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**EGLÉ KÖSTER**

Morphological and genetical  
variation within species complexes:  
*Anthyllis vulneraria* s. l. and  
*Alchemilla vulgaris* (coll.)



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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers which are referred to in the text by Roman numerals:

- I Puidet, E., Liira, J., Paal, J., Pärtel, M. & Pihu, S. 2005. Morphological variation in eight taxa of *Anthyllis vulneraria* s. lato (Fabaceae). *Annales Botanici Fennici* 42: 293–304.
- II Pihu, S., Hõimra, J., Köster, E. & Pärtel, M., 2009. Environmental dependent morphological variability in seven apomictic microspecies from *Alchemilla* L. (Rosaceae). *Folia Geobotanica* 44: 159–176.
- III Köster, E., Bitocchi, E., Papa, R., Pihu, S. 2008. Genetic structure of the *Anthyllis vulneraria* L. s. l. species complex in Estonia based on AFLPs. *Central European Journal of Biology*, 3(4): 442–450.

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Author's contributions to the papers:

	I	II	III
Original idea	*		*
Study design	*		*
Data collection	*		*
Data analysis	*	*	*
Manuscript preparation	*	*	*

# INTRODUCTION

## Species complexes

In many groups of organisms one can find taxa that are recognized by different authors on different levels. Self-pollination, vegetative propagation and agamosperous reproduction can lead to the genesis of taxa with continuous characters, which – depending on the taxonomic treatment – can either be recognized as individual species or defined together as a species complex (Squirrell et al. 2002).

These taxonomically-complex groups are found in numerous genera throughout the animal and plant kingdoms and have certain biological characteristics in common (Ennos et al. 2005). A cryptic species complex is a group of species that satisfies the biological definition of species, that is, they are reproductively isolated from each other but not reliably distinguishable morphologically (Stace 1989). These complexes can but need not be parapatric; quite often they are sympatric and sometimes allopatric, and are generally characterized by the presence of uniparental lineages and reticulate evolution (Ennos et al. 2005). Uniparental reproduction (e.g. self-fertilization, apomixes or gynogenesis) is usually present within a group and hybridization occurs to some degree among its members (Ennos et al. 2005).

Examples of taxonomically complex groups can be found in many plant families and genera: *Sorbus* (Robertson et al. 2004 a, b), *Rosa* (Joly & Bruneau 2007) and others in Rosaceae, *Anthyllis* (Brullo & del Caldo 2006), *Astragalus* (Mehrnia et al. 2005, Bacchetta & Brullo 2006), *Acacia* (Newmaster & Ragupathy 2009) and others in Fabaceae, *Ranunculus* (Burnier 2009, Hörandl 2009) Ranunculaceae, *Epipactis* (Squirrell et al. 2002, Hollingsworth et al. 2006), *Dendrobium* (Adams et al. 2006), *Corallorhiza* (Barrett & Freudenstein 2009) and *Dactylorhiza* (Hedrén et al. 2001) in Orchidaceae, *Taraxacum* and *Chondrilla* (Van Dijk et al. 2003) in Asteraceae, *Euphrasia*. (French et al. 2005) in Orobanchaceae, etc.

However, in some cases these species can be distinguished by micromorphology or anatomy. Still, the distinction of individual species within the complex is usually reliable only through non-morphological data, such as DNA sequence analysis, or thorough life history studies.

But, as taxon borders are themselves diffuse, even large amounts of genetic data will often not result in identification of discrete species. In cases of cryptic species complexes, genetic markers are more suitable as tools for elucidating the evolutionary processes generating taxonomic biodiversity, rather than being an extra source of taxonomic characters (Ennos et al. 2005).

The evolution of many groups of organisms that do not fit comfortably into conventional taxonomic frameworks might have been caused by deviations from random sexual mating systems (Stace 1998). One of the problems in resolving species complexes might be related to the fact that many of these

complexes consist of hybrids (Joly & Bruneau 2007). Hybrids bridge gaps between species, but they also can distort the true species relationships in phenograms (Heiser et al. 1965, Jensen and Eshbaugh 1976, McDade 1997). Although no clear evidence of hybridization impact on species complexes has been detected on the diploid level, it certainly complicates delimitating species boundaries at the polyploid level (Diamond 1992, Joly & Bruneau 2007).

Agamospermous polyploid complexes also pose challenging questions for both evolution and classification (Hörandl et al. 2009). Agamosperms are thought to be genetically invariant lineages originated by hybridization, and polyploidization of sexual species later fixed by apomixis (Stebbins 1950). Studies of population genetics show considerable genetic diversity within and among populations of agamospermous species (Hörandl & Paun 2007). Still, in many cases the pollen remains functional and facultative sexual reproduction (backcrossing of apomictic individuals with sexual individuals, hybridization between apomictic lineages and facultative sexuals) has been demonstrated in several apomictic complexes (Asker & Jerling 1992, Hörandl & Paun 2007, Hörandl et al. 2009). Therefore, apomicts with varying levels of stability and possessing distinct morphological, ecological and geographical features can be found in many genera (*Taraxacum*, *Rubus*, *Hieracium*, *Ranunculus*) (Van Dijk 2003, Hörandl 2006, Fehrer et al. 2007, Hörandl et al. 2009).

Species complexes can also be described as functional classes of organisms, which include taxonomic or ecological variants that replace one another across habitats or geographic regions, potentially providing advantages over individual species as indicators (Keddy 1992, Brazner et al. 2007). Still, geographic influences can be difficult to distinguish from taxon-level responses to climate, vegetation patterns and human disturbance, and differences in geographic influences on species and functional indicators will depend on the species selected (Brazner et al. 2007).

Hybridization and geographic distribution can be often related (Ennos et al. 2005). Endemic lineages formed by hybridization often occupy unique ecological niches that were previously unexploited, and individual lineages can be successful and have wider geographical distributions than their progenitors (Dufresne & Hebert 1997, Scali et al. 2003, Stenberg et al. 2003, Van Dijk 2003, Ennos et al. 2005).

There is also evidence that agamospermous species have wider geographical distributions than sexual species, while the former have potential to colonize areas consistently despite unreliable pollinators or disturbed environments (Richards 2003). These distributions are not parapatric – sexuals and agamosperms can generally be found growing together (Bierzychudek 1985, Van Dijk 2003, Hörandl 2006). Primary causes of this might be differences in ploidy level, as agamospermy is intimately associated with polyploidy (Bierzychudek 1985, Stenberg et al. 2003).

Although morphological characters rarely resolve species complexes (Ennos et al. 2005), one can find many examples in which morphological traits are used. As genesis of species complexes is often related to anomalies in the pro-



pagation system, in many cases characters used for discrimination of taxa in these complexes are associated with fertile parts of plants (Yeo 1978, Lukaszewska et al. 1983 a, b, Karlsson 1986, Adams et al. 2006, Holeski & Kelly 2006, Joly & Bruneau 2007). Hairiness characters are also considered to be beneficial for some groups of taxa (Bradshaw 1963, Sepp et al. 2000b, Roze 2004). For morphological analysis it is suggested to use as many characters as possible, all other vegetative characters are usually used concurrently (Lukaszewska et al. 1983 a, b, Karlsson 1986, Sepp et al. 2000 b, Roze 2004, Adams et al. 2006, Joly & Bruneau 2007).

In many cases, a lack of morphological individuality has led to the use of molecular characters. Different molecular markers are used to measure genetic variation, such as amplified fragment length polymorphism (AFLP) (Hedrén et al. 2001, Adams et al. 2006, Joly & Bruneau 2007, Burnier et al. 2009, Hörandl 2009), random amplified polymorphic DNA (RAPD) (Sepp et al. 2000a), rDNA ITS sequences (Allan & Porter 2000, Nanni et al. 2004), restriction fragment length polymorphism (RFLP) (Squirrell et al. 2002) and microsatellites (Robertson et al. 2004 a, b). In addition, isozymes (Kalinowski et al. 1983 a, b, Squirrell et al. 2002, Robertson et al. 2004 a) and ploidy level analysis (Hedrén et al. 2001, Mooring 2001, Stenberg et al. 2003, Joly & Bruneau 2007) are sometimes exploited.

Attempts to impose species-based conservation on such taxonomically complex groups are proving untenable. Species delimitation for so-called taxonomically complex groups can be ambiguous even for trained taxonomists. One solution could be to protect whole complexes (Ennos et al. 2005). Another possibility would be to protect habitats, which is sensible only if the complex is subsisting in limited conditions (Dolan 1995).

