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SILVIA SEPP

TARTU UNIVERSITY
PRESS

Chair of Botany, Institute of Botany and Ecology, University of Tartu, Tartu, Estonia

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I. Sepp, S. & Paal, J. 1998. Taxonomic continuum of *Alchemilla* (Rosaceae) in Estonia. — *Nord. J. Bot.* 18: 519–535.

II. Sepp, S., Bobrova, V. K., Troitsky, A. K. & Glazunova, K. P. 2000. Genetic polymorphism detected with RAPD-analysis and morphological variability in some microspecies of apomictic *Alchemilla*. — *Ann. Bot. Fenn.* 37: 105–123.

III. Sepp, S., Nahtman, T., Möls, T. & Paal, J. 2000. Study of the multivariate structure of the Estonian *Alchemilla* L. (Rosaceae) microspecies: an example of the structural indices approach. — *Proc. Estonian Acad. Sci. Biol. Ecol.* 49: 289–301 (in press).

IV. Sepp, S. & Paal, J. Patterns and relationships between and within the sections *Alchemilla* and *Ultravulgares* Fröhner of the genus *Alchemilla* L. (Rosaceae) in Estonia. (Accepted for publication in Nordic Journal of Botany).

Other relevant publications

Sepp, S. 1996. Some considerations of species problem in *Alchemilla*. — In: *Origin and Evolution*. Abstracts. Fifth Intern. Congress of Systematic and Evolutionary Biology. Budapest, p. 216.

Sepp, S. 1999. Perekond kortsleht — *Alchemilla* L. — In: *Eesti taimede määraja* (Ed. M. Leht), EPMÜ ZBI & Eesti Loodusfoto, Tartu, pp. 156–163.

Tikhomirov, V. N. & Sepp, S. 1993. Perekond kortsleht Eestis. — *Rukkilill* 4: 15–22.

1. INTRODUCTION

The genus *Alchemilla* L. (Fam. Rosaceae Juss., subfam. Rosoidae Focke) *sensu lato* consists of more than 1000 (micro)species (Fröhner 1995). Over 300 (micro)species have been described from Europe.

Grant (1971) has introduced the word 'microspecies' for apomictic organisms. Since then, hundreds and thousands of microspecies have been distinguished in apomictically reproducing plant genera like *Rubus, Taraxacum, Alchemilla,* etc. Stace (1998) argues that, pragmatically, we should use the species rank for these, in order not to loose information. Still, many of these "species" are probably single clones and several authors (*e.g.*, Dickinson 1998, Hörandl 1998) agree in not treating single clones of apomicts as species. Dickinson (1998) argues that sexual species involve several genotypes, and that the apomictic species should do that as well. Which then should be the criteria for separating such species? Hörandl (1998) stresses the constancy of progeny, being a product of the joint evolutionary process, considering similarity of phenotype and ecogeographical unity as consequences, while Dickinson (1998) still emphasises the phenotypic distinctness of species.

Already at the beginning of the twentieth century Murbeck (1901) and Strasburger (1905) discovered that many species of *Alchemilla* reproduce apomictically. Since then, most of the species are considered obligate agamosperms (apospory + parthenogenesis, Gustafsson 1947). Still, according to Glazunova (1977, 1983, 1987) and Izmailow (1984, 1986, 1994a, b), the majority of *Alchemilla* species are not obligate, but facultative apomicts.

Alchemilla species are high polyploids, their chromosome number is often aneuploid and varies widely within one species (Turesson 1957, Löve & Löve 1961, Bradshaw 1963, Wegener 1967, and Izmailow 1981, 1982). Most probably the variation in chromosome numbers is indicative of the hybridogenous origin of species and their genetic heterogeneity. Lundh-Almestrand (1958) and Turesson (1943, 1956, and 1957), in their experimental works, also detected genetic variants mainly within microspecies. Analysing DNA of *Alchemilla* with RAPD markers, Baeva et al. (1998) showed that populations within species are sometimes even genetically more dissimilar than different microspecies.

Due to its agamospermy and large variation, the genus *Alchemilla* has been an object of scientific interest since the end of nineteenth century. Most authors follow Buser (1894, 1895) in ranking *Alchemilla* microspecies on a species level (*e.g.* Lindberg 1909, Rothmaler 1935–1962, Juzepczuk 1941, Samuelsson 1943, and Plocek 1982). Some authors (Ascherson & Graebner 1900–1905, Turesson 1943, 1956, Löve 1960, 1961, 1975, Glazunova 1977, Tikhomirov *et al.* 1995) suggest that only a few collective species should be distinguished, but this is not a prevailing interpretation.

Of the different conceptions concerning the division of the genus *Alchemilla* into sections and series, the most widespread system originates from Buser (1891,

1901). This classification is further developed mainly by Rothmaler (1936, 1944), and Plocek (1982). Juzepczuk (1941) took the previous systems as his starting point and developed a more detailed system of sections, groups (series) and subgroups (subseries), but, since it is invalid according to the nomenclature rules, his system is not in use today.

Fröhner (1975, 1986, and 1995) has an interesting conception about origin and taxonomy of *Alchemilla*. He claims a hybridogenous origin of the genus from 4 pure genepools (in Europe) which have given all the possible hybrids between them. On that basis Fröhner proposed a new section-structure for the genus, taking into account morphological characters, chromosome numbers, ecology, and species distribution.

In practice, *Alchemilla* microspecies in nature are morphologically highly variable and their characters vary continuously. Nobody has checked whether using morphological characters for distinguishing the species really works and whether the species can be clearly distinguished. Up to now, numerical methods have been used very rarely for that purpose in the genus *Alchemilla* (*e.g.* Turesson 1956, Glazunova & Mjatlev 1990), though they have proved to be useful in analogous agamic complexes (*e.g. Amelanchier,* Dibble *et al.* 1998; *Antennaria,* Chmielewski 1995; *Potentilla,* Leht 1997; *Rosa,* Nybom *et al.* 1997; *Rubus,* Kraft & Nybom 1995). Because of the developmental and taxonomic complexity of the genus and the continuity of characters, numerical phonetics methods are mainly of use, since it is practically impossible to use cladistics in such cases (Duncan & Baum 1981, McNeill 1984). Walters (1987) has stressed that taxonomists should investigate this genus using biosystematical methods.

2. OBJECTIVES

In the present study the morphological and genetic variation and taxonomic continuum of 23 putative *Alchemilla* species represented in Estonia are analysed

- to assess the morphological variability of these taxa;
- to determine the distinctness of the microspecies from a statistical point of view using morphological characters;
- to ascertain, by means of different multivariate methods, patterns and relationships of species within and between the sections and series, and to indicate possible taxonomic consequences;
- to compile a set of morphological characters that discriminate between the analysed species most clearly;
- to find the most stable proportions between the variables according to the structural indices, and to assess how the structural indices distinguish microspecies;
- to assess the genetic variability of the microspecies, and the relationships of species using the RAPD method.

3. MATERIAL AND METHODS

3.1. Material

Analysis included 23 *Alchemilla* microspecies, which occur in Estonia and are widespread in Europe or Eurasia as well. According to Plocek (1982) they belong to the same section *Alchemilla* and subsection *Euvulgares* Camus (*Heliodrosium* Rothm.), but to four different series; while according to Fröhner (1995) they belong to six different sections (Table 1). The variation within these sections and series is well expressed in the chosen species.

Herbarium material from the Herbarium of the University of Tartu (TU), the Herbarium of the Institute of Zoology and Botany of the Estonian Agricultural University (TAA) and the Herbarium of Moscow State University (MW) was used. We also added material collected from different localities in Estonia in June 1995 and June 1996. Only material collected in Estonia was involved: altogether 598 specimens (whenever possible, at least 20 specimens of each species). The identification of specimens was checked by the author, in dubious cases additionally by K. P. Glazunova (Moscow).

Table 1. Classification of the studied species into sections and series according to the different concepts of the taxonomic structure of the genus.

Species	*Notation*	*Plocek (1982)*	*Juzepczuk (1941)*	*Fröhner (1995)*	*proposed by Sepp & Paal (1998)*
A. glaucescens Wallr.	GLC	ser. Pubescentes (Buser) Rothm.	sect. Pubescentes Buser	sect. Plicatae Fröhner	sect. Plicatae ser. Pubescentes
A. hirsuticaulis H. Lindb.	HIR	"	"	"	"
A. plicata Buser	PLI	"	"	"	"
A. monticola Opiz	MON	ser. Hirsutae (Lindb.) Rothm.	sect. Vulgares Buser [gr. Hirsutae Lindb. subgr. Barbulatae Juz.]	"	sect. Plicatae ser. Barbulatae
A. propinqua H. Lindb. ex Juz.	PRO	"	"	"	"
A. sarmatica Juz.	SAR	"	sect. Vulgares Buser [gr. Hirsutae Lindb. subgr. Imberbes Juz.]	"	" (?)
A. subglobosa C.G. Westerl.	SGL	"	"	"	doubtful
A. acutiloba Opiz	ACU	"	"	sect. Alchemilla	sect. Hirsutae ser. Alchemilla
A. micans Buser	MIC	"	"	"	"
A. xanthochlora Rothm.	XAN	"	"	"	"
A. lindbergiana Juz.	LIN	"	"	—	"
A. cymatophylla Juz.	CYM	"	"	sect. Ultravulgares Fröhner	sect. Hirsutae ser. Ultravulgares
A. subcrenata Buser	SCR	"	"	"	"
A. semilunaris Alechin	SEM	"	"	— (close to sect. Decumbentes Fröhner)	sect. Hirsutae ser. Decumbentes

Species	*Notation*	*Plocek (1982)*	*Juzepczuk (1941)*	*Fröhner (1995)*	*proposed by Sepp & Paal (1998)*
A. heptagona Juz.	HEP	"	sect. Vulgares Buser [gr. Hirsutae Lindb. subgr. Exuentes Juz.]	sect. Ultravulgares Fröhner	sect. Hirsutae ser. Ultravulgares
A. filicaulis Buser	FIL	"	"	sect. Plicatae Fröhner	sect. Coriaceae ser. Exuentes
A. glabricaulis H. Lindb.	GLI	ser. Glabrae Rothm. (Pawl.)	sect. Vulgares Buser [gr. Hirsutae Lindb. subgr. Glabricaules Juz.]	— (close to sect. Coriacea Fröhner)	sect. Coriaceae ser. Glabricaules
A. glomerulans Buser	GLO	ser. Subglabrae Rothm. (Pawl.)	sect. Vulgares Buser [gr. Subglabrae Lindb. subgr. Appressipilae Juz.]	sect. Coriaceae Fröhner	sect. Coriaceae ser. Coriaceae
A. glabra Neygenf.	GLA	"	sect. Vulgares Buser [gr. Subglabrae Lindb. subgr. Glabratae Juz.]	"	"
A. baltica Sam. ex Juz.	BAL	"	"	"	"
A. obtusa Buser	OBT	"	"	"	"
A. wichurae (Buser) Stefansson	WIC	"	"	"	"
A. murbeckiana Buser	MUR	"	"	— (close to sect. Coriacea Fröhner)	"

3.2. Morphometrics

Characters for morphometrics (Table 2) were chosen according to two criteria: (i) they should be relatively easy to measure in herbarium material, and (ii) they should be useful for species identification. Finally 43 characters were selected for analysis. An attempt was made to express most of the characters numerically; some nominal parameters were nevertheless included. Each character was measured three times on every specimen and the average or median values were used for further analysis. In addition, 12 ratios were calculated, since the latter are less dependent on environmental conditions, seasonal differences and other unspecified (noise) factors.

Table 2. Characters used in analysis of *Alchemilla* species.

Notation	Character	Type	States or units (in brackets degree of precision)
SILK	type of hairs	binary	0-not silky, 1-silky (sericeous)
STPOS	position of stem	nominal	1-decumbent, 2- bentform ascending, 3-erect
HRPOS	position of hairs on stem	nominal	1-deflexed, 2- patent, 3-erecto-patent, 4-appressed
LECOL	leaf colour	nominal	1-yellowish green, 2-grass green, 3-greyish green, 4-bluish green, 5-dark green
FLCOL	flower colour	nominal	1-reddish, 2-yellow, 3-yellowish green, 4-grass green, 5-greyish green

Notation	Character	Type	States or units (in brackets degree of precision)
STCOL	stipule colour	nominal	1-brown, 2-reddish, 3-green, 4-pale
INFSH	shape of inflorescence	nominal	1-narrow, 2-wide
FLGDN	density of flower glomeruli	nominal	1-sparse, 2-dense
LBTOP	shape of lobe apex (basal leaf)	nominal	1-obtuse, 2-acute
THTOP	shape of tooth apex (basal leaf)	nominal	1-obtuse, 2-acute
THSYM	symmetry of teeth (basal leaf)	nominal	1-symmetrical, 2-asymmetrical
CASH	shape of sepal apex	nominal	1-obtuse, 2-acute
INCDP	depth of incisions between lobes (basal leaf)	ordinary	0-missing, 1-shallow, 2-deep
LEFLD	leaf foldedness	ordinary	0-not folded, 1-slightly folded, 2-strongly folded
HYSH	shape of hypanthium	ordinary	1-tubular, 2-funnel-shaped, 3-campanulate, 4-round
STNR	number of flowering stems	interval (counted)	number per individual
LENR	number of basal leaves	interval (counted)	number per individual
LBNR	number of lobes (basal leaf)	interval (counted)	number per leaf
THNR	number of teeth (middle lobe, basal leaf)	interval (counted)	number per lobe
STLHR	number of hairs on the lowest internode of stem	interval (counted)	number per 1 mm of running length
STUHR	number of hairs on the upper part of stem (below inflorescence)	interval (counted)	number per 1 mm of running length
PETHR	number of hairs on petiole (basal leaf)	interval (counted)	number per 1 mm of running length
LEUHR	number of hairs on upper surface of basal leaf	interval (counted)	number per 1 mm^2
LELHR	number of hairs on lower surface of basal leaf	interval (counted)	number per 1 mm^2
VNHR	number of hairs on veins (lower surface of basal leaf)	interval (counted)	number per 1 mm of running length
PEDHR	number of hairs on pedicel	interval (counted)	number per 1 mm of running length
HYHR	number of hairs on hypanthium	interval (counted)	number per one side
CAHR	number of hairs on sepal	interval (counted)	number per sepal
LBCOR	angle between basal lobes (basal leaf)	metric	corner grade (5°)
STLN	length of flowering stems	metric	mm (5mm)
PETLN	length of petiole(basal leaf)	metric	mm (5mm)
SLELN	length (radius) of stem leaf	metric	mm (1mm)
LELN	length (radius) of basal leaf	metric	mm (1mm)

Notation	Character	Type	States or units (in brackets degree of precision)
LEWD	width of basal leaf	metric	mm (1mm)
LBLN	length of the middle lobe (basal leaf)	metric	mm (1mm)
LBWD	width of the middle lobe (basal leaf)	metric	mm (1mm)
TTHLN	length of the apical tooth (middle lobe, basal leaf)	metric	mm (0.1 mm)
STHLN	length of the tooth next to the apical (middle lobe, basal leaf)	metric	mm (0.1 mm)
STHWD	width of the tooth next to the apical (middle lobe, basal leaf)	metric	mm (0.1 mm)
HYLN	length of hypanthium	metric	mm (0.1 mm)
HYWD	width of hypanthium	metric	mm (0.1 mm)
CALN	length of sepal	metric	mm (0.1 mm)
OCALN	length of lobe of epicalyx	metric	mm (0.1 mm)
RPETLN	petiole length (divided) to stem length	ratio	
RSLELN	length of stem leaf (divided) to length of basal leaf	ratio	
CLESH	leaf length (radius) (divided) to leaf width	ratio	
RLBLN	lobe length (divided) to leaf radius	ratio	
CLBSH	lobe length (divided) to lobe width	ratio	
RLBWD	lobe width (divided) to leaf width	ratio	
TSTHLN	length of apical tooth (divided) to length of next tooth	ratio	
TTHLELN	length of apical tooth (divided) to leaf radius	ratio	
STHSH	length of side tooth (divided) to its width	ratio	
CHYSH	hypanthium length (divided) to its width	ratio	
RCALN	sepal length (divided) to hypanthium length	ratio	
ROCALN	length of outer sepals (divided) to length of inner sepals	ratio	

3.3. Data analysis

Specimens belonging to different conventionally identified species, series or sections were treated as separate clusters and the analysis of taxonomic continuum was carried out according to Paal and Kolodyazhnyi (1983) and Paal (1987, 1994)

with the original program SYNCONT 3.0 (compiled by A. Kink, S. Kolodyazhnyi, and J. Paal 1995). Coefficient of indistinctness (CI), which is the probability of α-criterion of Duda and Hart (1976), expressed in percentages, was calculated.

The covariance structure of variables was studied by principal component analysis using SAS/PRINCOMP procedure (SAS Institute Inc. 1998), and with CANOCO and CANODRAW packages (ter Braak 1988, 1990, Smilauer 1992). Eigenvectors of small eigenvalues, or structural indices, which represent the most stable proportions of variables (Möls & Paal 1998), were also calculated. The structural indices that correspond to the last five eigenvalues of the covariance matrix were used to test the difference between microspecies.

For correlation analysis, Spearman's rank correlation coefficient was used, obtained from the CORR procedure of the SAS package (SAS Institute Inc. 1998). The importance of each character in the separation of taxa was estimated by an analysis of variance — the ANOVA, MANOVA, and GLM procedures of SAS (SAS Institute Inc. 1998).

Classificatory discriminant analysis (DISCRIM procedure of SAS) was performed to investigate to what extent the empirical identification of taxa coincides with numerical classification. Stepwise discriminant analysis (STEPDISC procedure of SAS) was used to find a set of characters that maximises differences among the groups (Klecka 1980, SAS Institute Inc. 1998).

The classification package SYN-TAX 5.0 (Podani 1993) was used in order to find an optimal division of the species between sections and series. Methods such as UPGMA with Canberra distance, Manhattan distance, and Gower similarity distance for mixed data, and k-means' procedure were chosen. The latter method was started both from random seeds and from object sequences according to different existing classifications. Divisive Ward's method (MISSQ) was performed with the CLUSTER procedure of the SAS package (SAS Institute Inc. 1998).

3.4. RAPD analysis

In total, 51 plants of 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 genetically. On these plants the same morphological characters as in phenetic analysis (Table 2) were measured and coded for cladistic analysis.

DNA was extracted from dried or frozen leaves according to a slightly modified protocol of Doyle and Doyle (1987). DNA was amplified in 20 µl reaction mixtures containing 67mM Tris-HCl (pH 8,4), 16,6mM $(NH_4)_2SO_4$, 2,5mM $MgCl_2$, 0,01% gelatine, 100mM each of dATP, dCTP, dGTP and dTTP, 10 pmol primer, 2 units Taq polymerase (Sileks, Moscow, Russia) and 10–25ng of DNA template. Three primers were used for final 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). All the PCR reactions were repeated at least twice to confirm. 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 different RAPD bands and 48 morphological characters.

Phylogenetic trees were constructed with the unweighted pair-group method with the arithmetic average (UPGMA) and neighbour-joining (NJ) methods using the TREECON package (Van de Peer & De Wachter 1994). The genetic distances GD were calculated according to Link et al. 1995. For the NJ tree, bootstrap values were calculated.

Maximum parsimony (MP) analysis was carried out with the PAUP 3.1.1 programme (Swofford 1993) using heuristic search, random addition sequence (10 replicates), tree bisection-reconnection branch swapping, MULPARS option, and accelerated transformation 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.

The functional outgroup method was used in NJ and MP analysis.

4. RESULTS

4.1. Relationship of characters (Papers I and III)

According to Spearman's correlation coefficients, four groups, within which characters were strongly correlated, were formed, but correlation between the groups was insignificant or weak. The first group consisted of numerical characters describing the vegetative part of a plant, mainly the size of the specimens. Measurements of leaf teeth belonged to the second group; the third group was connected with plant hairiness. The fourth group united the metric characters of flowers. Ordination of characters with PCA resulted in the same groups of characters.

The ratio of leaf width and leaf length that describes the general shape of the leaf was the most stable combination of characters according to the structural indices. Stable combinations also existed between dimensions of the leaf and leaf lobe (LELN, LEWD, LBLN, LBWD), dimensions of the flower (HYLN, HYWD, CALN, OCALN) and leaf teeth (STHLN, TTHLN, STHWD).

4.2. Continuum of species (Papers I, III, and IV)

According to ANOVA, only 24% of all possible pairs of microspecies were statistically distinct by at least one character; MANOVA distinguished an additional 21% of species-pairs that were indistinct by ANOVA.

Only three species were totally distinct from all others according to continuum analysis (Fig. 1): *A. lindbergiana, A. plicata,* and *A. semilunaris*. In addition, two pairs of species were insignificantly distinct from each other, but well separated from the remaining ones: *A. glaucescens* and *A. hirsuticaulis*, *A. monticola* and *A. propinqua*. All other species formed a complicated network of mutually indistinct species-pairs.

Analysing sections separately, the results were slightly different. Specimens of *A. heptagona* and *A. cymatophylla,* as well as those of *A. subcrenata* and *A. cymatophylla,* were mutually indistinct. At the same time, the species of section *Alchemilla* were all significantly distinct from each other. Specimens of the three species of uncertain position, *A. semilunaris*, *A. lindbergiana* and *A. subglobosa,* were significantly separated from most of the specimens of sections *Alchemilla* and *Ultravulgares*, only specimens of *A. subglobosa* were indistinct from specimens of *A. subcrenata*. *A. semilunaris* and *A. lindbergiana* were insignificantly separated. From these sections, the classificatory discriminant analysis reclassified 20 conventionally identified specimens into different species. The largest number of them was moved from *A. acutiloba* to *A. micans* (six specimens), or *vice versa* (three specimens).

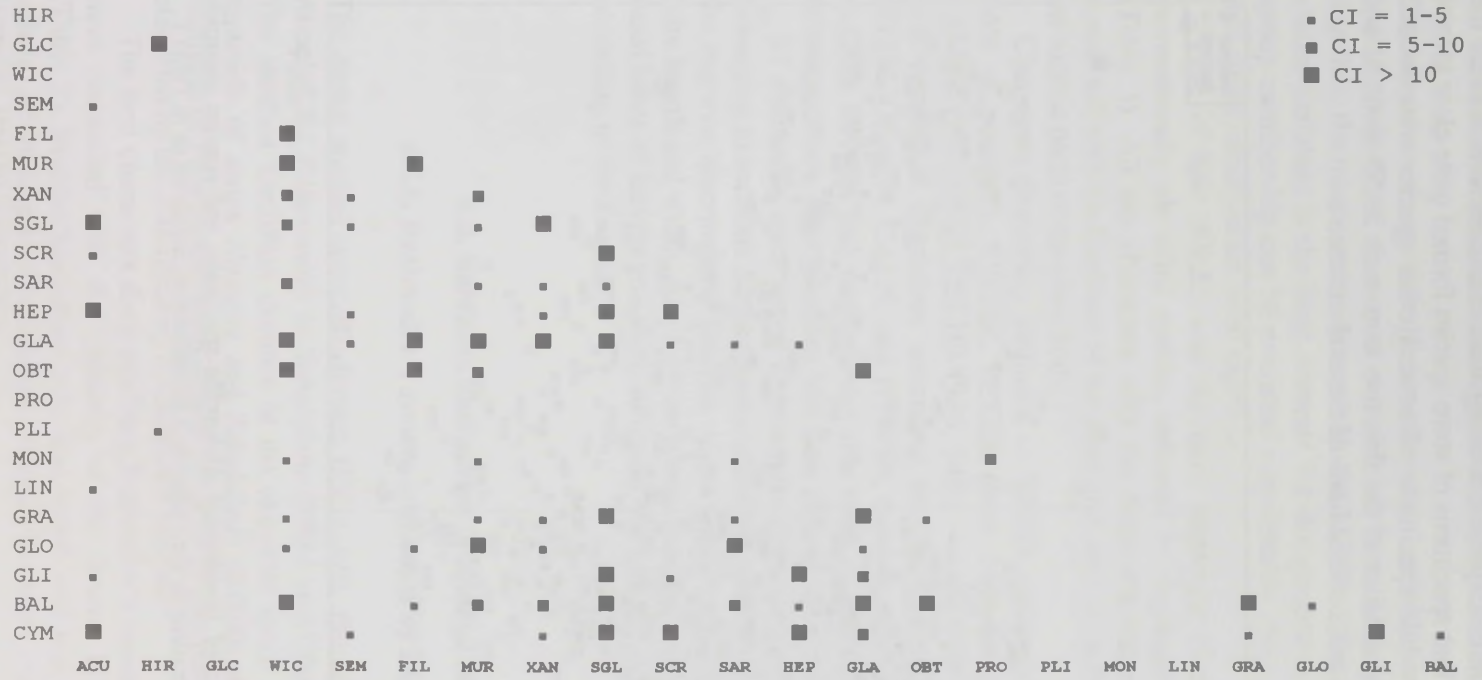

Figure 1. Coefficients of indistinctness (CI) between conventionally identified species. Notations of species names as in Table 1. If CI < 1, it is not marked.

PCA of empirically identified specimens along with calculated centroids of the species (Fig. 2) showed that specimens of some species formed quite clear groups around their centroids, while specimens of several other species were located indiscriminately. The eigenvalues of the first two axes were 39.5% (axis 1) and 11.0% (axis 2); the third axis covered 8.6% of the total variance.

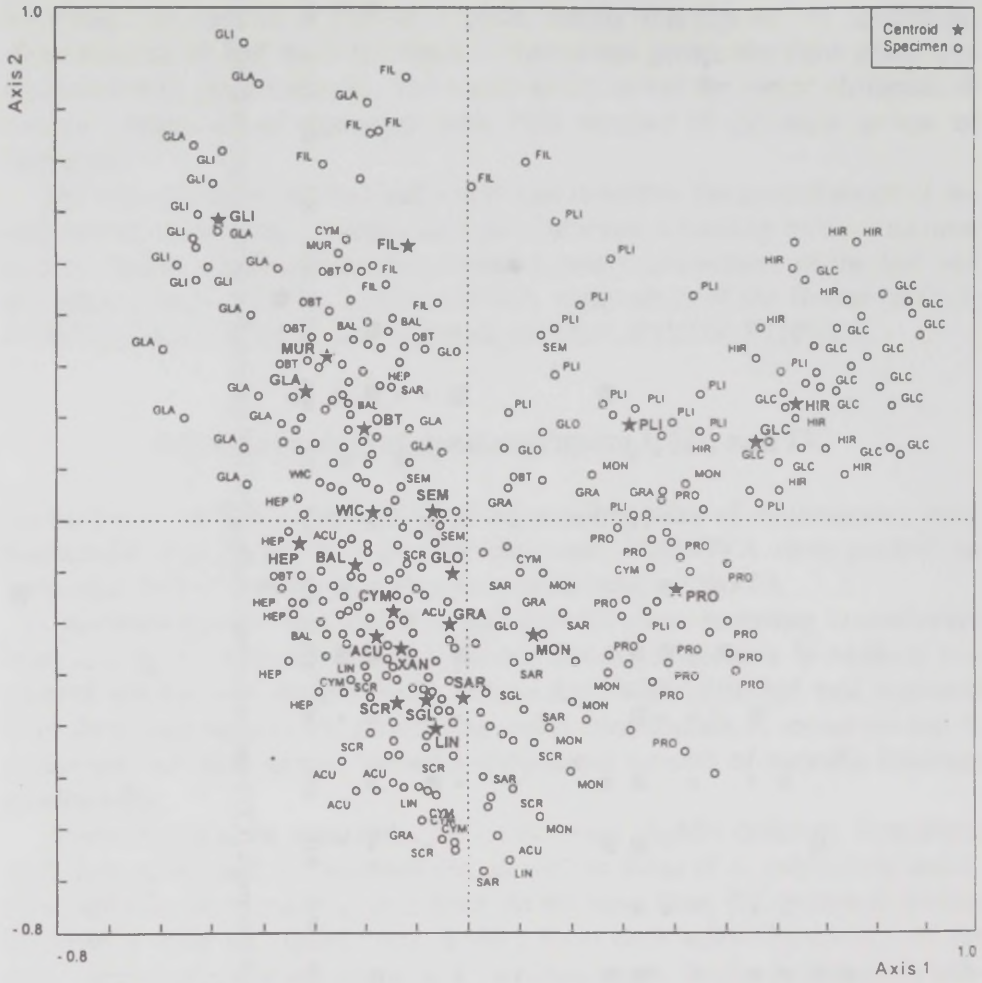

Figure 2. PCA ordination of investigated *Alchemilla* specimens and species centroids. Specimens are labelled according to their empirical identification, notations as in Table 1.

