Phylogenies and Secondary Chemistry in Arnica (Asteraceae)

CATARINA EKENÄS
Dissertation presented at Uppsala University to be publicly examined in Lindahlsalen, EBC, Norbyvägen 18A, Uppsala, Friday, March 14, 2008 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

The genus Arnica (Asteraceae) was investigated for phylogenetic relationships and sesquiterpene lactone (STL) content with the aims to trace the evolutionary history of the genus and to investigate possible congruencies between DNA sequence data, secondary chemistry, and biological activity.

Complex evolutionary patterns in Arnica are evident from phylogenetic analyses of chloroplast regions (the rpl16 and rps16 introns and the psbA–trnH, ycf4–cemA, and trnL–L spacers), nuclear ribosomal regions (the internal and external transcribed spacers) and the nuclear low-copy DNA region coding for the second largest subunit of RNA polymerase II (RPB2) between exons 17 and 23. Polymorphism was detected in nuclear ribosomal and low-copy regions, likely caused by polyploidy and agamospermy. Lineage sorting and/or hybridization is a possible explanation for incongruencies between topologies of the different DNA regions. None of the five subgenera in Arnica constitute a monophyletic group according to any of our analyses.

Sesquiterpene lactone profiles were compared to nuclear ribosomal DNA data using phylogenetic inference and principal component analysis for 33 accessions of 16 species. Clusters supported by both STL chemistry and ribosomal DNA sequence data consist of multiple accessions of the same species (e.g. A. montana and A. longifolia), indicating that these species are well defined both genetically and chemically, based on our sampling. Support for subspecies classification of A. chamissonis and A. parryi was found in chemical data. For the first time STLs are reported from subtribe Madiinae, sister to Arniciinae. Anti-inflammatory properties, as measured by inhibition of human neutrophil elastase release from neutrophils and inhibition of the binding of transcription factor NF-κB to DNA, were investigated for extracts of 12 Arnica species. Arnica montana, A. chamissonis and A. longifolia accessions show high inhibitory effects in both bioassays. Generally, species with a more diverse STL chemistry also possess the strongest inhibitory activity in the bioassays.

Keywords: Arnica, Asteraceae, phylogeny, ITS, ETS, RPB2, sesquiterpene lactones, PCA, bioassay, NF-κB, neutrophil, chloroplast

Catarina Ekenäs, Department of Evolution, Genomics and Systematics, Norbyv. 18C, Uppsala University, SE-75236 Uppsala, Sweden

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List of Papers

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


Paper I is reprinted with the kind permission of the publisher.

All papers included in this thesis are written by the first author, with comments and suggestions given by the co-authors. The studies were planned in cooperation with the co-authors. The laboratory work and analyses were conducted by CE with the exceptions that follow. In paper I KA did the nuclear ribosomal DNA laboratory work, in paper II JR did the PCA, in paper III AZ and BS did part of the laboratory work and TV the ANOVA, and in paper IV NH did most of the laboratory work.
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<td>secondary metabolite</td>
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<td>STL</td>
<td>sesquiterpene lactone</td>
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<tr>
<td>TBR</td>
<td>tree bisection-reconnection</td>
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Introduction

It has always been of interest to mankind to learn more about the world we live in – curiosity is the basis of science. Historically, natural sciences such as botany, medicine and chemistry were all included as one field of science, comprehensive enough for one person to grasp in a lifetime. Only a few centuries ago, a physician like Linnaeus was also a botanist, zoologist, and a geologist. With more knowledge accumulating, fields in science naturally get more specialized. The role of interdisciplinary studies is to link various fields of research and find patterns that would otherwise not be detected.

Plant systematics and phylogenetics

Systematics deals with the relationships and classification of organisms in groups. Classification of plants and animals based on appearance and other characteristics such as utility has probably existed in most traditional cultures worldwide. However, with Darwin’s theories of evolution as a base, new aspects of ancestry, relatedness and formation of new species were introduced (Darwin 1859). Phylogenetic studies were initially based mainly on morphology (e.g. Haeckel 1866, Fig. 1). With the emergence of molecular biology in the 20th century, hypotheses of relationships between taxa based on morphology could be tested by inferring phylogenetic relationships based on DNA sequence data. Since the mid-1990’s, molecular systematics has often been applied in attempts to resolve relationships at higher taxonomic levels, e.g. between tribes or families, as well as lower levels, e.g. between species within a genus.
Useful DNA regions

The majority of sequence data currently used in plant systematics originates from chloroplast DNA (cpDNA) or nuclear ribosomal DNA (nrDNA) data. Chloroplast DNA is mostly maternally inherited in flowering plants. Since the evolutionary history of the organism may differ from the gene based phylogenies due to e.g. hybridization (see below), it is recommended to be used in combination with at least one region from the nuclear genome. Non-coding chloroplast regions evolve relatively slowly in comparison to nuclear regions, resulting in low sequence variation in recently diverged groups and problems with lack of resolution in phylogenetic analyses (e.g., Small et al. 1998, Cronn et al. 2002, Hartman et al. 2002, Xu et al. 2000).

Nuclear ribosomal DNA, such as the Internal Transcribed Spacer (ITS) region has the advantage of being more variable than any non-coding cp region and has proven successful, especially in combination with cpDNA, to resolve relationships in several young groups (e.g., Soltis et al. 1996, Campbell et al. 1997, Yang et al. 2002). Nulear ribosomal DNA exists in tandem repeat multiple copies, which are believed to be homogenized by concerted evolution (Hillis et al. 1991).

Figure 1. One of the first published phylogenetic trees (Haeckel 1866).
In the search for other DNA regions for resolving phylogenetic relationships in recently diverged taxa, low copy nuclear DNA (lcnDNA) regions have been much investigated lately (e.g., Small et al. 1998, Popp and Oxelman 2001, Pfeil et al. 2004, Joly et al. 2006). Low copy nuclear DNA regions exist in just a few copies, making it possible to trace paralogues in the genome to infer the evolutionary history without homogenizing processes like concerted evolution.

Hybridization and lineage sorting

In low-level taxonomy, when relationships among recently diversified taxa are investigated, the influence of hybridization and lineage sorting as recent processes can complicate the picture. Hybridization, the interbreeding of lineages representing different taxa, has been proposed to be frequently occurring among plants (Ellstrand et al. 1996). When resulting in the formation of a new lineage, hybridization is often accompanied with a polyploidization event (i.e. a multiplication of the entire genome; allopolyploidization). Polyploidization as a result of a multiplication of the genome of a single species on the other hand (autopolyploidization) often results in sterility and propagation via agamospermy (asexual reproduction). In multi-copy regions prone to concerted evolution, such as in nrDNA, asexual reproduction can result in disrupted concerted evolution and polymorphisms between copies (e.g. Campbell et al. 1997).

Although hybridization can be reflected in gene phylogenies, other processes such as gene duplication and incomplete lineage sorting can result in similar patterns (Fig. 2).