## Objects of analysis

The investigative objects of the present work are two species complexes: *Anthyllis vulneraria* L. *sensu lato* and *Alchemilla vulgaris* L. (coll.): both growing throughout Europe and well abundant in Estonia.

### *Anthyllis* L.

The genus *Anthyllis* L. (Fam. Fabaceae Lindl.) is one of eight genera in the tribe *Loteae* DC. and has four subgenera: *Barba-Jovis* V. N. Tikhom. et D.D. Sokoloff, *Terniflora* V. N. Tikhom. et D. D. Sokoloff, *Anthyllis* and *Cornicina* (DC.) Akulova ex V. N. Tikhom. et D. D. Sokoloff (Cullen 1986, Sokoloff 2003). *Anthyllis vulneraria* L. *s. l.* belongs to the subgenus *Anthyllis* (Cullen 1986). *Anthyllis* is related closely morphologically and molecularly to the genus *Hymenocarpus* (Polhill 1994). This relationship has been confirmed in later

studies (Allan & Porter 2000, Allan et al. 2003), which substantiate the sister group relationship between *Anthyllis* and *Hymenocarpus xerxinnatus* (L.) Savi.

The exact number of *Anthyllis* species is controversial and depends on the interpretation of their morphological-geographical boundaries with respect to active speciation and hybridisation (Yakovlev et al. 1996). The genus is considered to range from 25 (Cullen 1986) to 60 (Minjaev & Akulova 1987) species. Although some species in the genus are well defined and universally accepted, many cryptic forms have been subject to different interpretations. Cullen (1968) divided *A. vulneraria* s. l. into three groups: subsp. *vulneraria*, subsp. *maritima* and subsp. *polyphylla*. In a subsequent revision of the group, 35 subspecies are recognized, some of which are further divided into varieties (Cullen 1986). Most authors do not differentiate all subspecies of *Anthyllis vulneraria* s. l. (Couderc 1971, 1975, 1980, Kalinowski et al. 1983a,b, Lukaszewska et al. 1983a, b, Kropf et al. 2002, van Glabeke et al. 2006), although different subspecies usually co-exist (Eichwald et al 1956, Krall 1983, Eglite & Krall 1996, Roze 2004).

The leading taxonomical approach in Estonia has been to interpret all taxa as species (Krall 1983, Eglite & Krall 1996, Kukk 1999, Leht 2007). Juzepczuk (1945) distinguished eight taxa in the Baltic Sea area. Subspecies are given with equivalents: *Anthyllis vulneraria* subsp. *vulneraria* – *Anthyllis vulneraria* L. s. str., *Anthyllis vulneraria* subsp. *polyphylla* (DC.) Nyman – *Anthyllis macrocephala* Wend. and *Anthyllis arenaria* (Rupr.) Juz., *Anthyllis vulneraria* subsp. *maritima* (Schweigg.) Corb. – *Anthyllis maritima* Schweigg., *Anthyllis vulneraria* var. *coccinea* L. – *Anthyllis coccinea* (L.) Beck. In addition, three hybrids are defined as species: *Anthyllis* x *colorata* Juz., *Anthyllis* x *baltica* Juz. ex Minjaev & Kolczkova and *Anthyllis* x *polyphyloides* Juz. (Juzepczuk 1945). As a species-based approach is traditional for given study area, it is also used in this study.

*Anthyllis vulneraria* L. s. l. occurs from the Volga River to England and from Northern Europe (including Iceland) to the Mediterranean area (North Africa) (Cullen 1986, Hultén & Fries 1986a). It has also been introduced into North America and New Zealand (Hultén & Fries 1986b).

Different authors have suggested that a different reproductive biology in the genus, such as autogamy, allogamy and geitonogamy, would make a mixture of reproduction modes possible, and for *A. vulneraria* s. l. various modes may prevail at different locations and/or infraspecific taxa (Couderc 1975, Duke 1983, Cullen 1986, Navarro 1999, 2000). Couderc (1980) distinguished between two breeding systems in *Anthyllis*: herbaceous species with autogamy and woody species with protandry precluding autogamy. Although the floral morphology of *A. vulneraria* apparently is adapted to cross-pollination by means of its pollen presentation mechanisms of shooting stamens and stigmas (Navarro 1999), it has been shown that the shooting mechanism also promotes autogamy (Couderc & Corenflot 1978). For example, in some subspecies of *A. vulneraria*, including subsp. *vulgaris*, the precocity of autogamy prevents cross-fertilization (Couderc 1971). Still, Navarro (1999) has shown that autogamy is

precluded in some populations by strong protandry, although he does not exclude geitonogamy.

No study has been undertaken to estimate the relative importance of autogamy and cross-fertilization in habitats of Estonia (Krall 1983). Three hybrid taxa in Baltic Sea area have been distinguished: *A. x colorata*, *A. x baltica* and *A. x polyphyloides* (Juzepczuk 1945, Eglite & Krall 1996, Kukk 1999, Leht 2007, Roze 2004).

Morphologically, *A. vulneraria* s. l. measures up to the essence of species complex as most of the studies acknowledge the difficulties of resolving these taxa according to morphological traits (Couderc 1971, 1975, Krall 1983, Lukaszewska et al. 1983a, b, Roze 2004). Still, several characters resolving *Anthyllis* species complex can be named. Bicoloured rufous calyx teeth demarcate *A. vulneraria*, s. str., *A. coccinea*, *A. x baltica* and *A. x colorata* from the other four species, which have concolorous, green calyces. Another readily detectable characteristic is hair disposition on the stem and petiole. *Anthyllis macrocephala*, *A. x polyphyloides* and *A. x colorata* have patent hairs on the stems and petioles, whereas the other species have appressed hair (Cullen 1968, Garcke 1972, Hegi 1975, Krall 1983, Eglite & Krall 1996, Leht 2007). *Anthyllis maritima* can be distinguished from the other species with concolorous calyces by sericeous calyx pubescence and some inflorescences with few flowers (sometimes not fully developed) (Krall 1983, Eglite & Krall 1996, Roze 2004). Inflorescences of this species also feature long peduncles. *A. arenaria* has well-developed inflorescences that are sessile (Eglite & Krall 1996, Leht 2007). Branches of this species form an acute angle with the stem (Roze 2004). *A. coccinea* is most readily distinguished from the species with bi-coloured calyces by its red corollas (Cullen 1968, Krall 1983, Eglite & Krall 1996, Leht 2007). *A. x baltica* has also some undeveloped inflorescences in axilla, such as *A. maritima* (Eglite & Krall 1996, Roze 2004). *A. vulneraria*, s. str., has unbranched stems and mainly apical inflorescences (Juzepczuk 1945, Eglite & Krall 1996, Leht 2007). There are also many infrequently used characteristics found in other studies (Cullen 1968, Lukaszewska et al. 1983a, b, Krall 1983).

*A. vulneraria* s. lato has been genetically investigated more than *Alchemilla vulgaris* (coll.). Several studies have been made to determine molecular traits that would resolve the complex. Still, the results of different molecular analysis of *A. vulneraria* s. lato are quite contentious (Kropf et al. 2002, Nanni et al. 2004, Honnay et al. 2006).

Molecular phylogeny of the genus was studied based on the sequences of the internal transcribed spacers ITS1 and ITS2 of the nuclear ribosomal DNA of ten *Anthyllis* species, including eleven subspecies of *A. vulneraria* and three subspecies of *A. montana* (Nanni et al. 2004). Additionally, polymorphic chloroplast SSR (simple sequence repeats) were used to quantify the genetic variation of *Anthyllis*. ITS sequences discriminate between some subspecies of *A. vulneraria*, but this genetic differentiation is inconsistency with taxonomic delimitation, based on morphological characters. cpSSRs showed some minor differences within *A. vulneraria*. These results suggest that the classification of

the subspecies of *A. vulneraria* should be revised according to their phylogenetic relationships (Nanni et al. 2004).

