloba* Opiz and *A. micans* Buser should be united as a single microspecies, *A. micans;* section *Plicatae* should be divided into two series *Pubescentes* and *Barbulatae*; and *A. heptagona* Juz. may be separated in *Exuentes* series of *Ultravulgares*.

Keywords: genetic variation, molecular taxonomy, morphological variation, random amplified polymorphic DNA

INTRODUCTION

Genus *Alchemilla* L. (Rosaceae) contains numerous microspecies, which are often treated as species. Most of the microspecies are high polyploids (Wegener 1967) reproducing apomictically (Strasbourger 1905), though in the recent works of Glazunova (1987) and Izmailow (1994) it was stated that the apomixis is facultative and probably hybridisation takes place from time to time. This makes the taxonomy very complicated. In fact, in practical geobotanical fieldwork all microspecies under investigation here are identified as a collective species *A. vulgaris* L. coll.

The microspecies have very different distributions. Endemic ones probably represent single or few clones, while widespread species are certainly not genetically homogenous. Experimental works of Turesson (1943, 1956, 1957) and Lundh-Almestrand (1958), where genetic variants were detected within microspecies prove the latter fact. According to Turesson (1943), the genetically distinct types within microspecies, called agamotypes, are specialised to different habitats.

The genus has not been very intensely investigated in the last few decades; the only noteworthy modern considerations of *Alchemilla* originate from Fröhner (1995 and earlier works). The latter are based on morphology and cytology (chromosome numbers and shapes), but they are purely classical, empirical works. We analysed the variation of the morphological features, and the distinctness of some microspecies and sections according to them, with multivariate statistical methods (Sepp & Paal 1998). However, we did not thoroughly investigate genetic variability within the genus. The rather old experimental works mentioned above and the very small-scale research of Baeva *et al*. (1998) are so far the only attempts. Walters (1987) stressed the need to use so-called biological systematics for solving the problems of this genus, but his advice is not followed very enthusiastically.

According to the statistical analysis of morphological features (Sepp & Paal 1998), lots of the pairs of microspecies are mutually indistinct. Experienced botanists know that several pairs of microspecies (e.g. *Alchemilla acutiloba* and *A. micans, A. glaucescens* and *A. hirsuticaulis*) have continuous variation also in nature. Considering the fact that several microspecies normally occur in the same habitat, and the conclusions of Turesson (1943), one may assume that they could have parallel variation, but this is not proved. Genetic variation within and among microspecies should be investigated to decide if it could be so, and if the morphologically indistinct pairs or even complexes of microspecies should be taxonomically united.

The development of "molecular markers", which reveal extensive polymorphism at the DNA or protein level, has greatly facilitated research in taxonomy, phylogeny and genetics. In recent years, a molecular technique called the random amplified polymorphic DNA (RAPD) assay (Williams *et al.* 1990), is increasingly used for detecting and estimating genetic diversity, in agamosperms and other species (e.g. Van Coppenolle *et al.* 1993, Wachira *et al.* 1995, Marillia & Scoles 1996, Brunell & Whitkus 1997, Crawford 1997). Intraspecific genetic variability and species borders are successfully investigated using RAPD (Weising *et al.* 1995, Bachmann 1997, Kokaeva *et al.* 1998). RAPD markers are generated by the amplification of anonymous genomic DNA segments with single, 10 base pair, arbitrary primers. Amplified DNA fragments are size-fractionated by agarose gel electrophoresis, and polymorphism is detected as the presence or absence of a particular band. The method is based on the statistical probability that complementary primer sites occur repeatedly in the genome. There may be problems with repeatability of the experiments, and with compatibility between laboratories, but these can be overcome by ensuring that the temperature profiles inside the tubes are identical (Penner *et al.* 1993). The main problem is that the markers are anonymous and one cannot be sure whether the annealing sites are really homologous (Quiros *et al.* 1995). Nevertheless, in comparison with some other analogous methods (restriction fragment polymorphism, minisatellite DNA fingerprinting) RAPD is much faster and simpler. In comparison with isozyme electrophoresis, the RAPD markers are always dominant and they give more information involving the whole genome (Penner *et al.* 1993, Hillis 1996).

In the current study, we used RAPD analysis

to assess the genetic relatedness of *Alchemilla* microspecies. Preliminary data showing the variability of RAPD patterns for 10 accessions of *A. vulgaris s. lato* (Baeva *et al*. 1998) revealed dissimilarity of populations within microspecies.

MATERIAL AND METHODS

In total, 51 plants from 12 *Alchemilla* microspecies (*A. acutiloba, A. baltica, A. cymatophylla, A. glabricaulis, A. glaucescens, A. micans, A. heptagona, A. hirsuticaulis, A. monticola, A. sarmatica, A. semilunaris, and A. subcrenata*) were analysed. Forty-two samples were collected in Estonia (Table 1). Voucher specimens are deposited in the Herbarium of the Institute of Botany and Ecology of Tartu University (TU). On these plants, the same morphological characters as in phenetic analysis (Sepp & Paal 1998) were measured and coded for cladistic analysis (Table 2). Nine samples were collected at the Biological station of Moscow University in Zvenigorod, Moscow district, Russia (Table 1), but without voucher specimens, hence no morphological data were recorded for those. All plants were identified or their identification verified by K. P. Glazunova. Classifications of the studied microspecies according to the different systems of genus *Alchemilla* are presented in Table 3.

DNA was extracted from quickly dried (40 °C) or frozen leaves according to a slightly modified protocol of Doyle and Doyle (1987). The concentration of the template was estimated on agarose minigel in comparison with a previously known DNA sample. DNA was amplified in 20 µl reaction mixtures containing 67 mm Tris-HCl (pH 8,4), 16,6 mm $(NH_4)_2SO_4$, 2.5 mm $MgCl_2$, 0,01% gelatin, 100 mm each of dATP, dCTP, dGTP and dTTP, 10 pmol primer, 2 units Taq polymerase (Sileks, Moscow, Russia) and 10–25 ng of the DNA template. From a set of primers initially tested for polymorphism, three gave good variation, and these were used for further analysis (primer 1: 5´ CTCACCGTCC 3´; primer 2: 5´ AGGCGGGAAC 3´; primer 3: 5´ ACGGTACCAG 3´). PCR reactions were carried out in a thermal cycler CycloTemp 6 (CTM, Russia). The programme consisted of 2 cycles of 94 °C for 4 min, 25 °C for 2 min, 72 °C for 2 min; 40 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 1 min; and 1 cycle of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 4 min. All the PCR reactions were repeated at least twice to confirm repeatability and if discrepancies occurred, PCR was repeated until two identical results were achieved. Amplified fragments were run on 2% NuSieve 3:1 agarose gels (FMC), stained with ethidium bromide and photographed on an UV transilluminator.

Altogether, 116 characters were considered: 68 RAPD bands over three primers and 48 morphological characters (characters in Table 2, data matrix in Appendix).

Phylogenetic trees were constructed with the unweighted pair-group method with the arithmetic average (UPGMA) and neighbour-joining (NJ) methods with the TREECON package (Van de Peer & De Wachter 1994). The genetic distances GD were calculated as follows (Link *et al*. 1995):

$$GD_{xy} = (N_x + N_y)(N_x + N_y + N_{xy})^{-1} \qquad (1),$$

where N_x is the number of bands in lane x and not in lane y, N_y is the number of bands in lane y and not in lane x, and N_{xy} is the number of bands in lanes x and y. For the NJ tree, bootstrap values were calculated.

Maximum parsimony (MP) analysis was carried out with the PAUP 3.1.1 programme (Swofford 1993). Heuristic search settings: random addition sequence (10 replicates), tree bisection-reconnection branch swapping, MULPARS option, and accelerated transformation were used for character state optimisation. Bootstrap values and Bremer's decay indices (Bremer 1988) were calculated. MP analyses were performed on three different data sets: RAPD data separately, morphological data separately and the combined data.