![Figure 2. Incomplete lineage sorting (left) and hybridization (right), are two underlying processes that can result in the same pattern.](image)

Where polymorphism occurs (i.e. multiple paralogues caused for example by gene duplication or polyploidy), lineage sorting can lead to extinction of all multiple paralogues within lineages, or a few (incomplete lineage sorting), which may lead to inference of wrong organismal relationships (see Doyle 1992).

By investigating several independent gene regions, it may be possible to distinguish between these processes. If the same typological pattern is evident from different regions of the genome, hybridization may be more likely
to be the cause, whereas if the pattern is detected in one region only, it is more likely to be caused by incomplete lineage sorting or gene duplication/loss.

Secondary metabolites in chemotaxonomy
Whereas primary metabolites are essential for cell survival, secondary metabolites (SMs) can be generally defined as compounds improving the fitness of the producing organism. These compounds can act for example as defence against microorganisms, protection against UV radiation, or as an attractant of pollinators. Secondary metabolites tend to be taxon-specific and various groups of SMs have been applied as taxonomic markers long before DNA markers were introduced (e.g., Abbott 1887). DNA sequence data is now more frequently used in phylogenetic studies, and phylogenies based on DNA sequence data can serve as a basis for interpretations of biosynthetic pathways of secondary metabolites (e.g., Wink and Mohamed 2003, Winsor et al. 2005).

Pharmacognosy
Secondary metabolites lead us to the field of pharmacognosy, which deals with the discovery, characterization, and evaluation of bioactive compounds or extracts originating from natural sources. Pharmacognosy is an interdisciplinary field of science that spans a wide range of fields, from ethnopharmacology to natural products chemistry. A model was recently proposed, summarizing interactions among aspects of the field of pharmacognosy today (Larsson 2007).

Figure 3. Model illustrating aspects of pharmacognosy (from Larsson 2007).
Secondary metabolites as bioactive compounds

Acting as a chemical defence in the producing organism, secondary metabolites possess properties that are of high interest in the search for pharmacologically interesting lead compounds (Müller-Kuhrt 2003, Larsen et al. 2005, and Larsson et al. 2007). Historically all drugs were based on natural products. The World Health Organization estimates that 25% of pharmaceuticals currently on the market still originate from traditional medicine (WHO 2003), and more than 45% originate from compounds isolated or derived from natural products (Cragg et al. 1997). So, a large percentage of modern pharmaceuticals originate from compounds that would also be classified as secondary metabolites. Although synthetic chemistry has enabled the production of large amounts of compounds to be screened in a relatively short time, it is unlikely that a complex molecule such as paclitaxel (Fig. 4) would have been synthesized in a chemistry lab. Natural products have the potential to serve as leads that, in combination with synthetic chemistry, can be modified and optimized.

![Paclitaxel](image)

*Figure 4. Paclitaxel, isolated from *Taxus brevifolia* (Taxaceae) and used in cancer chemotherapy (Wani et al. 1971, Cragg et al. 1993).*

Selection of organisms

With an estimation of several million species on earth (including the species-rich kingdoms of bacteria and fungi), how does one decide where to start looking for bioactive compounds? Random screening programs tend to be costly and time consuming and it is therefore advantageous to use an initial target approach when selecting objects for further investigations. A classical approach used in ethnobotany or ethnopharmacology is to investigate species that are or have been used in traditional medicine. A species that has been selected and used over long periods in human history to treat a certain disorder is more likely to contain bioactive compounds compared to randomly selected species.
A third approach that may be combined with the two above is to examine close relatives to species already known to synthesize pharmacologically interesting compounds. Theoretically, if SMs can be used as taxonomic markers (as discussed above), the opposite should also be true: there should be a higher chance of finding similar types of SMs in groups that are closely related, as inferred by a phylogeny based on DNA markers. As relationships between species are resolved by phylogenetic inference based on DNA markers, this can be tested by comparing a phylogeny based on DNA with a phylogeny based on chemical markers of the same species.
Arnica L. (Asteraceae) is a circumboreal genus of rhizomatous, perennial herbs with a center of diversity in western North America. Morphological features of the genus include simple, opposite leaves, epaleate receptacles, yellow (rarely whitish) to orange corollas, yellow or purple anthers, gray or brown to black cypselae, and usually a pappus of bristles. The base chromosome number for the genus is $x = 19$. In North America 32 minimal-rank taxa, including 26 species and six subspecies, were recognized by Wolf (2006). Including the species endemic to Europe (A. montana) and Asia (A. mallotopus and A. sachalinensis), 29 Arnica species are currently recognized worldwide.

Figure 5. Distribution of Arnica (from Maguire 1943).
Taxonomic history and phylogenetic position

The name *Arnica* was mentioned for the first time in Kräuterbuch by Jacobus Theodorus Tabernaemontanus (1625), where a woodcut of *A. montana* is displayed under the name *Caltha alpina* (Fig. 7), with the phrase “… aber von den Medicis, *Arnica*” added as a synonym. In the same reference,
*Ptarmica*, from the Greek word *ptaro* (= I sneeze), was also mentioned as a synonym, and could possibly be the origin of the name *arnica* (Mayer and Czygan, 2000). In the 17th-18th century, the European representatives of *Arnica* were commonly referred to under the name *Doronicum* (e.g. by Linnaeus in *Flora Lapponica*, 1737). However, in *Species plantarum* (1753), Linnaeus adopts the name *Arnica*, although of the six species he described only *A. montana* is maintained within the genus as recognized today.

![Figure 7. The first appearance of *A. montana* as a woodcut (Tabernaemontanus, 1625).](image)

Complex and diverse patterns of morphological variation in *Arnica* are reflected by widely differing taxonomic treatments. In a revision by Rydberg (1927) over 100 species were recognized as belonging in the genus. Polyploidy is widespread in many species and Barker (1966) established that there is a strong connection between polyploidy and agamospermy in *Arnica*. A hybrid origin of some species has also been proposed (e.g., Wolf and Denford 1984a). In the latest revision of the whole genus Maguire (1943) recognized 32 species and divided the genus into five subgenera; *Andropurpurea*, *Arctica*, *Arnica* (as: *Montana*), *Austromontana*, and *Chamissonis*. As a result of later revisions of subgenera *Arctica*, *Austromontana* and *Chamissonis*, the total number of species was reduced to 27 (Downie and Denford 1988, Wolf and Denford 1984b, Gruezo and Denford 1994). Later, in analyses by Baldwin et al. (2002), two species not included within the genus by
Maguire (1943), *A. dealbata* (A. Gray) B. G. Baldwin and *A. mallotopus* (Franch. & Sav.) Makino were nested within *Arnica*.

At the time of Maguire’s revision of the genus (1943), *Arnica* was considered to belong within tribe Senecioneae, subfamily Asteroidae, as proposed by Bentham (1873), but was later moved to tribe Heliantheae *sensu lato* (Nordenstam 1977, Robinson 1981) based on chemistry and morphology. In a discussion of the origin and biogeographical history of the genus, Maguire proposed that *Arnica* is of arctic origin and that it spread southward along four major routes. Using ITS sequence data, Baldwin and Wessa (2000) found that *Arnica* (subtribe Arnicinae) is sister to the tarweeds and silverswords (subtribe Madiinae) and nested within an otherwise temperate western North American lineage (tribe Madieae *sensu* Baldwin; see Baldwin et al. 2002; Fig. 8). Baldwin and Wessa suggested that the genus originated in temperate western North America, rather than in the arctic, as previously suggested by Maguire.