4.3. Characters for distinguishing species (Papers I, III, and IV)

The mean error rate for the set of metric variables in discriminating microspecies was 56%; for count variables it was 51%. Using all the variables together, the mean error rate was reduced to 32%. Consequently, the set of only metric variables is the least accurate for discriminating between microspecies, group membership can be predicted considerably more accurately if the metric and count variables are used together.

Type of hair (SILK) was the most important character for distinguishing conventionally identified species, indicated by the highest F-value by ANOVA (Table 3). All ten characters with the highest F-values, except THNR, were connected with the hairiness of the plant and most of them described hair numbers on various parts of the plant body.

Characters describing hairiness — VNHR (distinguishes 167 species-pairs, 66% of possible), STUHR, PETHR (both 160, 63%), STLHR (145, 57%), LEUHR (137, 54%), LELHR (136, 54%) —, and THNR (129, 51%) were the best numerical characters according to GLM. Flower characters (FPTHR, HYLN, OCALN, CALN, and HYWD), number of leaves and flowering stems (LENR, STNR), and width of the side teeth of leaf lobes (STHWD) were the least important, distinguishing less than 25% of species-pairs.

31 characters were found to be essential in distinguishing the microspecies belonging to sections *Ultravulgares* and *Alchemilla* (Fröhner 1995), according to the stepwise discriminant analysis where ratios were also included. The ratio of lobe length and width, which characterises the lobe shape, the angle between the basal lobes of leaves, and the position of hairs on the stem were most important, according to the F-value.

4.4. Sections and series (Papers I and IV)

4.4.1. Rothmaler's system corrected by Plocek (1982)

The series studied were all distinct (CI < 1.0), the highest adjacencies were recorded for *Pubescentes* to *Subglabrae* (85%) and *Glabrae* to *Hirsutae* (83%). The smallest Euclidean distance in the character space was found between the centroids of series *Hirsutae* and *Subglabrae* (0.068); the most apart were the centroids of *Pubescentes* and *Glabrae* (0.241). 19 specimens (5.1%) were wrongly classified by the classificatory discriminant analysis.

The best characters distinguishing Rothmaler's series according to ANOVA were connected with the hairiness of the flower: HYHR, CAHR, PEDHR (Table 3). Besides hair-characters, leaf length and leaf width also had high F-values. According to discriminant analysis the most important characters were HRPOS, HYHR and HYLN.

Table 3. Effectiveness of characters in distinguishing species and higher rank taxa according to the ANOVA F-value and F-value (for removal) of the stepwise discriminant analysis. The rank of the ten first characters is marked in brackets. Notation of characters as in Table 2.

Character	F-value for species	F-value for Rothmaler's series	F-value for remove of Rothmaler's series	F-value for Juzepzcuk's groups	F-value for remove of Juzepczuk's groups	F-value for Fröhner's sections	F-value for remove of Fröhner's sections	F-value for "corrected" sections	F-value for remove of "corrected" sections
SILK	123.72 (1.)	244.83 (4.)	–	129.37 (5.)	–	54.59 (9.)	–	170.39 (5.)	–
HYHR	100.38 (2.)	402.82 (1.)	33.14 (2.)	247.02 (1.)	19.92 (3.)	98.36 (3.)	NS	342.37 (1.)	25.70 (2.)
CAHR	94.77 (3.)	311.28 (2.)	5.37	198.49 (2.)	5.97	103.51 (2.)	3.17	265.37 (2.)	5.63
PEDHR	71.89 (4.)	266.29 (3.)	10.19 (9.)	135.70 (3.)	17.16 (4.)	39.62	10.85 (3.)	220.08 (4.)	14.74 (5.)
LELHR	51.94 (5.)	197.50 (5.)	12.93 (5.)	130.38 (4.)	9.64 (9.)	83.38 (4.)	3.32	252.79 (3.)	10.54 (6.)
HRPOS	41.22 (6.)	140.41 (7.)	86.77 (1.)	78.99 (6.)	45.98 (1.)	133.08 (1.)	71.79 (1.)	108.09 (9.)	55.94 (1.)
THNR	31.48 (7.)	143.47 (6.)	12.60 (6.)	77.14 (7.)	6.69	48.63	2.45	155.31 (6.)	9.90 (9.)
STUHR	24.84 (8.)	47.33	4.05	64.17 (9.)	20.62 (2.)	59.51 (7.)	NS	66.44	5.82
LEUHR	23.78 (9.)	81.28 (8.)	3.22	70.19 (8.)	5.63	77.15 (5.)	NS	111.02 (8.)	6.03
VNHR	22.95 (10.)	76.13 (9.)	3.40	59.23(10.)	NS	66.65 (6.)	NS	112.35 (7.)	2.44
LBNR	18.78	48.98	NS	37.16	3.60	42.89	3.98	48.86	2.52
PETHR	18.28	38.39	10.57 (8.)	26.76	6.74	31.68	6.81	64.45	8.64
LBCOR	17.71	3.97	4.88	7.92	3.77	21.22	7.34 (10.)	13.44	5.68
PETLN	16.77	37.79	8.29	30.26	5.86	58.09 (8.)	6.44	53.66	10.03 (8.)
INCDP	16.22	18.43	8.71	18.70	9.71 (8.)	18.83	8.25 (6.)	32.97	19.54 (3.)
SLELN	15.59	56.14	NS	39.40	NS	49.32(10.)	NS	72.43(10.)	NS
LBWD	15.55	41.02	NS	26.85	13.89 (5.)	35.06	22.18 (2.)	38.51	NS
LEWD	15.17	61.29(11.)	NS	35.04	NS	44.31	NS	62.99	NS
LELN	14.97	61.83(10.)	3.54	36.24	2.95	47.34	3.63	63.75	2.53
LBLN	14.03	40.57	4.64	22.17	6.24	25.11	7.62 (9.)	43.29	3.05
STLHR	13.64	28.99	NS	22.80	8.42 (11.)	26.06	NS	39.03	NS
STLN	13.60	29.01	NS	26.68	NS	43.44	NS	44.51	NS
FLGDN	12.92	22.78	NS	21.52	2.92	30.80	2.50	51.63	6.04
LBTOP	12.31	15.37	6.97	8.47	6.46	26.98	8.20	29.78	6.93
THTOP	9.58	14.91	5.06	7.41	7.48	21.55	5.45	14.88	NS
THSYM	8.59	NS	12.26 (7.)	4.22	11.35 (6.)	7.28	9.35 (5.)	12.11	9.52(10.)

INFSH	8.59	3.11	NS	NS	NS	10.21	5.80	4.36	NS
STCOL	7.99	11.45	9.03 (10.)	5.85	4.61	18.99	10.20 (4.)	11.61	6.10
FLCOL	7.20	5.99	NS	5.19	4.13	11.29	6.11	7.11	6.23
HYLN	7.10	31.25	18.12 (3.)	15.79	10.07 (7.)	5.38	6.29	33.13	16.74 (4.)
LECOL	6.86	5.48	NS	4.84	3.26	10.38	NS	3.89	NS
LEFLD	6.33	4.57	4.76	7.48	3.91	6.29	4.66	NS	2.98
STHLN	6.14	9.40	5.89	5.96	4.85	8.71	NS	19.77	6.96
TTHLN	6.10	8.36	4.39	6.48	6.02	9.02	2.57	19.81	6.04
STHWD	5.59	NS	3.06	2.50	2.40	11.10	NS	NS	2.39
HYWD	5.55	24.03	NS	12.26	NS	7.60	NS	21.44	NS
LENR	4.52	6.24	3.88	5.05	NS	10.22	NS	4.69	3.06
STPOS	3.50	8.13	NS	4.77	NS	2.79	3.38	4.48	2.76
CALN	3.47	8.98	14.57 (4.)	5.26	8.62 (10.)	7.58	8.67(6.)	9.34	10.21 (7.)
CASH	3.44	NS	NS	6.72	NS	5.22	NS	4.18	2.49
OCALN	2.75	6.77	4.57	4.64	5.74	7.58	7.66 (8.)	12.77	7.56
STNR	2.68	6.23	NS	4.08	NS	4.89	NS	5.28	NS
HYSH	2.10	5.42	NS	4.54	NS	3.46	NS	3.53	NS

4.4.2. Juzepczuk's (1941) groups

Of the groups, two pairs were separated non-significantly: *Exuentes* and *Glabricaules* (CI = 7.0), *Exuentes* and *Glabratae* (CI = 13.4), but the most adjacent were *Pubescentes* to *Barbulatae* (87%) and *Glabricaules* to *Imberbes* (58%). The largest Euclidean distance was found between the centroids of *Pubescentes* and *Glabricaules* (0.241), the smallest between the centroids of *Exuentes* and *Glabratae* (0.062). 31 specimens (8.3%) were re-identified into different subgroups by classificatory discriminant analysis.

The characters that were important in distinguishing these groups according to ANOVA were generally similar to those important for separating species and Rothmaler's series, being mainly hairiness characters (Table 3). Hairiness characters: HRPOS, STUHR, and HYHR are also the most important according to F-criterion in discriminant analysis.

4.4.3. Fröhner's (1995) sections

Of these sections only *Alchemilla* and *Ultravulgares* were statistically not reliably separated (CI = 18.4), with their centroids very close to each other (Euclidean distance 0.053). An additional statistical testing of the relationship of sections *Ultravulgares* and *Alchemilla* (excluding other species) proved even their reliable separation (CI = 0.00). The most adjacent were section *Plicatae* to section *Alchemilla* (83%) and species *A. lindbergiana* to section *Ultravulgares* (80%). 38 specimens (10.2%) were considered to be misidentified into sections, according to classificatory discriminant analysis.

The most important characters for distinguishing these sections according to ANOVA were different from those important in the previous two systems (Table 3): position of hairs on the stem (HRPOS), followed by some of the hair numbers, but also length of the stem leaves (SLELN) and length of the petiole (PETLN). HRPOS, LBWD, PEDHR, and STCOL were most important for separating Fröhner's sections according to F-criterion in discriminant analysis. Based on the analysed species, only 23 characters were necessary to separate the sections *Alchemilla* and *Ultravulgares*. The ratio of lobe width and leaf width appeared to be the most important.

4.5. Cluster analysis (Papers I and IV)

The results of clustering species centroids with different methods (UPGMA, Ward's method with different distances, and k-means clustering) seem to support Fröhner's system (1995). A group consisting of *A. glaucescens, A. hirsuticaulis* and *A. plicata* was constantly formed, and this was mostly well separated from the

other species. *A. propinqua* and *A. monticola* were closely related according to most of the clustering methods, but often they were accompanied by some other species, most frequently by *A. sarmatica*, occasionally also by *A. subglobosa*, *A. semilunaris,* and *A. micans.* Species belonging to the section *Coriaceae* formed a joint group or two different subgroups: *A. wichurae*, *A. baltica*, *A. glomerulans, A. murbeckiana* in one and *A. glabra*, *A. obtusa*, *A. filicaulis* in the other. *A. filicaulis* is closest to section *Coriaceae* (and not to the section *Plicatae*) according to cluster analysis, continuum analysis, and ordination. The results of some cluster analysis variants placed *A. baltica* closer to section *Alchemilla*, but in most cases it belonged to section *Coriaceae. A. glabricaulis* moved between section *Coriaceae* and section *Ultravulgares*, in some cases this species was located alone, separately from all others.

Cluster analysis of specimens of the sections *Ultravulgares* and *Alchemilla* by the MISSQ method classified the data into two big clusters: the first included mainly specimens of *A. acutiloba* and *A. micans*, the second comprised all other specimens.

4.6. RAPD analysis and cladistics (Paper II)

The clusters that appeared on the UPGMA phenogram of RAPD-data corresponded rather well with the Fröhner's sections. Section *Plicatae,* except the two plants in anomalous positions, was clearly one big cluster. It could be split further into two branches: *A. glaucescens* and *A. hirsuticaulis* together, and *A. monticola* with the only specimen of *A. sarmatica.* The two microspecies of section *Alchemilla* under analysis, *A. acutiloba* and *A. micans,* formed another large cluster. The next large cluster appearing on the UPGMA phenogram consisted of two branches. The first branch combined *A. baltica* and *A. glabricaulis* (belonging to Fröhner's section *Coriaceae*). The second one united *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 by the NJ method 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 on the UPGMA phenogram, corresponding to Fröhner's sections, could be seen on the NJ tree but, still, some changes should be emphasised. Section *Plicatae* was paraphyletic, consisting of two separate branches (*A. hirsuticalis* + *A. glaucescens* and *A. monticola* + *A. sarmatica*). Section *Coriaceae* (*A. baltica* + *A. glabricaulis*) was not separable from the section *Alchemilla* cluster. Section *Ultravulgares* formed a clearly separate cluster, and moreover, *A. heptagona* was strongly separated from all other species. It is noteworthy that the same pair of specimens of *A. subcrenata* and *A. cymatophylla* as in the UPGMA tree was again together with rather strong support (82%).

The MP method on RAPD data resulted in cladograms with many features in common with the UPGMA and NJ trees. The programme generated four shortest trees (384 steps, consistency index CI = 0.167, homoplasy index HI = 0.833). 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 two, mostly being equal to one. Still, the topology was practically the same in all trees, indicating that despite the weak support of branches the topology may be close to the true relationships. *A. glaucescens* and *A. hirsuticaulis, A. monticola* and *A. sarmatica* formed clades by pairs, but not all together. *A. heptagona* formed a clade which even had moderate support (bootstrap value 66, DI = 2). The section *Ultravulgares* as a whole could be considered to be an intergrade. *A. acutiloba* and *A. micans* were mixed with each other. *A. glabricaulis* and *A. baltica,* as representatives of the section *Coriaceae,* did not form a clade, but also an intergrade. The same two samples of different microspecies — *A. subcrenata* and *A. cymatophylla,* as in previous trees were most strongly supported as a clade (bootstrap value 90).

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

MP trees of combined data (Fig. 3, the consensus, and Fig. 4, one of 20 shortest trees, length 875 steps, CI = 0.190, HI = 0.810) gave the best-supported resolution of the microspecies. *A. glaucescens* and *A. hirsuticaulis* formed a clade together, which even had moderate support (bootstrap value 77). *A. monticola* plants of two geographically proximate populations were also weakly supported as a clade. The merging here of specimens of the same microspecies from other populations was not supported, but all plants of *A. monticola* formed an intergrade. All three microspecies behaved like a monophyletic group, too, but it was not well supported. The other groups were also not well supported, but again the topology was quite consistent through 20 trees. Sections *Ultravulgares* and *Coriaceae* together formed a clade, *A. acutiloba* and *A. micans* were mixed and formed an intergrade.

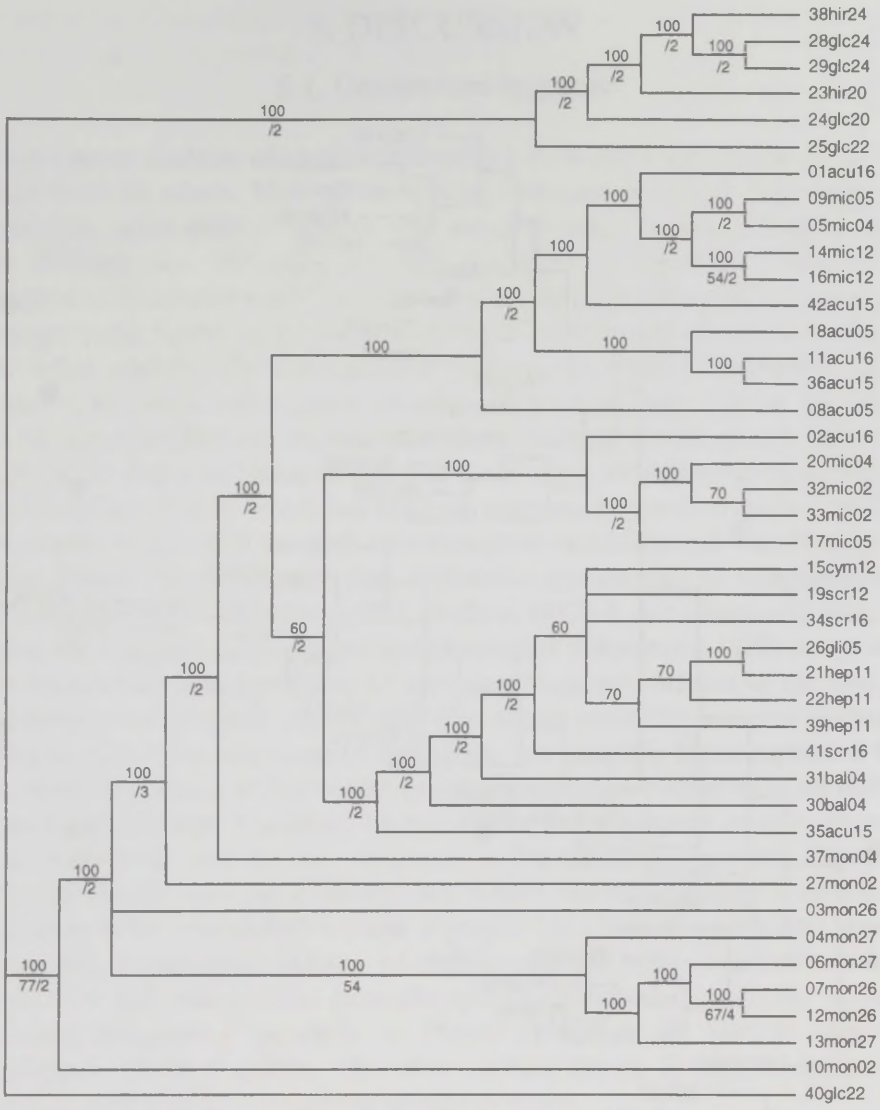

Figure 3. The 50% majority-rule consensus tree of 20 most parsimonious trees (length 875 steps, CI = 0.190, HI = 0.810) of combined (morphological and RAPD) data (42 specimens). Notations of species as in Table 1. Above the branch is marked the per cent of the most parsimonious trees with given topology, below the branch the bootstrap value (if over 50 of 100 replicates) / decay index (if over 2).

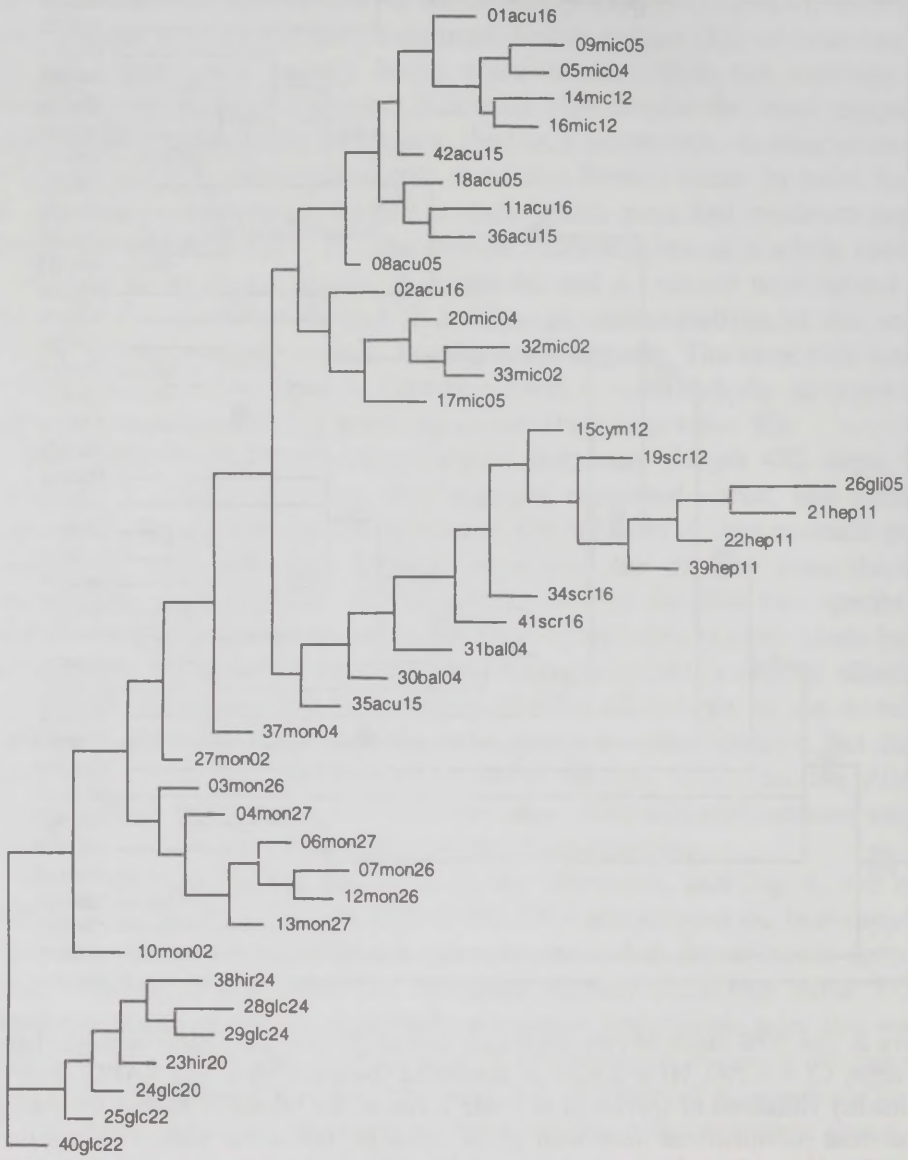

Figure 4. One of 20 most parsimonious trees of combined (morphological and RAPD) data (42 specimens). Notations of species as in Table 1.

5. DISCUSSION

5.1. Continuum in genus

The continuum analysis of species showed that only some species are completely distinct from all others. Most of the species could not be clearly separated, and, surprisingly, some pairs of species that are considered by several authors to be quite different, *i.e.* belonging to different series or sections, could not be distinguished from a statistical point of view. At the same time, some species-pairs that seem to be similar and hardly separable in nature were distinct according to multivariate analysis. The distinctness of close species could be the result of using herbarium material, which often consists of tendentiously typical specimens. Nevertheless, this does not explain the indistinctness of distant species pairs. One reason can be that a multitude of characters was used, including the measurements of leaf teeth and flower, which has not been common in previous studies. Still, the main reason is probably the high morphological variability and transitionality of species. Hence the assumption that *Alchemilla* species can be morphologically well distinguished (Tikhomirov 1967, Walters 1987) is not completely true, and treating these species as an agamo-sexual complex (Glazunova 1977) seems to be more reasonable. The continuum of species also gives evidence of the fact that hybridisation has occurred and probably also occurs nowadays between species.

Higher rank taxa, like sections and series, are generally better separated from each other. The series of Rothmaler according to Plocek (1982) were all distinct, but the series *Hirsutae* was rather heterogeneous, being adjacent on one side to the series *Subglabrae* and on the other side to the series *Pubescentes*. Groups of Juzepczuk (1941) were not all distinct, and from a statistical point of view some of them seem to be established without a proper basis, *e.g. Exuentes* and *Appressipilae*. All investigated sections of Fröhner (1995) were statistically reliably distinct. The different systems generally agree regarding the six species joined as the series *Subglabrae* according to Plocek (1982) or the section *Coriaceae* according to Fröhner (1995). The other authors except Fröhner also agree in placing *A. glabricaulis* in a separate section or series.

5.2. Characters

According to ANOVA, the most important characters for distinguishing species were connected with the hairiness of different parts of the plant body. Though Tikhomirov *et al.* (1995) also emphasise the role of these characters, we cannot leave out other characters, since different species and species groups can be distinguished using different characters. Most identification keys (*e.g.* Zamelis 1933, Juzepczuk 1941, Walters & Pawlowski 1968, Laasimer *et al.* 1996) also use many characters and give a different weight to the same character when identifying different species. Furthermore, many characters which have a high F-value

according to ANOVA, distinguished only some or even one of the 23 species clearly, and the remaining species cannot be distinguished. The results also showed that infraspecific variation of most morphological characters is extensive, as already found by Turesson (1956). Hence, in several species, character states partially overlap and this may cause species indistinctness, even though type specimens are distinct.

Most of the characters which, according to the discriminant analysis, appeared to be fundamental in species discrimination (*e.g.* CLBSH, LBCOR, HRPOS, PETHR, LEUHR), are emphasised as important in the identification keys as well (Juzepczuk 1941, Walters & Pawlowski 1968, Laasimer et al. 1996). The quite high effectiveness of leaf teeth characters (THTIP, THSYM) is surprising, since they are not considered to be essential in literature. Flower measurements did not seem to be so effective in the identification of these species, although they are used, *e.g.* by Walters and Pawlowski (1968). Generally, it appeared that the parameters of hairiness, as well as several nominal and ratio characters are better than the metric ones for species discrimination.

The most stable combinations of variables reflected in structural indices are in a good agreement with the main correlation groups of variables. The stability of leaf variables, for example, also shows that it is probably better to use ratios of metric variables in taxon discrimination, as already suggested by some authors (Fröhner 1995). If we use the structural indices or ratios instead of metric variables as such, there will probably be less individual differences between specimens of the same taxon.