We used the functional outgroup method in NJ and MP analysis. An outgroup for NJ analysis, a sample of *Alchemilla heptagona,* 21hep11, was chosen according to the UPGMA tree. An outgroup for MP analysis (*A. glaucescens* and *A. hirsuticaulis*, altogether 7 plants), was chosen as a monophyletic group of reasonable size, detected from an unrooted MP tree. The fact that these two microspecies are considered to be similar and belonging to one section by many authors was also taken into account.

RESULTS

The three analysed primers gave altogether 68 bands. Fig. 1 shows an example of RAPD amplification results with primer 1.

The clusters that appeared in the UPGMA phenogram of RAPD data (Fig. 2) correspond rather well to Fröhner's sections. Only 4 samples,

Table 1. *Alchemilla* accessions in analysis.

Notation	Sample	Species	Population
01acu16	1	*A. acutiloba*	16 = Estonia, Saaremaa Island, Loode, oak forest
11acu16	11	"	"
08acu05	8	"	5 = Estonia, Lääne county, Nõva, meadow
18acu05	18	"	"
35acu15	35	"	15 = Estonia, Põlva county, Valgjärve, park
36acu15	36	"	"
42acu15	42	"	"
v1acuMO	v1	"	MO = Russia, Moscow district, Zvenigorod
30bal04	30	*A. baltica*	4 = Estonia, Viljandi county, Tipu, meadow
31bal04	31	"	"
v2balMO	v2	"	MO (*see* above)
15cym12	15	*A. cymatophylla*	12 = Estonia, Põlva county, Wooded meadow
26gli05	26	*A. glabricaulis*	5 (*see* above)
v8gliMO	v8	"	MO (*see* above)
24glc20	24	*A. glaucescens*	20 = Estonia, Saaremaa Island, Kübassaare, meadow
25glc22	25	"	22 = Estonia, Saaremaa Island, Viidu, glade
40glc22	40	"	"
28glc24	28	*A. glaucescens*	24 = Estonia, Saaremaa Island, Kübassaare, alvar
29glc24	29	"	"
05mic04	5	*A. micans*	4 (*see* above)
09mic05	9	"	5 (*see* above)
14mic12	14	"	12 (*see* above)
16mic12	16	"	"
32mic02	32	"	2 = Estonia, Viljandi county, Halliselja, meadow
33mic02	33	"	"
v6mic MO	v6	"	MO (*see* above)
21hep11	21	*A. heptagona*	11 = Estonia, Põlva county, Valgjärve, scrub
22hep11	22	"	"
39hep11	39	"	"
v9hepMO	v9	"	MO (*see* above)
23hir20	23	*A. hirsuticaulis*	20 (*see* above)
38hir24	38	"	24 (*see* above)
v5hirMO	v5	"	MO (*see* above)
03mon26	3	*A. monticola*	26 = Estonia, Saaremaa Island, Loode, alvar
07mon02	7	"	"
12mon26	12	"	"
04mon27	4	"	27 = Estonia, Saaremaa Island, Loode, oak forest
06mon27	6	"	"
13mon27	13	*A. monticola*	27 = Estonia, Saaremaa Island, Loode, oak forest
10mon02	10	"	2 (*see* above)
37mon04	37	"	4 (*see* above)
v3monMO	v3	"	MO (*see* above)
v4sarMO	v4	*A. sarmatica*	MO (*see* above)
19scr12	19	*A. subcrenata*	12 (*see* above)
34scr16	34	"	16 (*see* above)
41scr16	41	"	"
v7se mm O	v7	*A. semilunaris*	MO (*see* above)

Table 2. Characters used in analysis.

Character Nr.	Denotation of corresponding phenetic character	Meaning	States and corresponding measured values
1–68		RAPD bands	1 = present, 0 = not
69	SILK	hairs sericeous	0 = no, 1 = yes
70	STPOS	position of stems	1 = decumbent, 2 = bentform ascending, 3 = erect
71	HRPOS	position of hairs on stem and petiole	1 = deflexed, 2 = patent, 3 = erecto = patent, 4 = appressed
72	LECOL	leaf colour	1 = yellowish green, 2 = grass green,3 = dark green
73	LECOL	leaf colour	1 = leaves bluish, 0 = not
74	LECOL	leaf colour	1 = leaves greyish, 0 = not
75	FLCOL	flower colour	1 = yellow, 2 = yellowish green, 3 = grass green
76	FLCOL	flower colour	1 = flowers reddish, 0 = not
77	STCOL	stipule colour	1 = brown, 0 = not
78	STCOL	stipule colour	1 = red, 0 = not
79	STCOL	stipule colour	1 = green, 0 = not
80	LEFLD	leaf foldedness	0 = not folded, 1 = slightly folded, 2 = strongly folded
81	INFSH	shape of inflorescence	1 = narrow, 2 = wide
82	FLGDN	density of flower glomeruli	1 = sparse, 2 = dense
83	LBTOP	shape of leaf lobe tops	1 = obtuse, 2 = acute
84	INCDP	depth of incisions between leaf lobes	0 = missing, 1 = shallow, 2 = deep
85	THTOP	shape of leaf teeth	1 = obtuse, 2 = acute tops
86	THSYM	symmetry of leaf teeth	1 = asymmetrical, 2 = symmetrical
87	CASH	shape of sepals	1 = obtuse, 2 = acute
88	HYSH	shape of hypanthium	1 = tubular, 2 = funnel = shaped, 3 = campanulate, 4 = round
89	STNR	number of flowering stems	1 = 0–1, 2 = 2–3, 3 ≥ 4
90	LENR	number of basal leaves	1 = 1–4, 2 = 5–6, 3 = 7–9, 4 ≥10
91	LBCOR	angle between basal lobes of leaf	1 = 0–10°, 2 = 10–30°, 3 = 30–60°, 4 ≥ 60°
92	STLN	length of flowering stems	1 ≤ 30cm, 2 = 30–50cm, 3 ≥ 50cm
93	STLHR	hairiness of the lower part of stem	1 = 0–20 mm^{-1}, 2 = 20–50, 3 = 50–80, 4 ≥ 80
94	STUHR	hairiness of the upper part of stem	1 = 0–5 mm^{-1}, 2 = 5–14, 3 = 15–40, 4 ≥ 40
95	PETHR	hairiness of petiole	1 = 0–20 mm^{-1}, 2 = 20–50, 3 = 50–80, 4 ≥ 80
96	SLELN	length of stem leaf	1 ≤ 15 mm, 2 = 15–25 mm,3 ≥ 25 mm
97	PETLN	length of petiole	1 ≤ 15cm, 2 = 15–25cm, 3 = 25–40cm, 4 ≥ 40cm
98	LBNR	number of lobes per leaf	1 = 6–8, 2 = 9, 3 = 10–11
99	LEUHR	hairiness of the upper surface of leaf	1 = 0–5 mm^{-2}, 2 = 5–7, 3 = 8–10, 4 ≥ 10
100	LELHR	hairiness of the lower surface of leaf	1 = 0–6 mm^{-2}, 2 = 6–20, 3 = 20–40, 4 ≥ 40
101	VNHR	hairiness of leaf veins	1 = 0–20 mm^{-1}, 2 = 20–40, 3 = 40–60, 4 ≥ 60
102	LELN	length of basal leaf	1 ≤ 30 mm, 2 = 30–40 mm, 3 = 40–55 mm, 4 ≥ 55 mm
103	LEWD	width of basal leaf	1 ≤ 60 mm, 2 = 60–90 mm, 3 = 90–100 mm, 4 ≥ 100 mm
104	LBLN	length of leaf lobe	1 ≤ 10 mm, 2 = 10–15 mm, 3 = 16–25 mm, 4 ≥ 25 mm
105	LBWD	width of leaf lobe	1 ≤ 20 mm, 2 = 20–25 mm, 3 = 26–35 mm, 4 ≥ 35 mm
106	THNR	number of leaf teeth	1 ≤ 15, 2 = 15–17, 3 = 18–19, 4 ≥ 20
107	STHLN	length of leaf tooth (not apical)	1 ≤ 1.4 mm, 2 = 1.4–1.8 mm, 3 ≥ 1.8 mm
108	TTHLN	length of the apical tooth	1 ≤ 1 mm, 2 = 1–1.3 mm, 3 ≥ 1.3 mm

Continued

Table 2. Continued.

