

**THE IMPACT OF POLYPLOIDY ON GENETIC STRUCTURE AND  
REPRODUCTIVE ISOLATION IN THE GENUS  
*LEUCANTHEMUM* Mill. (COMPOSITAE, ANTHEMIDEAE)**



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Evolution is a change from an  
indefinite, incoherent, homogeneity  
to a definite, coherent, heterogeneity,  
through continuous differentiations and integrations.

*Herbert Spencer*

Evolution is a change from a  
no-howish, untalkaboutable, all-alikeness  
by continuous sticktogetheration and somethingelsification.

*William James*

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## Abstract

Polyploidy is a prominent evolutionary process, particularly in plants. It is known to rapidly trigger a number of morphological, phenological, and ecological shifts, and may give rise to immediate post-zygotic isolation between the newly formed polyploids and their diploid progenitors. Consequently, it is considered to be the single most common mechanism of sympatric speciation. Various studies have either analyzed the phylogenetic patterns associated with polyploidy, or the mechanisms underlying polyploid speciation. By contrast, the thesis at hand combines these two approaches to provide a comprehensive picture of evolution by polyploidy in four species from the genus *Leucanthemum*, including one diploid, one tetraploid, and two hexaploid taxa. It particularly aims at the questions whether the investigated taxa are monophyletic, which species have been involved in the formation of the polyploids, and whether the members of the study group are reproductively isolated from each other. Sequencing of two markers from the chloroplast genome demonstrates that the diploid species *L. pluriflorum* represents the maternal parent of the three polyploid taxa, and further suggests that there were at least three independent genome duplication events. Furthermore, the analysis of ETS sequence variation shows that *L. pluriflorum* was formed presumably by homoploid hybrid speciation, and that the polyploids arose from this species by whole genome duplication. By contrast, the AFLP analysis reveals considerable genetic differentiation between the diploid and the polyploids, thereby indicating that other species might have played a role in the evolutionary history of the investigated polyploids. Finally, crossing experiments conducted between the four taxa rather showed that inter-ploidy crosses basically were capable of producing viable offspring. However, flow cytometrical analysis of 233 individuals demonstrates that inter-cytotype mating is rare, and consequently it can be

assumed that pre-zygotic barriers and reduced fitness of inter-cytotype hybrids play a decisive role in the reproductive isolation of polyploid *Leucanthemum* species.

## General Introduction

Whole genome duplication, commonly referred to as polyploidy, has long been recognized to be a prominent evolutionary process promoting the multiplicity of organisms on our planet. It can be found in archaea (Breuert et al. 2006), bacteria (Hansen 1978), and animals (e.g. Bogart 1979), and is exceedingly common in plants (e.g. Adams & Wendel 2005). Between 30 (Stebbins 1950) and 80 % (Goldblatt 1980) of all currently known angiosperm species are considered to be polyploid, and recent studies based on whole genome sequences from *Arabidopsis thaliana* and *Oryza sativa* suggest that there have been several rounds of genome duplication during the early evolution of angiosperms (Simillion et al. 2002, Zhang et al. 2005). These palaeopolyploidization events largely coincide with periods of rapid diversification, and Debodt et al. (2005) hypothesize that they might have provided the genetic raw material that triggered the evolution of insect pollination. Furthermore, polyploidy does not only generate the genetic basis for outstanding evolutionary novelties, but may also directly give rise to new species by producing strong reproductive isolation barriers between newly formed polyploids and their diploid progenitors (Grant 1971). Otto & Whitton (2000) estimate that 2 to 4 % of speciation events in flowering plants involve whole genome duplication, and Wood et al. (2009) even suggest that polyploid speciation could account for up to 15 %. Due to these insights, and fueled by the dramatic advance of molecular biology, the past 15 years have seen a revival in the study of polyploidy. Extensive research on evolution by polyploidy has been conducted in several angiosperm genera, including *Arabidopsis* (e.g. Beaulieu et al. 2009), *Chamerion* (e.g. Sabara 2009), *Gossypium*

(e.g. Adams & Wendel 2004), *Nicotiana* (e.g. Leitch et al. 2008), *Oryza* (e.g. Zhang et al. 2005), *Ranunculus* (e.g. Baack 2005), *Senecio* (e.g. Suda et al. 2007), *Silene* (e.g. Popp et al. 2005), *Spartina* (e.g. Ainouche et al. 2009), *Tragopogon* (e.g. Soltis et al. 2004b).

Thus, considerable progress has been made to understand the mechanisms of genome duplication (Ramsey & Schemske 1998, Ramsey & Schemske 2002), the establishment of newly formed polyploids (Husband 2000, Husband 2004, Baack 2005), the ecological aspects associated with polyploidy (Levin 1983, Bayer et al. 1991, Segraves & Thompson 1999), as well as its genetic, epigenetic, and genomic implications (Leitch & Bennett 1997, Bowers et al. 2003, Liu & Wendel 2003, Osborn et al. 2003).

### **Types of Polyploidy**

Basically, polyploids can be formed by intra-species genome duplication or following hybridization. In 1926, Kihara and Ono introduced the terms *autopolyploidy* (greek: αὐτό = 'self') and *allopolyploidy* (greek: ἄλλο = 'different') to differentiate between these types. Yet, these categories merely represent the two extremes of a continuum that is realized in nature, and soon the terminology had to be extended to better reflect the multitude of polyploid forms. In 1947, Stebbins (1950) discriminated between four categories of polyploids: (*strict*) *autopolyploids*, *segmental allopolyploids*, *true or genomic allopolyploids*, and *autoallopolyploids*. According to his definition, strict autopolyploids result from duplication of identical or highly similar genomes within a single species, true allopolyploids are derived from hybridization between two or more different species, and segmental allopolyploids represent an intermediate form between these two extremes. Autoallopolyploidy can be considered a minor category which includes hexaploids or

higher ploidies that formed by a combination of auto- and allopolyploidization. Although Stebbins included information about the formation process into his categorization, it is nearly as much connected to the species concept as the original terminology of Kihara and Ono, and proved to be problematic in many cases. However, the taxonomic approach represents a simple and straightforward approximation to a complex issue. Hence, it is still the most widespread one today.

### **Polyploid Formation and Establishment**

Several cytological mechanisms are known to induce whole genome duplication in plants. Somatic chromosome doubling in the zygote or in nonreproductive tissues immediately produces mixoploid chimeras, and subsequently, may result in polyploid lineages. While each of these somatic chromosome doubling mechanisms has been demonstrated in a number of polyploid plants (reviewed in Ramsey & Schemske 1998), polyploidization via the formation and fusion of unreduced gametes is considered to be the more common pathway for polyploid formation (Harlan & de Wet 1975, de Wet 1980). This mode of polyploidization mostly includes intermediate triploids (de Wet 1980, Ramsey & Schemske 1998), as the probability that an unreduced egg cell is fertilized by an unreduced sperm is quite low.

Following the genome duplication event, newly formed polyploids suffer from a number of severe disadvantages. On the one hand, early-generation polyploids are often characterized by reduced fitness which is mainly caused by cytological and genetical dysfunctions (Comai 2005). On the other hand, the 'minority cytotype exclusion principle' holds that they suffer from frequency-dependent selection that counteracts their establishment (Levin 1975). However, many factors are able to extenuate these disadvantages. For example, habitat differentiation may reduce

selective pressure on polyploids by their diploid progenitors (Thompson & Lumaret 1992), and the 'evolutionary novelty model' states that at least allopolyploids are often quite fit and highly successful in colonizing new habitats (Arnold 1997). Furthermore, mechanisms like pollinator-mediated assortative mating and breakdown of gametophytic self-incompatibility systems allow newly formed polyploids to effectively reproduce despite of their initial low frequency (Mable 2004, Husband 2000).

### **Multiple Origins of Polyploids**

Until fairly recently, the traditional view of speciation in plants suggested that individual species have a single evolutionary origin (reviewed in Grant 1971, Futuyma 1998, Levin 2000). This view was basically derived from the study of allopatric and sympatric speciation in diploids, and initially had been transferred to polyploids. Only during the past 20 years, there has been growing acceptance for the hypothesis that individual polyploid plant species often have multiple origins. A large number of studies found evidence for recurrent formation of both auto- and allopolyploids (Werth et al. 1985a, Werth et al. 1985b, Wyatt et al. 1988, Werth & Windham 1991, Brochmann et al. 1992a, Soltis et al. 1995, Allen & Eccleston 1998, Arft & Ranker 1998), and thus demonstrate that this phenomenon is the rule rather than the exception. Due to recurrent formation and post-speciation hybridization of the independently formed lineages, polyploid species can maintain a high level of genetic diversity that may drastically increase their evolutionary fitness (Soltis & Soltis 1999).



## Effects on Reproductive Biology

According to the biological species concept, the fundamental prerequisite for speciation is reproductive isolation between two groups of populations, causing a strong reduction or total elimination of geneflow between them. The traditional view of allopatric and sympatric speciation suggests that reproductive isolation is developed gradually by selection and genetic drift (Mayr 1942). Whole genome duplication, by contrast, is known to induce rapid morphological, phenological, and ecological shifts, and thus may produce isolation barriers within one or few generations (Stebbins 1950). As inter-cytotype hybrids are mostly sterile, it further may instantly cause reproductive incompatibility between the newly formed polyploid and its diploid progenitors (Grant 1971). Hence, whole genome duplication represents a potential pathway for rapid reproductive isolation.

## Thesis Outline

The focus of the present thesis lies on speciation by polyploidy in the genus *Leucanthemum* Mill. (Compositae, Anthemideae), which forms an impressive polyploid complex with ploidy levels ranging from diploid to dokosaploid (22 chromosome sets). The genus currently comprises 41 species (Euro+Med 2011) that naturally occur all over the European continent. For this thesis, four *Leucanthemum* taxa – subsequently referred to as '*L. pluriflorum* group' – from the NW Iberian Peninsula have been selected for analysis. The group includes the eponymous diploid species *L. pluriflorum* Pau, the tetraploid *L. ircutianum* subsp. *pseudosylvaticum* Vogt, and the two hexaploids *L. sylvaticum* (Brot.) Nyman and *L. merinoi* Vogt & Castrov. The distributional ranges of the four taxa partly overlap, and their morphology as well as preliminary cpDNA sequence

analyses strongly indicate that they share a common evolutionary history (Höfl 2006). Moreover, Vogt (1991) suggests that hybridization and introgression between at least two of the four taxa is frequent.

The three chapters of the thesis address different aspects of the main topic 'speciation by polyploidy'. The manuscripts are supposed to be published separately in appropriate scientific journals, and therefore they each feature an abstract, an introduction, a section explaining the methods, a presentation of the respective results, as well as an exhaustive discussion. Following the manuscripts, a synopsis of the three chapters is provided, as well as a list of the references used.

The aim of chapter 1 is to test the initial hypothesis of a close relationship between the members of the study group, and further to uncover potential crossbreeding with other sympatrically distributed *Leucanthemum* species. For this purpose, the four taxa of the study group were extensively sampled over their entire distributional range. In addition to this, populations of all other diploids that are found in the respective area were included in the analysis. The ploidy level of all populations was thoroughly checked using flow cytometry, and a phylogenetic analysis was conducted on the basis of cpDNA sequences and AFLP banding patterns.

The aim of chapter 2 is to clarify the origin of the three polyploid taxa of the study group. In particular, the question whether the polyploids are auto- or allopolyploids is addressed. In order to settle this matter, the sample set from chapter 1 was extended by specimens from all presently accepted diploid *Leucanthemum* species, and a genetic analysis using molecular cloning and sequencing of the ETS region from the nuclear ribosomal DNA was conducted.

Finally, reproductive isolation between the four taxa – particularly between different cytotypes – is investigated in chapter 3. According to the biological species concept, the members of a species form a reproductive community, and hence two species can be considered distinct when geneflow between their populations is

reduced or ceases altogether (Mayr 1942). To verify if polyploidy causes reproductive isolation and, therefore, triggers speciation in *Leucanthemum*, extensive crossing experiments between the members of the study group were performed. Subsequently, seed set and viability of seeds resulting from inter-cytotype crosses were measured and compared to fertilities following intra-cytotype pollinations to quantify the relative extent of reproductive isolation between the three cytotypes.