Kropf et al. (2002) investigated the ITS regions of nuclear ribosomal DNA by sequencing multiple accessions of *Anthyllis montana* L. and some closely related taxa. The ITS phylogeny implies a western Mediterranean origin followed by an eastward migration (Kropf et al. 2002). In addition, they analysed AFLP from 71 individuals of *A. montana* and revealed a major genetic (west/east) subdivision, probably caused by massive glaciations in the Alps during the last glacial period (Kropf et al. 2002). Also morphometric analysis (mainly based on floral characters) of *A. montana* reflected this major west-east differentiation and showed an intraspecific morphological differentiation in the context of the relatively recent biogeographical history (Kropf 2008).

Honnay et al. (2006) studied habitat fragmentation effects on the population genetic structure of *A. vulneraria* in the Viroin valley in southern Belgium. Their data show that the consequences of habitat fragmentation for genetic differentiation and diversity of *A. vulneraria* are relatively minor (Honnay et al. 2006). The same populations were used by Van Glabeke and workgroup (van Glabeke et al. 2006) to analyze the reproductive system of *A. vulneraria* by polymorphic microsatellite markers. The results of the study were not in accordance with a predominantly autogamous reproductive system (van Glabeke et al. 2006).

Also Kalinowski et al. (1983a, b) analyzed the geographic impact on the isoenzymatic variability of *A. vulneraria*, s. l. populations in Poland to determine whether there exists any differentiation between populations of inland and coastal areas. Different multivariate statistical methods all confirmed significant differences among populations depending on geographic distance (Kalinowski et al. 1983a, b).

The influence of ecological factors on *A. vulneraria* s. l. has not been studied so thoroughly. Becker (1912) distinguished two growth forms of *A. vulneraria*, s. l., (*Vulgaris* and *Vulneraria* types), which usually grow in similar conditions, but *Vulgaris* prefers moister habitats than *Vulneraria*. He also claimed that corollas are usually yellow in moister conditions, but principally red in dryer habitats. Despite its age, this research contradicts all traditionally used characteristics to distinguish *Anthyllis* species.

It can be said that all species in this genus inhabit relatively dry, open places (Cullen 1986). They are not particularly specialized to soil type or structure and grow equally well from sealevel to the high mountainous regions of the Pyrenees and Alps (Duke 1983). Estonian habitats are similar to those in Central and Southern Sweden, where several varieties of *Anthyllis* have been described and where populations of *Anthyllis* occur typically as hybrid complexes (Jalas 1950).

It is not so widespread to recognize *Anthyllis vulneraria* s. l. as a species complex; although the complexity of this taxon is evident (Krall 1983, Couderc 1975, Roze 2004). The species complex approach is used in some cases (Cullen 1976). Most authors do not differentiate subspecies of *Anthyllis vulneraria* s. l.

(Couderc 1971, 1975, 1980, Kalinowski et al. 1983a, b, Lukaszewska et al. 1983a, b, Kropf et al. 2002, van Glabeke et al. 2006), although usually different subspecies grow together (Eichwald et al. 1956, Krall 1983, Eglite & Krall 1996, Roze 2004). *A. vulneraria* has also been included in several molecular studies (Kropf et al. 2002, Nanni et al. 2004, Honnay et al. 2006), but in most cases the molecular variation between different subspecies has not been analyzed. The level of AFLP variation between and within seven intraspecific taxa of *Anthyllis vulneraria* s. l. in Estonia was studied.

### *Alchemilla* L.

Genus *Alchemilla* L. belongs to the family *Rosaceae* Juss., subfamily *Rosoidae* Focke, in the *Fragariinae* clade (Eriksson et al. 2003). In a strict sense it is widespread mainly in Eurasia (Rothmaler 1937, 1941; Fröhner 1995, Tihomirov et al. 1995), but according to molecular phylogeny (Gehrke et al. 2008), the genus should be treated in a wide sense, including also *Aphanes* and *Lachemilla* and thus it becomes cosmopolitan.

According to Linné, Genus *Alchemilla* L. was divided into three species: *A. vulgaris* L., *A. alpina* L. and *A. pentaphyllea* L. (Walters 1972). Nowadays this genus in a strict sense consists of over 1000 species and microspecies (Fröhner 1995, 1999); about 300 species have been described in Europe, most of which are thought to be microspecies of *A. vulgaris* species complex (Walters 1972, 1986). In Estonia 24 microspecies of *Alchemilla* have been identified, all belonging to the species complex *A. vulgaris* (Kukk 1999, Leht 2007).

As species of *Alchemilla* are relatively difficult to distinguish, accurate determination acquires material from the entire plant (Kask et al. 1966). Some characters as hairiness can change during the vegetation period (Eichwald et al. 1956). Fröhner (1995, 1999) suggests that the microspecies differ slightly but these differences are stable due to agamospermy and suggests that describing new species should be continued. In contrast, Turesson (1943, 1956, 1957) has demonstrated experimentally that the morphologically informative characters are very variable within the microspecies. The morphological differences between populations within a species are sometimes greater than inter-specific differences (Turesson 1943). Later works have also described high intraspecific variability of morphological characters and the indistinctness of microspecies (Sepp and Paal 1998, 2001).

The most important characters discriminating Estonian *Alchemilla* species are: hairiness of hypanthia, pedicels and leaves and arrangement of hair on leaves, shape of leaf lobe, hairiness of leaf veins on lower surface of leaves, angle between the basal lobes of leaf, length of leaf lobe apical tooth compared to the others, foldedness of leaves etc. (Eichwald et al. 1956, Kask et al. 1966, Leht 2007).

Molecularly *Alchemilla* has been investigated to a small degree and one can find few works describing molecular variation of the genus. The main work of

Gehrke and co-workers (Gehrke et al. 2008) presents the first molecular phylogeny of *Alchemilla* and gives a very good general overview of the genus. However, because it is so general, including also *Aphanes* and *Lachemilla*, the choice of microspecies of *Alchemilla vulgaris* (coll.) in analysis is not large and relationships within that are not really clarified. Additionally, RAPD method has been used a couple of times at a smaller scale (Baeva et al. 1998, Sepp et al. 2000a).

The agamospermous mode of reproduction in this genus was already evident at the beginning of the 20th century (Murbeck 1901, Strasburger 1905). Lady's mantles lack fertile pollen and they reproduce asexually, by somatic parthenogenesis (Koltunow 1993). However, at least some microspecies have some fertile pollen, thus hybridisation may occur (Glazunova 1977, 1983, 1987, Izmailow 1984, 1986, 1994a, b).

Agamospermous reproduction is also considered to be one reason why hundreds of microspecies have been described in genus and discrimination of these taxa is problematic (Fröhner 1995, 1999, Sepp 2000). In addition, *Alchemilla* species are highly polyploid and the number of chromosomes varies even within a single species (Turesson 1957, Löve & Löve 1961, Bradshaw 1963, Wegner 1967). It is presumed that most of the microspecies known probably result from relatively recent hybridisation events (Eichwald et al. 1956, Fröhner 1975, 1995).

It is known that – compared to sexually breeding species – agamosperms are less sensitive to environmental conditions and less varying (Bierzuchudek 1989). In many papers morphology and habitats are considered together to clarify geographical effects to species complexes (Dufresne & Hebert 1997, Peck et al. 1998, Mooring 2001, Dijk 2003, Stenberg et al. 2003). *Alchemilla* is no exception and some attempts to find correlation between habitat and morphological variation have been documented (Turesson 1943, 1956, 1957, Bradshaw 1963, Tihomirov 1967, Fröhner 1995, 1999). Still, the results are quite controversial.

The latest studies concerning *Alchemilla* have mainly been cytotaxonomic and floristic (e.g., Thiel 2004, Hayirlioglu-Ayaz et al. 2006, Hayirlioglu-Ayaz & Inceer 2009), or pharmacological (e. g., Shivastava et al. 2007, Falchero et al. 2009).

Different works made on *Alchemilla* give us generally an auspicious point of view; still some gaps remain in the whole picture. Also, several studies analyze the environmental influence on this species complex, results of which are controversial and further consideration is needed.

## Objectives

The present study was performed to investigate the morphological variation of *A. vulneraria* s. l. and *A. vulgaris* (coll.). As visually subtaxa of these two are

hardly separated, a morphometrical study is presented in order to address questions about the variability of taxa. Also, I tried to evaluate the efficacy of different morphological characters to identify the taxa.

To assess the plasticity of morphological characters under different environmental and experimental conditions and to determine how sensitive *Alchemilla* microspecies are to environmental changes, specimens were grown under manipulated conditions in a common garden experiment.