Character Nr.	Denotation of corresponding phenetic character	Meaning	States and corresponding measured values
109	STHWD	width of leaf tooth (not apical)	1 ≤ 1.9 mm, 2 = 1.9–2 mm, 3 ≥ 2 mm
110	PEDHR	hairiness of peduncle	1 = 0–10 mm^{-1}, 2 = 10–20, 3 = 20–40, 4 ≥ 40
111	HYHR	hairiness of hypanthium	1 = 0–10 per side, 2 = 10–30, 3 ≥ 30
112	HYLN	length of hypanthium	1 ≤ 1.2 mm, 2 = 1.2–1.6 mm, 3 = 1.6–2 mm, 4 ≥ 2 mm
113	HYWD	width of hypanthium	1 ≤ 0.8 mm, 2 = 0.8–1.1 mm, 3 ≥ 1.1 mm
114	CALN	length of sepal (inner circle)	1 ≤ 1 mm, 2 = 1–1.1 mm, 3 = 1.1–1.2 mm, 4 ≥ 1.2 mm
115	CAHR	hairiness of sepal	1 = 0–1 per sepal, 2 = 1–5, 3 = 5–25, 4 ≥ 25
116	OCALN	length of sepal of outer circle	1 = 0.8 mm, 2 = 0.8–0.9 mm, 3 = 0.9–1 mm, 4 ≥ 1 mm

Table 3. Classification of the studied *Alchemilla* species according to different authors. Notations of species as in Table 1.

Species	Plocek (1982)	Yuzepchuk (1941)	Fröhner (1990)	Proposed in Sepp and Paal (1998)
GLC, HIR	ser. *Pubescentes*	sect. *Pubescentes*	sect. *Plicatae*	sect. *Plicatae* ser. *Pubescentes*
MON	ser. *Hirsutae*	sect. *Vulgares* ser. *Hirsutae* gr. *Barbulatae*	"	sect. *Plicatae* ser. *Barbulatae*
SAR	"	sect. *Vulgares* ser. *Hirsutae* gr. *Imberbes*	"	"
ACU, MIC	"	"	sect. *Alchemilla*	sect. *Hirsutae* ser. *Alchemilla*
CYM, SCR	"	"	sect. *Ultravulgares*	sect. *Hirsutae* ser. *Ultravulgares*
SEM	Ser. *Hirsutae*	sect. *Vulgares* ser. *Hirsutae* gr. *Imberbes*	?	sect. *Hirsutae* ser. *Decumbentes*
HEP	"	sect. *Vulgares* ser. *Hirsutae* gr. *Exuentes*	sect. *Ultravulgares*	sect. *Hirsutae* ser. *Ultravulgares*
GLI	Sect. *Glabrae*	sect. *Vulgares* ser. *Hirsutae* gr. *Glabricaules*	sect. *Coriaceae*	sect. *Coriaceae* ser. *Glabricaules*
BAL	"	sect. *Vulgares* ser. *Subglabrae* gr. *Glabratae*	"	sect. *Coriaceae* ser. *Coriaceae*

marked with asterisks (v5hirMO, v3monMO, 30bal04, and 21hep11), were placed outside the clusters of their "own" sections. Section *Plicatae,* except the two plants in anomalous positions, is clearly one big cluster. It can be split further, into two branches joining *A. glaucescens* and *A.*

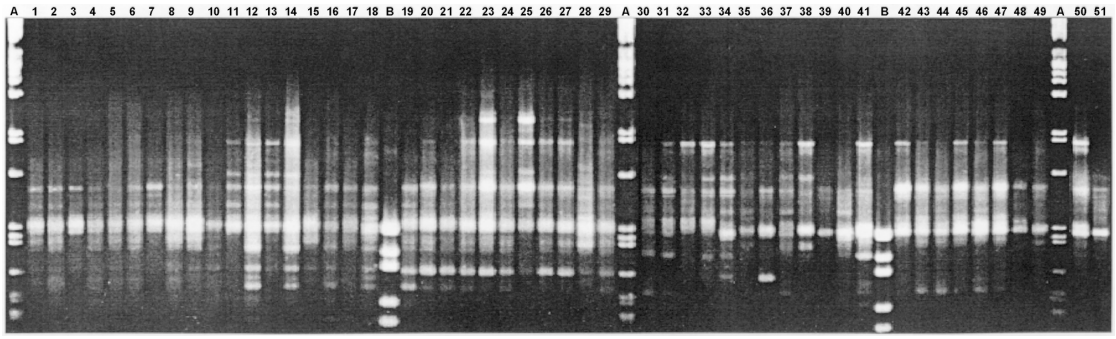

Fig. 1. RAPD profiles for *Alchemilla* accessions obtained with primer 1 (*see* Material and methods). 1: 08acu05, 2: 18acu05, 3: 35acu15, 4: 36acu15, 5: 42acu15, 6: 01acu16, 7: 02acu16, 8: 11acu16, 9: v1acuMO, 10: 32mic02, 11: 33mic02, 12: 05mic04, 13: 20mic04, 14: 09mic05, 15: 17mic05, 16: 14mic12, 17: 16mic12, 18: v6micMO, 19: 10mon02, 20: 27mon02, 21: 37mon04, 22: 03mon26, 23: 07mon26, 24: 12mon26, 25: 04mon27, 26: 06mon27, 27: 13mon27, 28: v3monMO, 29: v4sarMO, 30: 21hep11, 31: 22hep11, 32: 39hep11, 33: v9hepMO, 34: 19scr12, 35: 34scr16, 36: 41scr16, 37: 15cym12, 38: v7se mm O, 39: 30bal04, 40: v2balMO, 41: 26gli05, 42: 24glc20, 43: 25glc22, 44: 40glc22, 45: 28glc24, 46: 29glc24, 47: 23hir20, 48: 38hir24, 49: v5hirMO, 50: v8gliMO, 51: 31bal04. Denotations of samples are explained in Table 1. DNA molecular weight markers used are lambda DNA digested with PstI (lanes A) and plasmid pUC19 digested with MspI (lanes B).

hirsuticaulis together, and *A. monticola* with the single sample of *A. sarmatica.* The two microspecies of section *Alchemilla* under analysis, *A. acutiloba* and *A. micans*, form another large cluster. The next large cluster appearing in the UPGMA phenogram consists of two branches. The first one combines *A. baltica* and *A. glabricaulis* (belonging to Fröhner's section *Coriaceae*). The second one unites *A. subcrenata, A. cymatophylla, A. semilunaris* and *A. heptagona,* belonging, according to Fröhner (1995), to the section *Ultravulgares*.

The phylogenetic relationships inferred from RAPD data with the NJ method are shown in Fig. 3. Most of the clusters were not strongly supported by bootstrapping. A bootstrap value over 50% was demonstrated by only 13 groupings, none had very strong support. The same main clusters noted in the UPGMA phenogram, corresponding to Fröhner's sections, can be seen in the NJ tree but, still, some differences need to be emphasised. Section *Plicatae* is paraphyletic, consisting of two separate branches (*A. hirsuticalis* + *A. glaucescens* and *A. monticola* + *A. sarmatica*). One *A. monticola* sample collected from the Moscow district, v3monMO, is placed outside of its cluster and was an outlier in the UPGMA tree as well. Section *Coriaceae* (*A. baltica* + *A. glabricaulis*) is not separable from the section *Alchemilla* cluster. Section *Ultravulgares* forms a clearly separate cluster, and moreover, *A. heptagona* is strongly apart from all other species. It is noteworthy that the same pair of specimens of *A. subcrenata* and *A. cymatophylla* as in the UPGMA tree occurs again and with rather strong support (82%).