5.3. Analysis of sections and species

The section *Ultravulgares* appeared not to be monophyletic by RAPD-analysis, but in several other analyses it formed a separate cluster or an intergrade with section *Alchemilla*. Not many samples were included from this section either and, as the results are not in the best concordance, we cannot draw conclusions for the section as a whole. Specimens belonging to different species of the section *Ultravulgares* — *A. cymatophylla*, *A. subcrenata,* and *A. heptagona* — were, according to the continuum analysis, insignificantly distinct from each other and did not form separable species-clusters. But, according to structural indices, *A. heptagona* was indistinct from *A. filicaulis* and *A. subglobosa,* and on the canonical ordination plots, *A. heptagona* could visually be so clearly distinguished that one is tempted to place it together with *A. filicaulis* in a separate group (section?) *Exuentes,* as did Juzepczuk (1941). According to genetic data this microspecies is also mainly on a separate branch in the section. Still, its indistinctness from *A. cymatophylla* and adjacency to both *A. subcrenata* and *A. cymatophylla* convinces us that the species can also stay in the section *Ultravulgares*. *A. cymatophylla* specimens, on the contrary, are the most variable in the section *Ultravulgares,* and their identity is not always very clear.

The specimen of *A. cymatophylla* (15cym12), which is joined with a specimen of *A. subcrenata* in several RAPD trees, lies between *A. cymatophylla* and *A. subcrenata* in the morphology tree, with a little more similarity to the former. Still, genetically it is evidently close to *A. subcrenata.* It can be concluded that identification of *Alchemilla* microspecies by morphological features only is not always reliable.

The alleged similarity of *A. semilunaris* to the species of section *Ultravulgares* (Walters & Pawlowski 1968) is doubtful, though the only sample of *A. semilunaris* was close to section *Ultravulgares* according to RAPD-data. But, morphologically this species varied mainly towards *A. lindbergiana.* Specimens of *A. semilunaris* were set clearly apart from the specimens of all the other species in the character space. Maybe it is reasonable to include *A. semilunaris* in the section *Decumbentes,* since Fröhner (1995) considers it to be close to the species of this section, but, since we have not analysed any species belonging to the latter section, nothing certain can be said.

The three species of section *Plicatae*: *A. glaucescens*, *A. hirsuticaulis,* and *A. plicata* were grouped together and separated from the remaining species by all algorithms of cluster analysis. Intermixing of A. *glaucescens* and *A. hirsuticaulis* in all RAPD-trees, as well as their morphological indistinctness, indicates their close taxonomic relation, and, in fact, morphological features for discrimination of these two similar microspecies are not always clear-cut. Both 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. Therefore, the division of Fröhner's section *Plicatae* into two groups is suggested. One should consist of *A. glaucescens*, *A. hirsuticaulis, A. plicata* and similar microspecies (section *Pubescentes* in Plocek 1982, Rothmaler 1936, and Juzepczuk 1941) and the other of *A. monticola, A. propinqua, A. sarmatica,* and possibly some related microspecies.

A. filicaulis is, according to both continuum analysis and clustering, not adjacent to section *Plicatae*, as proposed by Fröhner (1995), but rather to section *Coriaceae,* where this species probably should be placed, or possibly in separate section *Exuentes* with *A. heptagona.*

Specimens identified as *A. subglobosa,* included also in section *Plicatae* by Fröhner (1995), vary a great deal, are non-significantly distinct from many other species, and belong to various groups according to the different types of cluster analysis. Typical specimens in the right phenophase are probably well distinguishable, while specimens collected in the "wrong" phenophase, or being atypical for some other reason, cannot be identified correctly. Additional research is needed on this species.

Specimens of different species in section *Alchemilla* are all significantly distinct from each other, though on the ordination plots they look quite mixed and form a joint "cloud". Even the specimens of *A. acutiloba* and *A. micans,* the affinity of which is stated to be rather high (Juzepczuk 1941, Walters &

Pawlowski 1968), and the identification of which is quite complicated in nature, are well separated from a statistical point of view. Still, according to both the classificatory discriminant analysis and the cluster analysis, many specimens are obviously intermediate. According to genetic characters *A. acutiloba* and *A. micans* are always intermixed as constituents of a single cluster or clade or at least intergrade. 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, A. xanthochlora* and *A. micans* belong to the section *Alchemilla*. RAPD data does not support the combining of them with some others in the *Hirsutae* series (Plocek 1982) nor in the *Imberbes* group (Juzepczuk 1941).

On the basis of our material *A. lindbergiana* is most closely related to *A. semilunaris*. This similarity is hard to interpret and could have been caused by our biased sample. According to the canonical discriminant analysis the specimens of *A. lindbergiana* are situated between the specimens of section *Alchemilla* and *A. semilunaris*. Still, some similarity to *A. xanthochlora* can be detected from both the continuum analysis and the canonical discriminant analysis. Whether this species can be placed in section *Alchemilla*, and whether its similarity with *A. semilunaris* is occasional, can only be determined for certain by means of molecular testing.

Section *Coriaceae* seems to be reasonable, though, using RAPD-data, it 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. *A. glabricaulis* differs remarkably from other species and can form a separate series *Glabricaules* in this section.

6. CONCLUSIONS

1. The study showed that *Alchemilla* microspecies are morphologically rather variable and the variation is continuous, thus, many of the microspecies are indistinct in nature.
2. The sections and series are better separated than species, and Fröhner's (1995) system appeared to be the most reasonable.
3. Different kinds of characters are necessary to discriminate between microspecies; the most effective are ratios, structural indices, and hair characters.
4. Genetic characters, for example RAPD markers, offer good prospects in attempts to find optimal taxonomic solutions for this genus.
5. Though the results depend on the sample, which is biased to some extent due to the fact that not all the species are sufficiently represented, and that not all species in the sections are involved, they indicate some possible taxonomic solutions, which can be finally accepted only after additional research involving type material:
   5.1. From section *Alchemilla,* intermixing of *A. acutiloba* and *A. micans* according to RAPD data, and their morphological similarity, probably allows them to be united in a single microspecies.
   5.2. Fröhner's section *Plicatae* should be divided into two series or sections (*Pubescentes* and *Barbulatae*) based on RAPD-data, morphological features, and the suggestions of Rothmaler (1936) and Juzepczuk (1941). *A. filicaulis* should be removed from the section, probably to series *Exuentes* of section *Ultravulgares.*
   5.3. Section *Ultravulgares* would also be better split. *A. heptagona* is very different from other species of that section, both by morphological and molecular traits, and should probably be separated into the series *Exuentes*, together with *A. filicaulis,* as previously proposed by Juzepczuk (1941).

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ABSTRACT

The morphological and genetic variation and taxonomic continuum of 23 putative *Alchemilla* species represented in Estonia were analysed to assess the morphological variability of these taxa; to determine the distinctness of the microspecies from a statistical point of view using morphological characters; to ascertain patterns and relationships of species within and between the sections and series; to compile a set of morphological characters that discriminate between the analysed species most clearly; to find the most stable proportions between the variables according to the structural indices, and to assess how the structural indices distinguish microspecies; and to assess the genetic variability and the relationships of the microspecies using the RAPD method.

The study showed that *Alchemilla* microspecies are morphologically rather variable and the variation is continuous, thus, many of the microspecies are indistinct in nature. The sections and series are better separated than species, and Fröhner's (1995) system appeared to be the most reasonable. Different kinds of characters are necessary to discriminate between microspecies; the most effective are ratios, structure indices, and hair characters. Genetic characters, for example RAPD markers, offer good prospects in attempts to find optimal taxonomic solutions for this genus.

Though the results depend on the sample, which is biased to some extent due to the fact that not all the species are sufficiently represented and that not all species in the sections are involved, they indicate some possible taxonomic solutions, which can be finally accepted only after additional research, involving type material:

1. Intermixing of *A. acutiloba* and *A. micans* probably allows them to be united in a single microspecies.
2. Fröhner's section *Plicatae* should be divided into two series or sections (*Pubescentes* and *Barbulatae*). *A. filicaulis* should be removed from the section, probably to series *Exuentes* of section *Ultravulgares*.
3. Section *Ultravulgares* would also be better split. *A. heptagona* should probably be separated into the series *Exuentes*, together with *A. filicaulis*.

SUMMARY IN ESTONIAN

Perekonna kortsleht (*Alchemilla* L., *Rosaceae*) morfoloogiline ja geneetiline varieeruvus Eestis

Sisukokkuvõte

Käesolevas töös uuriti 23 Eestis kasvava kortslehe mikroliigi 598 isendit morfomeetriliselt, kasutades mitmemõõtmelisi statistilisi meetodeid. Teostati ka väikesemahuline geneetiline uuring RAPD-meetodil. Töö eesmärgiks oli saada ülevaade kortslehtede morfoloogilisest varieeruvusest Eestis; hinnata mikroliikide statistilist eristuvust morfoloogiliste tunnuste alusel; vaadelda samadesse ja erinevatesse sektsioonidesse klassifitseeritud liikide omavahelisi suhteid ja seekaudu hinnata sektsioonide objektiivsust; koostada tunnuste pingerida hindamaks, millised neist on kõige efektiivsemad liikide eristamisel; leida kõige stabiilsemad tunnuste omavahelised suhted ja uurida struktuuriindeksite efektiivsust liikide eristamisel; hinnata mikroliikide geneetilist varieeruvust ja nende omavahelisi suhteid, kasutades geneetilisi tunnuseid — RAPD-markereid.

Töö tulemusena selgus, et kortslehe mikroliigid on väga varieeruvad ja kontinuaalsed, sageli esineb mikroliikide vahel pidev üleminek, hiaatus puudub ja liike on raske eristada. Sektsioonid eristuvad morfoloogiliste tunnuste alusel paremini, kõige optimaalsemaks osutus meie andmete põhjal Fröhneri (1995) süsteem.

Liikide eristamisel ei saa kõrvale jätta ühtegi tunnust, kuid kõige efektiivsemateks osutusid siiski taime karvasust iseloomustavad tunnused, samuti tunnuste suhtarvud ja struktuuriindeksid. RAPD-markerite vm geneetiliste tunnuste kasutamine perekonna probleemide lahendamiseks on kindlasti väga perspektiivne.

Kuigi kõik uuritud liigid ei olnud esindatud piisavas mahus, samuti ei olnud esindatud sektsioonide kõik liigid, võib siiski teha mõned esialgsed taksonoomilised järeldused, mis küll vajavad lõplikku kinnitust täiendava uurimise käigus, hõlmates ka tüüpmaterjali.

1. Teravahõlmise (*A. acutiloba*) ja küüt-kortlehe (*A. micans*) sarnasus morfoloogiliste tunnuste alusel ja mitte-eristumine RAPD-markereid kasutades lubaks nad liita üheks liigiks.
2. Fröhneri sektsioon *Plicatae* tuleks arvatavasti jagada kaheks seeriaks (*Pubescentes* ja *Barbulatae*). Niitjas kortsleht (*A. filicaulis*) ilmselt ei peaks sellesse sektsiooni kuuluma, vaid pigem sektsiooni *Ultravulgares* (seeriasse *Exuentes*).
3. Ka sektsiooni *Ultravulgares* võiks jagada kaheks seeriaks. Seitsmetine kortsleht (*A. heptagona*) tuleks eraldada koos niitja kortslehega seeriasse *Exuentes*.

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PUBLICATIONS

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Taxonomic continuum of *Alchemilla* (Rosaceae) in Estonia. —
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Taxonomic continuum of Alchemilla (Rosaceae) in Estonia

Silvia Sepp and Jaanus Paal

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23 widespread apomictic *Alchemilla* microspecies occurring in Estonia are analyzed to investigate whether the species and higher rank taxa are distinct, how variable these taxa are and which characters distinguish them better. Cluster analysis, discriminant analysis, analysis of variance, principal component analysis and continuum analysis are used for data processing. The characters form four correlative groups, describing (i) vegetative and (ii) generative parts of the plant body, (iii) hairiness characters and, (iv) leaf teeth measurements. The best characters according to analysis of variance for distinguishing species are hairiness characters, but often they distinguish only few species very clearly and cannot be used for the remaining ones. Hence the other characters cannot be excluded. From the studied species only *A. plicata*, *A. semilunaris* and *A. lindbergiana* are significantly distinct from all others. The remaining ones form a complicated network of mutually indistinct pairs. Higher rank taxa – sections and series according to Rothmaler, Fröhner and Yuzepchuk are better separated, containing very few mutually indistinct pairs. Results from species centroids' clustering are most congruent with Fröhner's system, but still some changes seem to be necessary and a corrected system is proposed here. Section *Plicatae* is split into two series: *Pubescentes* and *Barbulatae*, sections *Alchemilla*, *Ultravulgares* and *Decumbentes* are joined as three series of section *Hirsutae*, and *A. filicaulis* is moved from section *Plicatae* to section *Coriaceae*. *Coriaceae* should also be split into three series: *Exuentes (A. filicaulis)*, *Glabricaules (A. glabricaulis)* and *Coriaceae* (other species).

S. Sepp, Institute of Botany and Ecology, University of Tartu, 40 Lai St., EE-51005 Tartu, Estonia. –J. Paal, Institute of Botany and Ecology, University of Tartu, 40 Lai St., EE-51005 Tartu, Estonia.

Introduction

The genus *Alchemilla* L. (Fam. Rosaceae Juss., subfam. Rosoidae Focke) consists of more than 1000 (micro)species (Fröhner 1995), if *Lachemilla* (Focke) Rydberg is included as in Brummitt (1992). The genus is distributed mainly in Eurasia, but also in East Africa, and as an alien in North America and in Australia (Rothmaler 1937, 1941, Fröhner 1995, Tikhkhomirov et al. 1995). More than 300 (micro)species have been described from Europe, where large mountain ranges such as the Alps, the Caucasus, the Carpathians and others with many endemic species probably are their main distribution centers.

Already at the beginning of this century Murbeck (1901) and Strasburger (1905) discovered that many species of *Alchemilla* reproduce apomictically. From that time most of the species are considered to be obligate agamosperms (Khokhlov 1967, Rubtsova 1989, Koltunow 1994). Still, according to Glazunova (1977, 1983, 1987) and Izmailow (1984, 1986, 1994a,b), the majority of *Alchemilla* species are probably not obligate, but facultative apomicts.

Alchemilla species are high polyploids, the number of

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chromosomes ranging from 64 to 224 (Turesson 1957, Löve & Löve 1961, 1975, Bradshaw 1963, Wegener 1967, Izmailow 1981, 1982). The chromosome number is often aneuploid and varies widely within one species. One of the reasons may be count errors (as the number of chromosomes is high and they are small), but most probably the variation in chromosome numbers is indicative of the hybridogenous origin of species and their genetic heterogeneity. This interpretation was also reached by Lundh-Almestrand (1958) and Turesson (1943, 1956, 1957) in their experimental works, where they detected genetic variants mainly within microspecies. Analyzing DNA of *Alchemilla* with RAPD markers, Baeva et al. (1997) showed that populations within species are sometimes genetically more dissimilar than different microspecies.

Due to its agamospermy and large variation, the genus has been an object of widespread scientific interest since the last century. Most authors follow Buser (1894, 1895) in ranking *Alchemilla* microspecies on species level, as if they were sexual species (e.g. Lindberg 1909, Rothmaler 1935-1962, Yuzepchuk 1941, Samuelsson 1943, Plocek 1982). Also in "Flora Europaea" (Walters & Pawlowski 1968) and the "Illustrierte Flora of Mitteleuropa" (Fröhner 1990) this species level is used. This treatment originates from the assumptions that these "species" are clones (obligatory apomictic), they differ morphologically from each other, and they have different distribution areas and ecological niches. Some authors (Ascherson & Graebner 1900-1905, Turesson 1943, 1956, Löve 1960, 1961, 1975, Glazunova 1977, Tikhkhomirov et al. 1995) suggest that only a few collective species should be distinguished, but this is not a prevailing interpretation.

Of the different conceptions concerning the division of the genus into sections and series, the most widespread system originates from Buser (1891, 1901). This classification is further developed mainly by Rothmaler (1936, 1944), and it is presented (slightly modified) in "Flora Europaea" (Walters & Pawlowski 1968). Plocek (1982) has criticized and changed it according to nomenclature rules, but the corrections do not change the system as such. Yuzepchuk (1941) took the previous systems as his starting point and developed a more detailed system of sections, groups (series) and subgroups (subseries), but since it is invalid according to the nomenclature rules, his system is not in use today.

Fröhner (1975, 1986, 1995) has an interesting conception about origin and taxonomy of *Alchemilla*. He claims a hybridogenous origin of the genus from 4 pure genepools (in Europe) which have given all the possible hybrids between them. On that basis Fröhner proposed a new section-structure for the genus, taking into account morphological characters, chromosome numbers, ecology and species distribution.

Practically, *Alchemilla* species in nature are morphologically highly variable and their characters are varying continuously. The only basis for separating these (micro)species has been morphological difference, but nobody has checked whether these characters really work and whether the species can be clearly distinguished. Walters (1987) has stressed that taxonomists should investigate this genus with biosystematical methods, but he has also postulated that the species are discrete and easy to identify. Numerical methods have up to now been used very rarely for that purpose in the genus *Alchemilla* (e.g. Turesson 1956, Glazunova & Mjatlev 1990). Because of the developmental and taxonomic complexity of the genus and the continuity of characters, only numerical phenetic methods are of use, since it is practically impossible to use cladistics in such cases (Duncan & Baum 1981, McNeill 1984).

In the present study the variation and taxonomic continuum of 23 putative *Alchemilla* species, represented in Estonia, are analyzed with multivariate methods to answer the following questions:

Table 1. *Alchemilla* species and the number of specimens analyzed per species.

Notation	Species	Number of specimens
ACU	*A. acutiloba* Opiz	20
BAL	*A. baltica* Sam. ex Juz.	19
CYM	*A. cymatophylla* Juz.	20
FIL	*A. filicaulis* Buser	11
GLA	*A. glabra* Neygenf.	20
GLI	*A. glabricaulis* H.Lindb. fil.	12
GLC	*A. glaucescens* Wallr.	20
GLO	*A. glomerulans* Buser	13
GRA	*A. gracilis* Opiz	20
HEP	*A. heptagona* Juz.	16
HIR	*A. hirsuticaulis* H.Lindb. fil.	20
LIN	*A. lindbergiana* Juz.	10
MON	*A. monticola* Opiz	20
MUR	*A. murbeckiana* Buser	10
OBT	*A. obtusa* Buser	20
PLI	*A. plicata* Buser	20
PRO	*A. propinqua* H.Lindb. fil ex Juz.	20
SAR	*A. sarmatica* Juz.	20
SEM	A. *semilunaris* Alechin	10
SCR	A. *subcrenata* Buser	21
SGL	A. *subglobosa* C.G. Westerl.	12
WIC	A. *wichurae* (Buser) Stefans.	9
XAN	A. *xanthochlora* Rothm.	10
	TOTAL	373

Table 2. Classification of the studied species of *Alchemilla* into sections and series according to the different concepts of taxonomic structure of the genus. Notation of species names as in Table 1.

Species	Plocek (1982)	Yuzepchuk (1941)	Fröhner (1990)	proposed here
GLC, HIR, PLI	ser. Pubescentes	sect. Pubescentes	sect. Plicatae	sect. Plicatae ser. Pubescentes
MON, PRO	ser. Hirsutae	sect. Vulgares ser. Hirsutae gr. Barbulatae	-	sect. Plicatae ser. Barbulatae
SAR	-	sect. Vulgares ser. Hirsutae gr. Imberbes	-	-
SGL	-	-	-	doubtful
ACU, GRA, XAN	-	-	sect. Alchemilla	sect. Hirsutae ser. Alchemilla
LIN	-	-		-
CYM, SCR	-	-	sect. Ultravulgares	sect. Hirsutae ser. Ultravulgares
SEM	-	-	sect. Decumbentes	sect. Hirsutae ser. Decumbentes
HEP	-	sect. Vulgares ser. Hirsutae gr. Exuentes	sect. Ultravulgares	sect. Hirsutae ser. Ultravulgares
FIL	-	-	sect. Plicatae	sect. Coriaceae ser. Exuentes
GLI	sect. Glabrae	sect. Vulgares ser. Hirsutae gr. Glabricaules	sect. Coriaceae	sect. Coriaceae ser. Glabricaules
GLO	sect. Subglabrae	sect. Vulgares ser. Subglabrae gr. Apressipilae	-	sect. Coriaceae ser. Coriaceae
BAL, GLA, MUR, OBT, WIC	-	sect. Vulgares ser. Subglabrae gr. Glabratae	-	sect. Coriaceae ser. Coriaceae

How variable are these taxa morphologically?

How distinct are the species from a statistical point of view?

How do different conceptions of section and series rank structure work from a statistical point of view?

Which are the best morphological characters for distinguishing these taxa?

Table 3. Characters used in analysis of *Alchemilla* species.

Notation	Character	Type	States or units (in brackets degree of precision)
SILK	hairs silky (sericeous) or not	binary	0-no, 1-yes
STPOS	position of stem	nominal	1-decumbent, 2- bentform , ascending, 3-erect
HRPOS	position of hairs on stem	nominal	1-deflexed, 2- patent, 3-erecto-patent, 4-appressed
LECOL	leaf colour	nominal	1-yellowish green, 2-grass green, 3-greyish green, 4-bluish green, 5-dark green
FLCOL	flower colour	nominal	1-reddish, 2-yellow, 3-yellowish green, 4-grass green, 5-greyish green
STCOL	stipule colour	nominal	1-brown, 2-reddish, 3-green, 4-pale
LEFLD	leaf foldedness	ordinary	0-not folded, 1-slightly folded, 2-strongly folded
INFSH	shape of inflorescence	nominal	1-narrow, 2-wide
FLGDN	density of flower glomeruli	nominal	1-sparse, 2-dense
LBTOP	shape of lobe apices (basal leaf)	nominal	1-obtuse, 2-acute
INCDP	depth of incisions between lobes (basal leaf)	ordinary	0-missing, 1-shallow, 2-deep
THTOP	shape of tooth apex (basal leaf)	nominal	1-obtuse, 2-acute
THSYM	symmetry of teeth (basal leaf)	nominal	1-symmetrical, 2-asymmetrical
CASH	shape of sepal apex	nominal	1-obtuse, 2-acute
HYSH	shape of hypanthium	ordinary	1-tubular, 2-funnel-shaped, 3-campanulate,4-round
STNR	number of flowering stems	interval (counted)	number per individual
LENR	number of basal leaves	interval (counted)	number per individual
LBCOR	angle between basal lobes (basal leaf)	metric	corner grade (5°)
STLN	length of flowering stems	metric	mm (5mm)
STLHR	number of hairs on the lowest internode of stem	interval (counted)	number per 1 mm of running length
STUHR	number of hairs on the upper part of stem (below inflorescence)	interval (counted)	number per 1 mm of running length
PETHR	number of hairs on petiole (basal leaf)	interval (counted)	number per 1 mm of running length
SLELN	length (radius) of stem leaf	metric	mm (1mm)
PETLN	length of petiole(basal leaf)	metric	mm (5mm)
LBNR	number of lobes (basal leaf)	interval (counted)	number per leaf
LEUHR	number of hairs on upper surface of basal leaf	interval (counted)	number per 1 mm^2
LELHR	number of hairs on lower surface of basal leaf	interval (counted)	number per 1 mm^2
VNHR	number of hairs on veins (lower surface of basal leaf)	interval	number per 1 mm of running length
LELN	length (radius) of basal leaf	metric	mm (1mm)
LEWD	width of basal leaf	metric	mm (1mm)
LBLN	length of the middle lobe (basal leaf)	metric	mm (1mm)
LBWD	width of the middle lobe (basal leaf)	metric	mm (1mm)

Table 3 continued

THNR	number of teeth (middle lobe, basal leaf)	interval (counted)	number per lobe
TTHLN	length of the apical tooth (middle lobe, basal leaf)	metric	mm (0.1 mm)
STHLN	length of the tooth next to the apical (middle lobe, basal leaf)	metric	mm (0.1 mm)
STHWD	width of the tooth next to the apical (middle lobe, basal leaf)	metric	mm (0.1 mm)
PEDHR	number of hairs on pedicel	interval (counted)	number per 1 mm of running length
HYHR	number of hairs on hypanthium	interval (counted)	number per one side
HYLN	length of hypanthium	metric	mm (0.1 mm)
HYWD	width of hypanthium	metric	mm (0.1 mm)
CALN	length of sepal	metric	mm (0.1 mm)
CAHR	number of hairs on sepal	interval (counted)	number per sepal
OCALN	length of lobe of epicalyx	metric	mm (0.1 mm)

Material and methods

Material and measurement techniques

We included twenty-three *Alchemilla* species (Table 1), which occur in Estonia and are widespread in Europe or Eurasia, in our study. According to Plocek (1982) they belong to the series *Pubescentes* (Buser) Rothm., *Hirsutae* (Lindb.) Rothm., *Subglabrae* (Rothm.) Pawl. and *Glabrae* (Rothm.) Pawl., all of subsection *Euvulgares* Camus (*Heliodrosium* Rothm.), section *Alchemilla*. The whole variation of these series is well expressed in the chosen species. In other systems they are divided in slightly different ways (Table 2).

Herbarium material from the Herbarium of Tartu University (TU), the Herbarium of the Institute of Zoology and Botany (TAA) and the Herbarium of the Moscow State University (MW) was used. Whenever possible, 20 specimens of each species were measured. Altogether 373 specimens of 23 species were measured and analyzed (Table 1).

Characters for morphometrics were chosen according to two criteria: (i) they should be relatively easy to measure in herbarium material, and (ii) they should be useful for species identification. Several keys (Zamelis 1933, Yuzepchuk 1941, Eichwald 1962, Walters & Pawlowski 1968, Laasimer et al. 1996) were used for the selection of suitable characters. Initially more than 50 characters were chosen, but eight of them were dropped after a pilot study of the first 30 specimens due to very high correlation with other characters or as being non-significant both on a species and specimen level. Finally 43 characters were considered for analysis (Table 3). An attempt was made to express most of the characters numerically, for example leaf length and leaf width were measured rather than describing the leaf shape verbally. Some nominal parameters were nevertheless included. Each character was measured three times on every specimen and the average or median values (the latter in case of ordinary and nominal characters) were applied for further analysis. Metric characters were measured using a ruler and a binocular microscope MBS-2.

Data analysis

Specimens belonging to different conventionally identified species, series or sections were treated as separate clusters and the analysis of taxonomic continuum was carried out according to Paal & Kolodyazhnyi (1983) and Paal (1987, 1994) with the original program SYNCONT 3.0 (made by S. Kolodyazhnyi, J. Paal and A. Kink, 1995, in possession of authors).

Taxonomic continuum here does not mean all possible transitions between any pair of taxa, but relations between some of them that are more similar or adjacent in a multidimensional character space. For the estimation of clusters' adjacency the distances of all objects (specimens) from all centroids (except the cluster to which the specimen belongs) were calculated according to the postulate that the j-th cluster is considered to be adjacent to the i-th cluster if the distance between at least one of the objects of the i-th cluster and the centroid of the j-th cluster is smaller than the distance to the centroids of all other clusters (Paal & Kolo-

dyazhnyi 1983, Paal 1994). The definition of adjacency is non-symmetric: if the j-th cluster is adjacent to the i-th cluster, the latter will not necessarily be adjacent to the former. Adjacency is expressed relatively as percentage of objects in the i-th cluster for which the centroid of the compared cluster is adjacent.