## Chapter 1

# Analysis of Chloroplast Sequence Information and AFLP Fingerprints in Three *Leucanthemum* Mill. (Compositae, Anthemideae) Polyploids and their Putative Diploid Ancestors

### 1.1 Abstract

Evolution by genome duplication is widely accepted as a fundamental pathway in plant evolution, and enormous strides have been made to elucidate the mechanisms and consequences of polyploid speciation. In particular, modern techniques for high throughput genotyping and ploidy level estimation have contributed a lot to our understanding of this prevalent phenomenon. The present study uses flow cytometry, cpDNA sequencing and AFLP fingerprinting in order to better understand evolution by genome duplication in one tetraploid and two hexaploid *Leucanthemum* Mill. species from the Western Iberian Peninsula. Previous phylogenetic analyses of the entire genus showed that the investigated polyploids share a closely related chloroplast haplotype, and that they all presumably were derived from the diploid species *L. pluriflorum*, which is endemic to the coast of the Spanish region Galicia. Yet, the question remained unsettled whether there were other species involved in polyploid formation or not, and also whether the polyploids have a single or recurrent origin. Here, each of the four taxa was extensively sampled over its entire distributional range, and also populations of other diploids that are found in the study area were included. Chloroplast sequence data corroborate the hypothesis that *L. pluriflorum* represents the maternal parent of the polyploid taxa and further indicates that there were at least three independent genome duplication events. In addition to this, AFLP fingerprint data

suggest that one or more yet unknown diploid species were involved in the formation of the polyploids, and that geneflow between the polyploid lineages (and presumably also between cytotypes) has occurred.

## 1.2 Introduction

Hybridization and polyploidy have long been recognized to be a highly frequent evolutionary pathway promoting genetic diversity and speciation in plants (Stebbins 1950, Grant 1971, Masterson 1994, Rieseberg 1997). These processes give rise to complex patterns of reticulate evolution that can hardly be studied using classical systematic approaches, and they also decisively complicate molecular phylogenetics. Yet, the combination of DNA markers from the chloroplast and nuclear genome proved to be suitable for the analysis of these complex patterns, and consequently shed light on the relevance of polyploidy to speciation (Cronn et al. 2003, Doyle et al. 2004a, Albach 2007). Phenomena like multiple and recurrent formation of polyploids (Soltis et al. 2004b, Lihová et al. 2006) or cryptic barriers to geneflow within morphologically consistent species that are caused by cytotypic effects (Husband et al. 2002, Halverson et al. 2008) are today considered to be both prevalent and significant. In many genera, genome duplication triggered the emergence of extensive polyploid complexes, with a lot of different cytotypes being interrelated by vertical and horizontal geneflow. Examples for genera comprising several ploidy levels can be found in a number of angiosperm families (Grant 1971), and many of them are subject to intensive research [e.g. *Achillea* (Guo et al. 2008), *Dactylorhiza* (Pedersen 2006), *Glycine* (Doyle et al. 2004b), *Primula* (Guggisberg et al. 2009), and *Silene* (Popp & Oxelmann 2007)].

The present survey uses DNA sequence data from the chloroplast genome and AFLP nuclear genomic fingerprinting to reconstruct the evolutionary history of three polyploid species from the *Leucanthemum* Mill. polyploid complex.

The genus *Leucanthemum* belongs to the Circum-Mediterranean clade of the Compositae-Anthemideae (Oberprieler 2005). Its representatives are small perennial herbs that can be recognized by flower heads with white ray florets (greek: λευκος = white; ανθεμος = flower). They are characterized by simple or branched stems which grow from a rosette, and feature entire, toothed or pinnately lobed leaves (Figure 1A). The achenes are ribbed and furnished with mucilage cells as well as resin ducts (Figure 1A and 1B). At present, *Leucanthemum* comprises 41 species (Euro+Med 2011) which originally are found in Europe and Siberia, but also occur naturalized in many regions all over the world. While its presumptive sister genera exclusively contain diploid taxa (Oberprieler 2005), the genus *Leucanthemum* forms an impressive polyploid complex, with a basic chromosome number of nine and ploidy levels ranging from diploid to dodekaploid (Vogt 1991). One taxon even contains 198 chromosomes and hence is dokosaploid (22 chromosome sets). For ten taxa, ploidy levels are presently doubtful or unknown.

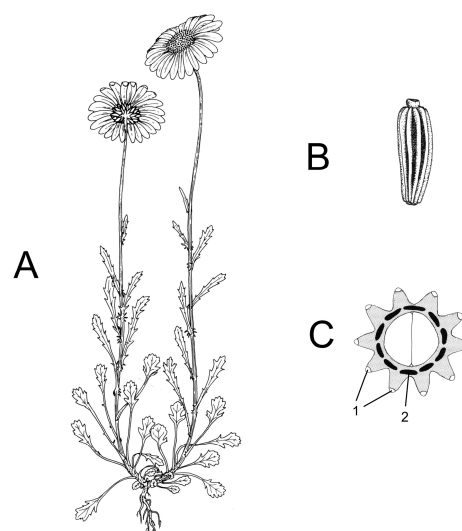


Figure 1: (A) Habitus of a typical *Leucanthemum* species (*L. gaudinii*). (B) An achene from *L. vulgare*, and (C) its semi-schematic cross-section. (1 = mucilage cells, 2 = resin ducts; illustrations have been modified after Vogt 1991)

The genus' center of diversity is located on the Iberian Peninsula, 16 of its species are exclusively found here. Vogt (1991) collected extensive data on morphological and cytological variation of *Leucanthemum* in Spain and Portugal, and revised the taxonomical classification on the basis of these data. Further, the Apennine Peninsula, the Alps and the Balkans feature a number of endemic *Leucanthemum* species. Until now, a detailed revision for these regions is pending, and therefore large parts of the genus' diversity still remain unsurveyed.

While nuclear ribosomal DNA data indicate that the entire genus is approximately 4.0-7.9 Ma old and originated in the Western Mediterranean, its recent species presumably developed throughout the Pleistocene, and hence genetic differentiation within *Leucanthemum* is rather weak (HöbI 2006). Yet, a molecular phylogeny of the genus based on chloroplast markers (HöbI 2006) identified a small monophyletic group of four taxa that are genetically clearly separated from all other *Leucanthemum* species: the diploid *L. pluriflorum*, the tetraploid *L. ircutianum* subsp. *pseudosylvaticum*, and the two hexaploid species *L. sylvaticum* and *L. merinoi*. These taxa are all endemic to the autonomous community of Galicia in NW Spain and to Portugal. While two of them are common at the coastline of Galicia (*L. pluriflorum* and *L. merinoi*), *L. ircutianum* subsp. *pseudosylvaticum* and *L. sylvaticum* are mainly distributed inland (c.f. Figure 2). Morphological differentiation of the four species is considerably low. Only the diploid taxon is well characterized by its leaf morphology, the polyploids are mainly discriminated by cytology or ecological traits. In particular, *L. sylvaticum* and *L. ircutianum* subsp. *pseudosylvaticum* are extremely similar to each other, indicating a close phylogenetic relationship of these two taxa.

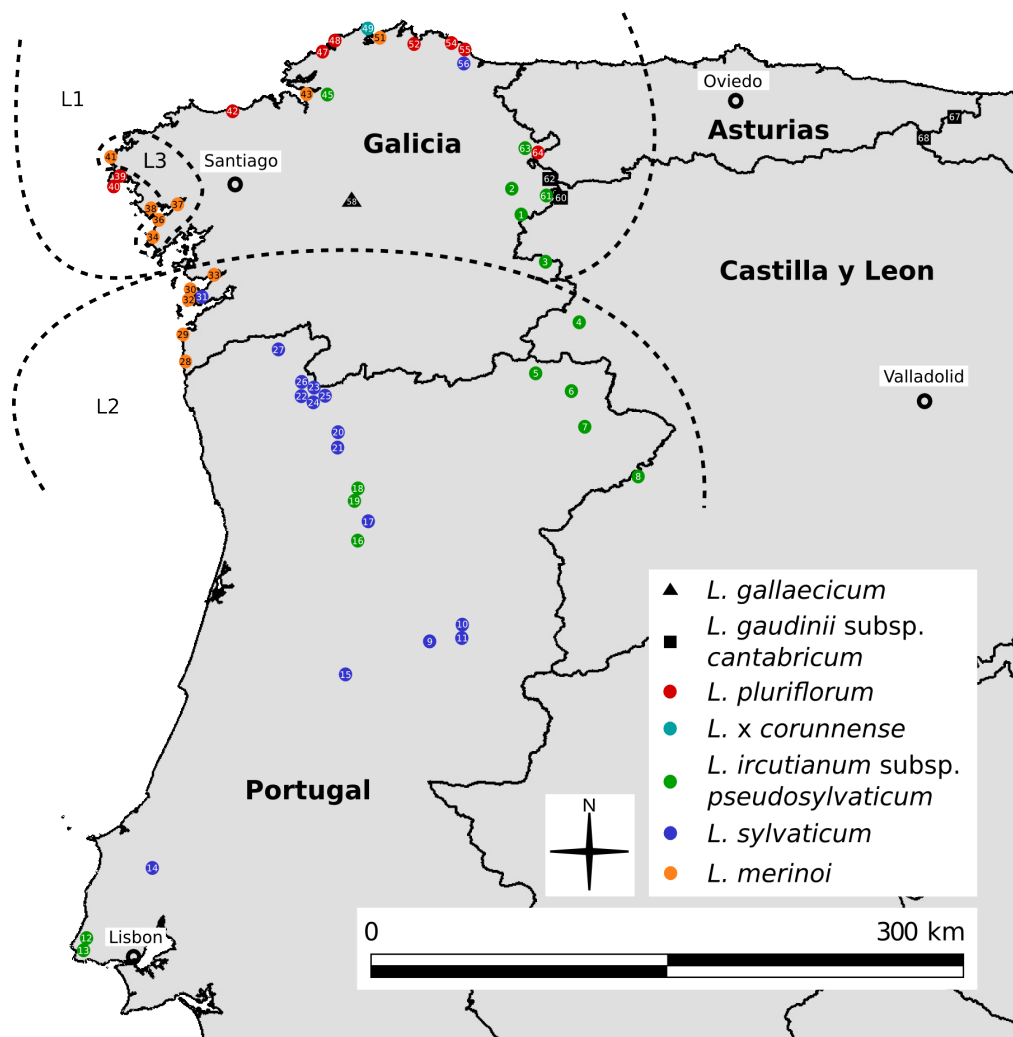


Figure 2: Geographical distribution of populations used in this study. Numbers in symbols represent population IDs (c.f. Table 1). Broken lines indicate affiliation to the three cpDNA lineages (c.f. Figure 3 and Figure 4).

Previous cytological investigations including the four species from the *L. pluriflorum* group suggest that there are large areas where two or more cytotypes co-occur (Vogt 1991). However, due to the high amount of work needed to determine ploidy levels by chromosome staining and counting, Vogt (1991) classified most plants mainly on the basis of morphological characters, and only a few populations of each taxon (6-10) were investigated cytologically. Hence, the emerging large scale geographical patterns of cytotype distribution are not well supported and require thorough revision. Further, the question whether cytotypes mix locally had to



remain completely unaddressed. In the current analysis, each of the four species from the *L. pluriflorum* group has been sampled across its entire distributional range, and several individuals per population have been investigated to increase the probability that mixed-ploidy stands are detected if present. In addition to the investigation of geographical patterns of cytotype distribution, the present work will focus on polyploid speciation and gene flow within the *L. pluriflorum* group as revealed by nuclear genetic fingerprinting and cpDNA sequence analysis.