![Figure 8](image_url)

*Figure 8. Placement of *Arnica* as subtribe Arnicinae, sister to subtribe Madiinae within tribe Madieae (from Baldwin and Wessa 2000, with permission from the authors).*
Traditional and modern use

Documented medicinal use of the European representative, *A. montana*, stretches back at least to the 14th century when it was mentioned in an encyclopedia by Matthaeus Sylvaticus (1317) for menstrual pains. From the 16th century it is widely mentioned as a “wound-remedy” to treat external injuries (Mayer and Czygan 2000). *Arnica* was also mentioned by Linnaeus as a substitute for tobacco in *Flora Suecica* (1755). The North American species *A. acaulis*, *A. angustifolia*, *A. cordifolia*, *A. latifolia*, and *A. discoidea* have also been used in traditional medicine according to a number of ethnobotanical surveys as summarized in Table 1. Interestingly, widely separated groups of people have used *Arnica* species to treat disorders that could be coupled to inflammation.

![Cultivation of A. montana](image)

*Figure 9. Cultivation of A. montana* (with permission from Weleda).

Flower heads of *A. montana* are still used today, applied externally as a tincture or oil to treat inflammatory-related topical disorders such as sprains, swellings, and bruises. The more easily cultivated *A. chamissonis* has been proposed as a substitute to *A. montana* (Cassells et al. 1999). In a randomised double-blinded study of 204 patients, the short-term use of *Arnica* gel was compared to ibuprofen gel (5%) in the treatment of hand osteoarthritis. The result was no significant perceived difference between the two treatments (Widrig et al. 2007).
Table 1. North American Arnica species for which there is documented traditional use.

<table>
<thead>
<tr>
<th>Species</th>
<th>Use</th>
<th>Preparation</th>
<th>Plant part</th>
<th>Ethnic group and location</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. acaulis</td>
<td>back pain (Speck 1937)</td>
<td>infusion</td>
<td>roots</td>
<td>Catawba, North Carolina</td>
</tr>
<tr>
<td>A. angustifolia</td>
<td>stomach problems (Alestine and Fehr 2002)</td>
<td>infusion</td>
<td>-</td>
<td>Gwich’in, Yukon and NWT</td>
</tr>
<tr>
<td>A. cordifolia</td>
<td>sore eyes (Palmer 1975)</td>
<td>-</td>
<td>-</td>
<td>Shuswap, BC</td>
</tr>
<tr>
<td>A. cordifolia and A. latifolia</td>
<td>bruises, swellings and cuts (Turner 1990)</td>
<td>poultice</td>
<td>whole plant</td>
<td>Thompson, BC</td>
</tr>
<tr>
<td>A. discoidea</td>
<td>wounds (Strike 1994)</td>
<td>-</td>
<td>roots</td>
<td>Okanagan-Colville, BC</td>
</tr>
</tbody>
</table>

NWT: Northwest Territories, BC: British Columbia

Sesquiterpene lactones

With more than 5000 known structures, STLs constitute one of the largest groups of natural products known (Schmidt 2006). Most of these compounds are isolated from species in Asteraceae and are believed to be responsible for at least part of the evolutionary success of this large group of taxa. These compounds have been investigated for potential use as chemotaxonomic markers for the family as a whole, using cluster analysis, principal component analysis (PCA), or other multivariate statistical methods (e.g., Alvarenga et al. 2001, Hristozov et al. 2007), and as a source of bioactive compounds (Schmidt and Heilmann 2002, Kanashiro et al. 2006, Wagner et al. 2006, Scotti et al. 2007).

Numerous STLs have been isolated and identified from Arnica species as summarized by Willuhn and co-workers (1995). Especially A. montana has been thoroughly investigated for STL contents and the anti-inflammatory effect of these compounds. Sesquiterpene lactones of the helenanolide type from A. montana and A. chamissonis have been shown to inhibit the release of human neutrophil elastase from neutrophils, and thereby intervene with the inflammatory process (Siedle at al. 2003). It has also been demonstrated that STLs prevent the binding of nuclear factor-κB (NF-κB) to DNA by alkylation of cysteine-38 in the p65/NF-κB subunit (Siedle at al. 2004), thereby preventing the transcription of inflammatory mediators. There are strong indications that this is a general mechanism for STLs possessing α,β-unsaturated carbonyl structures such as α-methylene-γ-lactones or α,β-unsaturated cyclopentenones (Lyss et al. 1998, Garcia-Pineres et al. 2001).
Aims

The work presented in this thesis was conducted at the Department of Evolution, Genetics and Systematics, Subdepartment of Systematic Biology, and at the Department of Medicinal Chemistry, Division of Pharmacognosy.

The specific aims of this thesis were to:

- Investigate phylogenetic relationships in *Arnica* using several independent DNA regions from cpDNA, nrDNA, and lcnDNA (I, II, and IV).
- Investigate the STL chemistry in *Arnica* and closely related taxa (II).
- Investigate possible congruencies between ribosomal DNA and STL data in *Arnica* (II).
- Evaluate anti-inflammatory activity of several species of *Arnica* using two bioassays and correlate the activity to STL content (III).
Materials and Methods

Taxon sampling
At least one representative of all species (29) belonging to *Arnica* are included in (IV), and in (I) all are included except *A. louiseana*. Four outgroup taxa; *Eatonella nivea*, *Hulsea algida*, *Raillardella argentea*, and *Venegasia carpesioides*, belonging to closely related lineages in tribe Madieae were included. Samples originated from herbarium specimens or from fresh specimens collected in the field, or in botanical gardens.

![Figure 10](image)

*Figure 10. Cultivation of *Arnica* species in Uppsala Botanical Garden. Photo by the author.*

All specimens included in (II) and (III) were cultivated outdoors in Uppsala Botanical Garden, Sweden. We collected seeds and rhizomes during
field trips to Alberta and California. Seeds were also obtained from specimens collected by others in the field and from botanical gardens. In (II) and (III), representatives of 16 and 12 Arnica species were included respectively. Two outgroup taxa, Layia hieracioides and Madia sativa, from the subtribe Madinae (sister to Arnica), were included in both studies.

Sequenced DNA regions and cloning

The internal and external transcribed spacers (ITS and ETS) of the nuclear ribosomal nrDNA, were sequenced in (I) and (II). When polymorphism was detected, PCR-products were cloned (10 clones) and sequenced. In (I), five cpDNA regions (the rpl16 and rps16 introns and the psbA–trnH, ycf4–cemA, and trnT–L spacers) were amplified and sequenced. In (IV) the region between exons 17 and 23 in the nuclear low-copy gene encoding the second largest subunit of RNA polymerase II (RPB2) was amplified, cloned (10-18 clones) and sequenced.