In order to measure statistically the degree of distinctness of two adjacent clusters the α-criterion of Duda & Hart (1976) was used:

$$\alpha = (1 - 2/\pi\, d - I_2/I_1) / \sqrt{2\,(1 - 8/\pi^2 d)/nd}, \qquad (1)$$

where

$$I_1 = \sum_{x \in X} \left| \left| \vec{x} - \vec{m}_i \right| \right|^2, \qquad (2)$$

$$I_2 = \sum_{i=1} \sum_{x \in X} \left| \left| \vec{x} - \vec{m} \right| \right|^2, \qquad (3)$$

I_1 – the sum of square distances between the objects (specimens) and the centroid of united complex of two clusters, I_2 – the sum of square distances between the objects and their cluster centroids after dividing the complex into two suboptimal parts, x – vector of objects, m – vector of the centroid of the united complex, m_i – vector of the cluster X_i centroid, d – dimensionality of the united complex, d = min (q, n-1), where q and n are the number of characters and objects (specimens) in the united complex, respectively.

To obtain a better interpretation of the estimates, the corresponding probabilities (expressed in percentages) rather than the direct values were estimated as following (Paal 1987, 1994):

$$CI = 100 / \sqrt{2\pi} \int_{\alpha} \exp(-x^2/2)\, dx\,. \qquad (4)$$

CI is called coefficient of indistinctness. If CI > 5, the two characters are considered to be significantly indistinct.

As we took into account both metric and non-metric characters, and many of them did not have a normal distribution, data was transformed before analysis. If not otherwise stated, standardization (by mean and standard deviation) was applied.

Ordination by principal component analysis (PCA) of log-transformed data (ln (1 + C), (C – the measured value of a character on an object) was carried out with CANOCO (ter Braak 1988, 1990) and CANODRAW (Smilauer 1992) packages .

As the variables were not normally distributed, for correlation analysis Spearman's rank order correlation coefficients was used, obtained from the CORR procedure of the SAS package (SAS Institute Inc. 1994),

Table 4. Spearman's rank correlation coefficients (r) between characters. Only character pairs that have significant ($p < 0.05$) correlation coefficients higher than 0.6 and that belong to correlation groups I and III are listed. Characters from correlation groups II and IV have few pairs with high correlation coefficient and are not presented here. Notation of characters as in Table 3.

Group I							
	LELN	LEWD	LBLN	LBWD	LBNR	THNR	STLN
LEWD	0.986						
LBLN	0.878	0.881					
LBWD	0.881	0894	0.789				
LBNR	0.664	0.668	0.603				
THNR	0.753	0.746	0.735	0.619	0.687		
STLN	0.702	0.708	0.608	0.646			
PETLN	0.793	0.800	0.712	0.720			0.768
SLELN	0.716	0.714		0.646		0.635	0.646
Group III							
	SILK	PEDHR	HYHR	PETHR	VNHR	LEUHR	LELHR
PEDHR	0.736						
HYHR	0.601	0.736					
STLHR				0.632			
VNHR				0.642			
LELHR			0.666		0.693	0.826	
CAHR		0.694	0.851				0.674

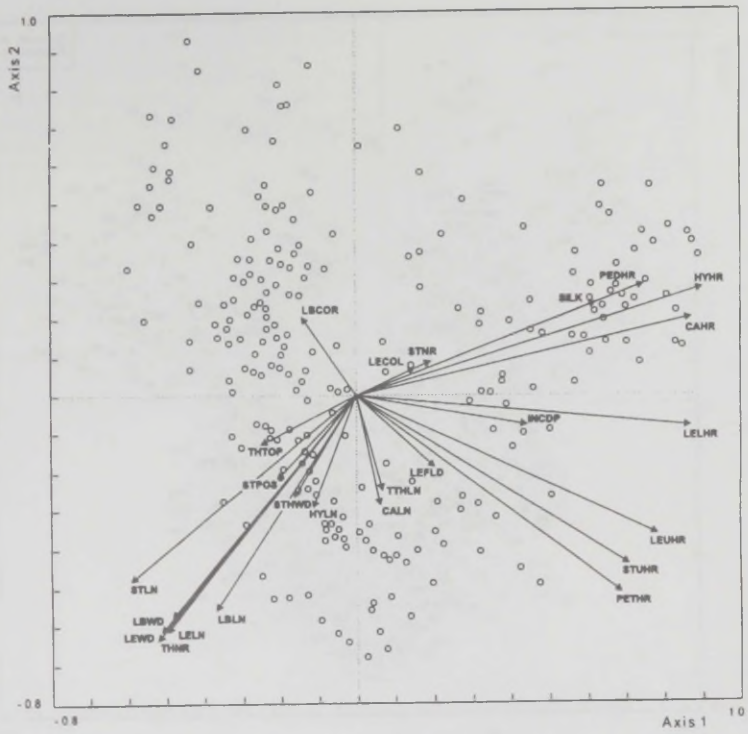

Fig. 1. PCA biplot of characters and specimens of *Alchemilla*. Notation for characters as in Table 3.

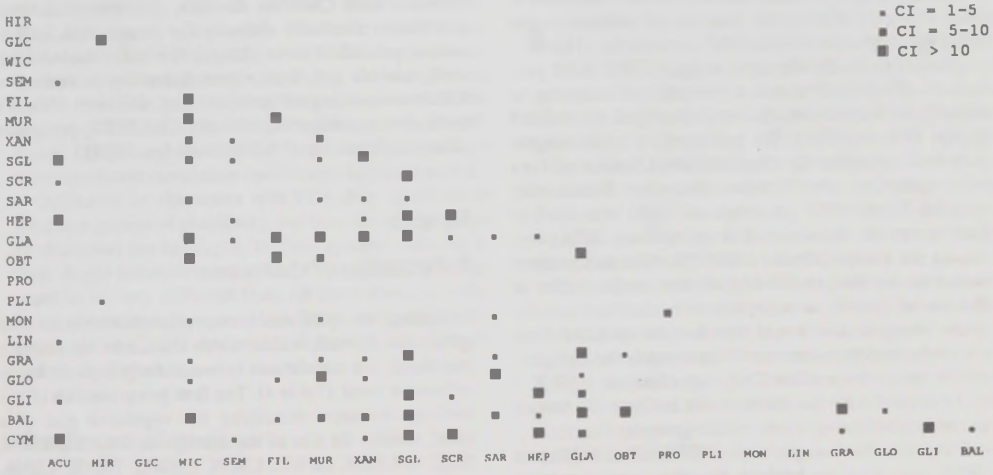

Fig. 2. Coefficients of indistinctness (CI) between conventionally identified species of *Alchemilla*. Notations of species names as in Table 1. If CI < 1, it is not marked.

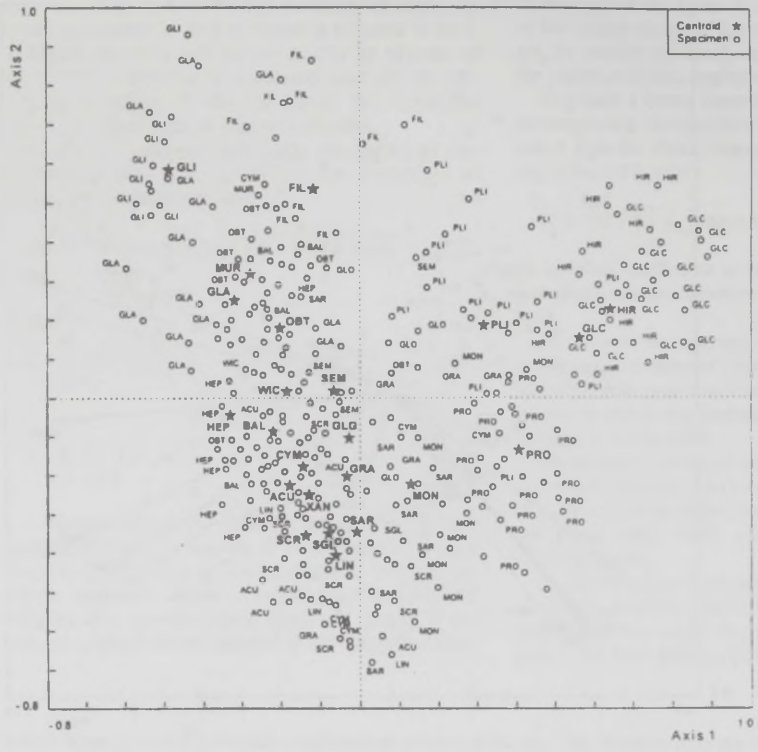

Fig. 3. PCA ordination of investigated *Alchemilla* specimens and species centroids. Specimens are labelled according to their empirical identification, notations as in Table 1.

The importance of each character in the separation of taxa was estimated by an analysis of variance – the ANOVA procedure of SAS (SAS Institute Inc. 1994).

Classificatory discriminant analysis (DISCRIM procedure of SAS) develops a discriminant criterion to classify each specimen into one of several pre-defined groups (Klecka 1980). We performed it to investigate how well coincides the empirical identification of taxa with numerical classification. Stepwise discriminant analysis (STEPDISC procedure of SAS) was used to find a set of characters that maximizes differences among the groups (Klecka 1980). Discriminant analysis was used for taxa ranked higher than species, since in the case of species too many characters had zero within-group variance, and would therefore be excluded from the study, which in turn would have made the comparison of results impossible. Only one character – SILK – was excluded from the discriminant analysis of sections and series, as having a zero within-group variance.

The classification package SYN-TAX 5.0 (Podani 1993) was used to analyze the data set of species centroids, in order to find an optimal division of the species between sections and series. Methods such as UPGMA with Canberra distance, Manhattan distance and Gower similarity distance for mixed data, and k-means' procedure were chosen, the latter started from random seeds and from object sequences according to different existing classifications. Divisive Ward's method was performed with the CLUSTER procedure of the SAS package (SAS Institute Inc. 1994).

Results

Relationship of characters

According to Spearman's correlation coefficients four groups are formed, within which characters are strongly correlated, but correlations between the groups are insignificant or weak (Table 4). The first group consists of numerical characters describing the vegetative part of a plant, mainly the size of the specimens: PETLN, STLN, SLELN, LELN, LEWD, LBLN, LBWD, THNR, LBNR. Measurements of leaf teeth – STHLN, TTHLN, STHWD – belong to the second group. The third group is connected with plant hairiness: LEUHR, LELHR, VNHR,

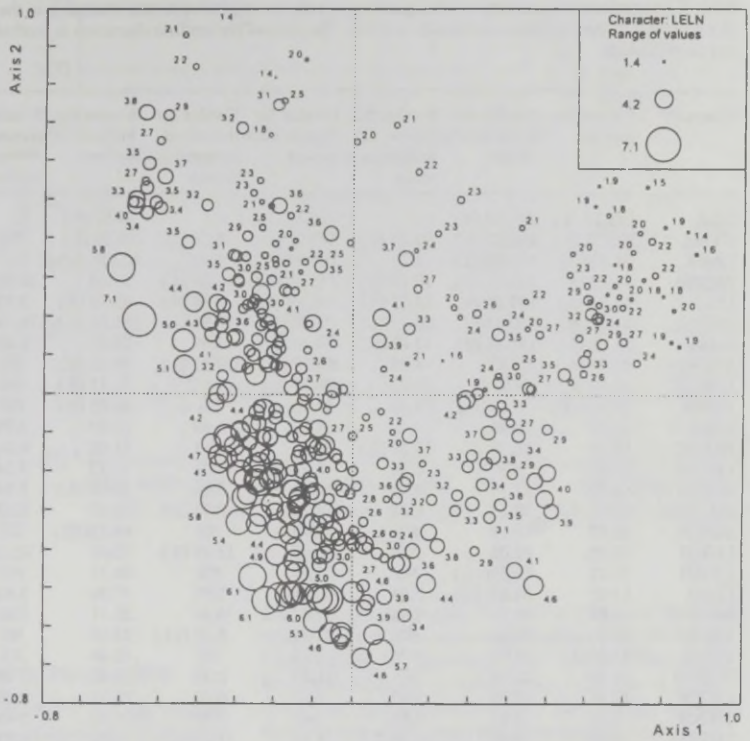

Fig. 4. Distribution of values for character LELN (leaf length) of *Alchemilla* in PCA ordination plot.

PETHR, STUHR, STLHR, HYHR, CAHR, FPTHR, SILK. The fourth group unites the metric characters of flowers: HYLN, HYWD, CALN, OCALN. Some characters are very strongly correlated (e.g. leaf length and width), but were not excluded from the research in the beginning because they are important for species identification. Character pairs not belonging to one group do not have significant correlation coefficients higher than 0.6.

Ordination of characters with PCA (Fig. 1) results in the same groups of characters, but here the relationships of characters not belonging to these groups come out as well. Angle between basal lobes of leaves (LBCOR) appears to be very different from all other metric characters (neither does it have a significant correlation with them). Most of the non-metric characters are close to leaf teeth characters.

Continuum of species

According to continuum analysis, only three species are totally distinct from all others (Fig. 2): *A. lindbergiana* Yuz., *A. plicata* Buser, and *A. semilunaris* Alechin. In addition, two pairs of species are insignificantly distinct from each other, but well separated from the remaining ones: *A. glaucescens* Wallr. and *A. hirsuticaulis* Lindb., *A. monticola* Opiz and *A. propinqua* Lindb. For specimens of either couple the counterpairing species is also the main neighbour in the character space. Remarkably high mutual adjacencies (>50%) have also *A. glaucescens* and *A. hirsuticaulis* (in both directions), *A. lindbergiana* to *A. acutiloba* Opiz, *A. propinqua* to *A. monticola*. All other species form a complicated network of more or less adjacent taxa, where many species-pairs are insignificantly separated.

PCA of empirically identified specimens along with calculated centroids of the species (Fig. 3) show specimens of some species form quite clear groups around their centroids, but specimens of some other species are located indiscriminately. The eigenvalues of the first two axes are 39.5% (axis 1) and 11.0% (axis 2), the third axis covers 8.6% of the total variance. Clear tendencies in the distribution of character values among the species can also be detected on ordination plot (e.g. LELN, Fig. 4).

For distinguishing conventionally identified species,

Table 5. Importance of characters in distinguishing species and higher rank taxa according to the ANOVA F-criterion and F-value (for removal) of the stepwise discriminant analysis. The rank of the ten first characters is marked in brackets. Notation of characters as in Table 3.

Character	F-value for species	F-value for Rothmaler's series	F-value for remove of Rothmaler's series	F-value for Juzepzcuk's groups	F-value for remove of Juzepczuk's groups	F-value for Fröhner's sections	F-value for remove of Fröhner's sections	F-value for "corrected" sections	F-value for remove of "corrected" sections
SILK	123.72 (1.)	244.83 (4.)	-	129.37 (5.)	-	54.59 (9.)	-	170.39 (5.)	-
HYHR	100.38 (2.)	402.82 (1.)	33.14 (2.)	247.02 (1.)	19.92 (3.)	98.36 (3.)	NS	342.37 (1.)	25.70 (2.)
CAHR	94.77 (3.)	311.28 (2.)	5.37	198.49 (2.)	5.97	103.51 (2.)	3.17	265.37 (2.)	5.63
PEDHR	71.89 (4.)	266.29 (3.)	10.19 (9.)	135.70 (3.)	17.16 (4.)	39.62	10.85 (3.)	220.08 (4.)	14.74 (5.)
LELHR	51.94 (5.)	197.50 (5.)	12.93 (5.)	130.38 (4.)	9.64 (9.)	83.38 (4.)	3.32	252.79 (3.)	10.54 (6.)
HRPOS	41.22 (6.)	140.41 (7.)	86.77 (1.)	78.99 (6.)	45.98 (1.)	133.08 (1.)	71.79 (1.)	108.09 (9.)	55.94 (1.)
THNR	31.48 (7.)	143.47 (6.)	12.60 (6.)	77.14 (7.)	6.69	48.63	2.45	155.31 (6.)	9.90 (9.)
STUHR	24.84 (8.)	47.33	4.05	64.17 (9.)	20.62 (2.)	59.51 (7.)	NS	66.44	5.82
LEUHR	23.78 (9.)	81.28 (8.)	3.22	70.19 (8.)	5.63	77.15 (5.)	NS	111.02 (8.)	6.03
VNHR	22.95 (10.)	76.13 (9.)	3.40	59.23(10.)	NS	66.65 (6.)	NS	112.35 (7.)	2.44
LBNR	18.78	48.98	NS	37.16	3.60	42.89	3.98	48.86	2.52
PETHR	18.28	38.39	10.57 (8.)	26.76	6.74	31.68	6.81	64.45	8.64
LBCOR	17.71	3.97	4.88	7.92	3.77	21.22	7.34 (10.)	13.44	5.68
PETLN	16.77	37.79	8.29	30.26	5.86	58.09 (8.)	6.44	53.66	10.03 (8.)
INCDP	16.22	18.43	8.71	18.70	9.71 (8.)	18.83	8.25 (6.)	32.97	19.54 (3.)
SLELN	15.59	56.14	NS	39.40	NS	49.32(10.)	NS	72.43(10.)	NS
LBWD	15.55	41.02	NS	26.85	13.89 (5.)	35.06	22.18 (2.)	38.51	NS
LEWD	15.17	61.29(11.)	NS	35.04	NS	44.31	NS	62.99	NS
LELN	14.97	61.83(10.)	3.54	36.24	2.95	47.34	3.63	63.75	2.53
LBLN	14.03	40.57	4.64	22.17	6.24	25.11	7.62 (9.)	43.29	3.05
STLHR	13.64	28.99	NS	22.80	8.42 (11.)	26.06	NS	39.03	NS
STLN	13.60	29.01	NS	26.68	NS	43.44	NS	44.51	NS
FLGDN	12.92	22.78	NS	21.52	2.92	30.80	2.50	51.63	6.04
LBTOP	12.31	15.37	6.97	8.47	6.46	26.98	8.20	29.78	6.93
THTOP	9.58	14.91	5.06	7.41	7.48	21.55	5.45	14.88	NS
THSYM	8.59	NS	12.26 (7.)	4.22	11.35 (6.)	7.28	9.35 (5.)	12.11	9.52(10.)
INFSH	8.59	3.11	NS	NS	NS	10.21	5.80	4.36	NS
STCOL	7.99	11.45	9.03 (10.)	5.85	4.61	18.99	10.20 (4.)	11.61	6.10
FLCOL	7.20	5.99	NS	5.19	4.13	11.29	6.11	7.11	6.23
HYLN	7.10	31.25	18.12 (3.)	15.79	10.07 (7.)	5.38	6.29	33.13	16.74 (4.)
LECOL	6.86	5.48	NS	4.84	3.26	10.38	NS	3.89	NS
LEFLD	6.33	4.57	4.76	7.48	3.91	6.29	4.66	NS	2.98
STHLN	6.14	9.40	5.89	5.96	4.85	8.71	NS	19.77	6.96
TTHLN	6.10	8.36	4.39	6.48	6.02	9.02	2.57	19.81	6.04
STHWD	5.59	NS	3.06	2.50	2.40	11.10	NS	NS	2.39
HYWD	5.55	24.03	NS	12.26	NS	7.60	NS	21.44	NS
LENR	4.52	6.24	3.88	5.05	NS	10.22	NS	4.69	3.06
STPOS	3.50	8.13	NS	4.77	NS	2.79	3.38	4.48	2.76
CALN	3.47	8.98	14.57 (4.)	5.26	8.62 (10.)	7.58	8.67(6.)	9.34	10.21 (7.)
CASH	3.44	NS	NS	6.72	NS	5.22	NS	4.18	2.49
OCALN	2.75	6.77	4.57	4.64	5.74	7.58	7.66 (8.)	12.77	7.56
STNR	2.68	6.23	NS	4.08	NS	4.89	NS	5.28	NS
HYSH	2.10	5.42	NS	4.54	NS	3.46	NS	3.53	NS

the most important character according to the highest F-value by ANOVA (Table 5) is sericeousness of hair (SILK). All ten characters with the highest F-values, except THNR, are connected with the hairiness of the plant and most of them describe hair numbers on various parts of the plant body. Unfortunately, comparison of means and standard errors (e.g. character HYHR, Fig. 5) shows that often the group of "best" characters (having high F-value by ANOVA) only distinguishes one or a few species from others very effectively, while the remaining species tend to have the same or very similar characteristics.

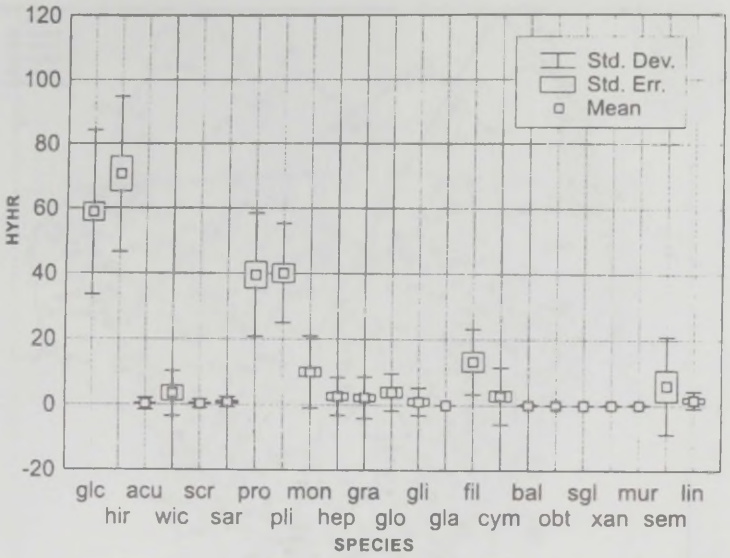

Fig. 5. Box and whisker plot for variable HYHR (hair number per one side of hypanthium) categorized by species of *Alchemilla*. Notations of species names as in Table 1.

Sections and series

Rothmaler's system corrected by Plocek (1982)

The series studied are all distinct (CI < 1.0), the highest adjacencies have *Pubescentes* to *Subglabrae* (85%) and *Glabrae* to *Hirsutae* (83%). The smallest Euclidean distance is found between the centroids of series *Hirsutae* and *Subglabrae* (0.068), the most distant are the centroids of *Pubescentes* and *Glabrae* (0.241).

PCA (Fig. 6) demonstrates a relatively clear separation of series in the ordination space and it is in good accordance with the estimation of adjacency.

19 specimens (5.1%) were wrongly classified by the classificatory discriminant analysis.

The best characters distinguishing Rothmaler's series according to ANOVA are connected with the hairiness of flower: HYHR, CAHR, PEDHR (Table 5). Besides hair-characters, leaf length and leaf width also had high F-values. According to discriminant analysis the most important characters were HRPOS, HYHR and HYLN. Some characters important by ANOVA (e.g. STLHR, LEWD) were nonsignificant according to F-criterion in discriminant analysis.

Yuzepchuk's (1941) groups

Of the groups two pairs are separated nonsignificantly: *Exuentes* Yuz. and *Glabricaules* Yuz. (CI = 7.0), *Exuentes* and *Glabratae* Yuz. (CI = 13.4), but the most adjacent are *Pubescentes* to *Barbulatae* Yuz. (87%) and *Glabricaules* to *Imberbes* Yuz. (58%). The largest Euclidean distance is between the centroids of *Pubescentes* and *Glabricaules* (0.241), the smallest between the centroids of *Exuentes* and *Glabratae* (0.062).

PCA (Fig. 7) also demonstrates that Yuzepchuk's groups are not very clearly separated from each other, and that some of them seem to be unjustified. *Pubescentes, Barbulatae* and *Glabrae* are clearly distinct, other groups are more transitional.

31 specimens (8.3%) were re-identified into different subgroups by classificatory discriminant analysis.

The characters which are important in distinguishing these groups according to ANOVA are generally similar to those important for separating species and Rothmaler's series, being mainly hairiness characters (Table 5). Also according to F-criterion in discriminant analysis the most important are hairiness characters: HRPOS, STUHR and HYHR.

Fröhner's (1990) sections

Of these sections only *Alchemilla* and *Ultravulgares* Fröhner are statistically not well separated (CI = 18.4) with their centroids very close to each other (Euclidean distance 0.053). The most adjacent are section *Plicatae* Fröhner to section *Alchemilla* (83%) and species *A. lindbergiana* to section *Ultravulgares* (80%). The

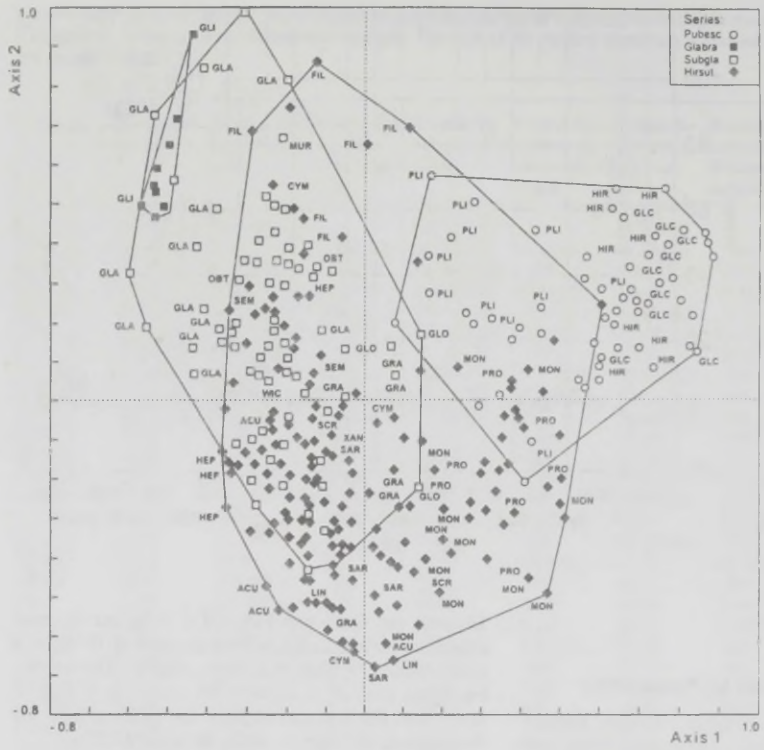

Fig. 6. Classification polygons of Rothmaler's series (according to Plocek 1982) superimposed onto PCA ordination of studied specimens of *Alchemilla*.

centroids of species *A. lindbergiana* and section *Plicata* are the most distant having Euclidean distance of 0.270.

According to PCA (Fig. 8) some of these sections are well separated, but *Ultravulgares, Decumbentes* and *Alchemilla* are mutually transitional. Section *Plicatae* seems very heterogeneous and thereby not clearly separated from the OTUs of some adjacent sections.

38 specimens (10.2%) are considered to be misidentified into sections according to classificatory discriminant analysis.

The most important characters for distinguishing these sections according to ANOVA are different from those important in the previous two systems (Table 5): position of hairs on the stem (HRPOS), followed by some of the hair numbers, but also length of the stem leaves (SLELN) and length of the petiole (PETLN). HRPOS, LBWD, PEDHR and STCOL are most important for separating Fröhner's sections acccording to F-criterion in discriminant analysis.