The Maximum parsimony method applied to RAPD data resulted in dendrograms with many features in common with the UPGMA and NJ trees. The programme generated 4 shortest trees (384 steps, consistency index CI = 0.167, homoplasy index HI = 0.833). The majority rule consensus tree and one of the shortest trees are presented in Fig. 4A and B. There was mostly low or no bootstrap support (Fig. 4A), only some small groups were moderately supported. Decay indices (DI) of branches also did not exceed 2, mostly were equal to 1. Still, the topology was practically the same in all trees indicating that, despite the weak support of the branches, the topology may be close to the true relationships. *Alchemilla glaucescens* and *A. hirsuticaulis, A. monticola* and *A. sarmatica* form clades by pairs, but not all together. *Alchemilla heptagona* forms a monophyletic group, which even has moderate support (bootstrap value 66, DI = 2). The section *Ultravulgares* as a whole can be considered to be an intergrade. *Alchemilla acutiloba* and *A. micans* are mixed with each other, and thus are not a monophyletic group. *Alchemilla glabricaulis* and *A. baltica,* as representatives of

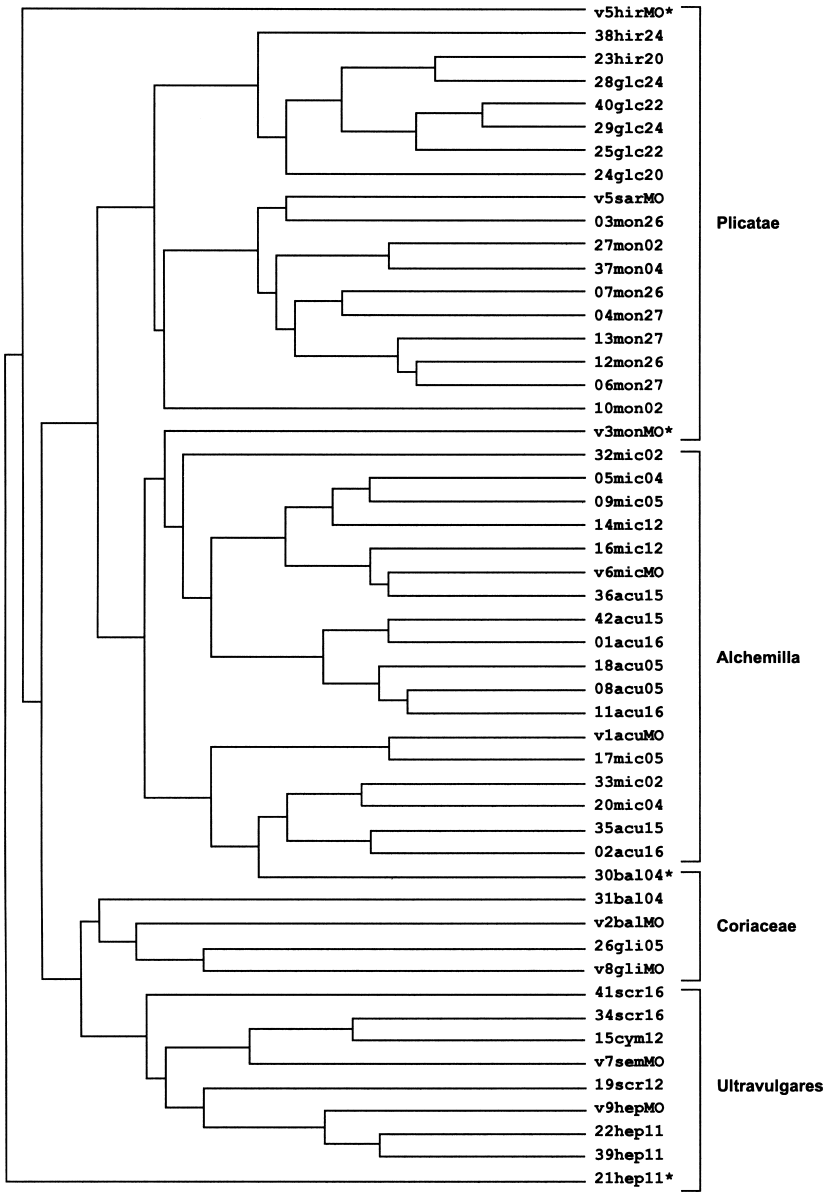

Fig. 2. UPGMA dendrogram based on RAPD data (3 primers). Denotations of accessions as in Table 1. Brackets on the right indicate the corresponding Fröhner sections. Plants marked with asterisks are located outside of their sections. The scale on the top shows genetic distance.

the section *Coriaceae,* do not form a clade, but also an intergrade. The same two samples of *A. subcrenata* and *A. cymatophylla,* as in previous trees, are most strongly supported as a clade (bootstrap value 90).

The MP trees based on morphological data only (Fig. 5A, the consensus and Fig. 5B, one of ten shortest trees, length 482 steps, CI = 0.212, HI = 0.788) were also not very strongly supported, and also the topology differed more in different trees. In six trees *Alchemilla hirsuticaulis* and *A. glaucescens* were separated from *A. monticola,* but in 4 trees the three microspecies were together (Fig. 5B). Still, separation of the first two has moderate support (bootstrap value 76, DI = 3), the four-species clade has no support, and therefore we consider these two microspecies monophyletic together, but not with *A. monticola.* It is also remarkable, that all but one of the *A. micans* specimens behave like a monophyletic group in tree topology, but only a smaller group of five specimens has some support. While section *Plicatae* is monophyletic at least on some trees, the other three analysed sections are all mixed up in all the trees