### 1.3 Material and Methods

*Sampling and DNA extraction.* – During a field trip in 2007, silica dried leaf material from the *L. pluriflorum* group was collected at 54 locations in NW Spain and Portugal, covering the entire distribution area of each of its five members (Table 1 and Figure 2). In addition to this, four populations from *L. gaudinii* subsp. *cantabricum* (distributed in the Cantabrian and Asturian Mountains, Vogt 1991) and one population of *L. gallaecicum* (endemic to C Galicia, Oubiña & Ortiz 1990) were sampled and included in the analyses. These two taxa represent the only *Leucanthemum* diploids that grow sympatrically with members of the *L. pluriflorum* group, and thus are putative parental species for polyploids in the study area. As the other three diploid *Leucanthemum* taxa known to occur on the Iberian Peninsula, *L. vulgare* subsp. *eliasii* (E Cantabrian Mountains), *L. vulgare* subsp. *pujiulae* (W Cantabrian Mountains), and *L. gracilicaule* (Valencia), are more than 300 km apart from the *L. pluriflorum* group, they very likely were not involved in recent polyploid formation in Galicia and Portugal. Hence, these taxa were not included in the present analysis. As a preliminary survey of cpDNA variability in the *L. pluriflorum* group indicated that populations do not comprise more than one chloroplast genotype, only one individual per population was

selected for sequencing. For AFLP fingerprinting, four to five individuals of each population were analyzed, for a total of 246 individuals. Only in exceptional cases of very small population sizes fewer individuals were examined. Due to the restricted distribution of the two diploid outgroup taxa, *L. gaudinii* subsp. *cantabricum* and *L. gallaecicum*, seven individuals (from four populations) and four individuals (from one population) were considered to be sufficient to represent these taxa in the AFLP analysis, respectively. The definite number of individuals from each population that were chosen for sequencing and fingerprinting is given in Table 1. In order to quantify AFLP genotyping errors, replicate profiles were generated for twelve randomly selected samples, representing 5 % of all samples. For all selected individuals, total genomic DNA was extracted from 10-20 mg leaf material according to a protocol based on the CTAB method of Doyle & Doyle (1987).

*Flow cytometry.* – For all populations used in this study, ploidy levels of several individuals were determined by flow cytometry (c.f. Table 1), either using silica dried leaf material or fresh leaves from cultivated plants. Measurements were performed by Plant Cytometry Services (Schijndel, NL) using DAPI as DNA stain and *Lactuca sativa* L. (6.38 pg/2C) as internal standard.

*Chloroplast DNA amplification and sequencing.* – For the analysis of chloroplast DNA sequences, the intergenic spacers *psbA-trnH* and *trnC-petN* were selected. PCR amplification of *psbA-trnH* was performed using primers published by Sang et al. (1997) and Hamilton (1999). The *trnC-petN* region was amplified with primers published by Lee & Wen (2004). Amplification setups basically followed the PCR protocol given in Hößl (2006), but annealing temperatures were modified according to the respective original publications. After amplification, the PCR products were purified with AMPure magnetic bead solution (Agencourt Bioscience Corporation, Beverly, USA) and Sanger sequencing was performed using the DTCS Quick Start Kit

from Beckman Coulter (Fullerton, USA), according to the manufacturer's protocol. The sequencing product was precipitated with ethanol and sodium acetate, and fragment detection was done on a CEQ8000 capillary sequencer (Beckman Coulter, Fullerton, USA). The resulting electropherograms were checked for sequencing errors and corrected manually if necessary. While *psbA-trnH* sequences were of high quality, a poly-A pattern in *trnC-petN* fragments caused severe sequencing problems. Hence, these fragments were sequenced in both directions to obtain full reading length.

*AFLP procedure.* – The AFLP fingerprinting method was conducted following the original two-step protocol of Vos et al. (1995), with some modifications. For the combined restriction-ligation reaction, 50 ng DNA was incubated at 37°C for 2 h, including 2.5 U of each MseI and EcoRI, 0.5 U T4 DNA ligase (all enzymes from Fermentas, St. Leon-Rot, Germany), as well as 1 µM and 0.1 µM of the original MseI and EcoRI adaptors, respectively. A pre-selective PCR was carried in 5 µl final volume containing 1 µl template DNA, 3.75 µl AFLP CoreMix from Applied Biosystems (Carlsbad, USA), and 0.5 µM of primers with one selective nucleotide (MseI-C and EcoRI-A). The selective PCRs were carried in 5 µl final volume containing 1 µl template DNA, 0.25 U Taq polymerase (PeqLab, Erlangen, Germany), 0.2 mM of each dNTP and 0.5 µM of primers with three selective nucleotides. Both PCR steps were performed according to the cycling protocols given in Meister et al. (2006). For each sample, three selective PCRs with differently labeled EcoRI primers were conducted, and suitable primer combinations were selected in an initial screening. Primer combinations were as follows: EcoRI-ACA/MseI-CAT, EcoRI-ACG/MseI-CAT, and EcoRI-AGC/MseI-CAT. The PCR products were precipitated and subsequently dissolved in a mixture of GenomeLab Sample Loading Solution and CEQ Size Standard 400 (both Beckman Coulter, Fullerton, USA). Fragment detection was performed on a CEQ8000 capillary sequencer (Beckman Coulter, Fullerton, USA).

Table 1: Taxa and populations included in chapter 1. Polyploid taxa are written in bold characters. For each population, sample sizes, geographical information, and collectors are provided. Collectors abbreviations: AH Andreas Hutschenreuther, NG Nina Greiner, RH Roland Hößl, SH Sven Himmelreich. Specimens for each population are deposited in the private herbarium of the author.

Taxon (ploidy level)	Population ID	Sample size			Country, province/district	Coordinates	Collectors
		Flow cytometry	Sequencing	AFLP			
<i>Leucanthemum gaudinii</i> subsp. <i>cantabricum</i> (Font Quer & Guinea) Vogt (2x)	60	1	1	1	Hs, Lugo	42.8315, -6.8569	RH 60
	62	2	1	2	Hs, Lugo	42.9249, -6.8657	RH 62
	67	3	1	2	Hs, Asturias	43.2712, -4.6363	RH 67 & SH
	68	2	1	2	Hs, Cantabria	43.1538, -4.8053	RH 68 & SH
<i>Leucanthemum gallaecicum</i> Rodr. Oubiña & S. Ortiz (2x)	58	3	1	4	Hs, Lugo	42.8205, -7.9504	RH 58
<i>Leucanthemum pluriflorum</i> Pau (2x)	39	4	1	5	Hs, A Coruña	42.9286, -9.2381	RH 39
	40	3	1	4	Hs, A Coruña	42.8838, -9.2726	RH 40
	42	3	1	5	Hs, A Coruña	43.3069, -8.6186	RH 42
	47	5	1	4	Hs, A Coruña	43.6418, -8.1225	RH 47
	48	3	1	4	Hs, A Coruña	43.7062, -8.0517	RH 48
	52	3	1	5	Hs, Lugo	43.6842, -7.6153	RH 52
	54	5	1	4	Hs, Lugo	43.6876, -7.4125	RH 54
	55	3	1	5	Hs, Lugo	43.6309, -7.3330	RH 55
64	3	1	2	Hs, Lugo	43.0797, -6.9559	RH 64	
<i>Leucanthemum X corunnense</i> Lago (4x)	49	3	1	4	Hs, A Coruña	43.7715, -7.8696	RH 49
<i>Leucanthemum icutianum</i> subsp. <i>pseudosylvaticum</i> Vogt (4x)	1	3	1	5	Hs, Lugo	42.7267, -7.0258	RH 1 & AH
	2	3	1	5	Hs, Lugo	42.8705, -7.0750	RH 2 & AH
	3	3	1	5	Hs, Ourense	42.4626, -6.8940	RH 3 & AH
	4	3	1	5	Hs, Zamora	42.1333, -6.7074	RH 4 & AH
	5	3	1	5	Lu, Bragança	41.8498, -6.9456	RH 5 & AH
	6	3	1	5	Lu, Bragança	41.7516, -6.7505	RH 6 & AH
	7	1	1	1	Lu, Bragança	41.5582, -6.6754	RH 7 & AH
	8	3	1	5	Hs, Salamanca	41.2843, -6.3787	RH 8 & AH
	12	3	1	5	Lu, Lisbon	38.7924, -9.4257	RH 12 & AH
	13	8	1	4	Lu, Lisbon	38.7972, -9.4384	RH 13 & AH
	16	3	1	5	Lu, Viseu	40.9372, -7.9255	RH 16 & NG
	18	3	1	4	Lu, Porto	41.1768, -7.9443	RH 18 & NG
	19	3	1	4	Lu, Porto	41.1803, -7.9344	RH 19 & NG
	45	1	1	1	Hs, A Coruña	43.3972, -8.0959	RH 45
	61	2	1	2	Hs, Lugo	42.8295, -6.8856	RH 61
63	6	1	5	Hs, Lugo	43.0943, -7.0045	RH 63	
<i>Leucanthemum sylvaticum</i> (Brot.) Nyman (6x)	9	3	1	4	Lu, Guarda	40.3934, -7.5289	RH 9 & AH
	10	3	1	4	Lu, Guarda	40.4434, -7.3463	RH 10 & AH
	11	3	1	5	Lu, Guarda	40.4309, -7.3491	RH 11 & AH
	14	5	1	5	Lu, Lisbon	39.1859, -9.0581	RH 14 & AH
	15	3	1	5	Lu, Coimbra	40.2165, -7.9954	RH 15 & NG
	17	3	1	5	Lu, Viseu	41.0392, -7.8684	RH 17 & NG
	20	3	1	5	Lu, Braga	41.4953, -8.0370	RH 20 & NG
	21	4	1	5	Lu, Braga	41.4811, -8.0379	RH 21 & NG
	22	3	1	4	Lu, Braga	41.7307, -8.1648	RH 22 & NG
	23	3	1	5	Lu, Braga	41.7057, -8.1673	RH 23 & NG
	24	3	1	5	Lu, Braga	41.7099, -8.2194	RH 24 & NG
	25	3	1	5	Lu, Braga	41.7260, -8.2165	RH 25 & NG
	26	9	1	5	Lu, Braga	41.7659, -8.2437	RH 26 & NG
	27	3	1	5	Lu, Viana do Castelo	41.9805, -8.3616	RH 27 & NG
31	3	1	5	Hs, Pontevedra	42.2756, -8.8038	RH 31 & NG	
56	6	1	4	Hs, Lugo	43.5774, -7.3412	RH 56	
<i>Leucanthemum merinoi</i> Vogt & Castro. (6x)	28	10	1	5	Hs, Pontevedra	41.9132, -8.8760	RH 28 & NG
	29	8	1	5	Hs, Pontevedra	42.0628, -8.8905	RH 29 & NG
	30	7	1	4	Hs, Pontevedra	42.2707, -8.8608	RH 30 & NG
	32	5	1	4	Hs, Pontevedra	42.2992, -8.8488	RH 32 & NG
	33	3	1	5	Hs, Pontevedra	42.3892, -8.7179	RH 33 & NG
	34	6	1	5	Hs, A Coruña	42.6013, -9.0563	RH 34
	36	2	1	3	Hs, A Coruña	42.6977, -9.0237	RH 36
	37	3	1	5	Hs, A Coruña	42.7835, -8.9231	RH 37
	38	3	1	4	Hs, A Coruña	42.7605, -9.0649	RH 38
	41	3	1	5	Hs, A Coruña	43.0471, -9.2873	RH 41
	43	3	1	5	Hs, A Coruña	43.4020, -8.2093	RH 43
51	1	1	1	Hs, A Coruña	43.7170, -7.8038	RH 51	

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*Sequence analysis.* – The *psbA-trnH* and *trnC-petN* sequences of each sample were combined in a single text file and a multiple alignment of all sequences was generated with Clustal W (Higgins & Sharp 1988) as implemented in BioEdit 7.0.5.3 (Hall 1999). In the next step, GapCoder (Young & Healy 2003) was used to code indels in a 0/1 matrix according to the simple gap coding method of Simmons & Ochoterena (2000). After some modifications on the gap-coded alignment ('1' and '0' were replaced by 'A' and 'T', original gaps were replaced by 'N'), the dataset was subjected to a parsimony analysis in PAUP\* 4.0b10 (Swofford 1998). A heuristic search was conducted using 1000 random sequence addition replicates, ACCTRAN character state optimization, TBR branch swapping and MulTrees option, and a 50 % majority rule consensus tree was constructed from the most parsimonious trees. Bootstrap support (Felsenstein 1985) was estimated with 1000 bootstrap replicates, TBR branch-swapping and simple sequence addition. In addition, a Neighbor-net diagram based on uncorrected p-distances was constructed and bootstrapped with 1000 replicates in SplitsTree4 (Huson 1998).