Cluster analysis

The results of clustering species centroids with different methods (UPGMA, Ward's method with different distances and k-means clustering) seem to support Fröhner's system (1990). A group consisting of *A. glaucescens, A. hirsuticaulis* and *A. plicata* is constantly formed, and this is mostly well separated from the other species. *A. propinqua* and *A. monticola* are closely related according to most clustering methods, but often they are accompanied by some other species, most frequently *A. sarmatica* Yuz., occasionally also *A. subglobosa* C. G.Westerl., *A. semilunaris* Alechin, and *A. gracilis* Opiz. Species belonging to the section *Coriaceae* form a joint group or two different subgroups: *A. wichurae* (Buser) Stefans., *A. baltica* Sam. ex Yuz., *A. glomerulans* Buser, *A. murbeckiana* Buser in one and *A. glabra* Neygenf., *A. obtusa* Buser, *A. filicaulis* Buser in the other. *A. filicaulis* is closest to section *Coriaceae* (and not to the section *Plicatae*) according to cluster analysis, continuum analysis and ordination. The results of some cluster analysis variants

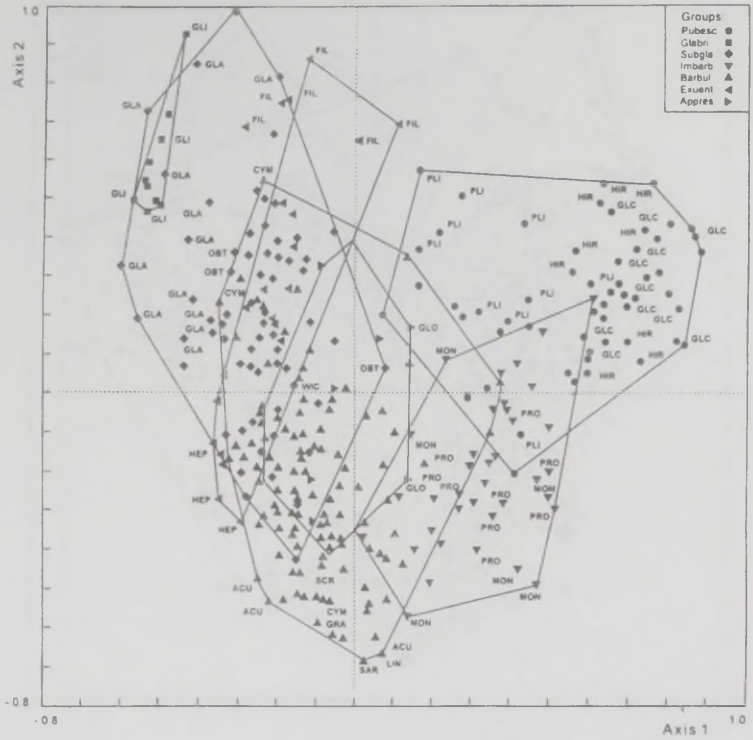

Fig. 7. Classification polygons of Yuzepchuk's groups (1941) superimposed onto PCA ordination of studied specimens of *Alchemilla*.

place *A. baltica* closer to section *Alchemilla*, but in most cases it belongs to section *Coriaceae*. *A. glabricaulis* Lindb. changes between section *Coriaceae* or section *Ultravulgares*, in some cases this species is located alone separately from all others. The species of sections *Ultravulgares* and *Alchemilla* mostly belong to separate clusters, but these sections as a whole are mutually indistinct according to continuum analysis.

Discussion

The continuum analysis of species shows that only some species are completely distinct from all others. Most of the species cannot be clearly distinguished, and surprisingly some pairs of species that are considered by several authors to be quite different, i.e. belonging to different series or sections, cannot be distinguished from a statistical point of view (Fig. 2). At the same time some species-pairs that seem to be similar and hardly distinct in nature, are distinct according to multivariate analysis. The distinctness of close species could be the result of using herbarium material, which often consists of tendentiously typical specimens. Nevertheless, this does not explain the indistinctness of distant species pairs. One reason can be that a multitude of characters were used, including the measurements of leaf teeth and flower, which is not common in previous works. But the main reason is probably the high morphological variability and transitionality of species. Hence the assumption that *Alchemilla* species can be morphologically well distinguished (Tikhkhomirov 1967, Walters 1987) is not completely true, and treating these species as an agamo-sexual complex (Glazunova 1977) seems to be more reasonable. The continuum of species gives also evidence of the fact that hybridization has occurred and probably occurs also nowadays between species.

According to ANOVA, the most important characters for distinguishing species were connected with the hairiness of different parts of the plant body. Though Tikhkhomirov et al. (1995) also emphasize the role of these characters, we cannot leave out other characters, since different species and species groups can be distin-

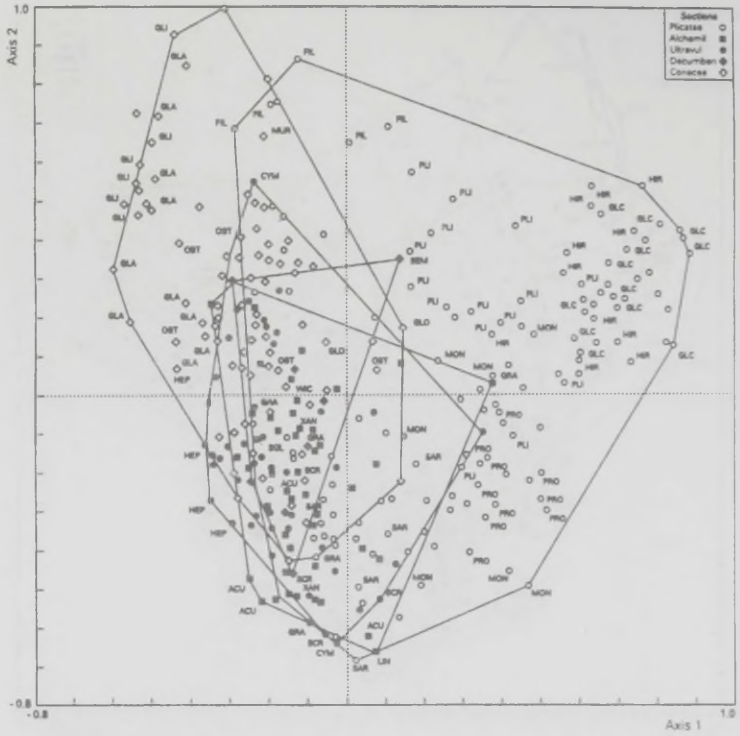

Fig. 8. Classification polygons of Fröhner's sections (1990) superimposed onto PCA ordination of studied specimens of *Alchemilla*.

guished using different characters. Most identification keys (e.g. Yuzepchuk 1941, Walters & Pawlowski 1968, Laasimer et al. 1996) also use many characters and give the same character a different weight in identifying different species. Additionally, many characters which have a high F-value according to ANOVA, distinguish only some or even one of the 23 species clearly, and the remaining species cannot be distinguished. This indicates that ANOVA does not seem to be a very suitable test for such purposes. Our results also show that infraspecific variation of most morphological characters is extensive, as already found by Turesson (1956). Hence in several species character states partially overlap and this causes species indistinctness, even though type specimens are distinct.

The correlation structure of characters is generally similar to the results of Glazunova & Mjatlev (1990), who analyzed the correlation structure of characters in *A. monticola*. Metric characters of the vegetative body and flower form clearly different groups as in the work mentioned, but apart from that all hair numbers also form a separate group. Leaf teeth measurements are not strongly correlated with other metric characters. One reason for the difference between our results and those of Glazunova & Mjatlev (1990) could be that our results summarize features common for several species, while cited authors studied only one microspecies.

Higher rank taxa like sections and series are generally better separated from each other. The series of Rothmaler according to Plocek (1982) are all distinct, but the series *Hirsutae* is rather heterogeneous, being adjacent on one side to the series *Subglabrae* and on the other side to the series *Pubescentes*. Groups of Yuzepchuk (1941) are not all distinct, and from a statistical point of view some of them seem to be established without a proper basis, e.g. *Exuentes* and *Appressipilae* Yuz. All investigated sections of Fröhner (1990) are significantly distinct, except sections *Ultravulgares* and *Alchemilla*. The different systems generally agree regarding the six species joined as the series *Subglabrae* according to Plocek (1982) or the section *Coriaceae* according to Fröhner (1990). The other authors except Fröhner also agree in placing *A. glabricaulis* in a separate section or series.

Results obtained by clustering species centroids with different methods were most congruous with Fröhner's

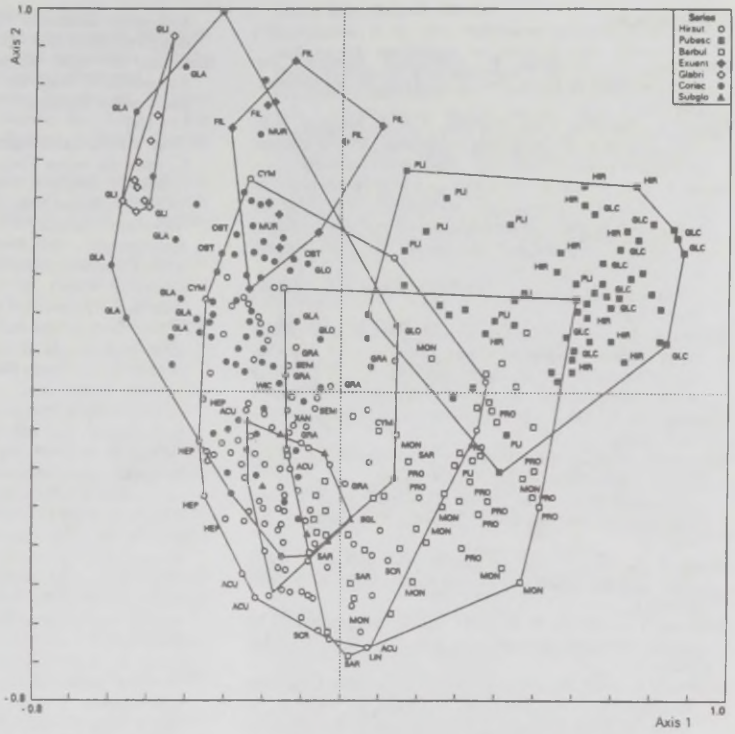

Fig. 9. Classification polygons of revised series superimposed onto PCA ordination of studied specimens of *Alchemilla*. The changes in taxonomic structure are stressed, thus section *Hirsutae* is presented as a whole, and species *A. subglobosa* is separated because of its doubtful position.

(1990) system. However, on the basis of multivariate analysis some corrections should be proposed (Table 2). One of the major changes proposed concerns *A. filicaulis*, which is, according to both continuum analysis and clustering, not adjacent to section *Plicatae*, but rather to section *Coriaceae*, where this species probably should be placed, possibly in separate series *Exuentes* Yuz. *A. glabricaulis* can also form a separate series *Glabricaules* Yuz. in section *Coriaceae*. It is reasonable to divide section *Plicatae* into two series. The first embraces the three species of series *Pubescentes* according to Plocek (1982) and Yuzepchuk (1941). These three species – *A. glaucescens*, *A. hirsuticaulis* and *A. plicata* – were also grouped together and separated from the remaining species by all algorithms of cluster analysis. The other series consisting of species *A. monticola*, *A. propinqua* and *A. sarmatica* may be called *Barbulatae* as in Yuzepchuk (1941). *A. subglobosa*, included also in section *Plicatae* by Fröhner (1990), needs more investigation, since it is nonsignificantly distinct from many other species and belongs to various groups according to the different types of cluster analysis. Sections *Ultravulgares* and *Alchemilla* are mutually indistinct and therefore should not be treated as different sections but rather as series of section *Hirsutae*. *A. lindbergiana* may be merged with series *Alchemilla* and thus in turn with this joint section, though it is well distinct, but there are no other proposals about this species. *A. semilunaris* can remain separately in series *Decumbentes*, as it is distinct from all other species, but in our opinion this series should also belong to section *Hirsutae*. The sections and series corrected in this way are well separated in the character space according to PCA (Fig. 9). Still, as the revised taxa are only based on the 23 investigated species, they cannot be accepted as a final decision. Nevertheless they show certain trends and will be used as a basis for the further research.

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II

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

Silvia Sepp, Vera K. Bobrova, Alex K. Troitsky & Klavdiya P. Glazunova

Sepp, S., Institute of Botany and Ecology, University of Tartu, Lai 40, 51005 Tartu, Estonia
Bobrova, V. K. & Troitsky, A. K., A. N. Belozersky Institute of Physicochemical Biology, Moscow State University, Moscow 119899, Russia
Glazunova, K. P., Biological Faculty of Moscow State University, Moscow 119899, Russia

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Sepp, S., Bobrova, V. K., Troitsky, A. K. & Glazunova, K. P. 2000: Genetic polymorphism detected with RAPD analysis and morphological variability in some microspecies of apomictic *Alchemilla*. — *Ann. Bot. Fennici* 37: 105–123.

Alchemilia 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):

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

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.*

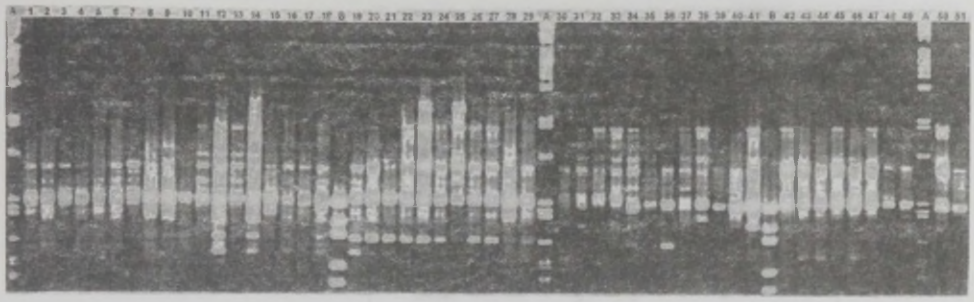

Fig. 1. RAPD profiles for *Alchemilla* accessions obtained with primer 1 (*see* Material and methods). 1: 08acu05, 2: 18acu05, 3: 35acu15, 4: 36acu15, 5: 42acu15, 6: 01acu16, 7: 02acu16, 8: 11acu16, 9: v1acuMO, 10: 32mic02, 11: 33mic02, 12: 05mic04, 13: 20mic04, 14: 09mic05, 15: 17mic05, 16: 14mic12, 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

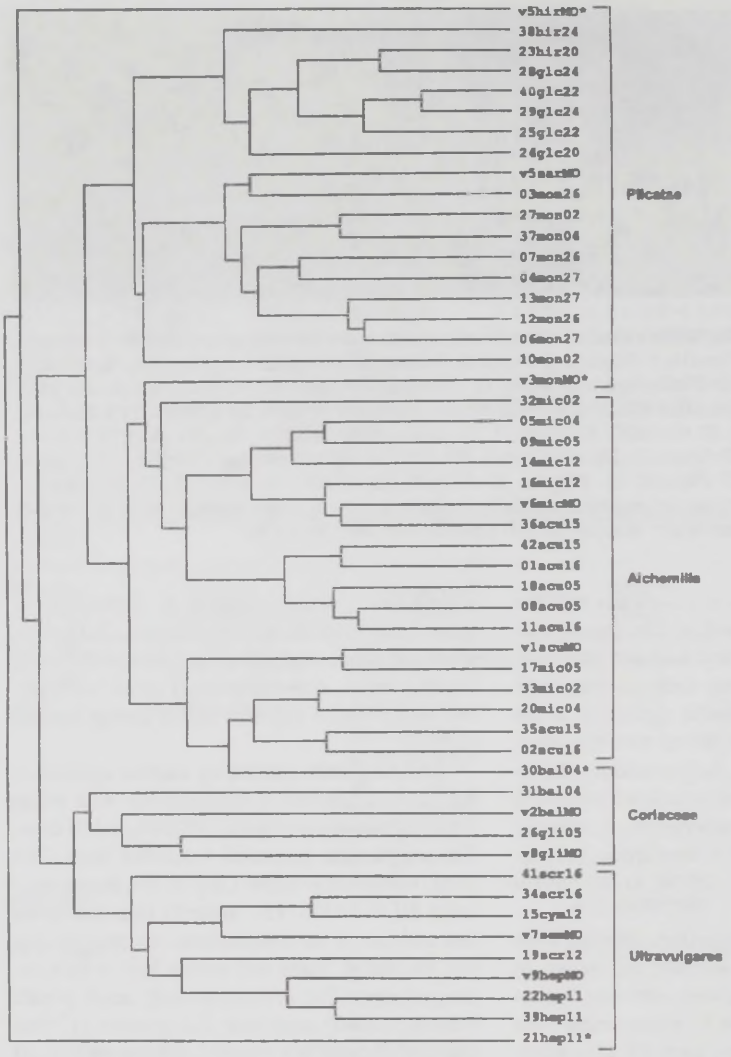

Fig. 2. UPGMA dendrogram based on RAPD data (3 primers). Denotations of accessions as in Table 1. Brackets on the right indicate the corresponding Fröhner sections. Plants marked with asterisks are located outside of their sections. The scale on the top shows genetic distance.

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

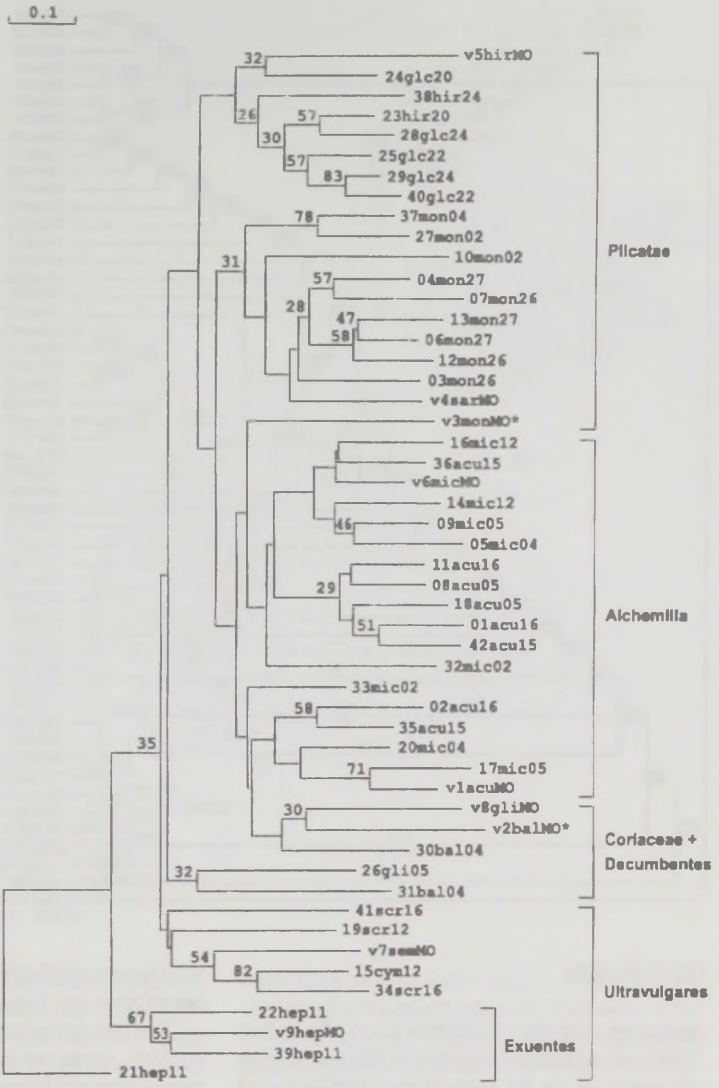

Fig. 3. NJ tree based on RAPD data (3 primers). Denotations of accessions as in Table 1. Brackets on the right indicate the corresponding Fröhner's sections; group *Exuentes* according to Yuzepchuk is also indicated. Plants marked with asterisks are located outside of their sections. The scale on the 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.

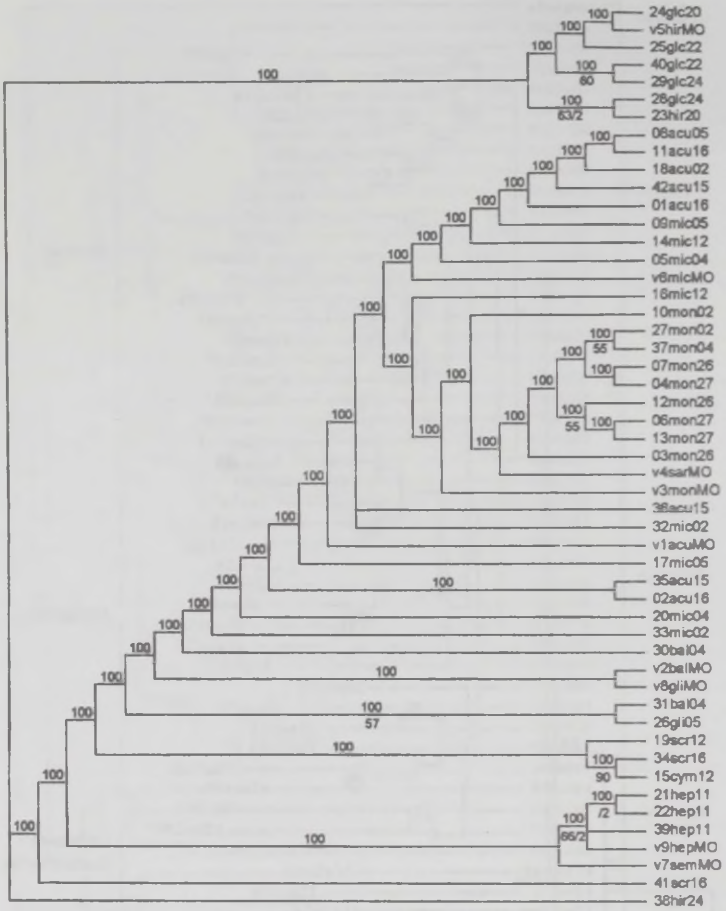

Fig. 4A. MP trees of RAPD data (3 primers, 51 samples). Notations as in Table 1. The 50% majority-rule consensus tree of 4 trees (length 384 steps, CI = 0.167, HI = 0.833). Above the branch is marked the per cent of the most parsimonious trees with given topology, below the branch the bootstrap value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

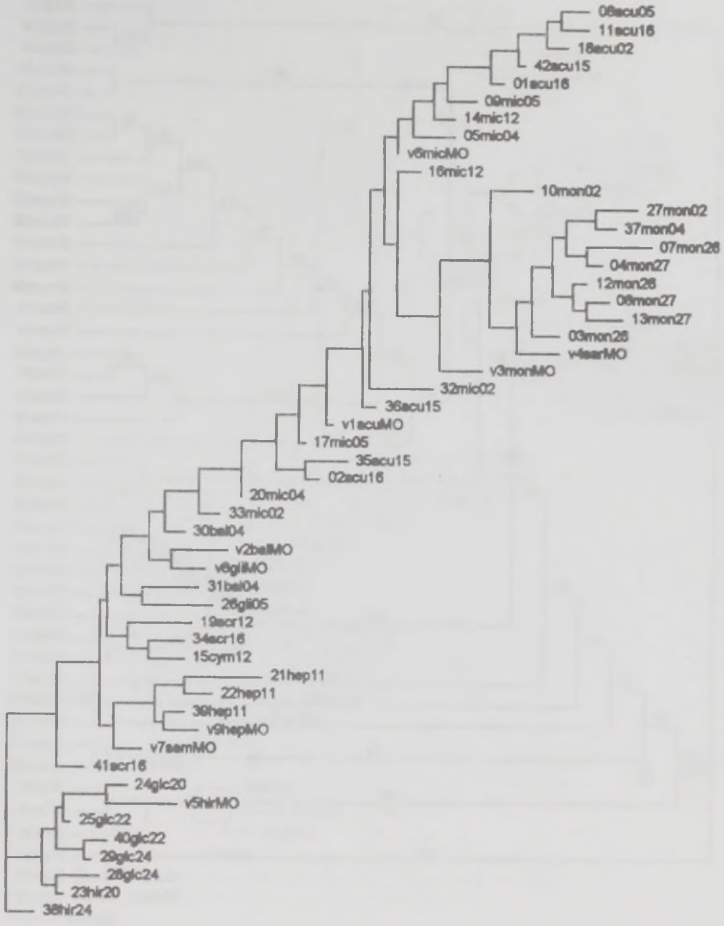

Fig. 4B. MP trees of RAPD data (3 primers, 51 samples). Notations as in Table 1. One of the four most parsimonious trees.

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

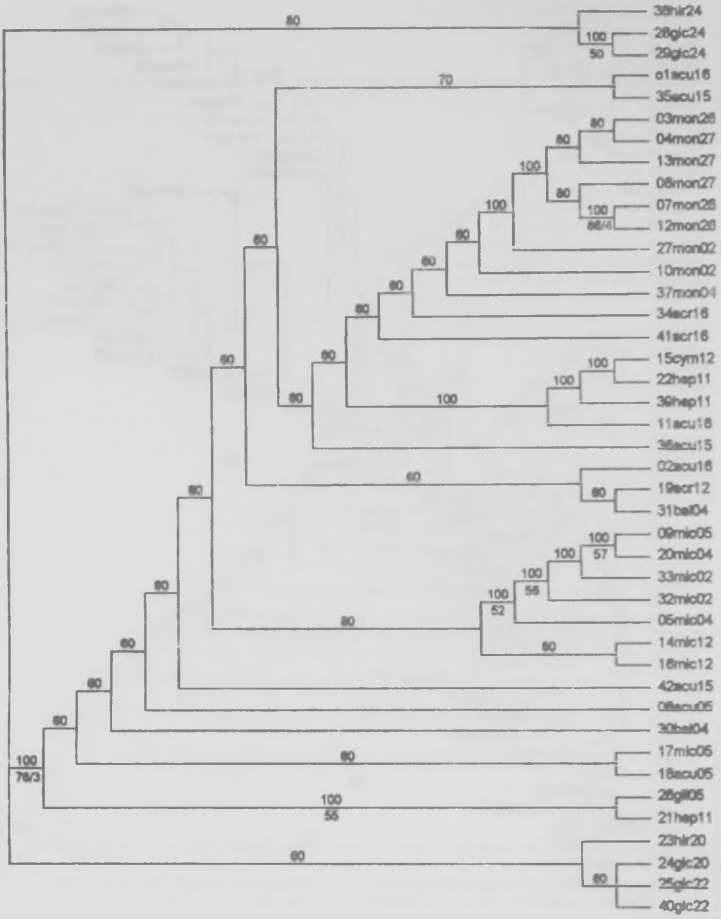

Fig. 5A. MP trees of morphological data (42 specimens). Notations as in Table 1. The 50% majority-rule consensus tree of 10 trees (482 steps, CI = 0.212, HI = 0.788). Support values as in Fig. 4A.