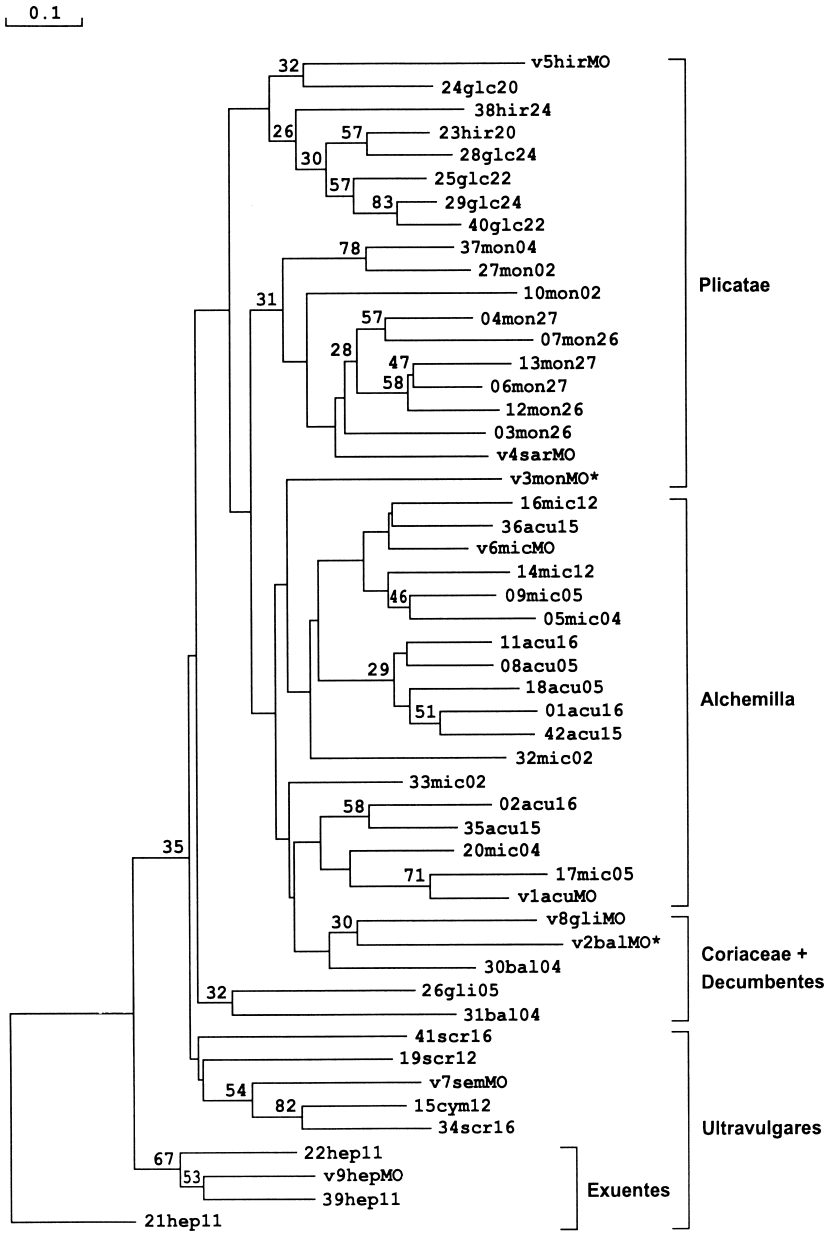

Fig. 3. NJ tree based on RAPD data (3 primers). Denotations of accessions as in Table 1. Brackets on the right indicate the corresponding Fröhner's sections; group *Exuentes* according to Yuzepchuk is also indicated. Plants marked with asterisks are located outside of their sections. The scale on the top shows genetic distance. Bootstrap values greater than 25% (from 100 replications) are indicated.

based only on morphological data.

MP trees of combined data (Fig. 6A, the consensus and 6B, one of 20 shortest trees, length 875 steps, CI = 0.190, HI = 0.810) gave the best-supported resolution of the microspecies. *Alchemilla glaucescens* and *A. hirsuticaulis* form a monophyletic group together, with moderate support (bootstrap value 77). *Alchemilla monticola* specimens of two geographically proximate populations are also weakly supported as a monophyletic group. The merging here of specimens of the same microspecies from other populations is not supported, but all plants of *A. monticola* form an intergrade. All three microspecies form monophyletic groups, too, but they are not well supported. The other groups are also not well supported, but again, the topology is quite consistent through 20 trees. Sections *Ultravulgares* and *Coriaceae* together are monophyletic, *A. acutiloba* and *A. micans* are mixed and form an intergrade.

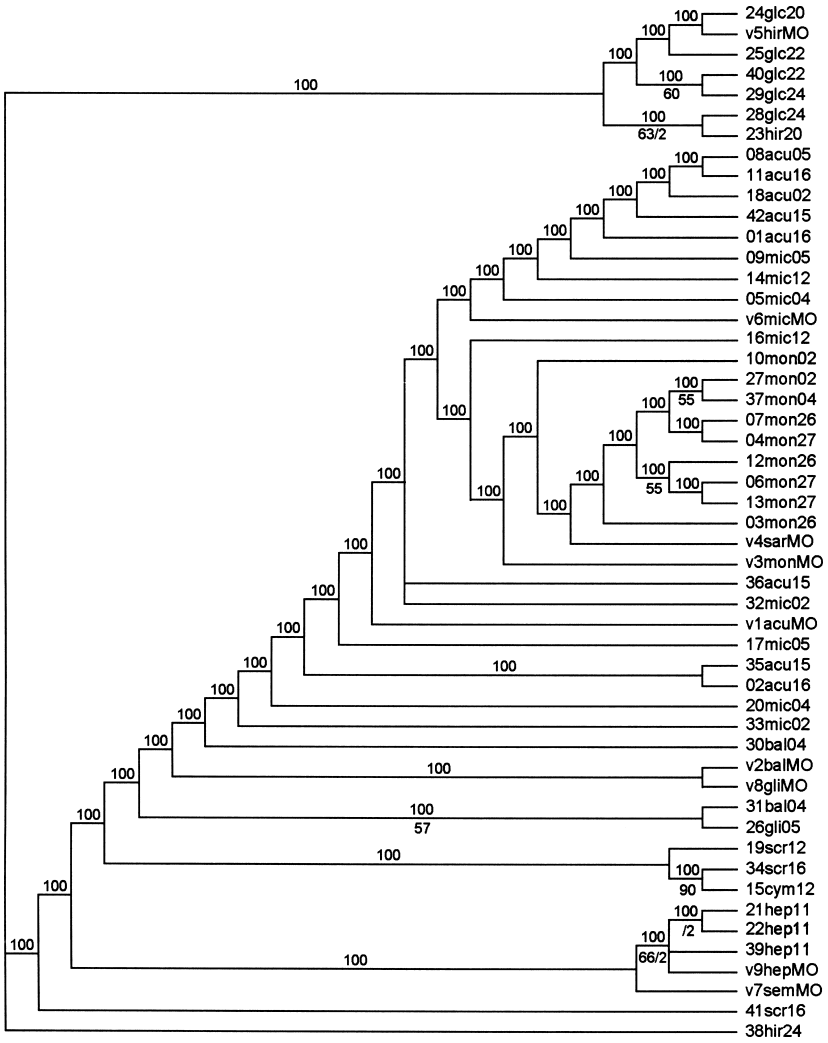

Fig. 4A. MP trees of RAPD data (3 primers, 51 samples). Notations as in Table 1. The 50% majority-rule consensus tree of 4 trees (length 384 steps, CI = 0.167, HI = 0.833). Above the branch is marked the per cent of the most parsimonious trees with given topology, below the branch the bootstrap value (if over 50 of 100 replicates)/decay index (if over 2).

DISCUSSION

An earlier computer simulation study (Jin & Nei 1991) revealed some advantages (bigger relative efficiency) of the NJ method over UPGMA and MP methods for obtaining a correct phylogeny for restriction-fragment data. Still, for comparison and when discussing the results we considered all the methods. The trees obtained with these methods differ in details, but for RAPD data, all methods agreed in main trends, e.g. intermixing of *Alchemilla acutiloba* and *A. micans,* separating *A. monticola* from the other species of section *Plicatae,* etc. There were major differences between RAPD and morphology trees. Hence, the morphological features are not necessarily in concordance with genetic similarity, and the diagnostic features can express just phenotypic plasticity.

Because the bootstrap values of MP trees were not high, we do not propose extensive taxonomic rearrangements. However, it has to be kept in mind that the use of bootstrapping procedure for estimation of the reliability of phylogeny inferred from RAPD data is not strictly valid, since RAPD data cannot be considered as a random sample of characters (Sanderson 1995). Thus we did not ignore the groups with low support value, especially if some tendencies were very clear and in good concordance with morphological data.

Intermixing of *Alchemilla glaucescens* and *A. hirsuticaulis* in all trees indicates their close taxonomic relation, and in fact, morphological fea-