As molecular data are often recommended for distinguishing hardly recognizable taxa within species complexes, a molecular study of *Anthyllis vulneraria* s. l. was carried out. The level of AFLP variation between and within seven intraspecific taxa of *Anthyllis vulneraria* s. l. in Estonia was investigated and the genetic differences of the taxa between four regions of contrasting edaphic and climatic conditions were quantified.

The specific aims were:

- i) to assess the morphological variation in *A. vulneraria* s. l. and *Alchemilla vulgaris* (coll.) species complexes;
- ii) to evaluate the efficacy of different morphological characters for identifying subtaxa of these two species complexes;
- iii) to estimate the differentiation of the intraspecific taxa under investigation;
- iv) to assess the plasticity of characters used to discriminate *Alchemilla* microspecies under different environmental and experimental conditions;
- v) to investigate the level of AFLP variation between and within intraspecific taxa of *A. vulneraria* s. l. and to quantify the genetic differences of these taxa between four regions of contrasting edaphic and climatic conditions.

## MATERIAL AND METHODS

### Plant materials of *Anthyllis*

Eight taxa belonging to *A. vulneraria* s. lato species complex were investigated: *A. arenaria* (Rupr.) Juz., *A. coccinea* (L.) Beck, *A. macrocephala* Wend., *A. maritima* Schweigg. and *A. vulneraria* L., s. str., *A. x colorata* Juz., *A. x baltica* Juz., *A. x polyphyloides* Juz.

For morphometric measurements of *Anthyllis vulneraria* s. l., herbarium material (198 individuals) 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 the Estonian Natural Museum (TAL) was used. In addition, the analysis included 300 specimens collected from different localities in Estonia in July 2001 and June 2002 (I).

Intraspecific genetic variation was analyzed of the seven above-mentioned taxa. As no materials of *A. x polyphyloides* were found, this taxon was excluded from molecular analysis. Specimens were collected in 2004 from four regions in Estonia. The four regions of collection were defined to analyze the genetic differentiation along a geographic gradient (Table 2 in III). The division was based on floristic division of Europe, climate conditions and bedrock (Eilart 1963, Raukas 1995, Kull et al 2002). Altogether 58 specimens were collected and included in AFLP analyses (III).

### Morphological analysis of *Anthyllis*

Twenty-three diagnostic characteristics were selected for analysis (Table 3 in I). Measurement techniques details are given in Article 1. In addition, ratios of length and width were calculated for the leaves, bracts and calyces.

General linear models (GLM, StatSoft Inc., 2001) were used to analyse metric and counted variables and their differences among species. As the second step in GLM analysis, Tukey's HSD test was used to estimate species differences by characters. Nominal and ordinal characteristics were analysed with nonparametric tests (Kruskal-Wallis ANOVA).

Linear discriminant analysis (StatSoft Inc., 2001) was used to determine the subset of characteristics that reliably distinguish the species. To achieve sub-optimal classification, iterative discriminant analysis was performed in which the initial classification was iteratively corrected according to the posterior probabilities until all the specimens were 100% correctly reclassified according to the classification matrix. Canonical discriminant analysis was used for ordination of species groups according to canonical roots

Least squared means with confidence intervals of taxonomically important features were calculated by one-way ANOVA for reclassified species groups.



## Plant materials of *Alchemilla* and experiment

Eight species belonging to the *Alchemilla vulgaris* (coll) species complex: *Alchemilla vulgaris* L. emend. Fröhner, *A. baltica* Sam. ex Juz., *A. glaucescens* Wallr., *A. micans* Buser, *A. monticola* Opiz, *A. plicata* Buser, *A. subcrenata* Buser and *A. hirsuticaulis* H. Lindb. were collected from 17 natural populations in different districts of Estonia in the summers of 1995, 1996 and 2000 (Table 1 in II). In total, 489 individuals were analyzed. All other vascular plant species in the 100 m<sup>2</sup> area were also identified and their coverage estimated. Environmental growing conditions for the study sites were estimated using Ellenberg indicator values (Ellenberg et al. 1991), and the average values were calculated.

Plants for the common garden experiments (*A. vulgaris*, *A. monticola*, *A. micans*, *A. glaucescens* and *A. hirsuticaulis*) were collected in the summer of 2001 from nine sites in different districts of Estonia. To characterize the biotopes of the source populations, Ellenberg indicator values of the other plant species at the sites were also used (Table 1 in II).

The field experiment was carried out at the Rapla district in Estonia in 2001-2002 on a ploughed agricultural field with medium-rich soil. The experiment consisted of two parts: the common garden experiment and the manipulated experiment.

In the common garden experiment, two species – *A. vulgaris* and *A. monticola*, were collected from three different sites (Table 1 in II), altogether 288 individuals of each species. The initial states of characters were measured in 2001 before planting and the final states were measured in 2002.

For the manipulated experiment, two species pairs were chosen: *A. vulgaris* – *A. micans* and *A. glaucescens* – *A. hirsuticaulis*. A total of 288 individuals from each species were collected. The plots were manipulated in 2002 by shading, irrigation and fertilization, singly and in combination; control plots were also included, and three replications of each manipulation were carried out. More precise description of manipulations can be found in II. The characters were measured in 2001 and 2002.

## Morphometric analysis of *Alchemilla*

Altogether, 30 characters were used: 16 in the natural populations (only vegetative characters) and 27 in the experimental (Table 2 in II). All details concerning character measurement are given in II. Additionally, several ratios were calculated and used as characters in the natural populations (Table 2 in II).

In the case of the natural populations, arithmetic averages were used of the characters of all individuals of the same species within same population. In the case of the experimental populations, arithmetic averages were used of the individuals planted in the same plot (12 individuals).

At first, forward stepwise discriminant analysis in the STATISTICA package (StatSoft Inc. 2001) was carried out for both character sets (natural

populations and experiment) and only the characters incorporated in the model (F-value to enter 3.5; see Table 3 in II) were used in further analysis. Linear discriminant analysis was exploited to estimate how the microspecies could be discriminated using the character set.

The influence of the factor ‘species’, as well as factors characterizing the environmental conditions expressed by Ellenberg indicator values or by factors and their interactions on the morphological characters was estimated with generalized linear models (GLM). For the experimental data, the repeated measurements ANOVA was used to detect the changes of the characters between years in the common garden experiment. The Tukey test was used for post-hoc comparison of the means and to correct  $\alpha$  for multiple testing, the Benjamini-Hochberg method (1995) suggested by Verhoeven et al. (2005) was used.

### **Molecular analysis of *Anthyllis***

Leaf material was collected in summer 2004. The leaves were dried in silica gel and crushed in a MM300 Mixer Mill (QIAGEN GmbH, Hilden, Germany). DNA was extracted using QIAGEN DNeasy 96 Plant Kit. The quality of DNA was deemed to be sufficient for the AFLP technique.

In addition, DNA of two species from *Fabaceae* family, *Lotus japonicus* and *Robinia pseudacacia*, was extracted and analysed with the AFLP technique, and used as outgroups.

A mass of 200ng of genomic DNA was digested with EcoRI and MseI, and double-stranded EcoRI and MseI adapters were ligated to the ends of the fragments (Vos et al. 1995). In the following two-step amplification, primers with one selective base (E+0, M+A) were used. In the last amplification step primer combinations: A14/P06, A05/P09 and C15/P13 were used (sequences of these given in III).

Selective amplification products were separated on 6% polyacrylamide gels (40% Acrylamide/Bisacrylamide, buffer TBE 1X and urea) on Genomix SC System (Beckman, Paulo Alto, CA. USA). Fluorescent fragments were scored on gels by visual observation.

Total genetic diversity was partitioned among regions and the seven taxa of *A. vulneraria* s. l. by carrying out a hierarchical analysis of molecular variance (AMOVA) based on pairwise genetic distances (Weir & Cockerham 1984, Excoffier et al. 2005). Allele frequencies and expected heterozygosity across the total dataset were used to calculate Nei's (1973) gene diversity. Regional genetic variation was estimated on the basis of Nei's (1972) genetic identity and genetic distance with Population Genetic Analysis (POPGENE 1.31).

Genetic relationships of analyzed individuals were visualised with the software package TREECON (Van de Peer and De Wachter 1994) by analysis of neighbour joining and simple matching. Neighbour-joining analysis was based on the genetic distances of Nei and Li (1979).