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-

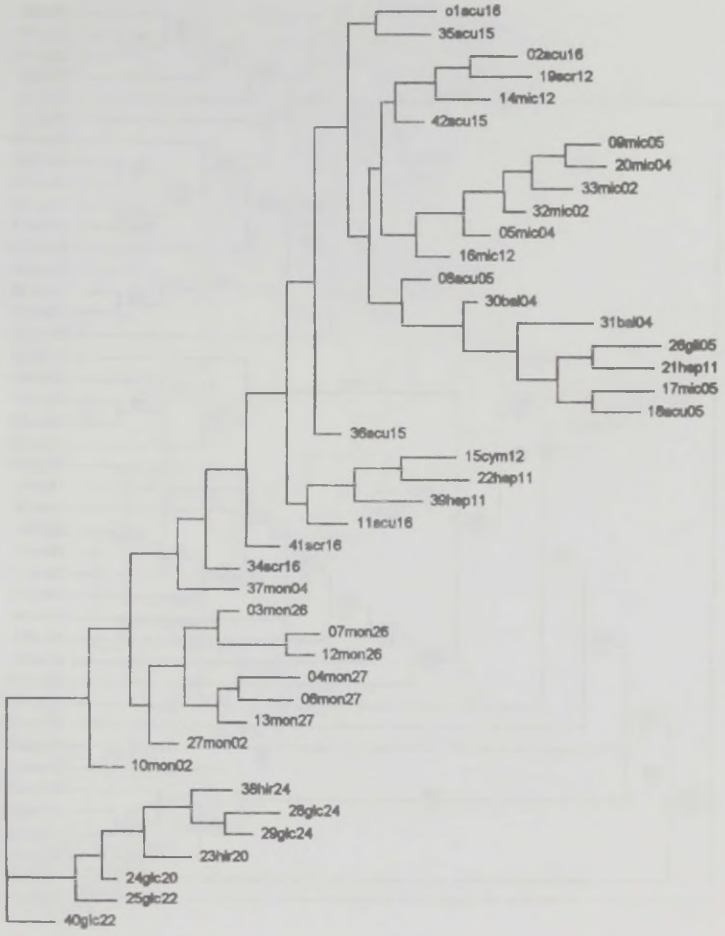

Fig. 5B. MP trees of morphological data (42 specimens). Notations as in Table 1. One of the ten most parsimonious trees.

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

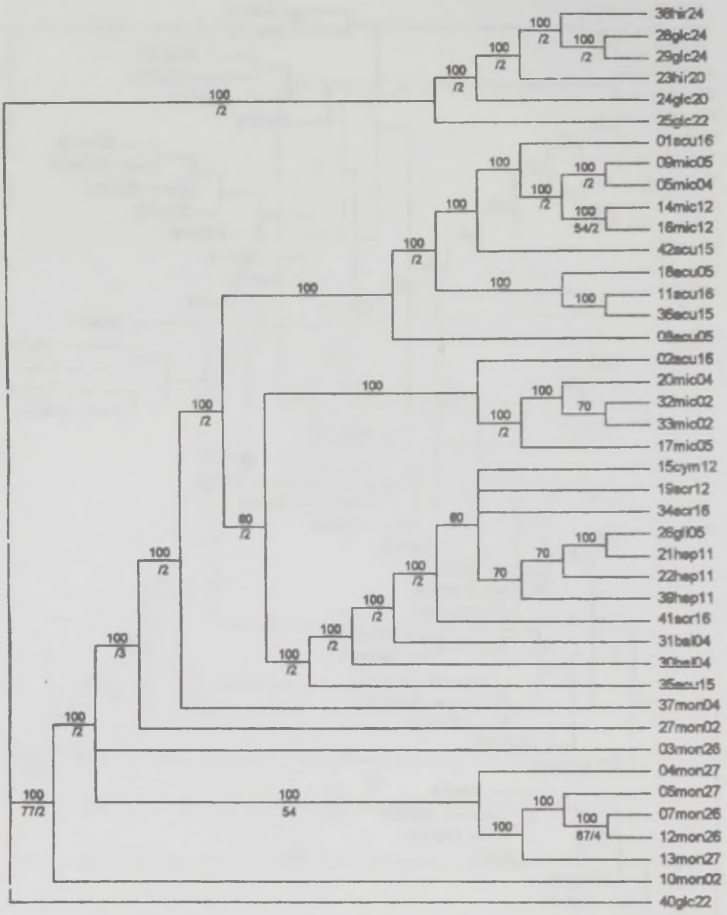

Fig. 6A. MP tree of combined (morphological and RAPD) data (42 specimens). Notations as in Table 1. The 50% majority-rule consensus tree of 20 trees (length 875 steps, CI = 0.190, HI = 0.810). Support values as in Fig. 4A.

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-

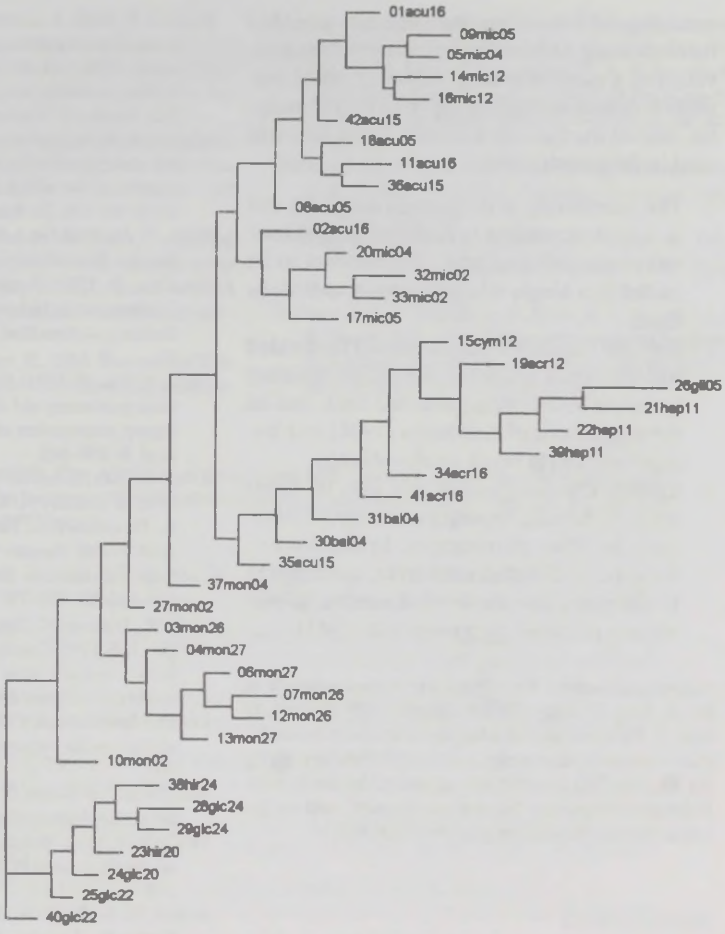

Fig. 6B. MP tree of combined (morphological and RAPD) data (42 specimens). Notations as in Table 1. One of 20 most parsimonious trees.

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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 01acu16 033100201001212222132143323343222322231111123414
0100000100110110111010011000011100000000000001111110110111010000000

 02acu16 032200201001212222222223324333222444333331123423
1111100101000110110000101000011010110000010000011110110111010000000

 03mon26 022200201102221221132321222223232222121111122232
1110110011001010100100011000011100110000000001011100110111011101000

 04mon27 032201211001211221133321323222324322121121223332
1110110011011111111100011000011100110110000000001110011011110111111000

 06mon27 022201311102221221132311333212324223222221122333
1110110011011001111000001000111111111011001000101110011011100110011000

 07mon26 022200201102121211122211334112424212122211222231
0000000011001101111100011000011101110110010001011100110111111111111000

 08acu05 032100201001212222221112323223112323232211123413
1111011101010101111110011100011110110000000001111110110111010000000000

 09gra05 023300210101212122222221223212322222122311143424
0100000100110011111110011000011101010000000011111110110111111111111000

 12mon26 022200201101121211322214341122232222223112222231
1110110011011000100000011000011101110110010001011100110111001101100000

 13mon27 022200311002211221131222333222424323221121322232
1100110011011001110001111011111101110110010001011100110111001101100000

 14gra12 013300300101212222223342324323422433232221123322
0100000100110010111100011000011101010000011001111111011010101110000000

 15cym12 012310301011211111132312213333111443322221122221
0111001100111011111100101000111101011000110111001010100001101110000000

 16gra12 012300300101212222222232222323322233323111122323
1110000100110010110010011000010101000000000000111110110101011100000000
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Continued

Appendix. Continued.

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 19scr12 012210201001211112222232123334224333322211224331110101101001111010010101101110101100011000010101010100111110000001
 20gra04 023300210102221122222321324222422222123311133423111110110101000111000000100001001011000000000001111011011111110000001
 30bal04 024200201001121222122112131131132222322211123313011110110111001111110010100001011101000000000001110010111100000000001
 31bal04 024200201011221221123421113323112443332321133424011010110111011111110010001111101101000000000000001000000100000000001
 32gra02 023200310100222122223231322222222323132311133424111010110011011010010010100001010001000000000111111011010100000000001
 33gra02 023200310102222121223222223223212322222311133413111010110111111111110010100001011011000000000001111011011111110000001
 34scr16 032200201000211122321222132322223233122211232221100010111011111110010100001110101101111110000101000000110110000000001
 35acu15 032200201002121222131242212233222333231111133414111100101110011111100101001011010110000010000011110110111000000000001
 41scr16 022200201001212122132132233232422443321121122221111100111110001111000001000011001010000000001010100010010011000000001
 42acu15 022200201002222222122242324223112333232111123323010001110011101111110011100011100000000000000111111011011010000000001
 38hir24 123300201001211212122232242121443212113323322344111111011110110111110001101001110111000000000000000110001110011000000001
 28glc24 113201201001111122121221243112443111112214321344111111011110110011111001101011110101001001101100111011001111110000001
 29glc24 113200201001121112121221232111443111113313312343110011001110001111111001101001111010100000001100111011001100110000001
 10mon02 032301200010221221231121232112212322212121122233110010001001001110001001100001110100000000001101110011011100110000001
 27mon02 033300201000221221122221232222322323232121122232111011101101111111111011010011110101101100000010111001101110011000001
 37mon04 033200201001211222221122232223222333343231222231111011101101111111111000010100111111101100000010111001101110000000001
 05gra04 023200200101212122323131223222222323232321133323010000010011000011000011110001010101000000001111111011011111111011000
 26gli05 014200201000211112133221111111211111111111123111011110010111001111100111101111101111000000001100101010000110111011001
 17gra05 022200200000212221133222212112121222122321132323111110010101000001000000100001001000000000000001111101101110100000001
 18acu05 022200200002212222132222112213112222132211123414111101110011010011001111001110100000000000011111101101110100000001
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Continued

Appendix. Continued.

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11100110011100101000110110100111000000000001100001110100100110000000

 22hepl1 021310201001221112132323112333111443422331133412
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 39hepl1 022310201101211022131112231233111332432322222333
11110011010110111111001010100111001100000000000000011010011011000000

 11acu16 022200201001212122133143121333111333242111112222
11111111000111111111100111000111110000000100011111101101110100000000

 36acu15 022200201000212122142142232323322333232111123214
11111001001100101100100111000101010100000000011111101101110100000000

 23hir20 133200210101221212122221421122321121133143113 43
11111101111011011111000110100111011100000110110011101100110011000000

 24glc20 123201201001212111211122421222321121133133113 41
11111101111011001111000010000100101100000000110011101100110011000000

 25glc22 123301201001221211231112242122232222213323312342
11111100111011111111100110100101010100000000110011101100110011000000

 40glc22 132201201010221121133111242122332111111212322242
11001100111000111111010110101111010100000000110011101100110001000000
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III

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Study of the multivariate structure of the Estonian *Alchemilla* L. (Rosaceae)
microspecies: an example of the structural indices approach. —
Proc. Estonian Acad. Sci. Biol. Ecol. 49: 289–301 (in press).

Proc. Estonian Acad. Sci. Biol. Ecol., 2000, **49**, 3, 289–301

STUDY OF THE MULTIVARIATE STRUCTURE OF THE ESTONIAN *Alchemilla* L. (ROSACEAE) MICROSPECIES: AN EXAMPLE OF THE STRUCTURAL INDICES APPROACH

Silvia SEPP[a*], Tatjana NAHTMAN[b], Tõnu MÖLS[b], and Jaanus PAAL[a]

[a] Institute of Botany and Ecology, University of Tartu, Lai 40, 51005 Tartu, Estonia
[b] Institute of Mathematical Statistics, University of Tartu, J. Liivi 2-411, 50409 Tartu, Estonia

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Abstract. The structural indices approach was used in the taxonomy of 23 microspecies of *Alchemilla* L. to overcome the problem of the statistical incorrectness caused by testing the objectivity of taxa applying the same morphometric variables as those used to define them. We tried to find answers to the following questions: How distinct are the microspecies according to the metric and count variables? How do the structural indices distinguish microspecies? What are the most stable proportions between the characters? Which characters are most informative in microspecies distinction? The structural indices proved to be better for taxon discrimination than the first principal components and single variables. The pairs of indistinct microspecies found in discriminant analysis were confirmed by structural indices analysis, but additionally many indistinct species pairs appeared. The hairiness characters were effective for microspecies discrimination while flower measurements were the poorest discriminators; all the metric variables and counts together were the most effective of all.

Key words: morphological variation, plant taxonomy, principal components analysis.

INTRODUCTION

The genus *Alchemilla* L. (Fam. Rosaceae Juss., subfam. Rosoidae Focke) consists of more than 1000 taxa (Fröhner, 1995), about 300 of which have been described in Europe. Because of its agamospermy and large variation, the genus has been an object of widespread scientific interest since the last century. Most authors rank *Alchemilla* microspecies on a species level; some suggest that only a

* Corresponding author, silvias@ut.ee

few collective species should be recognized. Numerical methods have been used quite rarely (Turesson, 1956; Glazunova & Myatlev, 1990; Sepp & Paal, 1998) for the analysis of this genus, though they have proved to be useful in analogous agamic complexes (e.g., *Amelanchier*, Dibble et al., 1998; *Antennaria*, Chmielewski, 1995; *Rosa*, Nybom et al., 1997; *Rubus*, Kraft & Nybom, 1995). Until recently nobody had investigated whether the morphological characters used in identification of these taxa really work and whether the microspecies are clearly distinct. From the first investigations of that kind (Sepp & Paal, 1998) it may be pointed out that sections are distinguished rather well, while among microspecies some are significantly distinct but some pairs or even bigger groups are taxonomically continuous.

A difficult problem in taxonomy is associated with the statistical incorrectness caused by testing the distinctness and objectivity of taxa using the same morphometric variables that are the basis of defining these taxa. To overcome this problem is a rather complicated task: it is equivalent to testing the reality of clusters in cluster analysis. The difference between taxonomy and cluster analysis is that the empirical definition of species is based on a visual univariate or bivariate analysis, whereas cluster analysis is mainly a multivariate technique, taking all the characters into consideration.

In component analysis eigenvectors corresponding to minimal eigenvalues were calculated instead of the standard principal components. These express stable proportions between variables, and are called structural indices (Möls & Paal, 1998). We argue that the eigenvector-based structural indices as multivariate tools are less dependent on the descriptive characters of taxa and give more objective criteria for taxon differentiation than other methods.

In the current paper 23 microspecies belonging to the collective species *Alchemilla vulgaris* L. (coll.) are analysed. All these microspecies are high polyploids and apomicts (apospory + parthenogenesis, Gustafsson, 1947). The questions to be answered in the current paper are:

1. How distinct are the microspecies according to the metric and count variables, and what is the error rate of classification based on these variables?
2. What are the most stable proportions between the variables according to the structural indices?
3. Which variables are the most informative for microspecies distinction?
4. How do the structural indices distinguish microspecies and by what measure is the pattern different from the one obtained by other methods?

MATERIAL AND METHODS

Altogether 598 specimens of 23 *Alchemilla* microspecies occurring in the Estonian flora (Laasimer et al., 1996) were analysed (cf. list of microspecies, notations and number of specimens in Table 1). Herbarium material from

the Herbarium of the University of Tartu and the Herbarium of the Institute of Zoology and Botany, as well as material collected by the authors, was used. The microspecies were identified using the key compiled by Tikhomirov (in Laasimer et al., 1996), in doubtful cases they were checked and/or reidentified by K. P. Glazunova.

Only metric variables and counts were taken into account, mainly for mathematical reasons, but also to see if the same pattern appears as with the whole set of variables, including qualitative ones (as used by previous researchers and also in Sepp & Paal, 1998). In all, 28 variables – 15 metric ones and 13 counts – of 43 assessed parameters (Sepp & Paal, 1998) were used in the analysis (Table 2). As a rule, each variable was measured three times on every specimen, and in data processing the means of these measurements were used. Metric variables were log or log+1 transformed to approximate their distribution to the normal distribution.

Microspecies assignments and the values of different variables in microspecies discrimination were checked by linear discriminant analysis, realized in SAS/DISCRIM release 6.12 (SAS Institute Inc., 1998). For each microspecies the misclassification probability, or error rate, was estimated by the cross-validation method using three different sets of predictor variables: only metric, only counts, metric and counts together. The mutual proximity of microspecies was detected from the discriminant analysis as the proportion of "erroneously" classified microspecies – if more than 5% of the specimens of one microspecies were classified as another certain microspecies, these two were considered to be close.

The covariance structure of variables was studied by principal component analysis using SAS/PRINCOMP procedure. Here, instead of the standard principal components, eigenvectors of small eigenvalues, or structural indices, were calculated. The eigenvalue of an eigenvector of the covariance matrix is equal to the variance of the corresponding linear combination of logarithmic morphometric variables. Consequently, the structural indices represent the most stable proportions of variables (Möls & Paal, 1998). For a given taxon, these proportions remain constant regardless of environment and age, being at the same time less influenced by single variables, including those used for defining the taxon.

The structural indices that correspond to the last five eigenvalues of the covariance matrix were used to test the difference between microspecies. Mean values of structural indices were estimated and compared for different microspecies by the SAS/GLM/LSMEANS option. If at least one index was significantly different, the two microspecies were declared to be "distinct by ANOVA", otherwise "indistinct by ANOVA". The Bonferroni adjustment for the significance level was requested because of multiple comparisons.

For each pair of "indistinct by ANOVA" microspecies, the five structural indices were additionally analysed as variables with the multivariate SAS/GLM/MANOVA procedure. Depending on the result of this test, the microspecies were considered to be either "distinct" or "indistinct by MANOVA".

Table 1. Value of different sets of variables (metric, counts, and both together) in *Alchemilla* species discrimination and their error rates.

Species	Notation	No. of specimens	No. of observations	Metric only		Counts only		Metric and counts together	
				Error rate, %	Reclassified into >5%	Error rate, %	Reclassified into >5%	Error rate, %	Reclassified into >5%
A. glaucescens	GLC	80	240	39	HIR	32	HIR, PLI	21	HIR
A. hirsuticaulis	HIR	20	60	44	GLC	15	GLC, PLI, OBT	17	GLC
A. acutiloba	ACU	81	243	38	SCR, SAR, MIC, BAL	70	MIC	35	MIC, BAL
A. wichurae	WIC	9	27	67	SAR, BAL, MUR	39	MIC, SCR, SAR, GLO, GLA, FIL, CYM, OBT, XAN	22	SAR, CYM, BAL, XAN
A. subcrenata	SCR	27	81	67	ACU, WIC, SAR, HEP, MIC, SGL	85	SAR, CYM, BAL, SGL	59	SAR, MON, HEP, MIC, CYM, SGL
A. sarmatica	SAR	22	66	67	ACU, SEM, MON, MIC, SGL	49	SCR, MIC, CYM	40	SCR, MON, MIC, SGL
A. semilunaris	SEM	10	30	28	PLI, HEP, SGL	50	LIN	23	PLI
A. propinqua	PRO	22	66	70	HIR, MON	24	SAR, MON, GLO	25	HIR, MON
A. plicata	PLI	29	87	40	GLC, PRO	31	GLC, HIR, MUR	21	GLC, HIR, PRO
A. monticola	MON	57	171	71	PRO, PLI, MIC, SGL	52	SCR, MIC, FIL, BAL, MUR	41	PRO, PLI, MIC
A. lindbergiana	LIN	10	30	23	HIR, MIC, SEM, SGL	44	CYM	20	CYM
A. heptagona	HEP	25	75	43	WIC	39	LIN, GLO, CYM, SGL	21	
A. micans	MIC	35	105	62	ACU, SAR, LIN, SGL	43	ACU, OBT, MUR	36	ACU, SAR

Table 1 continued

Species	Notation	No.of specimens	No.of observations	Metric only		Counts only		Metric and counts together	
				Error rate, %	Reclassified into > 5%	Error rate, %	Reclassified into > 5%	Error rate, %	Reclassified into > 5%
A. glomerulans	GLO	13	39	69	HIR, SAR, CYM	54	WIC, PRO, PLI, BAL, OBT, XAN	40	SAR, BAL
A. glabricaulis	GLI	14	42	49		27	HEP, FIL, CYM, SGL	7	OBT
A. glabra	GLA	23	69	84	SAR, GLI, BAL, OBT	77	MIC, WIC, MIC, BAL, OBT, XAN, MUR	51	SAR, GLI, BAL, OBT
A. filicaulis	FIL	11	33	69		24	HIR, PRO, PLI, MIC, BAL, OBT	16	BAL, OBT
A. cymatophylla	CYM	30	90	79	MIC, SCR, SAR, PRO, HEP, MIC, GLA, SGL	93	SCR, LIN, HEP, MIC, GLI	55	SAR, PRO, HEP, MIC, MUR, SGL
A. baltica	BAL	27	81	67	WIC, SAR, OBT, MUR	72	OBT, XAN	38	OBT, MUR
A. obtusa	OBT	20	60	68	WIC, MIC, GLA, BAL, MUR	7	WIC, GLA, FIL, BAL, SGL	45	MIC, GLA, BAL, MUR
A. xanthochlora	XAN	10	30	40	MIC, SCR, LIN, HEP	46	MIC, GLO	27	
A. murbeckiana	MUR	10	30	60	WIC, CYM, OBT	69	HIR, PLI, LIN, FIL, XAN	27	WIC
A. subglobosa	SGL	12	36	56	SAR, LIN, HEP, MIC, XAN	81	SCR, SAR, MON, HEP, GLI	52	SAR, LIN

Table 2. Variables used in analysis

Notation	Variable	Type	Precision
STNR	Number of (flowering) stems per plant	Count	
LENR	Number of basal leaves per plant	Count	
LBCOR	Angle between basal lobes of basal leaf	Metric	±5°
STLN	Length of stem	Metric	±5 mm
STLHR	Number of hairs per 1 mm length of lower part of stem (first internodes)	Count	
STUHR	Number of hairs per 1 mm length of upper part of stem (just below inflorescence)	Count	
PETHR	Number of hairs per 1 mm length of petiole (of basal leaf)	Count	
SLELN	Length (radius) of stem leaf	Metric	±1 mm
PETLN	Length of petiole (of basal leaf)	Metric	±5 mm
LBNR	Number of lobes per leaf	Count	
LEUHR	Number of hairs per 1 mm² area on upper surface of basal leaf	Count	
LELHR	Number of hairs per 1 mm² area on lower surface of basal leaf	Count	
VNHR	Number of hairs per 1 mm length of vein on lower surface of basal leaf	Count	
LELN	Length (radius) of basal leaf	Metric	±1 mm
LEWD	Width of basal leaf	Metric	±1 mm
LBLN	Length of apical lobe of basal leaf	Metric	±1 mm
LBWD	Width of apical lobe of basal leaf	Metric	±1 mm
THNR	Number of teeth of apical lobe of basal leaf	Count	
STHLN	Length of the tooth next to apical (of apical lobe of basal leaf)	Metric	±0.1 mm
TTHLN	Length of apical tooth (of apical lobe of basal leaf)	Metric	±0.1 mm
STHWD	Width of the tooth next to apical (of apical lobe of basal leaf)	Metric	±0.1 mm
PEDHR	Number of hairs per 1 mm length of pedicel	Count	
HYHR	Number of hairs per one side of hypanthium	Count	
HYLN	Length of hypanthium	Metric	±0.1 mm
HYWD	Width of hypanthium	Metric	±0.1 mm
CALN	Length of sepal	Metric	±0.1 mm
CAHR	Number of hairs per sepal	Count	
OCALN	Length of lobe of epicalyx	Metric	±0.1 mm

RESULTS

The mean error rate for the set of metric variables in discriminating microspecies was 56%; for count variables it was 51%. Using all the variables together, the mean error rate was reduced to 32%. Consequently, the set of only metric variables is the poorest option for discriminating between microspecies, group membership can be predicted considerably more accurately if the metric and count variables are used together (Table 1).

According to ANOVA, only 24% of all possible pairs of microspecies were statistically distinct (at least by one character); MANOVA distinguished an additional 21% of species pairs, which were indistinct by ANOVA (Table 3).

Table 3. Distinctness of the analysed *Alchemilla* species. A — species are distinct by ANOVA, at least by one structural index, M — indistinct by ANOVA, but distinct by MANOVA of five structural indices, I — species are indistinct by both ANOVA and MANOVA; d — species are distinct according to the discriminant analysis (not reclassified into one another), i — clearly indistinct (both reclassified into one another), (i) — indistinct (only one species reclassified into another, but not *vice versa*).

Species[a]																							
HIR	Ii																						
ACU	Ad	Id																					
WIC	Id	Md	Id																				
SCR	Ad	Ad	Ad	Md																			
SAR	Ad	Ad	Ad	M(i)	Ai																		
SEM	Id	Md	Id	Id	Id	Id																	
PRO	Ad	A(i)	Ad	Md	Ad	Md	Ad																
PLI	I(i)	I(i)	Id	Id	Ad	Id	M(i)	A(i)															
MON	Md	Ad	Ad	Md	I(i)	A(i)	Ad	Ii	I(i)														
LIN	Id	Md	Id	Id	Md	Md	Id	Ad	Id	Md													
HEP	Ad	Ad	Ad	Ad	A(i)	Ad	Ad	Ad	Ad	Ad	Ad												
MIC	Ad	Ad	Mi	Id	A(i)	Ii	Id	Md	Id	M(i)	Id	Ad											
GLO	Md	Ad	Id	Id	Id	I(i)	Id	Id	Id	Id	Id	Ad	Id										
GLI	Ad	Ad	Ad	Ad	Md	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad									
GLA	Id	Id	Id	Id	Id	A(i)	Id	Ad	Id	Id	Id	Ad	Id	Id	A(i)								
FIL	Id	Md	Ad	Ad	Id	Md	Ad	Md	Ad	Md	Ad	Id	Ad	Ad	Ad	Ad							
CYM	Id	Id	Ad	M(i)	A(i)	A(i)	Id	A(i)	Id	Md	I(i)	A(i)	A(i)	Ad	Ad	Id	Id						
BAL	Ad	Ad	A(i)	M(i)	Md	Ad	Ad	Id	Ad	Md	Ad	Ad	Ad	I(i)	Md	A(i)	M(i)	Ad					
OBT	Ad	Md	Md	Md	Ad	Ad	Md	Md	Ad	Id	Ad	Ad	A(i)	Id	I(i)	Ii	M(i)	Ad	Ii				
XAN	Md	Ad	Ad	I(i)	Ad	Ad	Md	Ad	Md	Ad	Md	Ad	Md	Md	Ad	Id	Id	Md	Ad	Md			
MUR	Id	Md	Id	I(i)	Md	Id	Id	Ad	Id	Md	Id	Ad	Id	Id	Ad	Id	Id	I(i)	A(i)	A(i)	Id		
SGL	Id	Md	Ad	Md	A(i)	Ai	Md	Ad	Md	Md	M(i)	Id	Ad	Ad	Ad	Md	Id	I(i)	Ad	Md	Id	Id	
	GLC	HIR	ACU	WIC	SCR	SAR	SEM	PRO	PLI	MON	LIN	HEP	MIC	GLO	GLI	GLA	FIL	CYM	BAL	OBT	XAN	MUR	

[a] See Table 1.