*AFLP analysis.* – Band scoring was performed manually with the software Genographer 1.6.0 (J. J. Benham, Montana State University, 1998). Only polymorphic, clearly scorable fragments in a range from 60 to 450 bp were analyzed. In past studies, a number of different methods were used to investigate AFLP data in polyploids. A comprehensive (but far from complete) review of 28 phylogenetic and population genetic studies using AFLP data in sample sets containing several ploidy levels clearly demonstrates the variety of methods available (Table 2).

Table 2: Statistical methods used for AFLP data analysis in 28 phylogenetic and population genetic studies including two or more ploidy levels.

Publication	Analysis of taxon specific bands	AMOVA	Parsimony	PCoA	Bayesian	NMDS	UPGMA	Neighbor-joining	Neighbor-net
Abdalla et al. (2001)				x			x	x	
Albach (2007)	x				x	x	x	x	
Bardy et al. (2010)				x	x		x	x	x
Dalirsefat et al. (2009)							x		
Dixon et al. (2008)									x
Emshwiller et al. (2009)						x			
Gobert et al. (2002)			x	x			x		
Goldmann et al. (2004)			x						
Guo et al. (2005)	x					x		x	
Guo et al. (2008)								x	
Halverson et al. (2008)		x						x	
Hedren et al. (2001)				x					
Hedren et al. (2007)						x	x		
Hodkinson et al. (2002)				x				x	
Kadereit et al. (2006)	x								
Kardolus et al. (1998)			x				x		
Kilian et al. (2007)								x	
Lihová et al. (2003)	x			x				x	
Lihová et al. (2004)				x				x	
Lihová et al. (2007)				x					
Liu et al. (2009)								x	
Ma et al. (2010)									x
Marhold et al. (2004)	x							x	
O'Hanlon et al. (1999)						x			
Ozkan et al. (2002)								x	
Rahimmalek et al. (2009)				x		x			
Tosto & Hopp (2000)							x		
Weiss-Schneeweiss et al. (2008)									x

Besides rarely used procedures like model-based Bayesian analysis or parsimony, similarity-based methods, both in combination with clustering (UPGMA, Neighbor-joining, Neighbor-net) and ordination techniques (PCoA, MDS), are the most prevalent approach to analyze mixed-ploidy data sets. While tree-shaped graphs are mainly used to visualize the phylogenetic history of diploids, splits graphs diagrams and ordination methods are more suitable when reticulate processes like hybridization or polyploidy are involved. Additionally, the analysis of taxon specific AFLP bands was successfully used to elucidate the evolutionary history of polyploids. However, there is no 'hard' criterion for choosing a certain approach, and several different methods should be used to reduce methodological bias. For the present study, a distance based ordination (PCoA; based on a simple matching coefficient) was performed in FAMD (Schlüter & Harris 2006), and a parsimony splits network was constructed in SplitsTree4 (Huson 1998). All analyses were conducted for each of the three primer combinations separately, and for the combined data

set. Yet, since the resulting patterns were congruent, the results of the individual analyses are neither shown nor discussed.

To test for the influence of band number in the distance analysis, in-silico hybridization was performed for all possible combinations of banding patterns from diploid individuals, and a second PCoA was conducted for these in-silico hybrids and the original data. Significant differences in band number of diploids, tetraploids, hexaploids, and synthetic hybrids was tested with an ANOVA and Scheffe's method for post-hoc analysis in the case of normally distributed data (Kolmogorov-Smirnov test  $P > 0.05$ ), or with the non-parametric Kruskal-Wallis one-way analysis of variance and subsequent Mann-Whitney  $U$  tests for normally distributed data (Kolmogorov-Smirnov test  $P \leq 0.05$ ).

#### 1.4 Results

*Flow cytometry.* - Both fresh leaves and silica-gel material was suitable for flow cytometry and produced good histograms (CVs < 5). On average, 3.58 individuals per population were investigated, for a total of 211 individuals. Each sample could be easily assigned to either diploid, tetraploid or hexaploid level, with mean relative DNA contents of 2.13-2.27, 3.62-4.29 and 5.28-5.85, respectively. None of the 59 investigated populations contained more than one cytotype, and neither triploids nor pentaploids were observed. A table showing the relative DNA contents of all samples is provided in the digital appendix (DA\_1\_4).

*Chloroplast DNA sequencing.* - Sequences of the chloroplast intergenic spacers *psbA-trnH* and *trnC-petN* were determined for a total of 59 individuals, representing 54 populations from the *L. pluriflorum* group (9x *L. pluriflorum*; 16x *L. ircutianum* subsp. *pseudosylvaticum*; 12x *L. sylvaticum*, 16x *L. merinoi*, and 1x *L. × corunnense*),

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and five populations from outgroup taxa (1x *L. gallaecicum*, 4x *L. gaudinii* subsp. *cantabricum*). The sequence alignment comprises 872 characters (391 and 464 characters from *psbA-trnH* and *trnC-petN*, respectively, plus 17 indels coded in a binary matrix at the end of the alignment). The alignment can be found in the digital appendix provided with this thesis (DA\_1\_2).

Within the 54 populations from the *L. pluriflorum* group, a total of 23 chloroplast haplotypes can be identified, i.e. nearly every second population represents an individual haplotype. Both the parsimony analysis (Figure 3) and the Neighbor-net diagram (Figure 4) show that the investigated populations from the *L. pluriflorum* group form a monophyletic group (bootstrap value: 100 %), and that they are well differentiated from their sympatric diploids. Within the *L. pluriflorum* group, three phylogenetic lineages can be recognized: lineage L1 comprises all diploid accessions and some polyploid populations, lineages L2 and L3 only contain polyploids. While the tetraploid *L. irtutianum* subsp. *pseudosylvaticum* is equally represented in lineage L1 and L2, the hexaploid *L. sylvaticum* is restricted to lineage L2. The second hexaploid, *L. merinoi*, is present in all lineages, and L3 exclusively contains populations from this taxon.



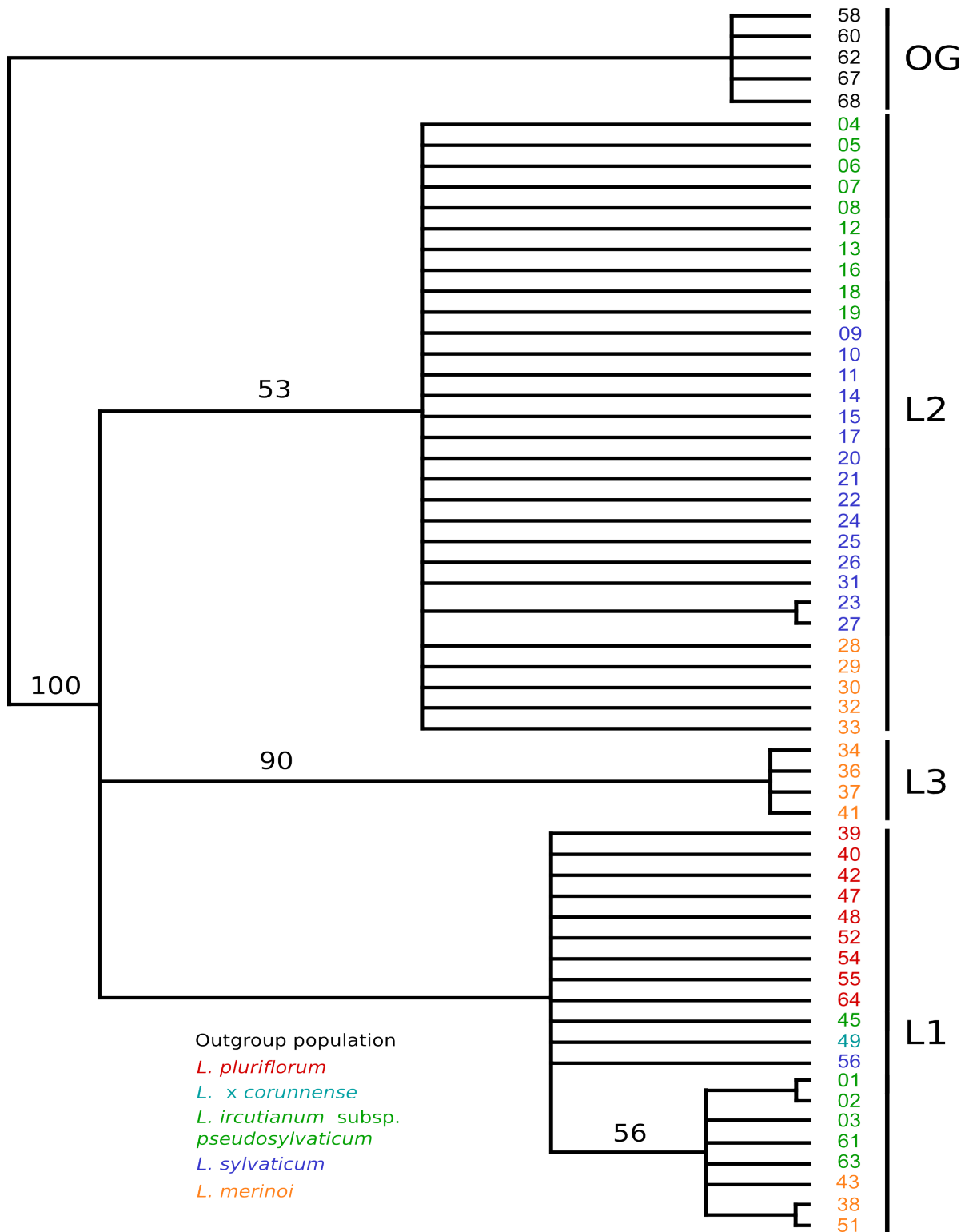
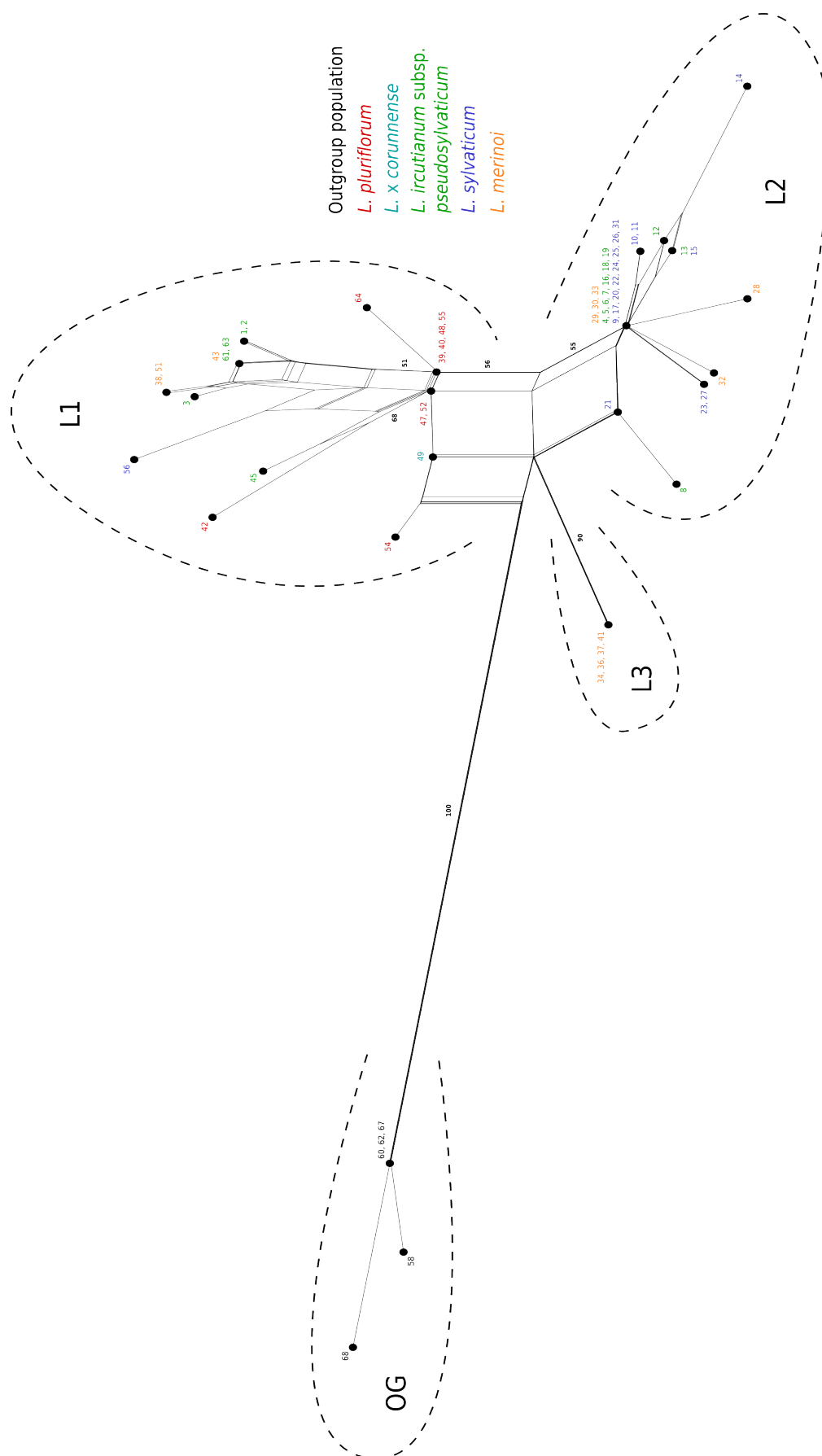