## RESULTS

### Morphometrical analysis of *Anthyllis*

Results of the univariate GLM analysis of metric and counted interval characteristics show that four species, *A. vulneraria*, *A. coccinea*, *A. x baltica* and *A. x polyphyloides*, are distinguishable from the other species (Table 4 in I). However, other species remain indistinguishable using the diagnostic characteristics. Characteristics that do not differ between any of the species pairs are ratio of leaf length and width, ratio of calyx length and width, and ratio of hypsophylls length and width.

Most of the nominal and ordinal characteristics are statistically significant (Table 5 in I). The Kruskal-Wallis test shows that only the shape of rosette leaves fails to distinguish species significantly. Linear discriminant analysis of all characteristics reveals that only 11 are statistically significant (Table 5 in I). Five metric characteristics are statistically significant according to GLM and discriminant analysis.

A scatterplot of canonical roots shows distinguishable mono-specific groups of *A. coccinea* and *A. x colorata* (Fig. 1 in I) and three overlapping pairs of species: *A. vulneraria* – *A. x baltica*, *A. arenaria* – *A. maritima* and *A. macrocephala* – *A. x polyphyloides*. After iterative canonical analysis with correction of identification, the ordination plot of canonical roots shows seven of the eight species form distinctive clusters (Fig. 2 in I). Cluster edges overlap to some extent, but most of the overlap is caused by the variability of *A. maritima*.

A comparison of the initial and final classification matrix reveals that *A. coccinea* is classified 100% correctly (Table 6 in I). Misclassification rate of other analysed species is from >90% to 3.4%. The most poorly classified species is *A. x polyphyloides*.

The least-square predictions of means of characteristics by species according to univariate ANOVA are presented in I Table 7. Mean plant height, number of inflorescences, number of stems, and leaf length differentiate species most successfully (Table 7 in I).

### Morphometrical analysis of *Alchemilla*

The forward stepwise discriminant analysis takes into the model 11 characters from the natural populations and 11 characters from the experiment if the F to enter is set to 3.5 (Table 3 in II).

According to the GLM, the factor ‘species’ is statistically significant for variability for five of eleven morphological characters, which are taken into model by discriminant analysis, and also for four of six ratios (Table 4 in II). Among the Ellenberg values, only light and moisture are found to be the statistically significant factors for at least some characters (Table 4 in II).

Many characters change in the common garden condition, even without manipulation (Table 5, in II). However, in several cases, one or two species are distinct from the others: the radius of the basal leaf in *A. monticola*, as well as the length of the middle leaf lobe changes opposite to that of the other species (Fig. 2 in II).

In the manipulated experiment the factor ‘species’ is significant for all analyzed characters. Fertilization affected five characters (Table 6 in II). Irrigation and shading have no significant effects. For two characters, the joint effect of fertilization and species is significant (Fig. 3 in II). Most of the characters change uniformly in different species. In some cases, one or two species change differently than others (Fig. 4 in II).

In the natural populations, *A. monticola* and *A. glaucescens* are the least reclassified, less than 10% (Table 7 in II). Contrastingly, more than half of the *A. micans* specimens and almost half of the *A. subcrenata* and *A. plicata* specimens are reclassified.

Before the common garden experiment in 2001, of 263 individuals of *A. hirsuticaulis*, one is reclassified as *A. glaucescens* and 19 of 305 from *A. glaucescens* to *A. hirsuticaulis*. Out of 258 individuals of *A. vulgaris*, 12 are reclassified as *A. micans* and 24 of 274 from *A. micans* to *A. vulgaris*. In 2002, the reclassification changes little, mainly occurring within the species pairs and in nearly the same proportions. Results of reclassifications are not taken as basis of further analysis, as the set of characters used is not representative.

## Molecular analysis of *Anthyllis*

The three AFLP primer combinations result in a dataset containing information on 131 AFLP loci for 58 samples and two outgroup specimens. Genetic characteristics obtained with AMOVA analysis for the seven subspecies and four regions are presented in Tables 3 and 4 (III). The maximum value of Nei’s gene diversity is 1.0 (Lowe et al. 2004). In my case the mean value for each analyzed region and taxon was less than 0.2 (see Tables 3 and 4 in III).

According to  $F_{ST}$ , only populations of the West and North do not differ significantly. All other p-levels of  $F_{ST}$  for regions are  $< 0.05$  (Table 2 in III).  $F_{ST}$  values of taxa are significant for distinguishing *A. vulneraria* from *A. maritima*, *A. x baltica* from *A. x colorata* (Table 4 in III).

Analysis of genetic variation based on Nei’s (1972) genetic distances support the results of AMOVA analyses of specimens from the four regions (Fig. 1 in III). Although the UPGMA dendrogram accentuates differences between Island and East regions of Estonia, values of genetic identity are very high and genetic distances between regions are insignificant (Table 5 in III).

A phenogram of all AFLP phenotypes based on a simple matching technique using the UPGMA clustering method (Fig. 2 in III) resolves all the *A. vulneraria* s. l. individuals in a single cluster, but the bootstrap support is less than

25%. The UPGMA dendrogram for *A. vulneraria* s. l. comprises many weakly supported clusters and no regional or taxonomic groups could be distinguished.

The same tendency can be seen on the neighbour-joining tree calculated from the genetic distances of Nei and Li (1979) (Fig. 3 in III).

## DISCUSSION

### Differentiation of taxa within *A. vulneraria* s. l. and *A. vulgaris* (coll.)

Results of morphological analyses support the deduction of the number of taxa inside *A. vulneraria* s. l. from eight to five: *A. coccinea*, *A. vulneraria*, *A. maritima*, *A. x colorata* and *A. macrocephala*. In morphometric analysis *A. coccinea* and *A. x colorata* are distinctly different from the others, which supports the distinction of two well-recognized taxa. Strongly expressed morphological similarity between all other species indicates the delimitation of a single species, *A. vulneraria*, s. l. (Cullen 1968, Hegi 1975, Lukaszewska et al. 1983c). The formation of subgroups (1 – *A. vulneraria* and *A. x baltica*, 2 – *A. arenaria* and *A. maritima* and 3 – *A. macrocephala* and *A. x polyphyloides*) supports the idea of three subspecies (subsp. *vulneraria*, subsp. *maritima* and subsp. *polyphylla*) proposed by Cullen 1968.

*A. arenaria* and *A. macrocephala* are considered to be the same taxon (*A. vulneraria* subsp. *polyphylla*) according to prevalent classification (Cullen 1968, Garcke 1972, Hegi 1975). According to the presented results these taxa did not form a common subgroup. *A. arenaria* was in the same subgroup with *A. maritima* and *A. macrocephala* joined the group with putative hybrid *A. x polyphyloides*.

Discrimination of some *Alchemilla* microspecies is also problematic but results of the current work support the distinction of the analyzed taxa. Discrimination of these seven microspecies according to the used character set is dependent on the taxa. Confusion of *A. micans* and *A. vulgaris* by both morphological and genetical characters has also occurred in earlier studies (Sepp & Paal 1998, 2001; Sepp et al. 2000a, b). Our results support these facts: *A. micans* can mainly be confused with *A. vulgaris*, and *vice versa*.

The obtained results distinguish rather well *A. monticola* from other species in natural populations and it was completely distinguishable from all other species grown. According to different authors the identification of *A. monticola* has been rather questionable. Tihomirov (1967) and Plocek (1976) claim a great variation in *A. monticola*, whereas some later studies find this species rather distinct (Sepp & Paal 1998, Sepp et al. 2000a, b). The second moderately well distinguished taxon was *A. baltica*. For this species only a restricted set of characters in natural populations was used. The discrimination could even be improved by adding hairiness characters (Sepp & Paal 1998, Sepp et al 2000b). *A. subcrenata* (included only in the analysis of the natural populations with the restricted set of characters) was also reclassified frequently, mainly as *A. vulgaris* and *A. monticola*. Similarities between *A. subcrenata* and *A. vulgaris* have been described earlier (Eichwald 1956). *A. glaucescens*, *A. plicata* and *A. hirsuticaulis*, [belonging to the section *Plicatae* Fröhner (1995) or to the section *Pubescentes* Bus. by older system (Juzepczuk 1941)], are generally distinct

according to the results presented. Some proximity of the three has also been previously noted (Sepp & Paal 1998, Sepp et al. 2000a, b). These taxa are generally well discriminated from other species.