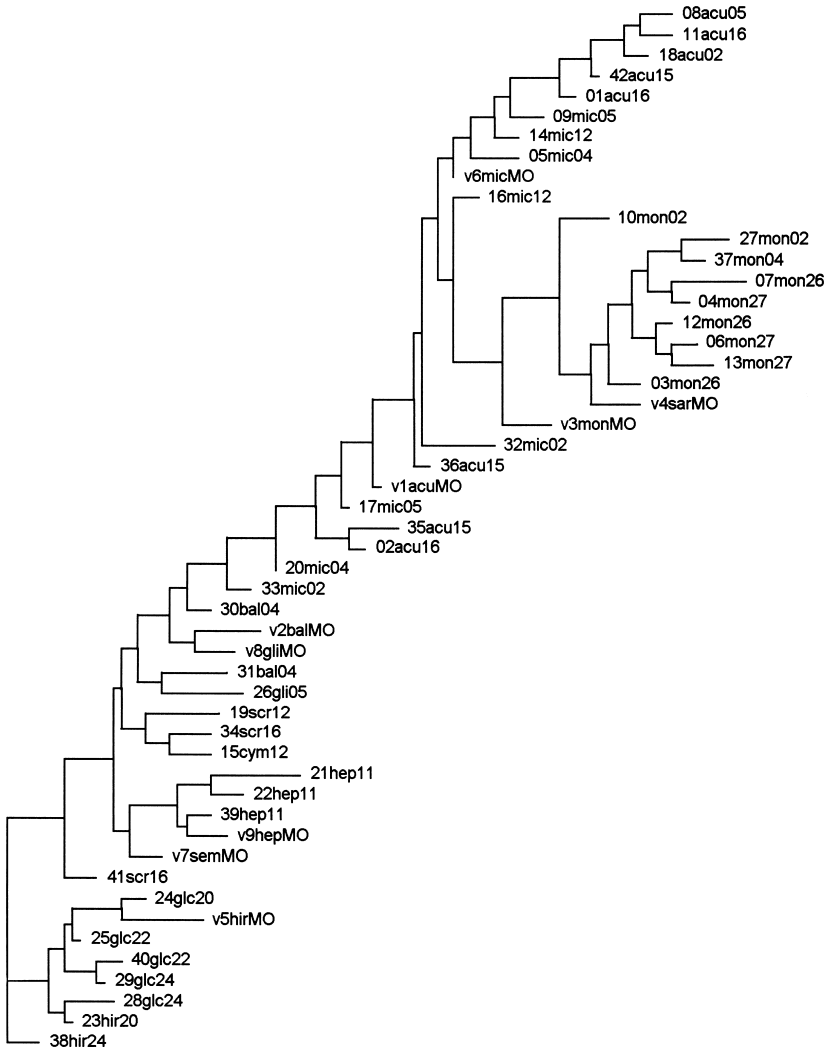

Fig. 4B. MP trees of RAPD data (3 primers, 51 samples). Notations as in Table 1. One of the four most parsimonious trees.

tures for discrimination of these two similar microspecies are not always clear-cut. The single studied sample of *A. sarmatica* was included in the *A. monticola* cluster/clade on RAPD trees. Both our morphological and RAPD data showed clustering of *A. glaucescens* and *A. hirsuticaulis* separately from *A. monticola* (and *A. sarmatica*), in some cases as a smaller branch of the larger *Plicatae* branch. The two-species branches were as a rule supported, while the four-species ones were not. Therefore we suggest the division of Fröhner's section *Plicatae* into two groups. One should consist of *A. glaucescens*, *A. hirsuticaulis* and similar microspecies (section *Pubescentes* in Rothmaler 1936, Yuzepchuk 1941, and Plocek 1982), and the other of A. *monticola, A. sarmatica* and possibly some related microspecies. The latter two are joined with many other microspecies in the *Hirsutae* series by Plocek (1982), but placed in different groups of the *Hirsutae* series by Yuzepchuk (1941).

Alchemilla acutiloba and *A. micans* were always intermixed as constituents of a single cluster or clade or at least intergrade; these two microspecies could not be separated from one another by morphological or genetic characters. Intermixing of these microspecies corresponds to the absence of reliable distinctions between them in the vast majority of morphological features. Probably it is sensible to join these microspecies. According to Fröhner (1995), *A. acutiloba* and *A. micans* belong to the section *Alchemilla*. RAPD

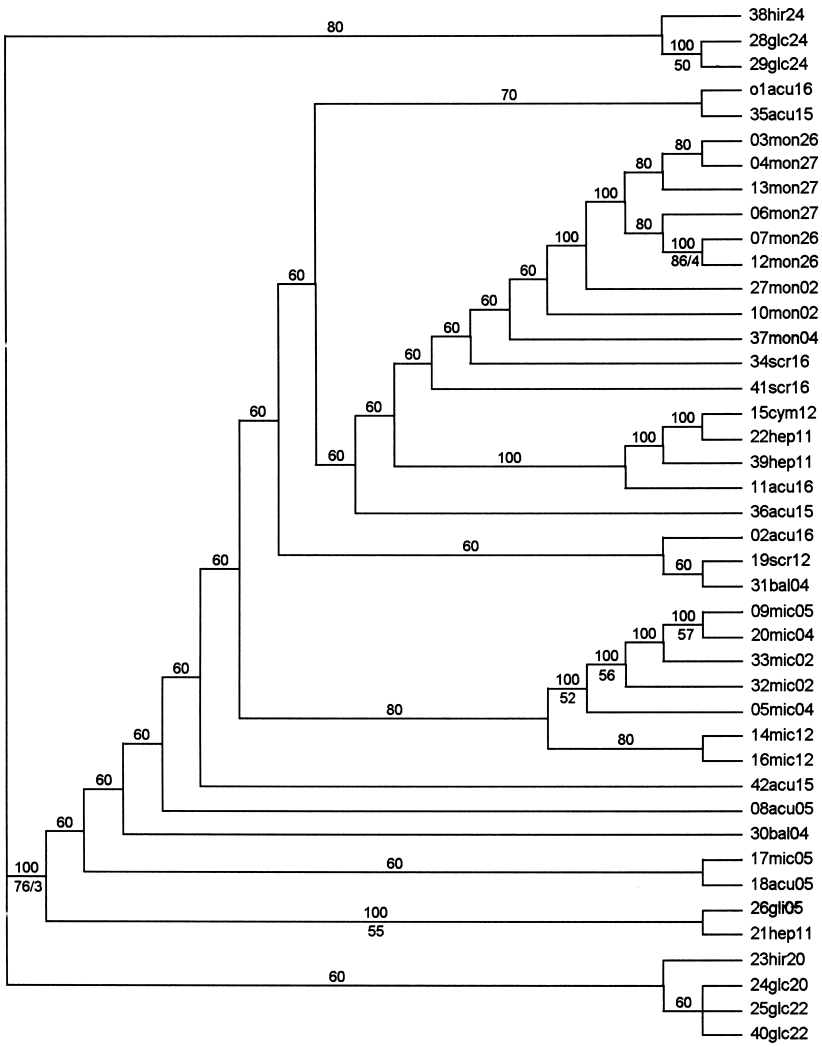

Fig. 5A. MP trees of morphological data (42 specimens). Notations as in Table 1. The 50% majority-rule consensus tree of 10 trees (482 steps, CI = 0.212, HI = 0.788). Support values as in Fig. 4A.

data did not support combining them with some others in the *Hirsutae* series (Plocek 1982) nor in the *Imberbes* group (Yuzepchuk 1941; *see* Table 2).

The section *Coriaceae* was never monophyletic as a whole, but often mixed with sections *Alchemilla* and/or *Ultravulgares.* Still, as quite few microspecies and samples were included in the analysis, no conclusions can be drawn.

The section *Ultravulgares* was mostly not monophyletic, but in some cases it formed a separate cluster or intergrade with section *Alchemilla*. Not many samples were included from this section either and, as the results were not highly concordant, we cannot draw conclusions for the section as a whole. Concerning *A. heptagona,* it is evident that this microspecies was mostly on a separate branch in the section, and hence may be separated from the other microspecies of this section. The only sample of *A. semilunaris* was close to section *Ultravulgares* according to RAPD data, but additional research is necessary to clarify this relationship. The specimen of *A. cymatophylla* (15cym12), which was joined with a specimen of *A. subcrenata* in several RAPD trees, laid between *A. cymatophylla* and *A. subcrenata* in the morphology tree, with a little more similarity to the former. Still, genetically it was evidently close to *A. subcrenata.* It can be concluded that identification of *Alchemilla* microspecies by morphological features alone is not always reliable.