Isolation of STL-rich fraction

For (II) and (III), flowerheads were collected from cultivated specimen in two batches (2005 and 2006). Extraction was followed by isolation of STL-rich fractions (see fractionation protocol, Fig. 11). These were subjected to gas chromatography and mass spectrometry (GC-MS) analyses in (II) and mass spectra and retention times of the resulting compounds were compared to those of already known STLs in Arnica.

Figure 11. Fractionation scheme for isolation of a STL-rich fraction (II and III).
Phylogenetic analyses and PCA

In (I), the two matrices of cp and nrDNA regions were subjected to separate and combined parsimony heuristic tree searches and bootstrap analyses. The combined matrix was also subjected to phylogenetic analysis using Bayesian inference. Three RPB2-d paralogues were detected in (IV) and each was aligned separately and subjected to separate parsimony heuristic tree searches and bootstrap analyses as well as Bayesian analyses.

In (II), nrDNA sequences and a matrix of STL data (in which peaks were coded as absent or present) were separately subjected to parsimony analyses and the matrix of STL data (peak areas) was also subjected to PCA.

Bioassay studies

In (III), the STL-rich fractions were tested for anti-inflammatory activity using two bioassays, one testing the capacity of extracts to inhibit the release of human neutrophil elastase (HNE) from neutrophils, and one testing the capacity to inhibit the binding of transcription factor NF-κB to DNA. The results were subjected to analysis of variance (ANOVA), comparing results between extracts as well as between results of the two bioassays.
Phylogenetic studies of *Arnica* (I, II, IV)

Phylogenetic investigations of plants are usually based on cpDNA, nrDNA, and/or lcnDNA regions. In this thesis we have sequenced DNA originating from all three types.

**Chloroplast regions – low variation (I)**

Variation in non-coding chloroplast regions of *Arnica* was found to be low leading to weakly supported basal nodes (Fig. 12). Five chloroplast regions (3710 bp) were sequenced which resulted in an average of 12 parsimony-informative characters. Similarly low variation in chloroplast DNA has been found in many recently diversified taxa, such as *Gossypium*, Malvaceae (Small et al. 1998, Cronn et al. 2002), *Lophocereus*, Cactaceae (Hartmann et al. 2002), and *Soja*, Fabaceae (Xu et al. 2000).
Figure 12. Strict consensus of 508 most-parsimonious trees based on cpDNA sequences (heuristic search, TBR, 100 replicates). Bootstrap values over 50% are indicated and major supported clades are marked with capital letters. Taxon samples are indicated by species and subspecies followed by population number and the first two letters of the subgenus in which it was placed by Maguire (MO: subg. Montana (now Arnica), AR: subg. Arctica, AU: subg. Austromontana, CH: subg. Chamissonis, and AN: subg. Andropurpurea). *Outgroup taxa. Missing sequences are indicated by superscript numbers (1: rpl16 intron, 2: rps16 intron, 3: trnT-L spacer, 4: PsbA-trnH spacer, and 5: ycf4-cemA spacer).

Polymorphism in nrDNA regions (I and II)

The nuclear ribosomal regions ITS and ETS were used in two studies. In (I), the aim was to compare phylogenies based on ribosomal DNA data (Fig. 13) and chloroplast data. In (II) ITS and ETS were sequenced for cultivated accessions of Arnica with the aim to compare the phylogeny (Fig. 19) with STL data of the same accessions. The two datasets are based on different accessions, with a more complete taxon sampling in (I). In (II) we detected three paralogues in the dataset for some species. Similar patterns of polymorphic ITS sequences have been found in a few taxa e.g., Amelanchier (Rosaceae), where it was explained by hybridization in combination with
reduced concerted evolution due to agamospermy (Campbell et al., 1997). It is likely that agamospermy in *Arnica* is responsible for a similar reduction in concerted evolution between different *ITS* and *ETS* copies and that this is causing the divergent paralogues to be maintained.

**Figure 13.** Strict consensus of 16 most-parsimonious trees based on nuclear ribosomal DNA (*ITS* and *ETS*) sequences (heuristic search, TBR, 100 replicates). Bootstrap values over 50% are indicated and clades discussed in (I) are marked with capital letters. Taxon samples are indicated by species and followed by population number and the first two letters of the subgenus in which it was placed by Maguire (MO: subg. *Montana* (now: *Arnica*), AR: subg. *Arctica*, AU: subg. *Austromontana*, CH: subg. *Chamissonis*, and AN: subg. *Andropurpurea*). *Outgroup taxa. **ETS sequence of A. mallotopus is from voucher source 2. Missing sequences are indicated by superscript numbers (1: *ITS* and 2: *ETS*).**

**RPB2** – three *d*-paralogues (IV)

The low-copy nuclear gene coding for the second largest subunit of RNA polymerase II (*RPB2*; Sawadogo and Sentenac 1990), has been successfully
used for resolving phylogenetic relationships at higher taxonomic levels (Denton et al. 1998, Oxelman and Bremer 2000, Oxelman et al. 2004, Ron-çal et al. 2005) as well as lower levels (e.g., Popp and Oxelman 2001, Pfeil et al. 2004, Goetsch et al. 2005, Thomas et al. 2006, Loo et al. 2006, Sun et al. 2007, Kool et al. 2007). Two copies of RPB2 are present in some angiosperm taxa, which has been suggested to be the result of a gene duplication near the origin of core eudicots. The RPB2-d copy is found almost universally in investigated angiosperms, while RPB2-i is scattered within two major clades of eudicots (Oxelman et al. 2004, Luo et al. 2007).

In Arnica, three RPB2-d paralogues were detected (RPB2-dA, RPB2-dB, and RPB2-dC; Fig. 14-16). Since the outgroup taxa (Venegasia, Hulsea, Eatonella, and Raillardella) are nested within the RPB2-d copies, the two duplication events within the d-copy must have occurred prior to the divergence of Venegasiinae, Hulseinae, Arnicinae, and Madiinae in Madieae. In a phylogenetic study of helenioid Heliantheae, Baldwin et al. (2002) suggest a polyploidization event at the base of the Heliantheae s.l. and Eupatorieae, which could possibly be coupled to at least one of the duplication events detected in RPB2 in Arnica and the outgroups. Multiple paralogues of RPB2-d in Hibiscus and Sidalcea have been explained as a result of a more recent duplication event within in Malvaceae (Pfeil et al. 2004, Stone and Andreasen, in prep.). Multiple RPB2-d copies have been found in Silene as result of polyploidization (Caryophyllaceae; Popp and Oxelman 2001).