Figure 3: Strict consensus tree constructed for 54 populations of the *L. pluriflorum* group and 5 outgroup populations (1 individual each), based on cpDNA sequence data from *psbA-trnH* and *trnC-petN* intergenic spacers (872 characters). Numbers on tips of the tree represent population IDs, their color provides taxonomic information. Numbers along branches are bootstrap values above 50%. The vertical bars indicate the outgroup (OG), and the three polyploid lineages recognized within the *L. pluriflorum* group (L1, L2, L3). Individual IDs can be found in the digital appendix provided with this thesis (DA\_1\_2).



Furthermore, the phylogenetic lineages show distinct patterns of geographical distribution, with populations from L2 in the South and members of L1 and L3 in the North (Figure 2).

*AFLPs.* – The AFLP process was applied to 246 individuals from 59 populations (54 populations from the *L. pluriflorum* group, five outgroup populations; c.f. Table 1), and the three primer combinations provided a total of 137 clearly scorable bands (EcoRI-ACA/MseI-CAT: 58; EcoRI-ACG/MseI-CAT: 38; EcoRI-AGC/MseI-CAT: 41). An average error rate of 5.6 % was estimated from the twelve replicates. The data matrix can be found in the digital appendix provided with this thesis (DA\_1\_3).

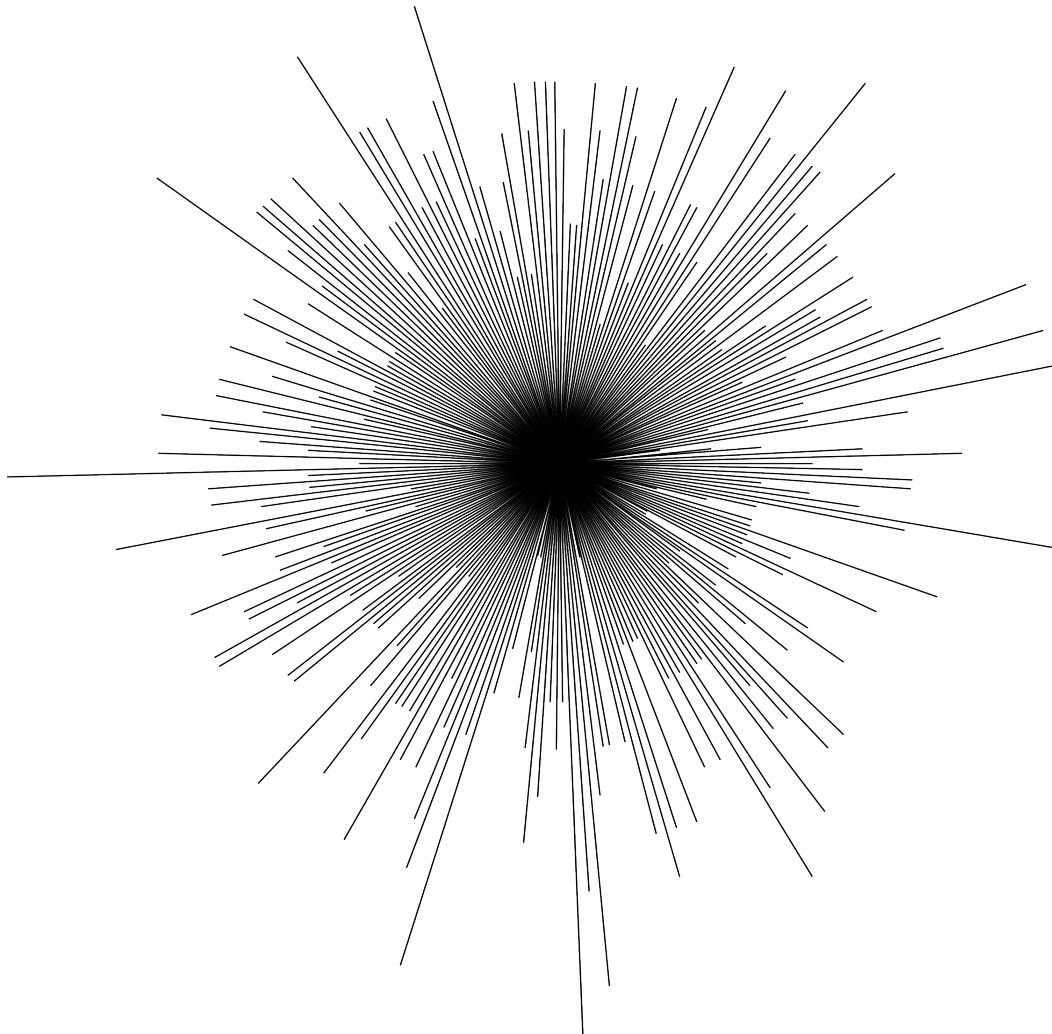


Figure 5: Parsimony splits analysis of 235 individuals from the *L. pluriflorum* group and 11 individuals from diploid outgroup taxa (*L. gaudinii* and *L. gallaecicum*), based on 137 AFLP fragments.

While the parsimony analysis did not reveal any structure in the data set (Figure 5), two distinct groups of individuals (G1 and G2) could be recognized in the scatter plot of PCoA axes 1 and 2 (Figure 6), with group G1 containing virtually all accessions from diploid populations (including all samples from the diploid outgroup *L. gallaecicum*, and all but one sample from *L. gaudinii*), and group G2 comprising all polyploids except the inter-cytotype hybrid *L. × corunnense* (which is found amongst the diploids). The first three axes represent 19 % of the total variation in the data set, with 11.8, 3.9, and 3.3 % for PCoA axes 1, 2, and 3, respectively. Student's *t*-test comparing mean band numbers of diploids and polyploids detected significant differences between these classes ( $P < 0.001$ ), with diploids and polyploids showing mean band numbers of 40.37 and 46.09, respectively. There was no significant difference between mean band numbers of tetraploids and hexaploids (both 46.09). The ANOVA of band numbers of the three ploidy levels was significant ( $P < 0.001$ ), and Scheffe's post-hoc test resulted that the differences are found between diploids and each of the polyploids. A Kruskal-Wallis analysis of mean band numbers in natural diploids, natural polyploids and in the synthetic profiles was significant ( $P < 0.001$ ; mean band number in in-silico hybrids: 59.07), and subsequent Mann–Whitney *U* tests showed that band numbers in all classes are significantly different from each other ( $P < 0.001$  in all tests). Despite of this relatively high number of bands in the synthetic profiles, the vast majority of the in-vitro hybrids did not ordinate with the natural polyploids in the second PCoA (Figure 7), but rather with samples of *L. pluriflorum*. Only four synthetic hybrids are similar to patterns from natural polyploids, but these in-silico hybrids are derived from the four diploids that also group with polyploids in the second PCoA (but not in the first one, where only one diploid individual is found amongst the polyploids; c.f. Figure 6).

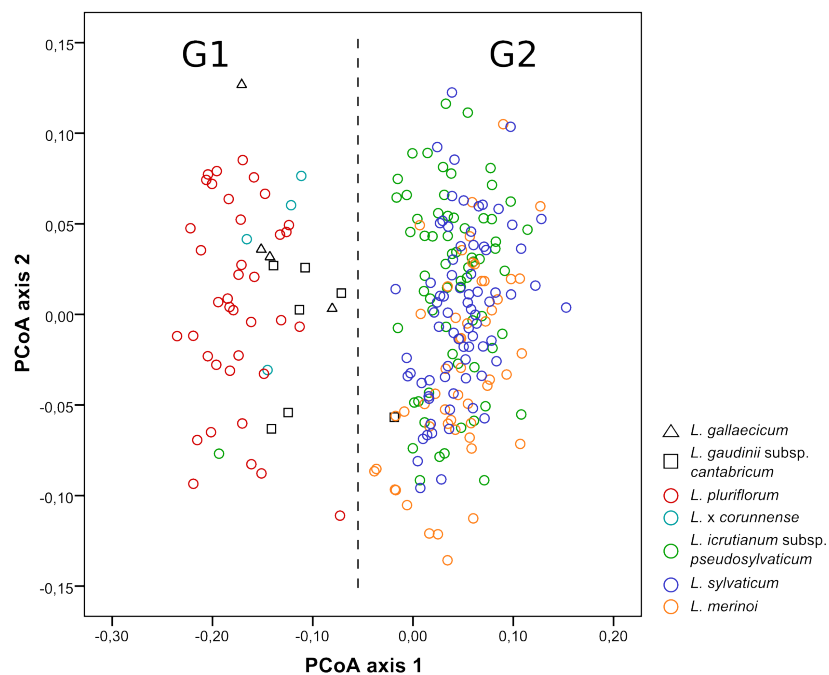


Figure 6: Principal coordinates analysis of 235 individuals from the *L. pluriflorum* group (colored icons) and 11 individuals from diploid outgroup taxa (*L. gaudinii* and *L. gallaenicum*; black icons), based on 137 AFLP fragments. The first two axes explain 11.8 and 3.9 % of the total variation. Groups of individuals mentioned in the text are denoted as G1 and G2.

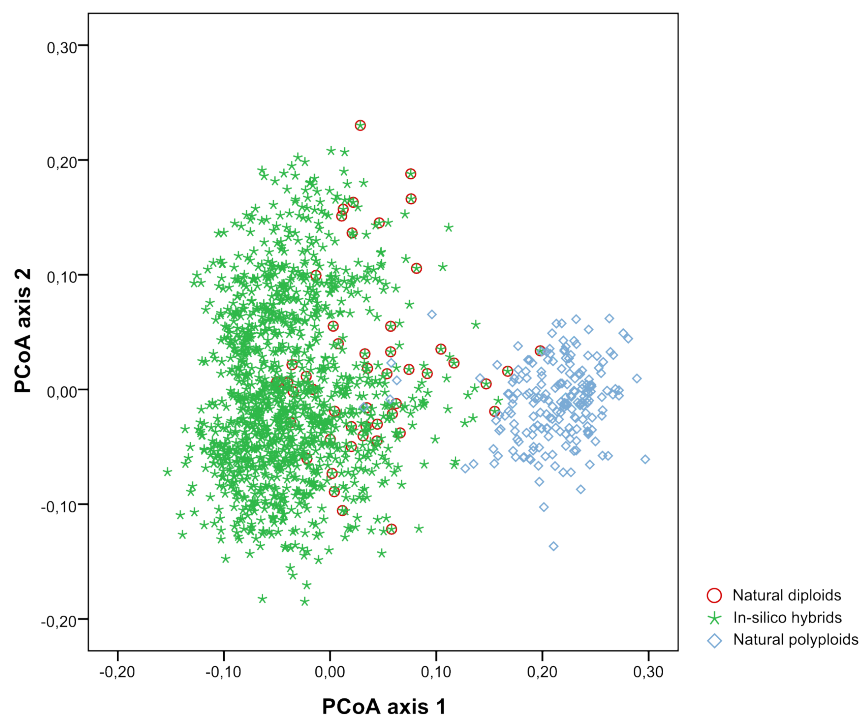


Figure 7: Principal coordinates analysis of 246 *Leucanthemum* individuals (red circles for diploids and blue diamonds for polyploids) and in-silico hybrids (green stars), based on 137 AFLP fragments. The first two axes explain 11.0 and 6.1 % of the total variation.