Though our sample was not large enough for population studies (only some samples were in-

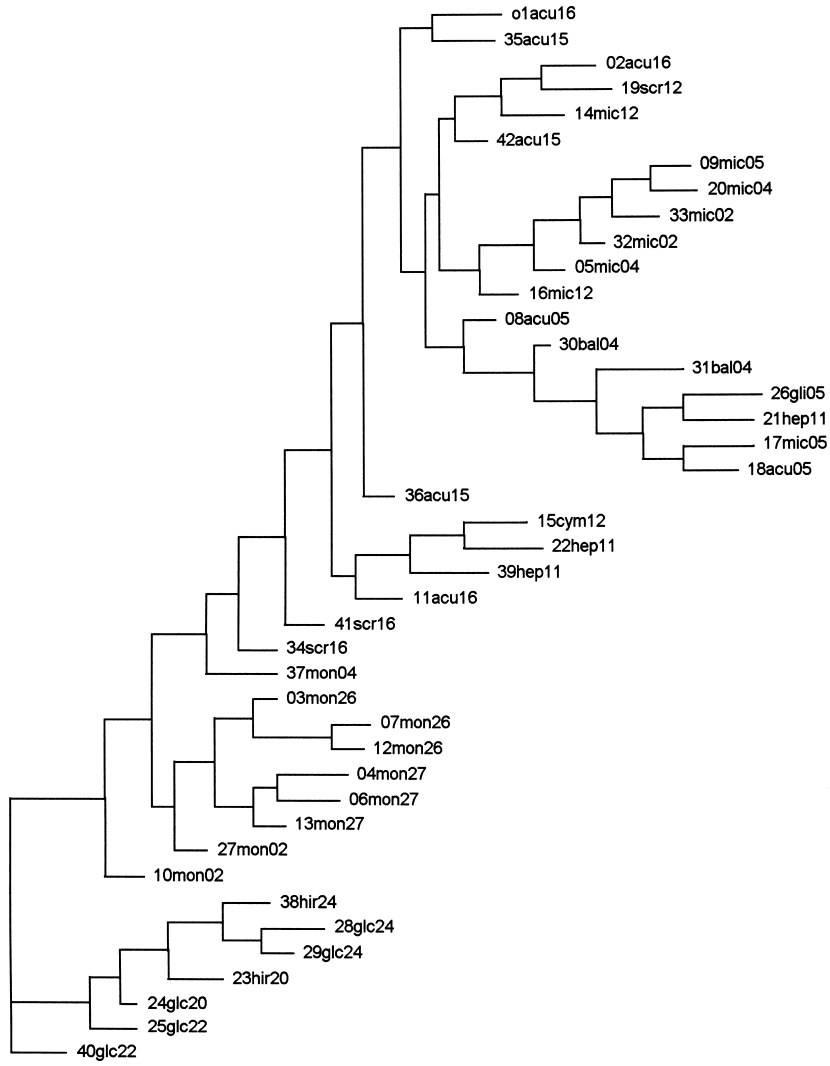

Fig. 5B. MP trees of morphological data (42 specimens). Notations as in Table 1. One of the ten most parsimonious trees.

cluded from each population), a tendency for samples grouped in trees more by microspecies, not by population could be seen. Thus, we can say that plants of the same microspecies are more similar than plants of the same habitat, but different microspecies. Naturally, samples from the plants of same microspecies from the same population were more similar than the plants of same species from different populations. Hence, our study generally confirms the conclusion of Turesson (1943) that microspecies are not genetically homogeneous and they have ecological variants. But our assumption about the parallel variation, that the agamotypes of different microspecies, which occur in the same habitat could be more similar than the agamotypes of the same microspecies, was not confirmed.

The "anomalous" position of a sample from the Moscow region, *Alchemilla monticola* 3monMO, on both UPGMA and NJ trees has to be noted. *Alchemilla monticola* is a microspecies with a wide geographical range and with a high variability of morphological features, and it is plausible that further investigations will lead to the separation of some new microspecies within *A. monticola* (V. N. Tikhomirov, pers. comm.), probably based on the different agamotypes.

Phenetic analysis of morphological data for 373 specimens of 23 *Alchemilla* microspecies from Estonia showed that only some of them are

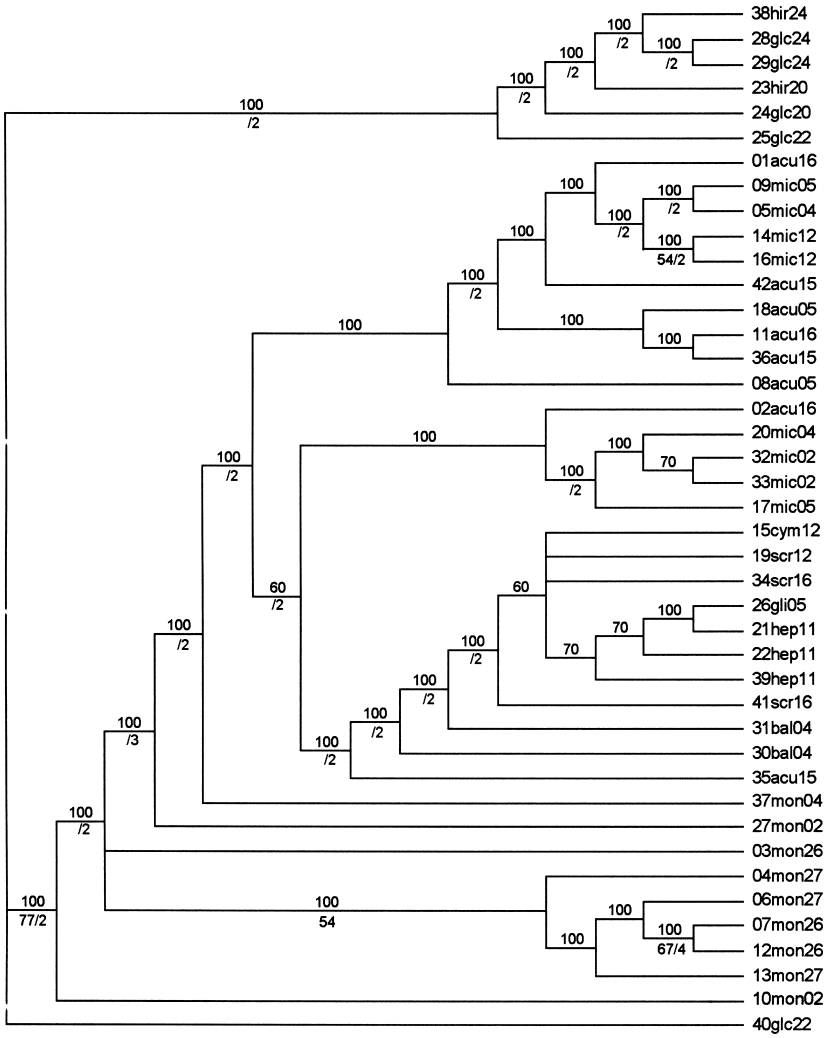

Fig. 6A. MP tree of combined (morphological and RAPD) data (42 specimens). Notations as in Table 1. The 50% majority-rule consensus tree of 20 trees (length 875 steps, CI = 0.190, HI = 0.810). Support values as in Fig. 4A.

completely distinct from all others, although sections and series are generally better separated (Sepp & Paal 1998). This study confirmed the earlier statement of extensive intraspecific variability of most morphological characters in *Alchemilla* (Turesson 1943). There was some congruence between morphological and RAPD analysis. Fröhner's system (1995), based on morphological traits, is in relatively good agreement with genetic relatedness inferred from DNA data. However, some results of RAPD analysis did not find support in morphological studies and vice versa.