As in ITS and ETS, polymorphism was also detected within the three paralogues. Although the tree topologies of the three paralogues are not completely congruent, 30% of the strongly supported clades uniting different species are present in trees of more than one paralogue.
Figure 14. One of 100,000 most parsimonious trees resulting from a maximum parsimony analysis of RPB2-dA (100 replicates with a maximum of 1000 trees saved per replicate). Species epithets are followed by clone denotation and the first two letters of the subgenus in which it was placed by Maguire (1943; MO: subg. Montana (now: Arnica), AR: subg. Arctica, AU: subg. Austromontana, CH: subg. Chamissonis, and AN: subg. Andropurpurea). This is followed by an accession number in cases of multiple representatives of the same species. Bootstrap support values > 50% are indicated above branches and Bayesian posterior probabilities >0.50 are indicated below branches. Dashed branches are not present in the parsimonious strict consensus tree. Branches uniting clades with bootstrap support > 80% are bold.
Figure 15. One of 4 208 most parsimonious trees resulting from a maximum parsimony analysis of RPB2-dB (100 replicates with a maximum of 1000 trees saved per replicate). Species epithets are followed by clone denotation and the first two letters of the subgenus in which it was placed by Maguire (1943; MO: subg. Montana (now: Arnica), AR: subg. Arctic, AU: subg. Austromontana, CH: subg. Chamissonis, and AN: subg. Andropurpurea). This is followed by an accession number in cases of multiple representatives of the same species. Bootstrap support values > 50% are indicated above branches and Bayesian posterior probabilities >0.50 are indicated below branches. Dashed branches are not present in the parsimonious strict consensus tree. Branches uniting clades with bootstrap support > 80% are bold.
Complex evolutionary patterns

Evolutionary relationships in *Arnica* are difficult to track, as expected in a young genus of recently diversified taxa, and in which processes such as polyploidy, agamospermy, and possibly hybridization are present. The phylogenetic trees resulting from analyses of the independent datasets of *RPB2-d* parologue, nrDNA, and cpDNA regions, have few strongly supported groups in common. Polymorphism within nrDNA and *RPB2-d* paralogues makes it difficult to concatenate matrices based on the different regions. It was therefore not possible to produce an analysis on the combined data matrix. However, a few groups are supported in trees of more than one region, such as the close (although weakly supported) relationship between *A. acaulis* and *A. fulgens* based on nrDNA, cpDNA, and *RPB2-dA* regions. These species are not regarded as close and are classified into separated subgenera: *A. acaulis* in *Arnica* and *A. fulgens* in *Arctica* (Maguire 1943). *Arnica sachalinensis*, *A. unalaschcensis*, and *A. lessingi* have been proposed to be closely related and share the synapomorphy of purple rather than yellow anthers. In our phylogenetic analyses, *A. sachalinensis* and *A. unalaschceni-
sis are sisters according to all RPB2-d paralogues, whereas in the tree based on ITS and ETS (Fig. 13), A. unalaschcensis, A. mallotopus and A. lessingi are found in a strongly supported group diverged from A. sachalinensis (although weakly supported). Arnica lanceolata and A. chamissonis have also been proposed to be closely related and end up close according to phylogenetic analyses based on rDNA data and RPB2-d paralogues A and C.

Barker (1966) noted a strong correlation between agamospermy and polyploidy in Arnica. He found all strictly diploid species in Arnica (A. montana, A. acaulis, A. unalaschcensis, A. sachalinensis, A. fulgens, A. sororia, A. cernua, A. viscosa, and A. venosa) to be also strictly amphimictic (i.e. only reproducing sexually) in all representatives investigated. Our results support the findings of Barker, since there is no polymorphism in any accessions of the species mentioned above in any of the regions we investigated. Polymorphism in both nrDNA and RPB2-d paralogues were found in accessions of A. angustifolia, A. chamissonis, A. gracilis, A. lanceolata, A. mollis, and A. parryi. It is likely that polymorphism observed in these accessions is connected to polyploidy, and that incomplete lineage sorting result in the clustering of these with different taxa in different regions or paralogues. This would explain the observed incongruencies between DNA regions and paralogues within regions.

Phylogeny and origin

Although patterns are complex, some trends can be identified from all datasets. In phylogenetic analyses resulting from nrDNA and cpDNA regions, there are strong support values for clades uniting all Arnica sequences. Regarding RPB2-dA and RPB2-dB, only one outgroup taxon was detected in each of the paralogues, and therefore we can draw no conclusions regarding monophyly of Arnica based on these paralogues. However, in the RPB2-dC parologue, A. viscosa is not nested within Arnica. A member of the tarweed genus, Raillardella, diverges in a clade after A. viscosa, making Arnica non-monophyletic in the RPB2-dC tree. Interestingly, the anomalous morphology of A. viscosa, noted by Maguire (1943), is superficially like that of Raillardella, as reflected by R. paniculata Greene, a synonym of A. viscosa. One explanation for this pattern could be introgression between A. viscosa and a taxon outside Arnica, e.g., in Raillardella or the lineage leading to Raillardella. Two species previously placed outside Arnica - A. mallotopus (Mallotopus japonicus) and A. (Whitneya) dealbata are confidently confirmed as members of Arnica in all analyses in which these are included.
Based on analyses of all regions, none of the subgenera proposed by Maguire (1943) constitute a monophyletic group. However, in all analyses, all members of subgenus *Chamissonis* are only present in branches diverging further up in the trees. Another trend is that basally diverging branches mostly consist of members of subgenus *Andropurpurea* (nrDNA, and cpDNA regions in I), *Austromontana* (RPB2-dC) or both (RPB2-dA and RPB2-dB). Maguire hypothesized that subg. *Arctica* is “the closest archetypal major group and is placed nearest the base” in *Arnica* (directly descended from his hypothetical “Protoarnica”). Although additional data will be necessary to ascertain the earliest diverging lineages in *Arnica*, none of the trees provide any indications that members of subg. *Arctica* constitute a deep grade or early diverging clade.

Maguire’s proposal of an arctic origin of *Arnica* is not supported by our analyses. Instead, the hypothesis suggested by Baldwin (2002) of an origin in temperate western North America, which coincides with the distribution of the sister group to *Arnica*, is more in agreement with our results. As discussed above, members of subgenus *Austromontana* are present in basally diverged clades and many of these are typically or strictly diploid species of restricted distribution in California and Oregon (e.g., the successively basally diverging taxa in RPB2-dC: *A. viscosa*, *A. dealbata*, *A. cernua*, and *A. spathulata*).

Members of subgenus *Andropurpurea* constitute early diverging clades in all of the trees based on cpDNA and mDNA (in I), which, although weakly supported, raises the intriguing possibility that an early lineage of *Arnica*
may have occurred in the northwestern Pacific Rim, where those taxa are endemic.

As discussed by Wolf and Denford (1984a), the East-West disjunct distribution of some species, and the circumboreal distribution of *A. angustifolia*, suggests that *Arnica* was part of the Arcto-Tertiary flora. Raven and Axelrod (1978) included *Arnica* in a group of genera that are well developed in California and at the same time widespread, suggesting that the spreading aridity from Upper Tertiary times which culminated in a full Mediterranean climate in the later Quaternary has affected these taxa. Our result of low support for basal branches, combined with short branch lengths, suggests that the spread of *Arnica* took place under rapid evolution, which may have coincided with this change in climate.
Sesquiterpene lactones in *Arnica* (II)

Identified STLs

Sesquiterpene lactones constitute one of the largest groups of structurally identified natural products and many STLs have been identified in *Arnica* species (e.g., Willuhn et al. 1995 and references therein, Schmidt and Willuhn 2000, Kos 2005). The total matrix of 239 peaks resulting from GC-MS data was subjected to phylogenetic analysis and PCA. Some peaks that were important for the formation of clusters were examined closer we were able to identify 29 STLs of seven core structures (Fig. 18, Table 2) by comparing with retention times and mass spectra of already structurally determined STLs.