The first three axes of the second PCoA explain 11.04, 6.13, and 4.89 % of the total variation in the respective dataset.

An analysis of band frequency in polyploids (*L. × corunnense* excluded) showed that nine of the 137 investigated fragments are frequent in one or more of the polyploids (frequency > 0.05), but rare or missing in either of the diploids (frequency ≤ 0.05). By contrast, only three bands were exclusive to one or more diploid taxa.

## 1.5 Discussion

*Geographical patterns of cytotype distribution.* - The present results corroborate Vogt's (1991) hypothesis of a partial sympatry of the four species. *L. pluriflorum* (2x) and *L. merinoi* (6x) grow sympatrically nearly all along the coast of Galicia, and regions exclusively inhabited by only one of the two species are scarce. Only the provinces of Pontevedra and Lugo lack areas of sympatry of *L. pluriflorum* and *L. merinoi*. By contrast, large scale geographical separation of *L. ircutianum* subsp. *pseudosylvaticum* (4x) and *L. sylvaticum* (6x) is more pronounced, with tetraploids and hexaploids predominantly growing in the northeastern and southwestern parts of the study region, respectively. Yet, areas of sympatry do exist, as both species can be found in the Districts of Porto, Viseu, and Lisbon in Portugal. Finally, also some populations of *L. pluriflorum* (2x) and *L. ircutianum* subsp. *pseudosylvaticum* (4x) grow close to each other (e.g. near A Coruña), even though these cases are the exception rather than the rule. An astonishing finding was that a diploid population from Southern Lugo (population 64) turned out to possess the cpDNA haplotype of the study group, and, consequently, was considered to be *L. pluriflorum*. This population was initially sampled as *L. gaudinii* subsp. *cantabricum* due to its morphology and habitat, and represents the first documented case of *L. pluriflorum* growing inland, sympatrically with the tetraploid *L. ircutianum* subsp. *sylvaticum*.

Concerning the two hexaploid species *L. sylvaticum* and *L. merinoi*, it is difficult to ascertain the degree of geographical overlap and hybridization, as morphological differentiation of these taxa is very low (*L. sylvaticum* is less woody than *L. merinoi*), and species delimitation is mainly based on ecological characters (*L. sylvaticum* is found in sparse forests, *L. merinoi* is a member of coastal vegetation types). Although two populations from the Galician coast have been classified as *L. sylvaticum* due to their morphology and habitat (populations 31 and 56), they would have been considered to be *L. merinoi* if being a little more woody and somewhat closer to the coast. For this reason, the focus of this study lies on inter-cytotype evolutionary processes, and questions on gene flow between the hexaploid species and its taxonomical consequences are not addressed.

*Genetic structure of the L. pluriflorum group and the origin of polyploids.* - The chloroplast DNA sequence data analyzed in the present study strongly support the monophyly of the four species *L. pluriflorum*, *L. irtutianum* subsp. *pseudosylvaticum*, *L. sylvaticum*, and *L. merinoi*, as both the parsimony analysis and the Neighbor-net diagram show high bootstrap support values for the split between the outgroup taxa and the study group. Yet, AFLP fingerprints only partially confirm this pattern. Here, samples of *L. pluriflorum* are more similar to *L. gaudinii* and *L. gallaecicum* than to the polyploids, therefore revealing incongruity between chloroplast and nuclear genome. Besides incomplete stochastic sorting of ancestral polymorphisms (Wendel & Doyle 1998), such discrepancies are usually considered to be an indication of reticulate evolution (Lihová et al. 2006). In most angiosperms, regions from the chloroplast genome are inherited only by the egg cell (Corriveau & Coleman 1988) and largely lack recombination. Therefore, these markers allow uniparental lineages to be traced quite easily, but they do not provide a complete evolutionary picture. On the contrary, nuclear markers are difficult to study due to

methodological issues, but they do represent both parental lineages and hence can be used to uncover past reticulation events.

In the present study, hypervariable plastid DNA and nuclear AFLP fingerprints complement each other and comprehensively illustrate polyploid evolution in *Leucanthemum*. On the one hand, chloroplast sequence data affirm the initial hypothesis that *L. pluriflorum* was involved in the formation of the polyploids, due to the fact that all investigated populations share a closely related chloroplast type. On the other hand, AFLP fingerprints show that there is considerable differentiation between the diploid and the polyploids, thereby weakening the interpretation that *L. pluriflorum* is the only parent for the polyploids.

Although the observed pattern could also have emerged through mutation and selection after an autopolyploid origin, this can be considered rather unlikely for two reasons. First, the banding patterns of the in-silico hybrids are quite similar to the banding patterns from the parental diploids rather than from the polyploids. This clearly demonstrates that the pattern seen in the original PCoA is not just caused by an increased number of bands in the polyploids, but by a phylogenetic signal in the data. Second, the polyploids are characterized by nine specific bands that are neither found in *L. pluriflorum*, nor in one of the other diploids included in this analysis. This points towards genetic contact to one or more other species which today may no longer be present in the study region. Nevertheless, an analysis of ETS sequence diversity in the *L. pluriflorum* group and in the whole genus *Leucanthemum* (chapter 2) did not identify any potential introgressive species among the diploids. Evidently, more molecular and morphological data are needed to clarify the origin of the polyploids. In particular, sequence data from low-copy nuclear loci should be obtained as it proved to be highly suitable to reconstruct the complex evolutionary history of polyploids (Joly et al. 2006, Brysting et al. 2007).



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*Geneflow between cytotypes.* - Past studies on inter-cytotype geneflow found that closely related species with different ploidy levels often are isolated from each other, either by pre- or by post-zygotic barriers or by a combination of both (Coyne & Orr 2004, Husband & Sabara 2004, Linder & Rieseberg 2004, Kennedy et al. 2006, Mallet 2007). Concerning *Leucanthemum*, Villard (1970) reported incompatibility of the widespread species *L. vulgare* (2x) and *L. ircutianum* (4x).

The present results indicate that isolation barriers between cytotypes do exist in the study group, but also show that geneflow is not necessarily eliminated by ploidy level change.

On the one hand, there seems to be relatively strong isolation between *L. pluriflorum* and the polyploids, as AFLP fingerprints show a clear dissimilarity between diploid and polyploid populations. As mentioned above, this dissimilarity may be caused by geneflow between the three polyploid species from the *L. pluriflorum* group and other yet unidentified species. However, if considerable geneflow had occurred between the polyploids and *L. pluriflorum*, populations that backcrossed with *L. pluriflorum* would become more 'diploid-like'. As there are large areas of sympatry of different cytotypes, there are a number of localities where hybridization is basically possible. Specifically, *L. pluriflorum* and *L. merinoi* are geographically very close to each other, thereby facilitating extensive cross-pollination between these cytotypes. Yet, only a single polyploid population was found that genetically resembled the diploids according to AFLP fingerprint data (population 49). This can be considered a strong indication that hybridization between these two cytotypes is rare. The results of crossing experiments between species from the study group (chapter 3) validate this hypothesis, and further suggest that the observed isolation barrier is based on pre-zygotic rather than post-zygotic mechanisms.

On the other hand, there is evidence that tetraploid and hexaploid species from the *L. pluriflorum* group are not completely isolated from each other, and that geneflow

happened after polyploid formation. Although chloroplast sequence data clearly shows that there were at least three independent polyploidization events, no genetic structure within the polyploids can be seen by AFLP. Furthermore, the observed pattern of geographical distribution of these polyploid chloroplast lineages suggests that the polyploidization events did not involve the same diploid parent populations in each of those events. Consequently, it can be assumed that the polyploid lineages originally were genetically distinct, and later got homogenized by geneflow between lineages. This homogenisation is decisively facilitated if isolation between ploidy levels is weak. Yet, the extent of reproductive compatibility of tetraploids and hexaploids cannot be determined from the results of this analysis, and homogenization of lineages could likewise have taken place without direct geneflow between cytotypes. Nevertheless, crossing experiments with members from the *L. pluriflorum* group have already been accomplished, and the results strongly point towards a weak post-zygotic isolation of tetraploids and hexaploids (chapter 3).

In conclusion, this analysis illustrates a complex pattern of evolution by polyploidy within a group of four closely related *Leucanthemum* species from the Western Iberian Peninsula. The *L. pluriflorum* group offers an excellent opportunity to study speciation by polyploidy, and to identify isolation barriers between the resulting cytotypes. Especially if supplemented by experimental approaches like crossing experiments or synthetic polyploids, a number of questions on reticulate evolution can be addressed by studying the *Leucanthemum* polyploid complex.

## Chapter 2

### Analysis of ETS Sequence Diversity in Three *Leucanthemum* Mill. (Compositae, Anthemideae) Polyploids and their Putative Diploid Ancestors

#### 2.1 Abstract

The present analysis uses cloning and sequencing of the external transcribed ribosomal spacer region ETS of the nuclear ribosomal DNA to disentangle evolution by hybridization and polyploidy in a group of four closely related *Leucanthemum* taxa from NW Spain and Portugal, comprising the diploid *L. pluriflorum*, the tetraploid *L. ircutianum* subsp. *pseudosylvaticum*, and the hexaploid species *L. sylvaticum* and *L. merinoi*. Along with a comprehensive set of samples from these four taxa, one population of the alleged hybrid *L. × corunnense* (*L. pluriflorum* × *L. merinoi*) was included, as well as specimens of all remaining 16 diploid taxa from the genus. The final dataset consisted of 540 sequences obtained from 73 plants. Among these sequences, 303 different ETS types were identified, and phylogenetic relationships between ETS types were estimated using Bayesian inference and phylogenetic network calculation. Both the presence of different ETS types within *L. pluriflorum* and its chimeric genetic constitution as uncovered by the phylogenetic analyses suggest a homoploid hybridogenous origin of this diploid. Further, the investigated polyploids did not contain any ETS lineages that were not already present in *L. pluriflorum*, indicating that they presumably formed by polyploidization of the hybrid diploid. Finally, the tetraploid *L. × corunnense* did not show the expected combination of ETS types from *L. pluriflorum* and *L. merinoi*, and hence

speciation by inter-ploidy hybridization involving these two species cannot be corroborated by the present results.

## 2.2 Introduction

Polyploidy has long been recognized to be a prominent evolutionary process. Particularly, it is an important mechanism of speciation in plants (Leitch & Bennett 1997, Otto & Whitton 2000, Soltis et al. 2009). Up to 70 % of all angiosperms have experienced whole genome duplication (Stebbins 1950, Grant 1971, Masterson 1994), and 2 to 15 % of all speciation events include changes in ploidy level (Otto & Whitton 2000, Wood et al. 2009). Extensive DNA sequencing further found traces of past polyploidization events in species commonly referred to as 'classical diploids' (The Arabidopsis Genome Initiative 2000, Bowers et al. 2003, Paterson et al. 2004, Tuskan et al. 2006). This abundance of polyploidy usually is attributed to an associated increase of plasticity (Leitch & Leitch 2008), fixed heterozygosity (Wendel 2000, Comai 2005), and novel patterns of gene expression (Adams et al. 2003, Osborn et al. 2003, Salmon et al. 2005, Tate et al. 2006, Akhunov et al. 2007, Flagel et al. 2008), that finally lead to higher morphological complexity and a lowered risk of extinction (Fawcett et al. 2009). By contrast, newly formed polyploids have to overcome fitness drawbacks caused by problems during meiosis (Cifuentes et al. 2010, Szadkowski et al. 2010, Yousafzai et al. 2010) and by minority cytotype effects (Levin 1975, Husband 2000, Baack 2005).