Contrastingly, the subspecies of *Anthyllis* are not readily distinguished, while microspecies of *Alchemilla* have small differences, they are still distinct. This can be caused by different breeding strategies: *Anthyllis vulneraria* s.l. is more or less autogamous and there are many putative hybrids, *Alchemilla* microspecies have prevailing agamospermy and there are no recent hybrids. The poor discrimination of taxa is characteristic for hybrid species complexes, such as *Fallopia* (Krebs et al. 2010, and *Salix* (Kehl et al. 2008). The mainly agamospermous taxa retain their small differences.

### **Efficacy of morphological characters**

Morphometric analysis of *Anthyllis vulneraria* s. l. revealed that some easily measured characteristics (number of rosette leaves and lengths of corolla and calyx), reliably distinguish these species, although they had not been acknowledged earlier. Also, plant height, shape of the hypsophyll apices, stem hairiness, and colour of corolla and calyx, which have also been recommended by different authors as suitable characters for distinguishing *Anthyllis* taxa (Cullen 1968, Juzepczuk 1945, Eglite & Krall 1996), were significant in our analysis. Additionally, the number of stems and inflorescences and hairiness of petiole and calyx, mentioned by Eglite & Krall (1996), distinguish the analyzed species.

Most of the used characters were significant for discriminating *Alchemilla* species. Some authors consider metric characters to be the most important (Fröhner 1995), whereas others stress the importance of using as wide a set of characters as possible (Sepp & Paal 1998, 2001; Sepp et al. 2000b). Many vegetative characters used for separation of Fröhner's (1995) sections (Sepp & Paal 1998, 2001) are abundant in natural populations, for example, the angle between the basal lobes. Ratios of less frequently used characters were found to be effective in discrimination of the *Alchemilla* microspecies, as previously described (Sepp & Paal 1998, 2001; Sepp et al. 2000b). Such ratios should probably be used more often in distinction of the *Alchemilla* microspecies, since they describe shapes better than metric characters. The nominal characters usually present in species descriptions (e.g., the color of flowers and leaves), but not widely used in identification keys (Juzepchuk 1941, Fröhner 1995, Sepp 2000), did not play an especially important role in species discrimination in the experiment.

The hairiness of flowers abundant for *Alchemilla* in the experiment; the importance was especially clear in resolving closely-related species pairs in our study. Hairiness characters have also been suggested by many authors for discriminating species complexes (Bradshaw 1963, Fröhner 1995, Tihomirov et al. 1995, Sepp 2000, Sepp et al. 2000b, Roze 2004). Bradshaw (1963) claims

that there is only one suitable character for *Alchemilla*: the hairiness of its flowers, which is in accordance with obtained results. Menemen and Hamzaoglu (2002 a, b) have also used hairiness characters to distinguish and describe new microspecies of *Alchemilla*.

Overlapping characters are often used to describe species complexes (Bradshaw 1963, Yeo 1978, Lukaszewska et al. 1983 a, b, Karlsson 1986, Sepp et al. 2000b, Roze 2004, Adams et al. 2006, Holeski & Kelly 2006, Joly & Bruneau 2007). In my case, some of the characters were overlapping, but there was a set of characters in both genera that can be reliably exploited for identification of these taxa.

### **Sensitivity of characters to manipulation**

Our common garden experiment on *Alchemilla* shows a tendency for plants to grow larger than in natural populations. However, most characters changed differently in different species. Both cultivation in common garden and manipulation of conditions therein did not affect the discrimination of microspecies. The most significant manipulation was fertilization (most characters were larger under manipulation), whereas shading and irrigation did not affect most characters. This could result from a low sensitivity of *Alchemilla* to environmental conditions – *Alchemilla* having co-called general-purpose genotypes that are not very dependent on environmental conditions, also found for some other apomicts (Bierzuchudek 1989). In the case of *Alchemilla*, the variability of several metric characters can also be, as was shown by both present and other studies (Turesson 1943, 1956, 1957; Lundh-Almestrand 1958). It can be said that in general microspecies as a factor was a much more significant factor than site or population.

Earlier experimental works on species complexes are mostly associated with the breeding system (Couderc 1971, 1980, Couderc & Coernfl 1978, Navarro 1999, 2000, Mooring 2001, Holeski & Kelly 2006). The number of comparative morphometrical studies is much less. Couderc (1975) grew some subspecies of *A. vulneraria* to study the variation in leaves. He found that *A. vulneraria* s. l. is very polymorphic as a species but this polymorphism is continuous and no clear groupings can be distinguished (Couderc 1975). Turesson (1943, 1956) studied the variation in the apomictic microspecies of *Alchemilla vulgaris* s. l. and established the variability of the morphologically informative characters within the microspecies. He found that the morphological differences between populations within a species might be greater than inter-specific differences (Turesson 1943). Current experiment added the knowledge that, although there is infraspecific variability and change of characters in cultivation of *Alchemilla*, it does not affect the species discrimination.

Physiological and morphological variations of the *Alchemilla* microspecies are caused both by genetic differences (Turesson 1956) and environmental variability (Lundh-Almestrand 1958). Probably both factors are important;



while characters changed in our experiment, species discrimination remained unaffected. It has been shown that agamosperms vary less than sexual species grown under normal conditions; however, under extreme conditions they adapt more quickly (Bierzychudek 1989). Smaller variability of asexuals could be explained by genetic uniformity, but their plasticity in some cases causes wide reaction norms within a single genotype (Fehrer et al. 2007, Hörandl & Paun 2007, Hörandl 2009). My results support the view that agamospermous micro-species of *Alchemilla* have small but persistent differences

## Molecular variability in species complexes

The data show that the (sub) species of *Anthyllis* cannot be differentiated by AFLP patterns, which is in concordance with the results of Nanni et al. (2004), who concluded from analysis of ITS sequences that different subspecies of *Anthyllis vulneraria* from a different origin and geographical distribution did not show significant differences. Nanni attributed these results to a complex mating system of *Anthyllis vulneraria* s. l. (Nanni et al. 2004). Other authors have suggested that a different reproductive biology in the genus, such as autogamy, allogamy and geitonogamy (Couderc 1975, Duke 1983, Cullen 1986, Navarro 1999, 2000), would make possible a mixture of reproduction modes and for *Anthyllis vulneraria* s. l. various modes may reign at different locations.

The variability of the analyzed populations shows an east-west trend, which is probably caused by different ecological conditions (climate and bedrock differences) occurring in the west and the east of Estonia (Eilart 1963, Raukas 1995). Thus interpopulational differentiation could represent more ecotypes than subspecies.

The west-east variability in the flora and vegetation of Estonia (Eilart 1963, Raukas 1995, Kull et al. 2002) is manifested by *Anthyllis* by the *P*-values of fixation indexes, which are generally significant for geographic regions except among specimens from the islands and northern Estonia. Genetic divergence was significant for several pairs of taxa; nevertheless, low values of  $F_{ST}$  and low mean values of Nei's gene diversity reflect nearly panmictic populations (the small probability that randomly chosen copies of the same gene are from different alleles) (Lowe et al. 2004).

Analyses of AFLP data clearly demonstrate that the analyzed specimens do not comprise groups of different subspecies. Thus, based on Nei's genetic distances the results do not support the recognition of the seven subspecies. Nor do AFLP results support the distinguishing of the five subgroups within *A. vulneraria* s. l., which were distinguished according to the morphometrical analysis.

Ennos et al. (2005) have suggested that for cryptic species complexes, genetic markers are better suited as tools for elucidating the evolutionary processes generating taxonomic biodiversity and their value as an extra source of taxonomic characters is doubtful. Still, several attempts have been made to

resolve species complexes by molecular markers (Hedrén et al. 2001, Squirrell et al. 2002, Robertson et al. 2004, Joly & Bruneau 2007, Burnier et al. 2009). Some attempts to detect genetic polymorphism within and among *Alchemilla* microspecies have been made using RAPD analysis (Baeva et al. 1998, Sepp et al. 2000a). Results reveal that several microspecies could be distinguished rather easily, whereas other species (e.g., *A. vulgaris* and *A. micans*) were relatively similar to each other (Sepp et al. 2000a). Nonetheless, more research is needed to clarify the relationships in this complex genus.

Thus, the AFPL variance and earlier works (Nanni et al. 2004, Joly & Bruneau 2007, Burnier et al. 2009) clearly support not recognizing the infraspecific taxa of *Anthyllis* at the species level, nor likely as a subspecies.