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**Figure 18.** Identified STLs in GC-MS profiles of *Arnica* and outgroup taxa.
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<th>Accession</th>
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See Fig. 18 for names of identified STLs. Superscript numbers indicate STL important for the cluster of: 1 the A. montana group, 2 the A. longifolia group, 3 the A. chamissonis (1 and 2) group, 4 the A. chamissonis (3-7) group, and 5 the A. gracilis group. This is also indicated by shaded areas. STLs are listed as derivatives of helenalin (He), dihydrohelenalin (DHe), arnifolin (Ar), chamissonolide (Cha), carabron (Ca), xanthanolide (Xa), florilenalin (Fl), and eudesmanolide (Eu). X: present, (x): present in trace amount (area under curve <5 % of internal standard), X: present in large amount (area under curve >150 % of internal standard) - : not detected. Accessions are listed according to the order they appear in the tree based on secondary chemistry in Fig. 19.
STLs new to *Arnica* species and Madiinae

*Arnica gracilis, A. latifolia, A. discoidea, A. fulgens,* and *A. griscomii* were investigated for STL content for the first time. Xanthalongin derivatives (particularly xanthalongin and dihydro-4H-xanthalongin) were found in accessions of *A. gracilis* and *A. latifolia,* whereas very few STLs were found in *A. discoidea, A. fulgens,* and *A. griscomii.*

Although *A. chamissonis* has been investigated for STLs, we found many of the xanthalongin type (e.g. 4H-xanthalongin, present in all *A. chamissonis* accessions). This type of STLs has previously not reported in this species.

Two STLs of the xanthalongin type (xanthalongin and dihydroxanthalongin) were identified in *Layia hieracioides,* belonging to the sister subtribe to Arnicinae (Madiinae; Baldwin and Wessa 2000). Although diverse in other closely related groups (e.g., Gaillardiiinae and Heliantheae; Ottosson 2003 and references cited therein), STLs have previously not been reported from subtribe Madiinae.

### STL chemistry within species

As discussed by Wink (2003), whereas a group of SMs usually dominates within a taxon, the pattern of SMs is complex and there are differences not only between individual plants or populations, but also between different tissues of the same individual. Still, STL chemistry was found to be very consistent within multiple samples of *A. montana* (five accessions) and *A. longifolia* (three accessions), each forming a distinct cluster in the PCA score plot and a strongly supported group in the phylogenetic tree (Fig. 19B and 20). The STL chemistry is somewhat less consistent in multiple accessions of *A. chamissonis* (seven accessions forming two groups) and *A. parryi* (two accessions diverged from each other; see below). The three *A. gracilis* accessions form a group of low support in the phylogenetic tree but do not form a distinct group in the PCA score plot. Major STLs supporting the formations of these clusters are indicated in Table 2. The two accessions of *A. angustifolia,* diverged in a basal clade of the tree and in the center of the PCA score plot, both contain less STLs. Based on our sampling, we can conclude that STLs can act as chemotaxonomic markers within *Arnica* species in which STLs are abundant.
Figure 19.

Figure 20.
Congruencies between DNA data and STL chemistry

Complex evolutionary patterns, as are evident from phylogenetic studies based on multiple regions in the genome of *Arnica*, motivate the search for additional phylogenetically useful information in other types of data, such as secondary chemistry. Ribosomal DNA sequence data and STL profiles were analysed for *Arnica* species, using phylogenetic inference and PCA (Fig. 19 and 20). Phylogenetic analyses resulted in the following strongly supported congruencies between STL data and nrDNA: clades I (100% bs) and J (99% bs), uniting all *A. montana* accessions, F (99% bs) and K (100% bs), uniting all *A. longifolia* accessions (although *A. gracilis* x *longifolia* is included in F), and D (100%) and N (86%), uniting five of the *A. chamissonis* accessions (although *A. lanceolata* is included in D). Clade H (94% bs), uniting all *A. gracilis* accessions with *A. gracilis* x *longifolia*, is also present in the STL chemistry tree, but with low support.

Another congruency between the datasets is that representatives present in the three paralogues of the nrDNA strict consensus tree (*A. angustifolia*, *A. chamissonis*, *A. gracilis*, *A. lanceolata*, *A. longifolia*, *A. mollis*, and *A. parryi*) also form a clade in the tree based on STL data (although also weakly supported and with *A. montana* included and *A. angustifolia* and *A. mollis* excluded). Hence, it seems as taxa containing more diverse STL profiles (see Table 2), also are more closely associated based on nrDNA data.

Moreover, of the taxa mentioned above, *A. chamissonis*, *A. lanceolata*, *A. longifolia*, and *A. parryi* belong to subg. *Chamissonis*, of which sequences are only present in branches diverging further up in the trees in studies based on cpDNA and nrDNA and lcnDNA regions. According to identified STLs of the present study (see Table 2), taxa of this subgenus generally possess more diverse sesquiterpene lactone chemistry compared to taxa of subgenera *Andropurpurea*, *Arctica* and *Austromontana* (also shown by Willuhn et al. 1995), which can be interpreted as additional support for the circumscription of this subgenus.

![Figure 19](image-url) Phylogenetic trees based on (A) DNA sequence data and (B) secondary chemistry data for the 33 *Arnica* accessions and accessions of *Layia hieracioides* and *Madia sativa* (outgroup taxa). Numbers are assigned to multiple accessions of the same species. Multiple accessions of *A. chamissonis*, *A. gracilis*, *A. longifolia*, *A. montana*, and *A. parryi* are marked by colour. Bootstrap values over 50% are indicated and branches with more than 75% bootstrap support are marked as bold. Major clades discussed are marked with capital letters. A. Strict consensus of 432 most parsimonious trees based on nuclear ribosomal DNA (ITS and ETS) sequences (heuristic search, TBR, 100 replicates). Sequences of polymorphic taxa form three groups (I, II, III). *No ITS sequences were found in these positions. B. The single most parsimonious trees based on a matrix of 239 peaks (compounds) of STL-rich extracts (heuristic search, TBR, 100 replicates).

![Figure 20](image-url) Score plot of principal component 2 vs component 4 of the PCA. Multiple accessions of *A. chamissonis*, *A. gracilis*, *A. longifolia*, *A. montana*, and *A. parryi* are marked by colour.
Support for subspecies classification

Although not recognised in the latest revision of subgenus Chamissonis (Gruezo and Denford 1994), *A. chamissonis* and *A. parryi* have each been classified into subspecies, based on morphology.