Thus, by linearly combining structural indices, a new structural index can be constructed, which discriminates between microspecies most effectively.

The characters that have larger coefficients in a structural index (for example, >0.2) are considered to be the main components of the respective structural index. The most stable combination in our case is the ratio of leaf width and leaf length (V_1, Table 4), which describes the general shape of the leaf. Stable combinations also exist between dimensions of the leaf and leaf lobe (V_2: LELN, LEWD, LBLN, LBWD), dimensions of the flower (V_3 and V_4: HYLN, HYWD, CALN, OCALN) and leaf teeth (V_5: STHLN, TTHLN, STHWD). An orthogonal rotation of the five-dimensional space of the indices V_1–V_5 could generate other structural indices, which might emphasize different stable proportions between the same metric variables.

The best numerical characters for distinguishing microspecies according to GLM are mainly hairiness characters: VNHR (distinguishes 167 species pairs, 66% of possible), STUHR, PETHR (both 160, 63%), STLHR (145, 57%), LEUHR (137, 54%), LELHR (136, 54%), and THNR (129, 51%). The least important (distinguish less than 25% of species pairs) are flower characters (FPTHR, HYLN, OCALN, CALN, HYWD), number of leaves and flowering stems (LENR, STNR), and width of the side teeth of leaf lobes (STHWD).

Table 4. The structural indices defined by the five last eigenvectors V_1–V_5 of the covariance matrix of 15 variables

Variable	Eigenvectors (with their eigenvalues)				
	V_1 (0.002)	V_2 (0.008)	V_3 (0.011)	V_4 (0.013)	V_5 (0.014)
LBCOR	0.005	0.029	0.007	0.006	–0.001
STLN	–0.002	0.017	–0.009	–0.057	–0.004
SLELN	–0.004	0.020	0.012	0.044	0.028
PETLN	–0.005	0.049	–0.006	0.0003	0.024
LELN	–0.694	–0.467	–0.060	–0.016	0.013
LEWD	0.720	–0.433	–0.053	–0.037	0.014
LBLN	0.002	0.214	0.010	0.101	–0.028
LBWD	–0.018	0.710	0.156	–0.006	–0.070
STHLN	–0.004	0.026	0.018	–0.092	0.783
TTHLN	0.005	–0.059	–0.069	0.180	–0.528
STHWD	–0.009	–0.063	–0.056	–0.062	–0.214
HYLN	–0.009	0.004	0.162	–0.697	–0.052
HYWD	0.010	0.042	–0.293	0.592	0.198
CALN	0.004	–0.143	0.827	0.301	–0.032
OCALN	–0.001	0.103	–0.407	–0.109	–0.105

DISCUSSION

Comparison of the ordinary discriminant analysis and the analysis of the variance of structural indices (Tables 1 and 3) revealed that the results agree in certain points. In both cases some results are in good concordance with the opinions of taxonomists about the similarity of microspecies, for example, the indistinctness of *A. acutiloba* and *A. micans*, *A. glaucescens* and *A. hirsuticaulis*. But some of the indistinct pairs in both cases are rather surprising, for example *A. lindbergiana* and *A. cymatophylla*, *A. plicata* and *A. semilunaris*, since these microspecies are generally considered to belong to different sections.

As a rule, the results of the discriminant analysis were in relatively good concordance with classical systematics. One reason is that the structural indices approach uses only metric variables while the discriminant analysis also takes into consideration the count variables. According to discriminant analysis, microspecies from different sections are seldom indistinct while microspecies of the same section are more often statistically continuous. For example, in addition to the "classically" continuous species pairs mentioned above, several species pairs in the section *Coriaceae* are not well separable in the discriminant analysis. As a rule, if the two microspecies are indistinct, and one or some authors have placed them into different sections, by other author(s) these microspecies are included into the same section. For example, according to Yuzepchuk (1941), Rothmaler (1962), Walters & Pawlowski (1968), and Plocek (1982) *A. plicata* and *A. monticola* belong to the separate sections or series *Pubescentes* and *Hirsutae*, but Fröhner (1995) merged them in the section *Plicatae*. Still, there are also some "odd" indistinct pairs, whose similarity has not been stated before (cf. Table 1).

The structural indices approach and discriminant analysis also differ if we take into account that it is a mathematical nonsense to use a variable both for the taxon definition and to show that the taxa are well defined and distinct. Split, for example, a homogenous population into two parts, according to whether the value of the variable x is smaller or larger than its mean value. Declare, thereafter, the two parts to be two taxa and check their difference using Student's test. The test will definitely confirm the distinctness of these taxa. Unlike single variables, structural indices depend simultaneously on many variables and cannot be easily followed on certain specimens. For the latter reason, the network of indistinct pairs from the structural index analysis can not always be directly biologically interpreted. Nevertheless, from a statistical point of view, it is safer to test distinctness of taxa using structural indices.

In our case, pairs of indistinct microspecies found in discriminant analysis were mostly confirmed by structural indices analysis. Besides that, according to structural indices numerous indistinct species pairs appeared, often from different sections. It is noteworthy that according to structural indices *A. heptagona* is indistinct from *A. filicaulis* and *A. subglobosa*. The systematic position of the last one is doubtful and it is indistinct from many other microspecies, but Yuzepchuk

(1941) segregated *A. heptagona* and *A. filicaulis* into a separate group: *Exuentes*. No other authors have agreed with him, but the preliminary genetic data (Sepp et al., 2000) also suggest that *A. heptagona* is exceptional in the section *Ultravulgares* Fröhner.

We can also use structural indices for descriptive purposes, to get a general idea of taxon separation according to each structural index. Visual inspection of Fig. 1 indicates that, for example, V_4 and V_5 account for separation between *A. xanthochlora* and *A. propinqua*, but separation between *A. propinqua* and *A. subglobosa* by the same indices appears to be slightly less clear (Fig. 2). The

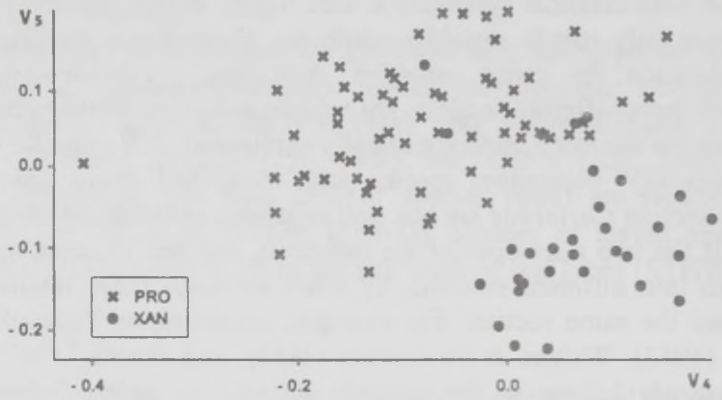

Fig. 1. Ordination plot of *Alchemilla propinqua* and *A. xanthochlora* by the structural indices V_4 and V_5 (see Table 4).

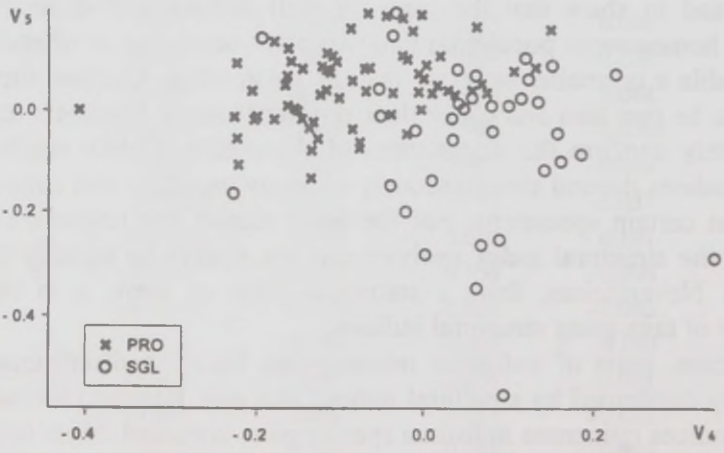

Fig. 2. Ordination plot of *Alchemilla propinqua* and *A. subglobosa* by the structural indices V_4 and V_5 (see Table 4).

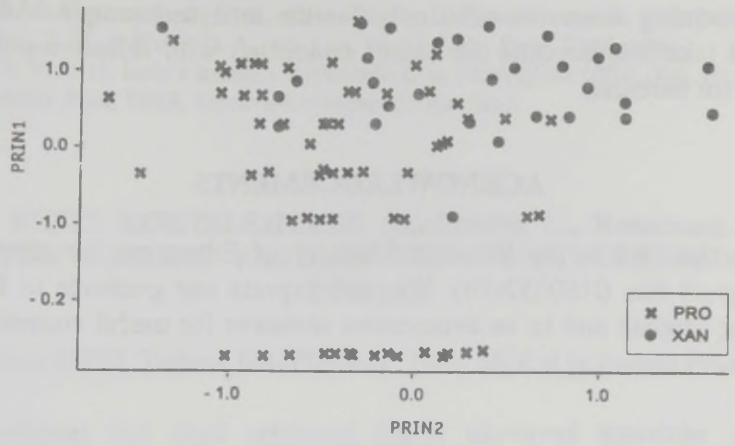

Fig. 3. Ordination plot of *Alchemilla propinqua* and *A. xanthochlora* by the first two principal components (proportions of eigenvalues are 0.63 and 0.22, respectively).

first principal components (Fig. 3, for *A. propinqua* and *A. xanthochlora*) discriminate between microspecies much less clearly. One must certainly be wary of drawing conclusions based only on the visual analysis of ordination plots. If there are many observations for each taxon the plots can show rather undifferentiated groups.

Concerning the variables, general patterns obtained using metric and count variables are similar to those revealed by all kinds of different variables (Sepp & Paal, 1998); for example, they agree in showing the hairiness variables as the most effective and flower measurements as the least effective discriminators. Still, as it appeared from discriminant analysis, the metric variables and counts together are more effective than the hairiness or metric variables alone. However, although the metric variables distinguish a minority of microspecies, these can be just the ones which hair characters are not capable of discriminating.

The most stable combinations of variables reflected in structural indices are in good agreement with the main correlation groups of variables (Sepp & Paal, 1998). The stability of leaf variables, for example, also shows that it is probably better to use ratios of metric variables in taxon discrimination, as already suggested by some authors (e.g., Fröhner, 1995). If we use the structural indices or ratios instead of metric variables as such, there will probably be less individual differences between specimens of the same taxon.

As a concluding remark, we should be cautious with extrapolation of the results to the microspecies as a whole. For more abundant microspecies, the material originated from different populations all over Estonia, and the conclusions are more or less trustful for this part of their distribution area. However, the material is not representative for rare microspecies, and thus the

results concerning them are valid only for the analysed sample. Additionally, one should take into account the biases connected with selective collection of specimens for herbaria.

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EESTI KORTSLEHTEDE (*Alchemilla* L., Rosaceae) PALJUTUNNUSELINE VARIEERUVUS STRUKTUURIINDEKSITE PÕHJAL

Silvia SEPP, Tatjana NAHTMAN, Tõnu MÖLS ja Jaanus PAAL

On selgitatud, kui hästi eristuvad Eestis kasvavad kortslehe mikroliigid meetriliste ja loendustunnuste alusel, samuti hinnatud, missugused on stabiilseimad tunnuste kombinatsioonid ja missugused tunnused on liikide eristamiseks kõige informatiivsemad. Arvestades seda, et matemaatiliselt on ebakorrektne kasutada taksonite objektiivsuse hindamiseks samu tunnuseid, mille põhjal toimub nende defineerimine, rakendati taksonoomiliseks analüüsiks uudset struktuuriindeksite meetodit. Struktuuriindeksid eristasid mikroliike paremini kui esimesed peakomponendid või üksikud tunnused. Samas osutusid diskriminantanalüüsiga selgitatud indistinktsed liigipaarid kontinuaalseteks ka struktuuriindeksite alusel; lisaks tuvastati viimaste abil veel mõned teineteisest halvasti eristatavad liigipaarid. Mikroliikide eristamisel osutusid kõige efektiivsemaks taimede karvasust iseloomustavad tunnused, õite parameetrid aga kõige kehvemateks tunnusteks. Parim tulemus saavutati nii meetriliste kui ka loendustunnuste koos kasutamisel.

IV

Sepp, S. & Paal, J.
Patterns and relationships between and within the sections *Alchemilla* and *Ultravulgares* Fröhner of the genus *Alchemilla* L. (Rosaceae) in Estonia.
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Patterns and relationships between and within the sections *Alchemilla* and *Ultravulgares* Fröhner of the genus *Alchemilla* L. (Rosaceae) in Estonia

Silvia Sepp (corresponding author)

Institute of Botany and Ecology, University of Tartu, 40 Lai St., 51005 Tartu, Estonia, e-mail: silvias@ut.ee; fax: (372 7) 376 222; phone: (372 7) 376 221

Jaanus Paal

Institute of Botany and Ecology, University of Tartu, 40 Lai St., 51005 Tartu, Estonia, e-mail: jpaal@ut.ee; fax: (372 7) 376 222; phone: (372 7) 376 220

Abstract

Nine Estonian *Alchemilla* species belonging to the sections *Ultravulgares* and *Alchemilla,* or considered being close to them, are analysed. When the analysed specimens are divided into sections, the latter are statistically distinct and form separable groups in character space. When the specimens are grouped on species level, *A. cymatophylla, A. subcrenata* and *A. heptagona* are insignificantly distinct, but in character space specimens of *A. heptagona* are visually well apart from the other two. Specimens of *A. acutiloba, A. micans* and *A. xanthochlora* form confidentally distinct ($p < 0.01$) species-clusters, but they form a joint cloud in the character space, indicating the compactness of section *Alchemilla*. Specimens of *A. semilunaris* are close to *A. lindbergiana,* and not as has previously been supposed to the section *Ultravulgares.*. *A. semilunaris* should possibly be kept in a separate section *Decumbentes*. Specimens of *A. lindbergiana* are on one hand close to *A. semilunaris,* on the other hand close to the species of section *Alchemilla*; including it in the latter section is still doubtful. *A. subglobosa* is indistinct from *A. subcrenata*, but can generally be separated from sections *Alchemilla* and *Ultravulgares*. Of the 41 tested characters 35 are useful for species discrimination; counts, nominal and ratio characters are better than metric ones. According to the cluster analysis, specimens of *A. acutiloba* and *A. micans* form one big cluster, all other specimens belong to another.

Key words: continuum, distinctness, multivariate analysis, taxonomy, character ranking, morphological variation.

Introduction

There are several different concepts of the infrageneric structure of the genus *Alchemilla* (Buser 1901, Rothmaler 1944, Walters & Pawlowski 1968, Plocek 1982, Fröhner 1995). Fröhner's system seems to be the most suitable for the species occurring in Estonia — according to our earlier investigations the sections *Alchemilla*, *Ultravulgares* Fröhner, *Plicatae* Fröhner, and *Coriaceae* Fröhner can be distinguished rather well (Sepp & Paal 1998).

Section *Ultravulgares* comprises altogether five species: *Alchemilla heptagona* Juz., *A. cymatophylla* Juz., *A. subcrenata* Buser, *A. gaillardiana* Buser, and *A. heteropoda* Buser (Fröhner 1995), the first three of which are found in Estonia (Laasimer *et al.* 1996). The section is considered to be one of the four basic gene pools of the whole genus (Fröhner 1986). Fröhner considered the species *A. semilunaris* Juz. to be close to *A. maureri* from section *Decumbentes* Fröhner, but according to Juzepczuk (1941) and Walters & Pawlowski (1968) it is more similar to *A. cymatophylla, A. heptagona* and *A. subcrenata*; therefore, in the current paper this species is treated together with the section *Ultravulgares*.

The section *Alchemilla* consists of ten species (Fröhner 1995), of which *A. acutiloba* Opiz (= *A. vulgaris* L. *sensu* Fröhner), *A. micans* Buser (*A. gracilis* auct. p. p.) and *A. xanthochlora* Rothm. are found in Estonia (Laasimer *et al.* 1996). Section *Alchemilla* is supposed to be hybridogenous originating from two gene pools, section *Ultravulgares,* and section *Erectae* Fröhner (Fröhner 1986). The latter section is not represented in the Estonian flora. The analysis also included specimens of *A. lindbergiana* Juz., since, according to Walters & Pawlowski (1968), this species is close to *A. micans*.

The sections *Ultravulgares* and *Alchemilla* are analysed together in the current paper for two reasons: they are closely related according to Fröhner (1995), and they appeared to be indistinct by an earlier multivariate analysis (Sepp & Paal 1998). In addition, we compared *A. subglobosa* C. G. Westerl. with species of these two sections, although Fröhner (1995) includes it in the section *Plicatae*. The reason for this is that, according to an earlier continuum analysis (Sepp & Paal 1998), it was closer to the species of these sections than to the species of the section *Plicatae*.

The aims of this study were:

- to analyse the mutual distinctness of specimens representing the Estonian microspecies of the sections *Ultravulgares* and *Alchemilla*;
- to evaluate the relationship of three problematic species — *Alchemilla semilunaris, A. lindbergiana* and *A. subglobosa* — with the established members of sections *Ultravulgares* and *Alchemilla;*
- to ascertain, by means of different multivariate methods, patterns and relationships of specimens within and between these sections, and to indicate possible taxonomic consequences;

– to compile a set of characters that discriminate between the analysed species most clearly.

Material and methods

Estonian specimens of nine *Alchemilla*-species were analysed: *A. heptagona* (HEP), *A. cymatophylla* (CYM) and *A. subcrenata* (SCR) from the section *Ultravulgares; A. acutiloba* (ACU), *A. micans* (MIC) and *A. xanthochlora* (XAN) from the section *Alchemilla; A. subglobosa* (SGL) from the section *Plicatae;* and *A. lindbergiana* (LIN) and *A. semilunaris* (SEM), which were not classified by Fröhner (1995). Herbarium material from the Herbarium of Tartu University (TU), the Herbarium of the Institute of Zoology and Botany (TAA) and the Herbarium of the Moscow State University (MW) was used. We added also material collected from different localities in Estonia in June 1995 and June 1996. If possible, specimens collected in June or July were selected from herbarium material, because material which is collected too late or too early, may sometimes not be identifiable (Fröhner 1995). Totally, 240 specimens were measured. Of each species at least 10 specimens were included, the actual number depended on the number of specimens available in herbaria.

Altogether 41 morphological characters, which are mentioned in literature as applicable for identification of *Alchemilla* species, were taken into account (Table 1). Initially the number of characters was higher, but some of these were excluded from further study. Fröhner (1995) argues that for the purpose of distinguishing between species it is better to use a large number of characters. In practical taxonomy, however, it is very inconvenient to use 200–300 characters for species discrimination. One reason for omitting some characters was their non-applicability for herbarium material; for example leaf and flower colours are not exactly the same for fresh and herbarium material. According to a pilot study, several characters appeared to be insignificant both on species and specimen levels, or had very high correlation (more than 0.9) with others; these were also left out. Some of the initially metric characters were recalculated as ratios, since the latter are less dependent on environmental conditions, season and other unspecified (noise) factors. Still, some of the initial metric characters were retained for comparison. Nominal and hair characters (counts) were used without recalculation.

Cluster analysis was performed with programme packages SYN-TAX 5.02 (Podani 1993) with various algorithms. The best interpretable structure was achieved by the method of incremental sum of squares (MISSQ) or Ward's method (Podani 1994), with Manhattan distance as a similarity measure.

Stepwise and classificatory discriminant analyses of the SAS package (SAS Institute Inc. 1996) were used to estimate how well the discrimination function separates conventionally identified taxa as well as clusters obtained by multivariate analysis, and to find an optimal set of characters necessary for each classification (Klecka 1980). Canonical discriminant analysis was used as an

ordination method, which derives a combination of canonical variables having the highest possible multiple correlation with the groups (SAS Institute Inc. 1996).

Continuum analysis was carried out according to Paal and Kolodyazhnyi (1983) and Paal (1987, 1994) with an original SYNCONT 3.0 programme. As a measure of the distinctness of the clusters, the probability of α-criterion (Duda & Hart 1976), called coefficient of indistinctness (CI) was utilised. In addition, for elucidating the mutual relationship between clusters in the multivariate character space, the adjacency matrix was calculated. The adjacency is expressed as the percentage of specimens in the considered cluster for which the centroid of the compared cluster is the closest (Paal 1987, 1994, Sepp & Paal 1998).

To visualise the transitions between some problematic clusters, and to show the distribution of specimens located between the centroids of the clusters considered, the split window method was applied as in Paal *et al.* (1998).

Results

Statistical testing of the relationship of sections *Ultravulgares* and *Alchemilla* proves their reliable separation (CI=0.00). Fig. 1 shows that, although the projection probability distribution of specimens of these sections is partly overlapping, the curve maxima are well apart. Thus, there obviously exist some intermediate specimens, but most specimens can be clearly classified to either one of the sections. If all specimens of these two sections are merged into one cluster, then the curve of projection probability distribution has a clear depression, indicating that a hiatus exists between the sections.

Continuum analysis on the species level (Table 2) shows that in section *Ultravulgares*, specimens of *A. heptagona* and *A. cymatophylla,* as well as those of *A. subcrenata* and *A. cymatophylla* are mutually indistinct. At the same time, the species of section *Alchemilla* are all significantly distinct from each other. Specimens of the three species of uncertain position, *A. semilunaris*, *A. lindbergiana* and *A. subglobosa,* are significantly separated from most of the specimens of sections *Alchemilla* and *Ultravulgares*, only specimens of *A. subglobosa* are indistinct from specimens of *A. subcrenata* (CI = 6.89, Table 2). *A. semilunaris* and *A. lindbergiana* are insignificantly distinct (CI = 21.60, Table 2, Fig. 2).

The adjacency matrix (Table 3) gives additional information on the relationships of the investigated species. The specimens of *A. lindbergiana* and *A. semilunaris* are remarkably mutually adjacent; the centroids of both species are the closest centroids for most of specimens of the other species. There are also two asymmetrically adjacent species-pairs. 60% of *A. xanthochlora* specimens recognise *A. lindbergiana* as an adjacent cluster, while this is true for only 10% of specimens of the latter species towards *A. xanthochlora.* For 72%

of the *A. heptagona* specimens the centroid of *A. cymatophylla* is the closest, while only 43% of the specimens of the latter species recognise *A. heptagona* as the most adjacent species. *A. subglobosa* deserves attention, since the specimens of this species are adjacent to the centroids of several different species.

The classificatory discriminant analysis reclassified 20 conventionally identified specimens into different species. The largest number of them was moved from *A. acutiloba* to *A. micans* (6) or *vice versa* (3). Five *A. cymatophylla* specimens were reclassified into several different species.

The canonical discriminant analysis of species (Fig. 3) demonstrates an obvious separation of specimens of *A. semilunaris*, *A. subglobosa* and *A. heptagona* from the others. Specimens of different species of section *Alchemilla* are intermixed on the ordination plot, although *A. lindbergiana*'s position is marginal and it can be separated from the other three species. *A. subcrenata* and *A. cymatophylla* are also mixed. The specimen clusters of sections *Alchemilla* and *Ultravulgares* overlap slightly, but the sections can still be considered to be relatively well separable.

Cluster analysis by the MISSQ method classified the data into two big clusters (Fig. 4): the first includes mainly specimens of *A. acutiloba* and *A. micans*, the second comprises all other specimens. The first of these can be divided into three subclusters. Cluster 1A consists almost exclusively of *A. acutiloba* specimens (+ one specimen of *A. subglobosa)*, probably the typical ones. The second cluster, 1B, comprises the putative intermediate specimens identified as *A. acutiloba* or *A. micans*. In cluster 1C, specimens of *A. micans* are prevailing, but some (supposedly *micans*-like) *A. acutiloba* specimens also belong there. All these clusters are distinct from each other as well as the subclusters of the second big cluster. To split these clusters further has no biological meaning.

The second big cluster (Fig. 4) is more heterogeneous, and it can be split into a rather large number of small clusters. If we start with numerous small subclusters that mainly contain specimens of one species only, and check their statistical reliability by continuum analysis, it appears that some of them are indistinct. After the mutually indistinct subclusters are merged, nine distinct subclusters can be accepted. Cluster 2A consists of specimens of *A. lindbergiana* and *A. semilunaris*. Clusters 2B and 2D are very homogenous, respectively representing *A. xanthochlora* and *A. heptagona*. Cluster 2C contains specimens of all three species of section *Ultravulgares*. Most of the members of the clusters 2E and 2F also belong to the section *Ultravulgares*, but some *A. acutiloba* and *A. micans* specimens are also found here. Cluster 2G consists predominantly of *A. cymatophylla* specimens, but there are also two specimens of *A. lindbergiana*. Like cluster 2A, cluster 2H consists of two equally represented species, in this case *A. cymatophylla* and *A. subglobosa*. Cluster 2I is especially heterogeneous, there are represented specimens of all analysed species except *A. lindbergiana*.

In distinguishing the conventionally identified species, 31 characters were found to be essential according to the stepwise discriminant analysis (Table 4). Most important, according to the F-value, are the ratio of lobe length and width which characterises the lobe shape, the angle between the basal lobes of leaves, and the position of hairs on the stem. Based on the analysed species (excluding the three questionable species), only 23 characters are necessary to separate the sections of Fröhner (1995). The most important appears to be the ratio of lobe width and leaf width, the following characters in importance are the same as those used for discriminating species.

The 37 statistically reliable characters for distinguishing the 12 multivariate clusters are partly different from those required for conventionally identified species (Table 4). For the clusters, as for species, the most decisive character is the ratio of lobe length and lobe width, but for clusters, it is followed by length (radius) of the basal leaf, and hairiness of the petiole.

Discussion

In the character space, specimens of sections *Ultravulgares* and *Alchemilla* are on the whole not largely intermixed, and form slightly overlapping, but generally separable "clouds". The sections are also clearly distinct according to continuum analysis.

Specimens belonging to different species of the section *Ultravulgares* — *A. cymatophylla*, *A. subcrenata* and *A. heptagona* — are, according to the continuum analysis, insignificantly distinct from each other, and do not form separable species-clusters. But on the canonical ordination plots, *A. heptagona* can visually be so clearly distinguished, that one is tempted to place it in a separate group (section?) *Barbulatae,* as was done by Juzepchuk (1941). Still, its indistinctness from *A. cymatophylla,* and adjacency to both *A. subcrenata* and *A. cymatophylla,* convinces us that the species should stay in the section *Ultravulgares,* in which it appears to be the most clearly separable species. *A. cymatophylla* specimens on the contrary, are the most variable in the section *Ultravulgares,* and their identity is not always very clear.