Since the beginning of the 1990s, DNA sequence analysis has contributed a lot to our understanding of evolution involving hybridization and polyploidy. Since then, plastid and nuclear markers have successfully been used to disentangle phylogenetic relationships in a number of polyploid complexes (reviewed in Soltis et al. 2004a). Especially the internal and external transcribed spacers (ITS, ETS) of the

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nuclear ribosomal DNA (nrDNA) have proven to be instrumental due to various methodological conveniences. ITS and ETS are part of ribosomal DNA arrays that code for ribosomal RNA, which in turn is the main structural and catalytic component of the ribosome. These arrays are organized in large tandem repeats found at one or several loci in each haploid genome (reviewed in Álvarez & Wendel 2003). The number of repeat units in plants varies from 150 to 26,000 and is positively correlated with genome size (Ingle et al. 1975, Álvarez & Wendel 2003, Richard et al. 2008). By reason of the high frequency within the genome, this DNA region can easily be amplified by PCR. Furthermore, the ribosomal genes contained in each tandem repeat are highly conserved, and primer binding sites located in these genes can be used in many organisms (Baldwin et al. 1995). Finally, concerted evolution causes homogenization of different nrDNA sequences (Zimmer et al. 1980, Dover 1982), and hence these multi-copy DNA elements show many typical characteristics of low-copy loci.

Yet, while nrDNA is prominent in phylogenetics, several considerations have to be made when using this highly repetitive region as a evolutionary marker. First, it has to be ensured that orthologous rather than paralogous sequences are analyzed. Two loci are said to be orthologous if their relationship originated from organismal cladogenesis, while gene duplication gives rise to paralogs. Although orthology of investigated loci can be supported by FISH and GISH experiments (e.g. Hodkinson et al. 2002, Mishima et al. 2002, Chung et al. 2008, Malinska et al. 2010), the presence of paralogous sequences in phylogenetic datasets can never definitely be ruled out and possibly lead to phylogenetic incongruences. For instance, Mayol and Rosselló (2001) demonstrated that paralogs may cause long-branch attraction in Neighbor-joining analyses, and tree topologies inferred from a mixture of orthologous and paralogous sequences contradict those obtained from 'pure' datasets. Second, repeats at some loci may undergo pseudogene formation. As these non-functional duplicates will evolve independently from their genetic

ancestors, pseudogene sequences will have strong impact on phylogenetic analyses (Mayol & Rosselló 2001). Yet, unlike functional paralogs, pseudogenes can be identified quite easily as they usually show several characteristics caused by relaxed evolutionary pressure. When compared to functional copies, pseudogenes typically have lowered secondary structure stability, an increase in AT content, and a higher relative substitution rate particularly in conserved regions (Buckler et al. 1997). Finally, genetic recombination may produce chimeric nrDNA sequences that combine motifs from different paralogs and/or pseudogenes (e.g. Volkov et al. 1999, Nieto Feliner & Rosselló 2007). Unequal crossing-over may also result in the complete loss of array types, thereby blurring the genetic traces of hybridization and polyploid speciation. Despite these limitations of nrDNA for phylogenetic reconstruction, nrDNA genes and spacers have successfully been used to infer the evolutionary history of polyploids in a number of recent studies (e.g. Muir et al. 2001, Barkman & Simpson 2002, Koch et al. 2003, Albach & Chase 2004, Hörandl et al. 2005, Fehrer et al. 2009, Garcia-Jacas et al. 2009, Guggisberg et al. 2009, Bao et al. 2010, Liu et al. 2010).

In the present analysis, extensive cloning of the external transcribed spacer (ETS) is used to reconstruct the evolutionary history of three polyploid *Leucanthemum* taxa from the Iberian Peninsula. The genus *Leucanthemum* belongs to the Circum-Mediterranean clade of the Compositae-Anthemideae (Oberprieler 2005), presently comprises 41 species (Euro+Med 2011), and is distributed all over the European continent (Vogt 1991). Its center of diversity is located on the Iberian Peninsula, 16 species are exclusively found here. *Leucanthemum* includes 14 diploid species, as well as many polyploid taxa with ploidy levels ranging from tetraploid to dokosaploid (22-ploid). Due to a high frequency of hybridization and polyploidization, the evolutionary history of the genus is quite complex, and early attempts to systematically structure *Leucanthemum* on the basis of morphological traits soon had to be revised (Vogt 1991). With the introduction of extensive

cytological investigation, species circumscription got more clear, but still did not necessarily reflect phylogenetic relationships among *Leucanthemum* species. Within the last years, the use of molecular methods like DNA sequencing and genomic fingerprinting provided insight into the intricate patterns of reticulation that characterize the evolutionary history of the genus *Leucanthemum* (Höfl 2006, chapter 2). An analysis of cpDNA sequence variation within the whole genus by Höfl (2006) identified a well-supported monophyletic group consisting of one diploid, one tetraploid, and two hexaploids in the Western part of the Iberian Peninsula, particularly in the Spanish autonomous community of Galicia and in Portugal (Höfl 2006). While two of the taxa are common at the coastline of Galicia [*L. pluriflorum* (2x) and *L. merinoi* (6x)], *L. irtutianum* subsp. *pseudosylvaticum* (4x) and *L. sylvaticum* (6x) are mainly distributed inland (c.f. Figure 2). Morphological differentiation of the four species is considerably low. While the diploid *L. pluriflorum* can be identified by its leaf morphology, the polyploids are merely discriminated by cytological and ecological traits. In particular, *L. sylvaticum* and *L. irtutianum* subsp. *pseudosylvaticum* are very similar to each other, and Vogt (1991) strongly suggests a close phylogenetic relationship of these two taxa. Based on this morphological and phylogeographical evidence, the four taxa included in this '*L. pluriflorum* group' were selected for the present phylogenetic analysis in order to clarify their evolutionary history. All taxa were extensively sampled over their complete distributional range, and several populations of other sympatrically distributed diploids were included in the analysis. Particularly, four populations of the Galician diploid *L. gaudinii* subsp. *cantabricum* were sampled. This diploid is morphologically very similar to *L. pluriflorum*, indicating a close phylogenetic relationship of these two taxa. Further, one representative of each diploid taxon of the genus *Leucanthemum* was analyzed to check for hybridization between species currently not present in the study area. Finally, one accession of the alleged hybrid tetraploid species *L. × corunnense* [*L. pluriflorum* (2x) × *L. merinoi* (6x)] was

included. For each individual, comprehensive cloning, PCR amplification, and sequencing of ETS was performed. The resulting sequence data were analyzed with Bayesian and Neighbor-net algorithms to trace hybridization events and to elucidate the origin of the polyploids.

### 2.3 Material and Methods

*Plant material.* – Samples for the present analysis were taken from either (i) silica-dried leaf material collected from populations of the *L. pluriflorum* group (including one population of *L. × corunnense*) and their sympatric diploids (*L. gaudinii*, *L. gallaecicum*), or (ii) from herbarium specimens of all other diploid species in the genus *Leucanthemum*. Specimen information (herbarium; collectors and collection numbers; coordinates) for all accessions is given in Table 3. For species that comprise two or more subspecies (*L. vulgare*, *L. gaudinii*), additional accessions were included to detect subspecific variation. The sample collection is covering the entire distributional range of all taxa from the *L. pluriflorum* group.

*DNA isolation, cloning, and sequencing of nrDNA external transcribed spacer (ETS) region.* – For all individuals, total genomic DNA was extracted from 10-20 mg leaf material according to a protocol based on the CTAB method of Doyle & Doyle (1987).

The 3'-ETS region was amplified by PCR with the primers 18S-ETS (Baldwin & Markos 1998) and L-ETS (Lee et al. 2002) with the following temperature profile: (i) 94 °C/5 min, (ii) 74 °C/7 min, (iii) 30 cycles of 94 °C/45 s, 50 °C/45 s, 72 °C/40 s, and (iv) 72 °C/7 min. For almost every individual, direct sequencing of the resulting 500-600 bp fragment produced highly ambiguous electropherograms, indicating the presence of two or more ETS copy types. To facilitate sequencing of the different



ETS copies, PCR products were purified with Agencourt AMPure XP (Beckman Coulter, Krefeld, Germany), and cloned into NEB Turbo *E. coli* (New England Biolabs, Frankfurt am Main, Germany) with the CloneJET PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany). All reactions were conducted following the manufacturers' protocols. After 12 h of incubation at 37 °C, several clones of each cloning reaction were picked, dissolved in water, and used as templates in a second PCR reaction. The primers and temperature profile were identical to the initial PCR. Following another purification step, the cloned amplicons were sequenced with the 18S-ETS primer using the DTCS QuickStart Kit and the CEQ 8000 Genetic Analysis System (both Beckman Coulter, Krefeld, Germany). The resulting chromatograms were checked for sequencing errors manually. Sequences containing ambiguous character states were excluded from the analysis.

*Data processing and phylogenetic analyses.* – In order to reduce phylogenetic bias caused by Taq-induced errors, polymorphisms observed in only one clone were removed from the analysis (Joly et al. 2006, Bao et al. 2010). Subsequently, all identical sequences were collapsed into single representatives ('ETS types'). The sequences of the ETS types were imported into the BioEdit sequence alignment software (Hall 1999). In addition to the newly acquired data from the genus *Leucanthemum*, ETS sequences of four other diploid genera from the Mediterranean clade of the Anthemideae tribe have been downloaded from NCBI nucleotide sequence database GenBank and included in the present analysis as outgroup taxa (taxon names and GenBank accession numbers: *Achillea schmakovii* AB359892.1, *Anthemis arvensis* EU747088.1, *Argyranthemum winteri* AF123545.1, *Rhodanthemum hosmariense* AB359891.1). A full multiple alignment of all ETS types was performed using the ClustalW algorithm (Thompson et al. 1994) as implemented in BioEdit. Indel character states were coded with GapCoder (Young &

Healy 2003) according to the simple gapcoding method of Simmons and Ochoterana (2000).

In addition to the dataset containing the sequences from all taxa, a second gapcoded alignment was prepared that only contained sequences from diploid *Leucanthemum* taxa, as well as from the outgroups. This was done due to the fact that hybridization and polyploidy may facilitate inter-genomic recombination by combining divergent genomes in a single individual (i.e. allopolyploidy). Furthermore, identical effects may arise from in-vitro recombination during PCR and molecular cloning of polyploid samples. Consequently, chimeric ETS types may be produced that possibly blur phylogenetic patterns. The 'diploid dataset' contained sequences from 31 *Leucanthemum* samples and the four outgroup sequences.

For both datasets, two phylogenetic analyses were carried out: a Bayesian inference (BI) analysis in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), and a splits graphs analysis in SplitsTree (Huson 1998). The Bayesian inference was performed implementing a GTR + I + G model and without fixing rates and nucleotide frequencies, as these parameters are estimated from the data during the analysis. Substitution model parameters and rates of substitution were allowed to vary among the parameters (“unlink” command and “ratepr = variable”) and a binary model (“Lset coding = variable”) was applied to the coded gaps (Ronquist & al. 2005). The analyses were carried out with four heated chains and one cold chain (chain heating parameter value: 0.1). The MCMC chains were run for 100,000,000 generations (standard deviation of split frequencies < 0.01), with trees sampled every 1000th generation. The analysis was repeated for a total of two runs. The quality of the analyses was checked by comparing likelihood values and parameter estimates from different runs in Tracer v.1.3 (Rambaut & Drummond, 2003). Finally, the first 25,000 sampled trees were discarded as burn-in, and the remaining trees were used to compile the posterior-probability (PP) distribution on the basis of a 50 %-majority-rule consensus. The Neighbor-net analysis in SplitsTree was based on

uncorrected p-distances, and bootstrap values were estimated from 1000 replicates.