Based on Maguire’s classification (1943), the two *A. chamissonis* accessions (1 and 2; originating from California) that are clearly separated from the remaining *A. chamissonis* accessions based on secondary chemistry data, are determined as *A. chamissonis* ssp. *foliosa*. The remaining representatives would be classified as *A. chamissonis* ssp. *chamissonis* (as: *genuina*). This is also supported by earlier studies, e.g., Kresken (1984) where subspecies of *A. chamissonis* have been distinguished based on isolated STLs. We also find differences between the two groups in genetic and cytometric data. In the ribosomal DNA tree, *A. chamissonis* 1 and 2 are only detected in paralogue I, whereas the remaining *A. chamissonis* accessions are detected in paralogues I and II.

Similarly the two representatives of *A. parryi* (1 and 2; originating from Colorado and California), are differentiated based on secondary chemistry data and are determined as *A. parryi* ssp. *parryi* (as: *genuina*) and *A. parryi* ssp. *sonnei* respectively based on Maguires classification (1943). In a study by Gruezo and Denford (1995), relatively few flavonoids were found in *A. parryi* ssp. *parryi*, which is in line with our findings of a low secondary metabolite content in *A. parryi* 1. Additional support for the separation of the *A. parryi* accessions is found in ribosomal DNA data. In paralogue II the repre-
sentatives are separated with *A. parryi* 1 associated with *A. lanceolata*, and *A. gracilis* 3 and *A. parryi* 2 associated with *A. chamissonis* representatives.

*Arnica parryi* 2 (= *A. parryi* ssp. *sonnei*) is nested within a strongly supported group with *A. chamissonis* 1 and 2 (= *A. chamissonis* ssp. *foliosa*; Fig. 19B) and contains many of the same STLs, but in smaller amounts (Table 2). These findings are consistent with a study by Merfort et al. (1986) in which a high number of flavonoids were detected in *A. parryi* ssp. *sonnei*, with a profile very similar to that of *A. chamissonis* ssp. *foliosa*. Jepson (1925) transferred *A. parryi* ssp. *sonnei* to *A. foliosa* (= *A. chamissonis* ssp. *foliosa*) based on morphological similarities. A close connection between *A. chamissonis* ssp. *foliosa* and *A. parryi* ssp. *sonnei*, is evident based on chemistry and ribosomal DNA results, and supported by earlier studies. Perhaps hybridization events between these two geographically overlapping taxa (both taxa have a restricted distribution in California) have resulted in chemical and morphological similarities. Alternatively, the taxonomy of these two taxa is in need of revision.

**A possible hybrid**

Similarly, based on morphological characters one accession collected in Alberta, Canada, is suggested to be a hybrid of *A. gracilis* and *A. longifolia* (*A. gracilis* × *longifolia*). It would be classified as *A. gracilis* except for leaf morphology, which is similar to *A. longifolia* (e.g., leaves are sessile and lanceolate rather than petiolate and ovate, as in *A. gracilis*). Genetic and chemical data of the present study also suggest that it may be a hybrid of these two species. If so, ribosomal DNA data indicate that directional concerted evolution has caused the *ITS* and *ETS* copies to converge towards each of the two parental types in paralogues I and III, while the affinity in paralogue II remains uncertain (*A. longifolia* is not represented in this clade).

In the tree based on chemical data, *A. gracilis* × *longifolia*, forms a weakly supported group with the rest of the *A. gracilis* representatives. The accession also has many STLs in common with each of its suggested parent species. In the PCA score plot it ends up alone in a position in between the groups consisting of *A. gracilis* and *A. longifolia* representatives. Using PCA based on secondary metabolite profiles (quinolizidine alkaloids) in *Ulex* (Fabaceae), Maximo et al. (2006) propose the possibility of detecting hybrids in intermediate positions between the parent species.
Several *Arnica* species have been used in traditional medicine. Extracts of *A. montana* – used in herbal medicine today – have been thoroughly investigated for anti-inflammatory properties, but most species in the genus have not been investigated. We tested extracts of several *Arnica* species for anti-inflammatory properties by evaluating inhibitory effects on release of human neutrophil elastase HNE by neutrophils and inhibitory effects on the binding of transcription factor NF-κB to DNA.

![Graph showing inhibition of HNE release and NF-κB binding](image)

*Figure 22.* Results of the inhibition of HNE release (induced by PAF and fMLP) and the inhibition NF-κB for all extracts with 95% confidence intervals. Accession numbers correspond to those in Table 2 and Figs. 19 and 20.

Comparing the inhibitory capacities of the tested extracts, *Arnica montana*, *A. longifolia* and two of the *A. chamissonis* accessions show high inhibitory effects in both bioassays (80-100%; Fig. 22). Extracts of *A. lanceolata*, *A. griscomii*, *A. sachalimensis*, and *Layia hieracioides* in the HNE bioassay, and *A. gracilis* and *Madia sativa* in the NF-κB bioassay possess intermediate inhibition (60-80%). This can be correlated to the PCA score plot (Fig. 20; based on the total matrix of compounds), where the more active accessions
(of *A. montana*, *A. chamissonis* and *A. longifolia*) are present in the fringes of the PCA score plot as these are more diverse in STLs.

Since the same STL-rich extracts possess strong inhibitory activity in both the NF-κB and the HNE release bioassays, the possibility that HNE release inhibition is mediated by inhibition of NF-κB or downstream events cannot be excluded. NF-κB is involved in the transcription of Interleukin-8 (IL-8), which is also known to be involved in neutrophil activation (Fig. 23), and there may be other inflammatory mediators linking the two assays. The NF-κB bioassay seems to be more sensitive at low concentrations of active compounds since all extracts possess at least 40% inhibition even though extracts were tested in lower concentrations in this assay.

![Figure 23. Possible connection between the two bioassays. The inhibition of HNE release from neutrophils (1) may be linked to the inhibition of NF-κB binding to DNA (2).](image)

**Correlations between activity and STL content**

Accessions of species containing STLs of the helenanolide type (e.g., *A. montana*, *A. chamissonis*, and *A. longifolia*) were found to possess strong inhibitory activity in both bioassays (Table 2, Fig. 22). This is in agreement with a previous study by Siedle et al. (2003) in which two dihydrohelenalin derivatives from *A. montana* were active in the HNE release bioassay at micromolar concentrations. In Siedle et al. (2004), it was also demonstrated that isolated helenalin and dihydrohelenalin derivatives inhibit NF-κB binding to DNA. However, in two extracts of intermediate activity in the HNE bioassay (*A. griscomii* and Layia hieracioides), only STLs of the xanthalongin core structure could be identified. Xanthalongs are also the major expressed STLs in *A. longifolia* and *A. gracilis*. This suggests that STLs of this type may also be potent inhibitors.
Activity within species