Specimens of different species in section *Alchemilla* are all significantly distinct from each other, though on the ordination plots they look quite mixed and form a joint "cloud". Even the specimens of *A. acutiloba* and *A. micans,* the affinity of which is stated to be rather high (Juzepczuk 1941, Walters & Pawlowski 1968), and the identification of which is quite complicated in nature, are well separated from a statistical point of view (CI = 0.00). Although in the character space the distance between the centroids of these species is comparatively short, the adjacency between them (Table 3) is not remarkably big. Still, according to both the classificatory discriminant analysis and the cluster analysis, many specimens are obviously intermediate. One can certainly argue about proper identification of some specimens, but as it has occurred that

expert identified specimens of these two species are also unseparable by genetic characters (Sepp *et al.* 2000), separating of these species becomes at all doubtful.

The alleged similarity of *A. semilunaris* to the species of section *Ultravulgares* (Walters & Pawlowski 1968) is doubtful. Instead this species varies mainly towards *A. lindbergiana* (Table 3). At the same time, specimens of *A. semilunaris* are clearly apart from the specimens of the other species in the character space. Maybe it is reasonable to include *A. semilunaris* in the section *Decumbentes,* since Fröhner (1995) considers it to be close to the species of this section, but, since we have not analysed any species belonging to the latter section, nothing certain can be said.

On the basis of our material, *A. lindbergiana* is most closely related to *A. semilunaris.* This similarity is hard to interpret, and could have been caused by our biased sample. According to the canonical discriminant analysis, the specimens of *A. lindbergiana* are situated between the specimens of section *Alchemilla* and *A. semilunaris.* Still, some similarity to *A. xanthochlora* can be detected from both the continuum analysis and the canonical discriminant analysis. Whether this species can be placed in section *Alchemilla,* and whether its similarity with *A. semilunaris* is occasional, can only be determined for certain by means of molecular testing.

Specimens of *A. subglobosa* vary a great deal, although they are statistically indistinct only from *A. subcrenata* (Table 2). On the canonical ordination plot they are also mixed with specimens of section *Ultravulgares* (Fig. 3). Typical specimens in the right phenophase are probably well distinguishable, while specimens collected in the "wrong" phenophase or being atypical for some other reason cannot be identified correctly. Additional research is needed on this species, preferably including genetic testing.

As expected, the cluster analysis results do not coincide entirely with the empirical classification. Specimens of the section *Ultravulgares* in particular tend to show a pattern of variation in which specimens of conventionally identified species can be subdivided into smaller units, which may be close to the specimens of various other species. Surprising is that the cluster analysis results support the similarity of the specimens of *A. semilunaris* and *A. lindbergiana.*

Most of the characters, which, according to the discriminant analysis, appeared to be fundamental in species discrimination (*e.g.* CLBSH, LBCOR, HRPOS, PETHR, LEUHR), are emphasised as important in the identification keys as well (Juzepczuk 1941, Walters & Pawlowski 1968, Laasimer *et al.* 1996). Surprising is the quite high rank of teeth characters (THTIP, THSYM), since they are not considered to be essential in the literature. The flower measurements do not seem to be so effective in the identification of these species, although they are used, e.g. by Walters and Pawlowski (1968). Generally, it appears that the hairiness, and the nominal and ratio characters are better than the metric ones for species discrimination. The character ranking obtained in the

current analysis could be useful in compilation of identification keys, but it should be kept in mind that these results only hold for certain within the set of species considered here.

The characters that participate in separating the clusters, differ partly from those significant for conventionally estimated species. The noticeable features are the higher position of the metric characters (LERAD) and the flower characters (HYLN, PEDHR, and HYHR) that are not so relevant in species discrimination. This indicates that the multivariate cluster structure is based partly on a different set of characters than the conventional taxonomic structure.

One must bear in mind that the results depend on the sample, which is biased to some extent due to the fact that not all the species are sufficiently represented, and that not all species in the sections are involved. However, the results show the problems and patterns in the variation of these groups of species and indicate some possible taxonomic solutions.

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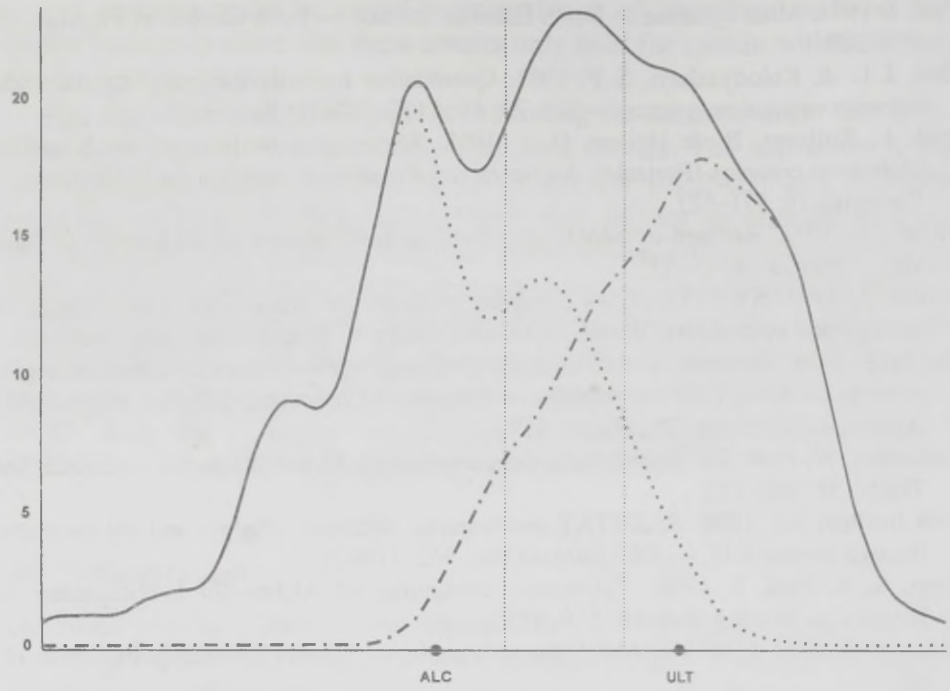

Figure 1. Projection probability distribution of the specimens of sections *Alchemilla* and *Ultravulgares* on the axis passing through the centroids of the section-clusters (denoted as ALC and ULT, respectively), according to the split window method. The curve marked with a dotted line portrays the *Alchemilla* section; the dash-and-dot-line curve corresponds to the *Ultravulgares* section. The curve above them represents the specimens' projection probability distribution of the merged cluster. Two dotted lines perpendicular to the axis mark the transition zone.

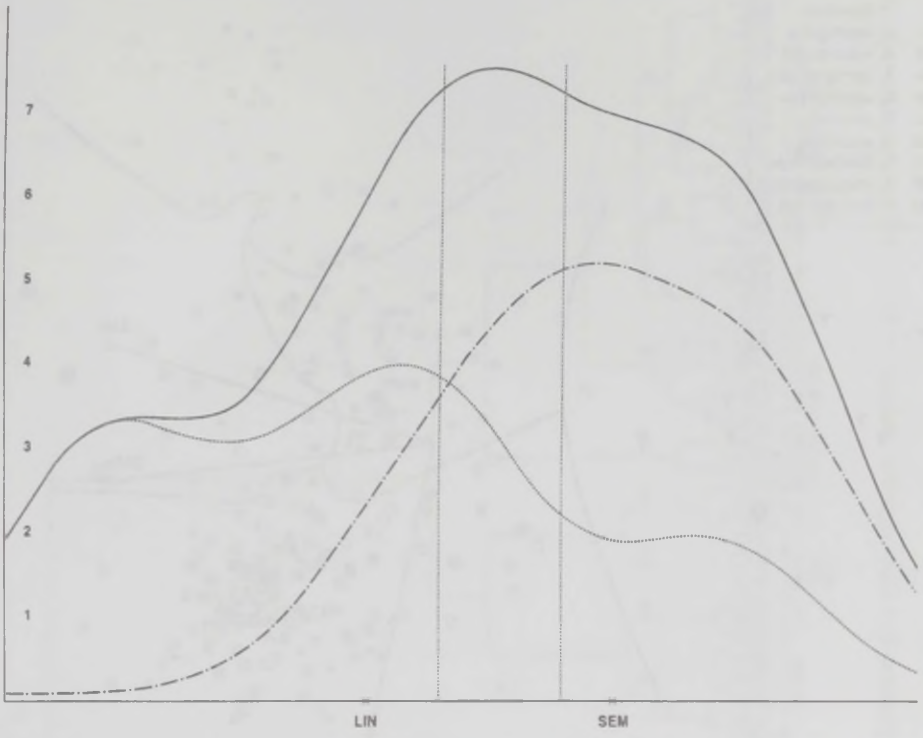

Figure 2. Projection probability distribution of the specimens of species *A. lindbergiana* and *A. semilunaris* (denoted as LIN and SEM, respectively), according to the split window method.

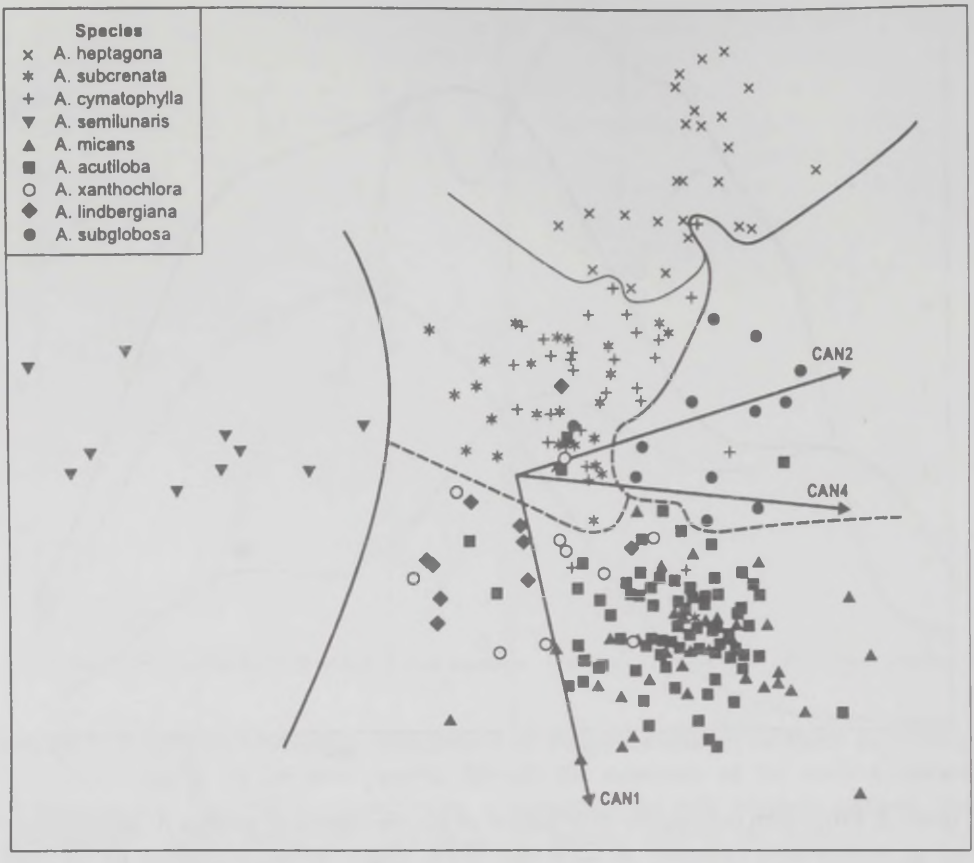

Figure 3. Ordination of data by canonical discriminant analysis. Canonical axes 1, 2 and 4 characterise 38%, 17% and 11% of the total variance, respectively. Specimens are grouped according to the conventional identification of species. *A. heptagona* is separated from the other two *Ultravulgares* species by a narrow line, a fat line indicates a borderline between sections *Ultravulgares* and *Alchemilla*, and separates *A. subglobosa* and *A. semilunaris* from the others.

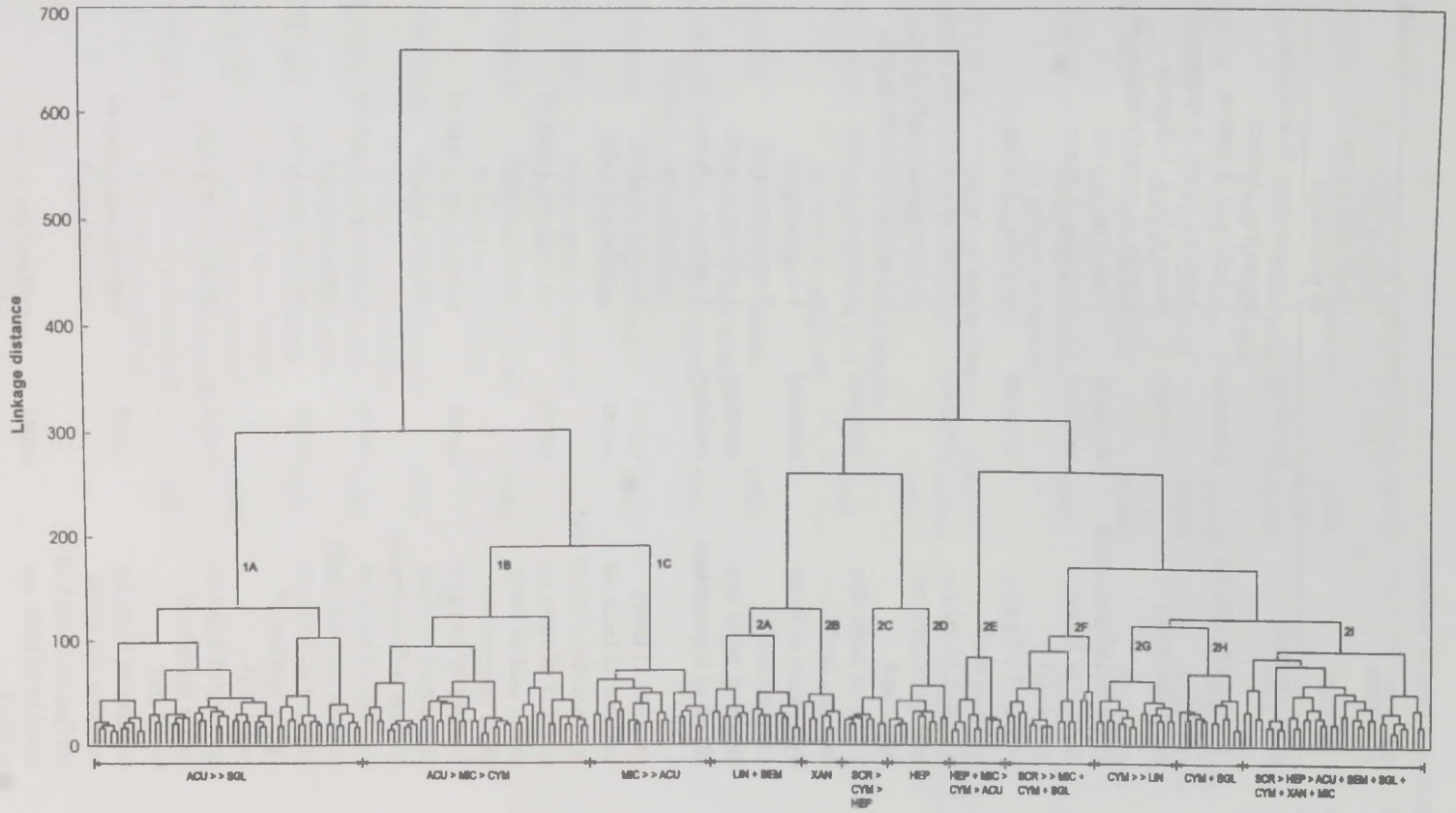

Figure 4. Dendrogram of clustering by MISSQ, with Manhattan distance as a resemblance measure. Clusters are marked at two levels, large ones with numbers 1 and 2, their subclusters as 1A, 1B, etc.

Table 1. Characters used in analysis.

Denotation	Character	Type	States or units (in brackets degree of precision)
STSH	shape of stem	nominal	1-decumbent, 2-bentform ascending ,3-erect
HRPOS	position of hairs on stem	nominal	1-deflexed, 2- patent 3-erecto-patent, 4-appressed
LEFLD	leaf foldedness	ordinary	0-not folded, 1-slightly folded, 2-strongly folded
INFSH	shape of inflorescence	nominal	1-narrow, 2-wide
FLGDN	density of flower glomeruli	nominal	1-sparse, 2-dense
LBTIP	shape of lobe tips of basal leaf	nominal	1-obtuse, 2-acute
INCDP	depth of incisions between lobes on basal leaf	ordinary	0-shallower than teeth length, 1-about teeth length, 2-much longer (twice teeth length)
THTIP	shape of teeth tips of basal leaf	nominal	1-obtuse, 2-acute
THSYM	symmetry of teeth of basal leaf	nominal	1-symmetrical 2-asymmetrical
CASH	shape of sepal tips	nominal	1-obtuse, 2-acute
HYSH	shape of hypanthium	ordinary	1-tubular, 2-funnel-shaped 3-campanulate, 4-round
LBNR	number of lobes	count	number per leaf
THNR	number of teeth on apical lobe of basal leaf	count	number per lobe
STLHR	number of hairs on lower part of stem (first internodes)	count	number per 1 mm of running length
STUHR	number of hairs on upper part of stem (below inflorescence)	count	number per 1 mm of running length
PETHR	number of hairs on petiole (of basal leaf)	count	number per 1 mm of running length
LEUHR	number of hairs on upper surface of basal leaf	count	number per 1 mm^2
LELHR	number of hairs on lower surface of basal leaf	count	number per 1 mm^2
VNHR	number of hairs on veins on the lower surface of basal leaf	count	number per 1 mm of running length
PEDHR	number of hairs on pedicel	count	number per 1 mm of running length

Table 1 (continued)

HYHR	number of hairs on hypanthium	count	number per one side
CAHR	number of hairs on sepal	count	number per sepal
LBCOR	angle between basal lobes of basal leaf	metric	angle grade (5°)
STLN	length of stem	metric	mm (5mm)
SLELN	length (radius) of stem leaf	metric	mm (1mm)
LERAD	length (radius) of basal leaf	metric	mm (1mm)
STHLN	length of tooth next to apical (of apical lobe of leaf)	metric	mm (0.1 mm)
HYLN	length of hypanthium	metric	mm (0.1 mm)
CALN	length of sepal	metric	mm (0.1 mm)
RPETLN	petiole length divided to stem length	ratio	
RSLELN	length of stem leaf divided to length of basal leaf	ratio	
CLESH	leaf length (radius) divided to leaf width	ratio	
RLBLN	lobe length divided to leaf radius	ratio	
CLBSH	lobe length divided to lobe width	ratio	
RLBWD	lobe width divided to leaf width	ratio	
TSTHLN	length of apical tooth divided to length of next tooth	ratio	
TTHLELN	length of apical tooth divided to leaf radius	ratio	
STHSH	length of side tooth divided to its width	ratio	
CHYSH	hypanthium length divided to its width	ratio	
RCALN	sepal length divided to hypanthium length	ratio	
ROCALN	length of outer sepals divided to length of inner sepals	ratio	

Table 2. Coefficients of indistinctness between conventionally identified species. Species are considered to be indistinct, if CI>5, which corresponds to the significance level 0.05. For denotations of species names, see "Material and methods".

CYM	8.42							
SCR	0.00	18.84						
SEM	0.00	0.00	0.00					
ACU	0.00	0.00	0.00	0.00				
MIC	0.00	0.00	0.13	0.00	0.00			
XAN	0.00	0.00	0.00	1.40	0.00	0.00		
LIN	0.00	0.00	0.00	21.60	0.00	0.00	3.39	
SGL	0.00	1.07	6.89	0.82	0.00	2.80	0.62	0.04
	HEP	CYM	SCR	SEM	ACU	MIC	XAN	LIN

Table 3. The adjacency matrix of conventionally identified species. Figures in the matrix indicate the percentage of specimens of the analysed species (rows) to which the centroid of the compared species (columns) is the nearest in the character space. For denotations of species names, see "Material and methods".

	HEP	CYM	SCR	SEM	ACU	GRA	XAN	LIN	SGL
HEP	×	72.0	–	–	–	–	–	–	24.0
CYM	43.3	×	30.0	–	10.0	–	–	–	10.0
SCR	43.3	22.2	×	–	–	11.1	–	–	37.0
SEM	10.0	–	–	×	–	–	10.0	60.0	20.0
ACU	–	–	48.8	–	×	45.0	–	–	–
GRA	–	–	5.7	–	37.1	×	–	–	51.4
XAN	20.0	–	–	10.0	10.0	–	×	60.0	–
LIN	10.0	10.0	–	70.0	–	–	10.0	×	–
SGL	8.3	25.0	25.0	8.3	–	25.0	8.3	–	×

Table 4. The importance of characters in the identification of conventionally identified species, sections and numerically established clusters, according to the stepwise discriminant analysis. Char — character, F — F-value of discriminant analysis, p — probability of F-value. Character denotations as in Table 1.

	Species		Sections		12 clusters	
Char	F	p	F	p	F	p
CLBSH	76.8 (1)	0.00	6.6(13)	0.00	46.0 (1)	0.00
LBCOR	23.3 (2)	0.00	31.2 (2)	0.00	2.9(26)	0.00
HRPOS	20.5 (3)	0.00	28.2 (3)	0.00	7.7(11)	0.00
THTIP	15.0 (4)	0.00	19.1 (4)	0.00	9.6(10)	0.00
PETHR	14.8 (5)	0.00	14.0 (5)	0.00	30.0 (2)	0.00
LEUHR	13.5 (6)	0.00	–		14.7 (5)	0.00
THSYM	11.2 (7)	0.00	8.7 (8)	0.00	5.4(13)	0.00
STLHR	9.1 (8)	0.00	5.0(16)	0.00	11.9 (7)	0.00
RLBWD	8.8 (9)	0.00	94.6 (1)	0.00	1.5(36)	0.13
STUHR	6.9(10)	0.00	5.7(14)	0.00	10.8 (9)	0.00
LBTIP	6.0(11)	0.00	–		4.3(18)	0.00
CLESH	5.7(12)	0.00	2.8(19)	0.04	4.0(19)	0.00
INFSH	4.7(13)	0.00	11.0 (6)	0.00	2.6(28)	0.00
LERAD	4.4(14)	0.00	8.0 (9)	0.00	20.2 (3)	0.00
CHYSH	4.2(15)	0.00	7.5(10)	0.00	1.8(32)	0.06
RLBLN	4.1(16)	0.00	9.2 (7)	0.00	11.1 (8)	0.00
LEFLD	3.9(17)	0.00	7.4(12)	0.00	1.9(31)	0.04
FLGDN	3.7(18)	0.00	–		4.4(17)	0.00
LELHR	3.3(19)	0.00	7.5(11)	0.00	1.6(34)	0.10
INCDP	3.3(20)	0.00	–		2.8(27)	0.00
CALN	2.6(21)	0.01	–		4.7(16)	0.00
ROCALN	2.6(22)	0.01	–		1.6(33)	0.10
PEDHR	2.6(23)	0.01	–		17.0 (4)	0.00
STHLN	2.3(24)	0.02	–		3.1(24)	0.00
STSH	2.2(25)	0.03	1.9(22)	0.13	3.1(23)	0.00
VNHR	2.1(26)	0.03	3.4(18)	0.02	5.0(15)	0.00
CAHR	2.0(27)	0.05	–		2.2(29)	0.02
STHSH	1.9(28)	0.06	2.4(20)	0.07	3.0(25)	0.00
HYSH	1.9(29)	0.06	–		–	
TSTHLN	1.9(30)	0.06	3.6(17)	0.01	1.5(35)	0.12
RPETLN	1.7(31)	0.10	–		3.9(20)	0.00
RSLELN	–		5.0(15)	0.00	–	
CASH	–		2.4(21)	0.07	–	
LBNR	–		1.9(23)	0.13	6.9(12)	0.00
HYLN	–		–		11.9 (6)	0.00
HYHR	–		–		5.1(14)	0.00
TTHLEL	–		–		3.7(21)	0.00
STLN	–		–		3.1(22)	0.00
THNR	–		–		2.1(30)	0.02
RCALN	–		–		1.5(37)	0.15

CURRICULUM VITAE

Silvia Sepp

Born on August 19th, 1966, in Pärnu, Estonia. Estonian citizen. Co-habitated, a son.
Address: Institute of Botany and Ecology, University of Tartu, Lai 40, 51005 Tartu, Estonia
Telephone: + 372 7 376 221, fax: + 372 7 376 222, e-mail: silvias@ut.ee

Education

1984	I Secondary School of Pärnu
1989	University of Tartu, Faculty of Biology and Geography, biologist-botanist
1999	MSc in botany, University of Tartu
1992–1999	PhD student at the Institute of Botany and Ecology, University of Tartu

Professional employment

1989–1992	Estonian Naturalists' Society, senior bibliographer
Since 1989	(with breaks) Institute of Botany and Ecology, University of Tartu, research assistant

Scientific work

Fields of research: plant taxonomy and evolution, especially Rosaceae and genus *Alchemilla,* molecular taxonomy; apomixis; flora and vegetation of Estonia, especially of towns (Pärnu).

Organizational activities

Estonian Naturalists' Society, vice-president of the Section of Botany
International Association of Plant Taxonomists, member

Teaching

I have participated in teaching of the following courses in TU: Evolution of Plants, Field Course in Floristics, Practical Course in Plant Taxonomy, Botany.

ELULOOKIRJELDUS

Silvia Sepp

Sündinud 19. augustil 1966 Pärnus. Eesti kodakondsus. Vabaabielus, üks laps.
Aadress: Botaanika ja ökoloogia instituut, Tartu Ülikool, Lai 40, 51005 Tartu
Telefon: 07 376 221, faks: 07 376 222, e-post: *silvias@ut.ee*

Haridus

1984	Pärnu 1. Keskkool
1989	Tartu Ülikool, bioloogia-geograafiateaduskond, bioloog-botaanik
1999	*MSc* botaanikas, Tartu Ülikool
1992–1999	doktorant TÜ botaanika ja ökoloogia instituudis

Teenistuskäik

1989–1992	Eesti LUS, vanembibliograaf
Alates1989	(vaheaegadega) Tartu Ülikooli botaanika ja ökoloogia instituut, vanemlaborant

Teadustegevus

Uurimisvaldkonnad: taimesüstemaatika, eriti roosõieliste sugukond, sh perekond kortsleht, molekulaarsüstemaatika; apomiksis; Eesti floora ja taimkate, eriti linnades (Pärnu)

Teaduslik-organisatsiooniline tegevus

Eesti Looduseuurijate Selts, botaanikasektsiooni aseesimees
Rahvusvahelise Taimesüstemaatika Assotsiatsiooni liige

Õppetöö

Õpetanud TÜs järgmisi kursusi: taimede evolutsioon, floristika välipraktika bioloogiaüliõpilastele, botaanika (farmaatsiaüliõpilastele), taimesüstemaatika praktikum

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