## Perspectives

The results of morphometrical analysis of this work support the fact that both *Anthyllis vulneraria* s. l. and *Alchemilla vulgaris* (coll.) can be considered species complexes, while intraspecific taxa of these two are morphometrically complex and continuous.

However, based on my results the differences between investigated *Alchemilla* microspecies are stable and characters do not merge through cultivation. It can be said that the current work supports the existing system and these species are morphologically rather distinct. Additional genetic analysis would still be a benefit.

Better discrimination of *Alchemilla* microspecies can be attributed to different mating systems. As *Alchemilla* is mainly agamosperous plant, the hybridization is almost excluded. *Anthyllis* on the other hand has several hybrids, which cause the continuity in characteristics.

In contrast, obtained results support the deduction of taxa distinguished within *A. vulneraria* s. l. My attempts to solve the difficulties in distinguishing taxa within *Anthyllis vulneraria* s. l. according to morphological and molecular characteristics did not reveal clearly-grouped intraspecific variation. It was found reasonable to distinguish at the subspecies or even variety level five morphological taxa. Still, similar common garden experiment and manipulations as used on *A. vulgaris* species complex would make a better basis for final conclusions.

## CONCLUSIONS

(i) Eight subtaxa of *Anthyllis vulneraria* s. l. growing in Estonia were under investigation. It was found that according to morphological characters the discrimination of five subtaxa is justified. *A. coccinea* and *A. x colorata* were two well separated taxa. Other taxa formed three subgroups consisting *A. vulneraria* and *A. x baltica*, *A. arenaria* and *A. maritima*, *A. macrocephala* and *A. x polyphyloides*. It is noteworthy that *A. arenaria* is in one group with *A. maritima* although this species is generally considered to be a subtaxon of *A. macrocephala*.

(ii) According to my results analyzed *Alchemilla* microspecies are, in most cases, discriminated using a suitable set of characters. *A. micans* can be confused with *A. vulgaris*, although experimental data show they are distinguishable in most cases. Rather well separated microspecies were *A. monticola* and *A. baltica*. Other *Alchemilla* microspecies were also generally distinct.

(iii) Better discrimination of *Alchemilla* microspecies compared to *Anthyllis* subtaxa can be related to their different reproductive system. As *Alchemilla* is mainly an apomict, the hybridization between taxa is almost excluded. Hybrids between different subspecies may be the reason of continuity inside *A. vulneraria* s. l.

According to the morphometrical analysis, the most efficient characters to distinguish subtaxa of *A. vulneraria* are plant height, shape of the hypsophyll apices, stem hairiness, and color of corolla and calyx. Also, the number of stems and inflorescences, hairiness of petiole and calyx, number of rosette leaves and lengths of corolla and calyx distinguish these species. Hairiness characters are useful for distinguishing *Alchemilla* microspecies as well. Nominal characters often used in keys and floras (e.g., the colour of flowers and leaves) do not play an important role. In addition to traditionally used characters, ratios are effective for discrimination of *Alchemilla* microspecies.

(iv) In a common garden experiment, *Alchemilla* plants grew larger than in natural populations, but individuals of different species did not become more similar. Of different treatments (shading, fertilization and irrigation), fertilization had the most significant effect on *Alchemilla* microspecies: metrical characters increased in all species. However, the lack of effects of shading and irrigation could also be caused by stability of traits across environmental conditions and the low sensitivity of *Alchemilla* as an apomict to them. I found that changing characters did not affect species discrimination significantly.

(v) Data showed that AFLP patterns do not discriminate taxa within *A. vulneraria* s. l., thus, according to the results presented here, the recognition of seven species within *A. vulneraria* s. l. growing in Estonia is not justified. Nor do AFLP results support the distinguishing of the five subgroups within *A. vulneraria* s. l., which were distinguished according to morphometrical analysis. The variability of the analyzed populations shows an east-west trend, which is probably caused by different ecological conditions (climate and bedrock differences) occurring in eastern and western Estonia. It was found that interpopulational differentiation can be interpreted as ecotypes rather than subspecies.

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## SUMMARY IN ESTONIAN

### Morfoloogiline ja geneetiline varieeruvus liigikompleksides: *Anthyllis vulneraria* s. l. ja *Alchemilla vulgaris* (coll.)

Bioloogilisele liigi kontseptsioonile vastavalt on liik reaalselt või potentsiaalselt ristuvate isendite populatsioonide kogum, mis esineb teatud ökoloogilistel tingimustel. Taimede puhul on selline liigi käsitlus sageli sobimatu kuna taimedel esineb lisaks seksuaalsele paljunemisele asekuaalne paljunemine, hübriidiseerumine ja polüploidus. Liigikompleksid, milledest antud töös juttu tuleb, on just sellised erandid. Neid iseloomustavad vegetatiivne paljunemine, agamosperma, hübriidiseerumine ja viimasest tingitud polüploidus. Sellised kõrvalekalded seksuaalsest paljunemisest põhjustavad nende taksonite morfomeetrilise varieeruvuse langust ja üleminevate tunnuseisundite teket, mis omakorda viib taksonite käsitlemiseni erinevatel taksonoomilistel tasemetel. Liigikompleksi võibki defineerida kui taksonoomiliselt keerukat isendite gruppi millele on iseloomulik morfoloogiline kontinuum. Samuti võib neid kirjeldada kui organismide funktsionaalseid klasse, mille koosseisus olevad taksonoomilised ja ökoloogilised teisendid asendavad üksteist kasvukohtade või geograafilise asukoha vahetudes. Ökoloogiliste ja geograafiliste mõjude osakaalu on aga raske hinnata, kuna sageli sõltub see valitud liigist.

Näiteid liigikomplekside kohta võib leida nii taime- kui loomariigis üsna sageli. Taimede hulgas on tuntumad liigikomplekse sisaldavad perekonnad näiteks *Sorbus* ja *Rosa* sugukonnas *Rosaceae*, *Epipactis*, *Dendrobium* ja *Dactylorhiza* sugukonnas *Orchidaceae*, *Taraxacum* ja *Chondrilla* sugukonnas *Asteraceae*, *Euphrasia* spp. sugukonnas *Orobanchaceae* jne.

Antud töös on liigikompleksidena võetud vaatluse alla kahe sugukonna kahe perekonna liigid. Harilik koldrohi – *Anthyllis vulneraria* s. l. (*Fabaceae*) ja harilik kortsleht – *Alchemilla vulgaris* (coll.) (*Rosaceae*) sarnanevad teineteisega oma liigisiseste taksonite raskesti määratavuse ja rohkuse poolest. Morfoloogiliselt on nii hariliku koldrohu alamliigid kui hariliku kortslehe mikroliigid raskesti eristatavad ja määramistabelites kasutatavate tunnuste seisundid eristatud taksonitel üleminevad ning tihti on taksonite määramine looduses raskendatud. Paljunemistüübilt on harilik kortsleht valdavalt apomikt, kuid mõnedel mikroliikidel on leitud ka fertiilset õietolmu, mis viitab hübriidiseerumise võimalikkusele. Hariliku koldrohu puhul on võimalikuks peetud autogaamiat, allogaamiat ja geitonogaamiat ning on tõenäoline, et erinevatele piirkondadele on iseloomulikud erinevad paljunemistüübid.

Käesolevas töös analüüsiti hariliku koldrohu ja hariliku kortslehe liigikomplekside morfoloogilist ja geneetilist varieeruvust.

Hariliku koldrohu puhul kasutati looduslikest populatsioonidest kogutud isendeid ja lisaks herbaarmaterjale Eesti erinevatest herbaariumidest (TU, TAA, TAL), millel hinnati kokku 23 tunnust. Saadud mõõtmistulemusi analüüsiti

statistiliste meetodite abil (üldised lineaarsed mudelid, Tukey's HSD test, Kruskal-Wallis test, dispersioonanalüüs, diskriminantanalüüs, kanooniline diskriminantanalüüs) (I).