Inhibitory capacity is very high in multiple accessions of *A. montana* and *A. longifolia*. As discussed above, the STL chemistry within these two species is also very homogenous. Two of the *A. chamissonis* representatives (2, collected in California and 6, cultivated and from unknown origin) also possess very high inhibitory activity, whereas the third (collected in Alaska) is much less active. According to subspecies classification within *A. chamissonis* discussed previously, *A. chamissonis* 1 belongs to *A. chamissonis* ssp. foliosa whereas the remaining two would be classified as *A. chamissonis* ssp. chamissonis. Therefore the difference in effect can not be correlated with subspecies classification. The PCA score plot (Fig. 20) reveals that the STL chemistry of *A. chamissonis* 4 (which is the blue spot closest to the center of the plot) is removed from the remaining *A. chamissonis* accessions, which shows that the difference in activity is accompanied with a difference in STL chemistry. As previously discussed, although a group of secondary metabolites dominates within a taxon, contents between populations and individuals vary depending on the origin and ecological niche of the plant. This has also been shown in studies comparing extracts of different origins from *A. montana* (e.g. Klaas et al 2002).
Conclusions

Phylogenetic studies of *Arnica*

Complex evolutionary patterns in *Arnica* are evident from phylogenetic analyses of chloroplast, nuclear ribosomal and low-copy DNA regions. Polymorphism was detected in both the nuclear ribosomal and low-copy regions in accessions of *A. angustifolia*, *A. chamissonis*, *A. gracilis*, *A. lanceolata*, *A. mollis*, and *A. parryi*, probably caused by polyploidy, agamospermy and possibly hybridization.

No polymorphism was detected for any of the diploid and strictly amphimictic species (*A. montana*, *A. acaulis*, *A. unalaschcensis*, *A. sachalinensis*, *A. fulgens*, *A. sororia*, *A. cernua*, *A. viscosa*, and *A. venosa*). Incongruencies between topologies of the different regions may be due to processes such as lineage sorting.

Taxa belonging to subgenus *Chamissonis* are not present in any early diverging branches in the phylogenetic trees of any investigated DNA region, which suggests that these species diverged later in the evolutionary history of *Arnica*. Species of subgenera *Austromontana* (and *Andropurpurea*) constitute early diverging lineages in all regions, which is in agreement with the hypothesis of an origin of *Arnica* in temperate Western North America.

Sesquiterpene lactones in *Arnica*

Several STLs of the xanthalongin type were found in *A. chamissonis*, *A. gracilis* and *A. latifolia*, previously not reported for these species. For the first time STLs are also reported from subtribe Madiinae, since xanthalongin and 4H-xanthalongin were also found in large amounts in *Layia hieracioides*. It would be of interest to examine additional taxa from Madiinae in order to further evaluate STL chemistry of the subtribe.

Biological activity of *Arnica* species

Two bioassays evaluating anti-inflammatory activity by measuring interactions with the inflammatory pathway show that accessions of *A. chamissonis* and *A. longifolia* are equally active as *A. montana*. *Arnica longifolia* has
previously not been investigated for biological activity and would be interesting to further evaluate for STL contents.

**Correlations: phylogeny – STLs – biological activity**

Generally, species rich in STLs (*A. montana, A. longifolia, A. chamissonis,* and to some degree *A. lanceolata* and *A. gracilis*) also possess the strongest inhibitory activities in two bioassays measuring interactions with the inflammatory pathway. It is therefore likely that mainly STLs are responsible for the anti-inflammatory activity as measured by these bioassays in these species of *Arnica*.

According to the phylogenetic analyses, clades supported by both STL chemistry and DNA sequence data consist of multiple accessions of the same species (*A. montana, A. chamissonis, A. longifolia,* and *A. gracilis*). These species are therefore well defined genetically and chemically, based on our sampling. Support for subspecies classification of *A. chamissonis* and *A. parryi* was found in STL data and differences between subspecies could also be detected in cytometry data and nrDNA data.
Svensk sammanfattning


Arnica är ett växtsläkte som består av 29 arter och hör till familjen Asteraceae, korgblommiga växter. Arnica har delats in i fem undersläkten: Arctica, Arnica, Andropurpurea, Austromontana och Chamissonis. Slåttergubbe (Arnica montana) och fjällarnika (Arnica angustifolia) är de svenska namnen på våra två representanter av släktet. Slåttergubbe finner man i delar av Europa och fjällarnikan över de nordliga delarna av hela norra halvklotet, men de flesta Arnica-arten förekommer endast i västra Nordamerika. Vissa av arterna är väldigt variabla morfologiskt vilket resulterat i att släktet ibland delats in i fler än 100 arter!

Vi undersökte flera oberoende DNA-regioner för att få fram en hypotes om hur släktet har utvecklats och fann komplexa mönster. I flera regioner hittade vi polymorfitser, vilket betyder att det verkar finnas flera olika kopior av en och samma genregion. Men de olika kopiorna visar inte på samma släktspårikhet i de olika genregionerna, vilket troligen är resultatet av en process som gör att kopier slumpvis försvinner. Inget av de fem undersläktena som vanligen nämns inom Arnica bildar en enhetlig grupp i någon av våra analyser. Arter från undersläktet Austromontana utgör flera basala grupper i vissa av analyserna. Det stämmer överens med en hypotes om att hela släktet skulle ha uppstått i tempererade västra Nordamerika där
de närmaste släktingarna till *Arnica* finns och där en del av arterna inom *Austromontana* har en begränsad utbredning.

Förr utgjorde naturen den enda källan till läkemedel och än idag härstammar mer än 45% av våra moderna läkemedel från ämnen som kommer från naturen, många av dessa från traditionellt använda växter. Unika kemiiska föreningar i växter, så kallade sekundärmetaboliter, som används som markörer inom kemosystematik är också vanligen de ämnen som är biologiskt aktiva. *Arnica montana* har sedan länge använts som medicinalväxt inom Europeisk folkmedicin och används fortfarande utvärdes för att behandla exempelvis sträckningar, stukningar och blåmärken. Det finns även dokumenterad traditionell användning av flera av de nordamerikanska arterna i släktet, t ex mot ryggsmärter, blåmärken, svullnader och för sårläkning. Man har undersökt *Arnica montana* med avseende på dess verkamma ämnen och kommit fram till att en grupp sekundärmetaboliter, så kallade seskviterpenlaktoner, står för den huvudsakliga antiinflammatoriska aktiviteten.


För att närmare studera om sekundärmetaboliter kan hjälpa till att utreda släktspä i växter som uppvisar komplexa evolutionära mönster, och om man kan använda ett släktråd baserat på DNA för att förutsäga förekomsten av seskviterpenlaktoner, jämförde vi dessa två typer av data för 16 arter av *Arnica*. I kemi-data kunde vi bland annat se stöd för tidigare underartsklassificeringar av *Arnica chamissonis* och *Arnica parryi*. Vi fann även stöd i morfologi, DNA och kemi för att en av de prover som insamlats var en korsning (hybrid) mellan de två arterna *Arnica gracilis* och *Arnica longifolia*. Vissa grupperingar av flera exemplar av samma art (*Arnica montana, Arnica longi-
folia, Arnica chamissonis och Arnica gracilis) sammanfaller mellan släktträdet baserat på DNA och släktträdet baserat på sesquiterpenlaktoner, vilket betyder att dessa är väl definierade såväl kemiskt som genetiskt, baserat på vårt urval.

Figure 24. The author collecting A. viscosa at Mt Shasta, California. Photo: Mike Park.
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