It may be concluded that RAPD analysis may give new impetus to studies of the systematics and evolution of *Alchemilla* as well as other apomictic plants. The main difficulties in obtaining the phylogenetic relationships among *Alchemilla* microspecies are that different microspecies and groups are distinguished by their different morphological characters; the same character has a different weight in different species, and the vast majority of characters used are quantitative. The RAPD technique, as a DNA-based method of inferring phylogenetic relationships, is free of such kinds of problems. Genealogical links can be found and estimated quantitatively using the same range of molecular data for the whole set of taxa. Though the RAPD data has its own problems of reproducibility and homology (Penner *et al.* 1993, Quiros *et al.* 1995), it adds a considerable new aspect to the taxonomy of *Alchemilla.*

The investigation of genus *Alchemilla* as a rep-

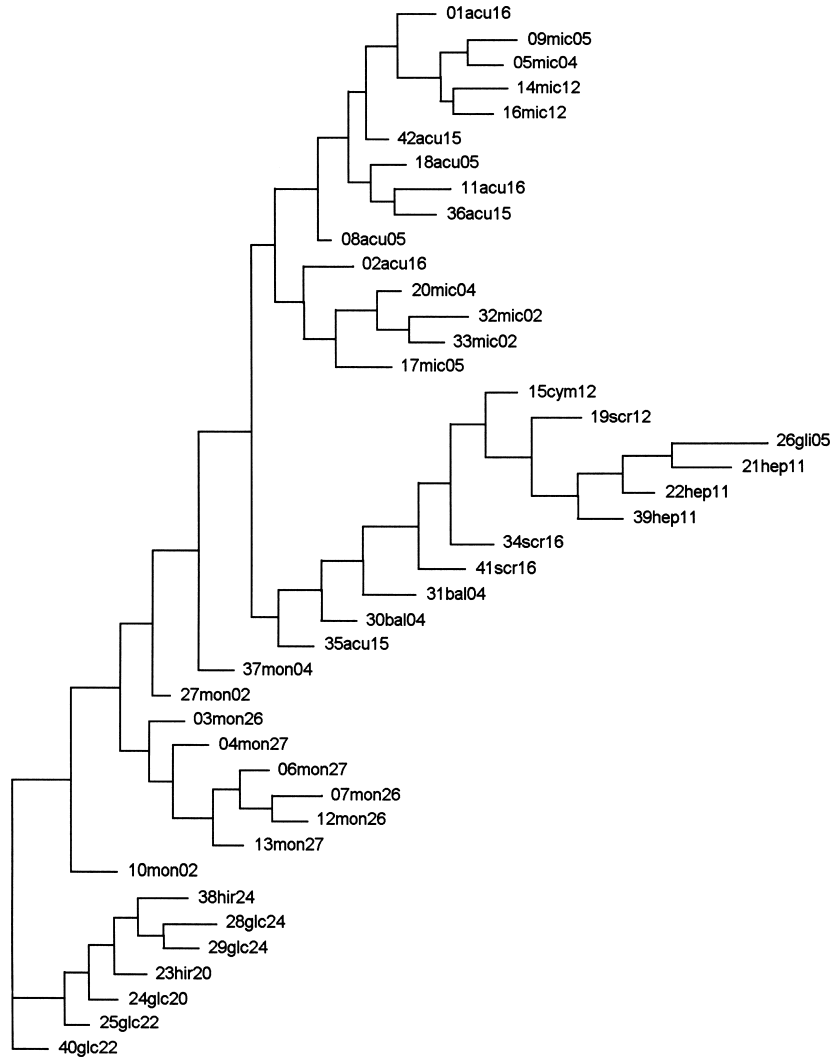

Fig. 6B. MP tree of combined (morphological and RAPD) data (42 specimens). Notations as in Table 1. One of 20 most parsimonious trees.

resentative of facultatively apomictic genera is of great interest for solving questions about the biology, evolution and species concepts in apomicts and their relation to species of amphimicts. The fact that facultative rather than obligate apomixis occurs among agamospecies of the *A. vulgaris* group (Glazunova 1987, Tikhomirov *et al.* 1995) leads to the conclusion that there is a possibility of exchange of genetic material among individual plants of distinct sympatric agamospecies. Agamospecies evidently represent morphologically distinct groups within populations. The *A. vulgaris s. lato* population is polymorphic in numerous traits, which are different for distinct agamospecies. The treatment of agamospecies as a morphologically distinct group of plants in a population, and species as a unit joining several agamospecies is not generally accepted, but the new evidence favours this point of view. At present, it is not clear which and how many taxa in the genus *Alchemilla* should be distinguished in Europe, and which specific agamospecies should be included into which taxon. RAPD analysis and other molecular methods may be informative for elucidating the relationships of agamospecies and other taxa of *Alchemilla*.

It must be noted that the samples used in RAPD analysis were not specially collected from plants close to the type of microspecies by morphological criteria, but were collected in the course of random sampling from natural populations, and the number of microspecies and plants involved

was not great. It is evident that more data is needed for elucidating *Alchemilla* phylogeny and the derivation of a modern system. However, some tentative conclusions can be made from RAPD analysis, despite the fact that a limited set of data was used in the investigation.

1. The intermixing of *Alchemilla acutiloba* and *A. micans* according to RAPD data, and their morphological similarity, allows them to be united in a single microspecies, *A. acutiloba* Opiz.
2. Fröhner's section *Plicatae* should be divided into two series (*Pubescentes* and *Barbulatae*) based on RAPD data presented here, and on the suggestions of Rothmaler (1936) and Yuzepchuk (1941) based on morphology.
3. Section *Ultravulgares* would also be better split. *Alchemilla heptagona* is very different from the other microspecies, by its morphological as well as molecular traits, and it should be separated into the series *Exuentes*, as previously proposed by Yuzepchuk (1941).

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Appendix. Data matrix. The notations of specimens as in Table 1. Characters of every specimen are arranged in two rows. The 48 morphological characters are in the first row, the second row contains 68 RAPD bands.

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010000010011011011101001100001110000000000000111111011011101000000000

 02acu16 032200201001212222222223324333222444333331123423
111110010100011011000010100001101011000001000001111011011101000000000

 03mon26 022200201102221221132321222222323222121111122232
111011001100101010010001100001110011000000000101110011011101110110000

 04mon27 032201211001211221133321323222324322121121223332
111011001101111111110001100001110011011000000001110011011111011111000

 06mon27 022201311102221221132311333212324223222221122333
111011001101100111100000100011111111011001000101110011011100110110000

 07mon26 022200201102121211122211334112424212122211222231
000000001100110111110001100001110111011001000101110011011111111111000

 08acu05 032100201001212222221112323223112323232211123413
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 09gra05 023300210101212122222221223212322222122311143424
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 12mon26 022200201101121211132221434112223222222311222231
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 13mon27 022200311002211221131222333222424323221121322232
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 14gra12 013300300101212222223342324323422433232221123322
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 16gra12 012300300101212222222232223232322333231111122323
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Appendix. Continued.

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01111011011100111111001010000101110100000000000011100101111000000000

 31bal04 0242002010112212211234211133231124433323211334 2 4
01101011011101111111001000111111011010000000000000100000010000000000

 32gra02 0232003101002221222232313222222223232132311133424 4
11101011001101101001001010000101000100000000011111101101010000000000

 33gra02 0232003101022221212232222232232123222223111333413 4
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 34scr16 0322002010002111223212221323222323312221123222 3 3
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 35acu15 0322002010021212221312422122332223332311111334 1 4
11111001011100111111001010010110101100000100000111101101110000000000

 41scr16 0222002010012121221321322332324224433211211222 2 1
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 42acu15 0222002010022222221222423242231123332321111233 2 3
01000111001111011111100111000111000000000000011111101101110100000000

 38hir24 1233002010012112121222322421214432121133233223 4 4
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 28glc24 1132012010011111221212212431124431111122143213 4 4
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 29glc24 1132002010011211121212212321114431111133133123 4 3
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 27mon02 0333002010002212211222212322223223232321211222 3 2
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 17gra05 0222002000002122211332222121121212222122321132323
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11110111001101001101100111100111010000000000011111101101110100000000
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Continued

Appendix. Continued.

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 39hep11 022310201101211022131112231233111332432322222333
11110011010110111111001010100111001100000000000000001101001101100000

 11acu16 022200201001212122133143121333111333242111112222
11111111000111111111110011100011111000000010001111110110111010000000

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 24glc20 123201201001212111121112242122232112113313311341
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