Ka hariliku kortslehe morfomeetrilisteks analüüsideks kasutati looduslikest populatsioonidest kogutud isendeid, kuid peamiselt analüüsiti geograafilisi ja ökoloogilisi mõjusid morfoloogilistele tunnustele. Kasvukohtade iseloomustamiseks arvutati Ellenbergi väärtarvude kaalutud keskmised vastavalt liigi ohtrusele. Keskkonna mõjude paremaks hindamiseks kontrolliti liikide eristamisel kasutatavate tunnuste püsivust erinevates populatsioonidest pärit taimedel keskkonnatingimuste suhtes. Aiaeksperimendis kasvatatud taimi mõjutati erinevate töötlustega (väetamine, varjutamine ja niisutamine ning nende koosmõjud) (II). Saadud andmete analüüsiks kasutati samuti erinevaid meetodeid (diskriminantanalüüs, üldised lineaarsed mudelid, dispersioonanalüüs, Tukey test) (II). Tasa-kaalustamiseks statistilisi võrdlusi parandati  $\alpha$  väärtusi Benjamini ja Hochbergi (1995) meetodil.

Lisaks hinnati hariliku koldrohu geneetilist mitmekesisust molekulaarsete tunnuste alusel kasutades valikulist restriksiooni fragmentide amplifikatsiooni polümeraasi ahelreaktsioonil (AFLP) (III). Analüüsideks kasutati samuti looduslikest populatsioonidest kogutud materjali. Välisrühmana kasutati *Lotus japonicus* (Regel) Larsen ja *Robinia pseudacacia* L. – harilik robiinia (*Fabaceae*) DNA-d. Amplifikatsiooni produktid eristati polüakrüülamiidgeelil ja loendati visuaalselt. Saadud andmeid analüüsiti hindamiseks geneetilist mitmekesisust taksonite vahel ja geograafilisel gradiendil (III).

Saadud tulemused toetavad mõlema uuritud liigi käsitlemist liigikompleksidena. Morfomeetrilise analüüsi tulemusel võib tõdeda, et nii *Anthyllis vulneraria* s. l. kui *Alchemilla vulgaris* (coll.) liigisiseseid taksonid iseloomustavad üleminevad tunnuste seisundid. Samas ei saa väita, et liigisiseseid taksonid ei esine.

Morfoloogiliste tunnuste põhjal võib eristada viite taksonit *Anthyllis vulneraria* s. l. liigikompleksis. Kõige paremini eritusid teistest *A. coccinea* ja *A. x colorata*. Lisaks võis eristada kolme kaheliigilist gruppi: *A. vulneraria* ja *A. x baltica*, *A. maritima* ja *A. arenaria* ning *A. macrocephala* ja *A. x polyphyloides* (Joon. 1, I). Neid alamgrupe võib võrrelda Culleni (1968) poolt eristatud alamliikidega – subsp. *vulneraria*, subsp. *maritima* ja subsp. *polyphylla*. Üllatuslik on ka *A. arenaria* ja *A. maritima* sattumine samasse gruppi. *A. arenaria* on eristatud liigina endise NSVL territooriumil, Lääne-Euroopas kuulub see liik alamliiki *subsp. polyphylla*. Viimase vaste on Ida-Euroopas kehtivas süsteemis *A. macrocephala*.

Koldrohtudele iseloomulike tunnuste varieeruvust ja püsivust võiks kontrollida ka keskkonna tingimuste suhtes nagu seda on tehtud kortslehe mikroliikide puhul (II). Selgus, et kõik uuritud kortslehe mikroliigid olid keskkonna tingimuste suhtes püsivate tunnustega nii sarnastel tingimustel kui ka erinevate manipulatsioonide (väetamine, varjutamine ja niisutamine) korral. Eksperimendi käigus kasvatatud taimed olid suuremad kui looduslikes tingimustes, viimast eriti väetatud katsealadel. Muus osas oli manipulatsioonide mõju tun-

nustele vähemärgatav. Tunnused küll muutusid, kuid muutused ei mõjutanud liikide eristatavust. Kuigi kaks liikide paari (*A. vulgaris* ja *A. micans* ning *A. glaucescens* ja *A. hirsuticaulis*) osutusid morfoloogiliselt sarnasteks, oli nende eristamine siiski võimalik (II). Kortslehe mikroliike hästi eristavatest tunnustest võiks välja tuua meetriliste tunnuste suhtarvud. Näiteks kirjeldab lehe kuju pikkusest ja laiuusest oluliselt paremini pikkuse-laiuse suhe. Ordineeritud tunnustest osutusid kasulikuks erinevate taimeosade karvasused. Värvustunnused, mida sageli kasutatakse kirjeldustes, olid vähetähtsad. Töö tulemusel võib ka väita, et erinevate mikroliikide eristamiseks tuleks kasutada erinevaid mitte ühte tüüpi tunnuseid.

AFLP andmeid kasutatakse sageli raskesti eristatavate taksonite vaheliste suhete analüüsiks. Kahjuks ei andnud meie töö oodatud tulemust. Analüüsitud isendid ei moodustanud alamliikide grappe. Ka morfomeetrilise analüüsi käigus eristunud viite liigisisest taksonit ei olnud võimalik saadud tulemuste põhjal eristada. Siiski esines teatav korrelatsioon geneetilise varieeruvuse ja geograafilise kasvukoha vahel. Viimast võib põhjendada peamiselt aluspõhja erinevusega Lääne ja Ida Eestis. Sellist geneetilise variatsiooni puudumist on põhjendatud koldrohtudele omase paljunemisega, kuid ei saa alahinnata ka ökoloogiliste tegurite mõjusid.

Kokkuvõtvalt võib öelda, et nii *A. vulneraria* s. l. kui *A. vulgaris* (coll.) on liigikompleksid, mida iseloomustavad keerukas paljunemise süsteem, üleminevate seisunditega tunnused, kompleksi kuuluvate taksonite raskestimääratavus ning koldrohtude puhul ka arvatavate hübriidide esinemine. Kortslehtede puhul on tegemist püsivate tunnustega erinevates keskkonnatingimustes ning mikroliikide eristamine seega antud tulemuste põhjal igati õigustatud. Koldrohtude liigikompleksis aga on liikide eristumine vähem tõestatud – morfomeetrilisel analüüsil saadud tulemused toetavad viie mitte kaheksa liigisisese taksoni eristamist. Samade taksonite molekulaarne analüüs aga ei erista neist ühtegi. Seega on ilmselt vajalikud edasised uuringud morfoloogiliste tunnuste stabiilsuse hindamiseks sarnastel tingimustel või erinevate manipulatsioonide korral.

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## **PUBLICATIONS**

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## II. Scientific and research activity

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Morphological and molecular variation of genus *Anthyllis*. Morphological variation and ecological impacts to the morphometrics of genus *Alchemilla*.

### Publications

- Puidet, E., Liira, J., Paal, J., Pärtel, M. & Pihu, S. 2005. Morphological variation in eight taxa of *Anthyllis vulneraria* s. lato (Fabaceae). *Annales Botanici Fennici* 42: 293–304.
- Köster, E., Bitocchi, E., Papa, R., Pihu, S. 2008. Genetic structure of the *Anthyllis vulneraria* L. s. l. species complex in Estonia based on AFLPs. *Central European Journal of Biology*, 3(4): 442–450.
- Pihu, S., Hõimra, J., Köster, E. & Pärtel, M., 2009. Environmental dependent morphological variability in seven apomictic microspecies from *Alchemilla* L. (Rosaceae). *Folia Geobotanica* 44: 159–176.

**Grants and nominations.**

Archimedes Foundation, Kristjan Jaak grant 2005.

**III. Supervisions and teaching****Supervisions**

2007 Jane Oja – Hariliku koldrohu (*Anthyllis vulneraria* L. s. lato, Fabaceae) tolmlimine ja noortaimede morfoloogiline varieeruvus. (bakalaureusetöö)

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### Publikatsioonid

Puidet, E., Liira, J., Paal, J., Pärtel, M. & Pihu, S. 2005. Morphological variation in eight taxa of *Anthyllis vulneraria* s. lato (Fabaceae). *Annales Botanici Fennici* 42: 293–304.  
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**Saadud uurimistoetused ja lepingud (finantseerija, programm, maksumus, tähtaeg)**

SA Archimedes, Kristjan Jaagu stipendium (osaline õpe välismaal), 2005.

### **III. Õppetöö**

#### **Juhendamine**

2007 Jane Oja – Hariliku koldrohu (*Anthyllis vulneraria* L. s. lato, Fabaceae) tolmlamine ja noortaimede morfoloogiline varieeruvus. (bakalaureusetöö)

### **IV. Erialane enesetäiendus**

2005 DELTA (Description Language for Taxonomy) kursus, Islandi Ülikool.

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