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Table 3: Taxa and populations included in chapter 2. Polyploid taxa are written in bold characters. For each population, geographical information, collectors, and herbarium information are provided. Additionally, the number of sequences obtained from each sample is specified, as well as the results of the Neighbor-net analysis. Stars indicate coordinates that were determined from specimen data rather than by a GPS device. Collectors abbreviations: AH Andreas Hutschenreuther, NG Nina Greiner, RH Roland Hößl, SH Sven Himmelreich.



## 2.4 Results

*Cloning and sequencing of nrDNA ETS.* – The nrDNA ETS region was amplified and cloned for 73 representatives of the genus *Leucanthemum*, including 57 individuals from the *L. pluriflorum* group and 16 plants from other diploid taxa. For diploid, tetraploid, and hexaploid taxa, an average of 4.8, 8.4, and 9.8 clones have been sequenced, respectively, resulting in a total of 540 sequences. An overview on the sequencing results (number of sequences analyzed per sample; number of different ETS types found per individual) is given in Table 3.

*Sequence collapsing and alignment.* – After exclusion of PCR artefacts, collapsing of identical sequences resulted in 303 different *Leucanthemum* ETS types. Of these 303 *Leucanthemum* ETS types, 246 were represented by a single sequence, while the remaining 57 contained two or more sequences. Of the latter, 34 ETS types comprised sequences from several different individuals, and 25 even contained different taxa. Of those 25 ETS types with two or more different taxa, five exclusively contained sequences from diploids, 13 exclusively from polyploids, and 7 from both diploids and polyploids. Table 4 shows ETS types that contain sequences from different individuals or taxa. The alignment of the ETS types comprised 544 sequences with 481 characters for the complete dataset, and 154 sequences with 476 characters for the reduced dataset. The input files for all phylogenetic analyses as well as a detailed table documenting the sequence collapsing process can be found in the digital appendix provided with this thesis (DA\_2\_2 - DA\_2\_9).

*Bayesian inference.* – The results of the Bayesian analysis of the dataset including ETS types from all samples (303 ETS types from 73 *Leucanthemum* samples plus 4 outgroup ETS types) shows only a small number of supported groups (Figure 8). While all sequences from the genus *Leucanthemum* form a well-supported

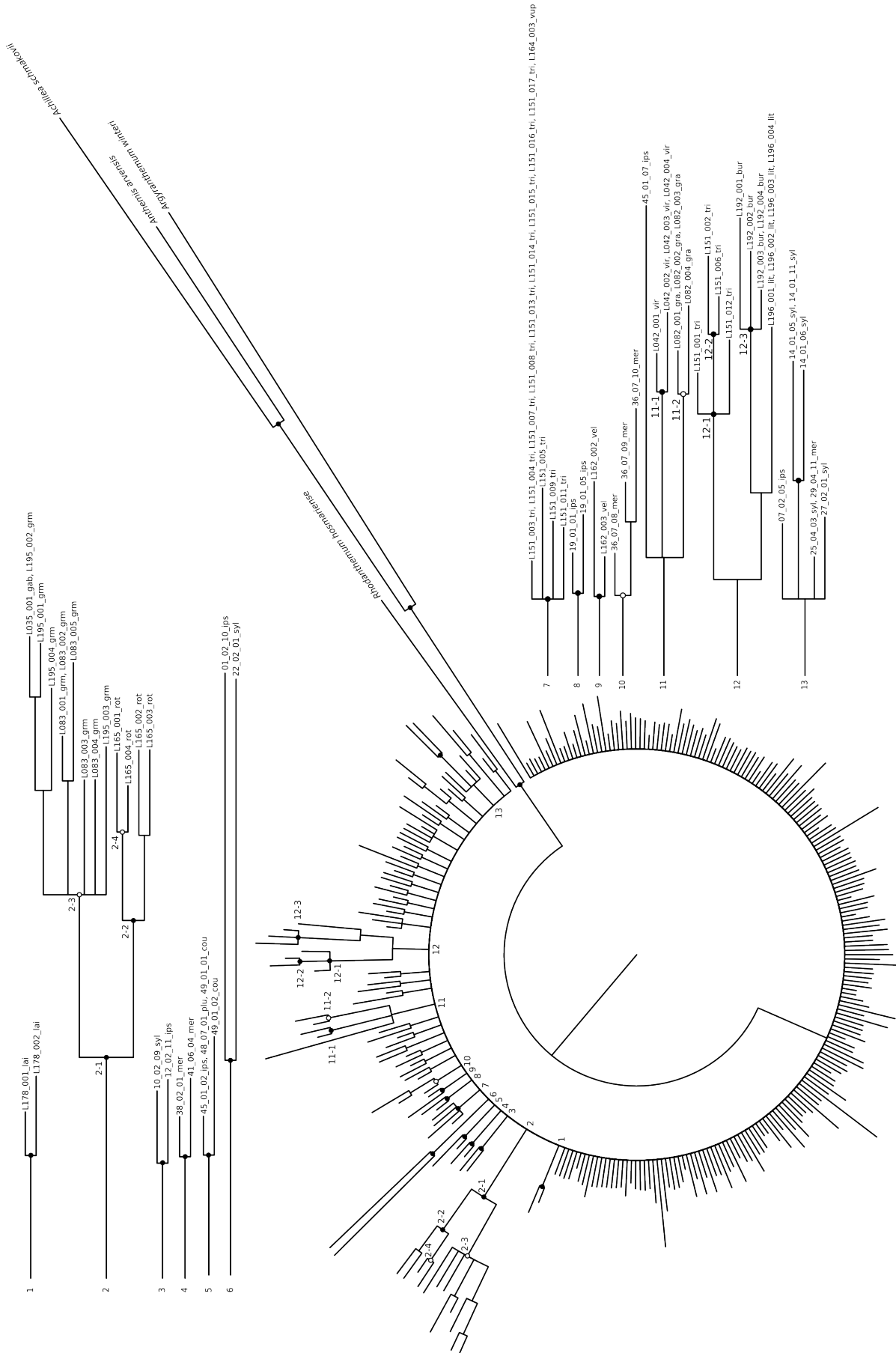
monophyletic group ( $PP \geq 0.99$ ), no major structure can be found at infrageneric level. Most of the supported amplicon groups (i.e. groups with  $PP \geq 0.95$ ) merely comprise sequences obtained from single individuals (clade 1, 2-2, 7, 8, 9, 10, 11-1, 11-2, 12-1, 12-2 12-3, 13-1), and hence do not provide a phylogenetic signal. Yet, there are some supported groups that indicate phylogenetic relationships between different populations and taxa.

The analysis of the reduced dataset containing only amplicons from diploid samples (92 ETS types from 31 *Leucanthemum* samples plus 4 outgroup ETS types) corroborates the results from the comprehensive analysis (Figure 9). The monophyly of amplicons from *Leucanthemum* samples is backed up by a high posterior probability, and all well-supported groups of diploids found in Figure 8 can also be seen the 50 %-majority-rule consensus tree of the reduced dataset.

*Neighbor-net analysis.* – Like the results of the Bayesian analyses, also the splits graph diagrams show that differentiation among identified nrDNA ETS types is low, as indicated by a high number of parallel edges found in the phylogenetic networks of both datasets (Figure 10 and Figure 11). However, a clear – yet unsupported – bipartite structure can be recognized, splitting the datasets into two subnetworks (shown in red and green) and an unassigned region between them (shown in black). The number of clones in each subnetwork for each sample is given in Table 3.

Table 4: ETS types including different individuals or different taxa. For reasons of clarity, sequences are not specified. A complete list of all ETS types and sequences can be found in the digital appendix (DA\_2\_4).

ETS type ID	Taxa	Individuals
2	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i>	01_01, 03_04 34_02
4	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i>	01_01, 16_01 33_02
5	<i>L. x corunnense</i> <i>L. gaudinii</i> subsp. <i>cantabricum</i> <i>L. gaudinii</i> subsp. <i>gaudinii</i> <i>L. gallaecicum</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. pluriflorum</i> <i>L. vulgare</i> <i>L. vulgare</i> subsp. <i>puijulae</i>	49_01 68_01 L033 L194 01_01, 02_01, 45_01 46_02, 48_07, 52_02 L028 L164
7	<i>L. gaudinii</i> subsp. <i>barrelieri</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i>	L035 01_01
11	<i>L. gaudinii</i> subsp. <i>cantabricum</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i> <i>L. pluriflorum</i> <i>L. sylvaticum</i>	60_01 01_01, 04_02, 05_01, 19_01 28_04 64_01 09_02, 21_03, 24_03, 26_03
13	<i>L. gaudinii</i> subsp. <i>cantabricum</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i> <i>L. pluriflorum</i>	60_01 02_01 41_06 39_05, 46_02, 54_04, 55_01
16	<i>L. x corunnense</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. pluriflorum</i>	49_01 02_01, 45_01, 61_01 47_02, 48_07
17	<i>L. gaudinii</i> subsp. <i>cantabricum</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. pluriflorum</i>	67_01 02_01 39_05
21	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i> <i>L. sylvaticum</i>	03_04, 04_02, 05_01, 06_01, 12_02, 13_02, 16_01, 18_01, 09_02, 10_02, 11_08, 14_01, 15_03, 17_04, 20_02, 21_03, 23_02, 31_02, 56_01 28_04, 29_04, 30_04, 32_03, 33_02, 34_02, 36_07, 37_08, 38_02, 41_06, 43_02
28	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i>	04_02, 16_01
29	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. sylvaticum</i>	05_01 27_02
39	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i>	06_01, 18_01, 19_01
40	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. sylvaticum</i>	06_01, 12_02 11_08, 14_01, 31_02
43	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i> <i>L. sylvaticum</i>	07_02 28_04, 36_07 20_02
49	<i>L. sylvaticum</i>	09_02, 10_02
50	<i>L. sylvaticum</i>	09_02, 15_03
60	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. sylvaticum</i>	13_02 10_02, 15_03
65	<i>L. merinoi</i> <i>L. sylvaticum</i>	32_03 10_02
79	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i>	13_02 32_03
81	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i> <i>L. sylvaticum</i>	13_02 33_02 14_01
114	<i>L. merinoi</i> <i>L. sylvaticum</i>	29_04 25_04
129	<i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. merinoi</i>	61_01 28_04
132	<i>L. merinoi</i>	28_04, 34_02
133	<i>L. merinoi</i> <i>L. sylvaticum</i>	29_04, 34_02 31_02, 56_01
156	<i>L. merinoi</i>	32_03, 43_02
164	<i>L. merinoi</i>	34_02, 43_02
168	<i>L. merinoi</i>	36_07, 41_06
195	<i>L. gaudinii</i> subsp. <i>cantabricum</i> <i>L. pluriflorum</i> <i>L. halleri</i>	68_01 42_06, 52_02, 55_01 L039
196	<i>L. gaudinii</i> subsp. <i>cantabricum</i> <i>L. pluriflorum</i>	68_01 42_06
203	<i>L. x corunnense</i> <i>L. ircutianum</i> subsp. <i>pseudosylvaticum</i> <i>L. pluriflorum</i>	49_01 45_01 48_07
207	<i>L. pluriflorum</i>	46_02, 54_04
228	<i>L. gallaecicum</i> <i>L. vulgare</i> subsp. <i>puijulae</i>	58_02 L164
251	<i>L. gaudinii</i> subsp. <i>barrelieri</i> <i>L. graminifolium</i>	L035 L195
267	<i>L. tridactylites</i> <i>L. vulgare</i> subsp. <i>puijulae</i>	L151 L164





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Figure 8: 50% majority rule consensus tree from the Bayesian analysis of the complete dataset comprising 303 *Leucanthemum* ETS types and 4 outgroup sequences. Filled and open circles designate nodes with posterior probabilities  $PP \geq 0.95$  and  $PP \geq 0.99$ , respectively. Well supported clades of the main tree are shown in detail at the upper left and lower right of the figure. Taxon short names provided after sample numbers follow Table 3.

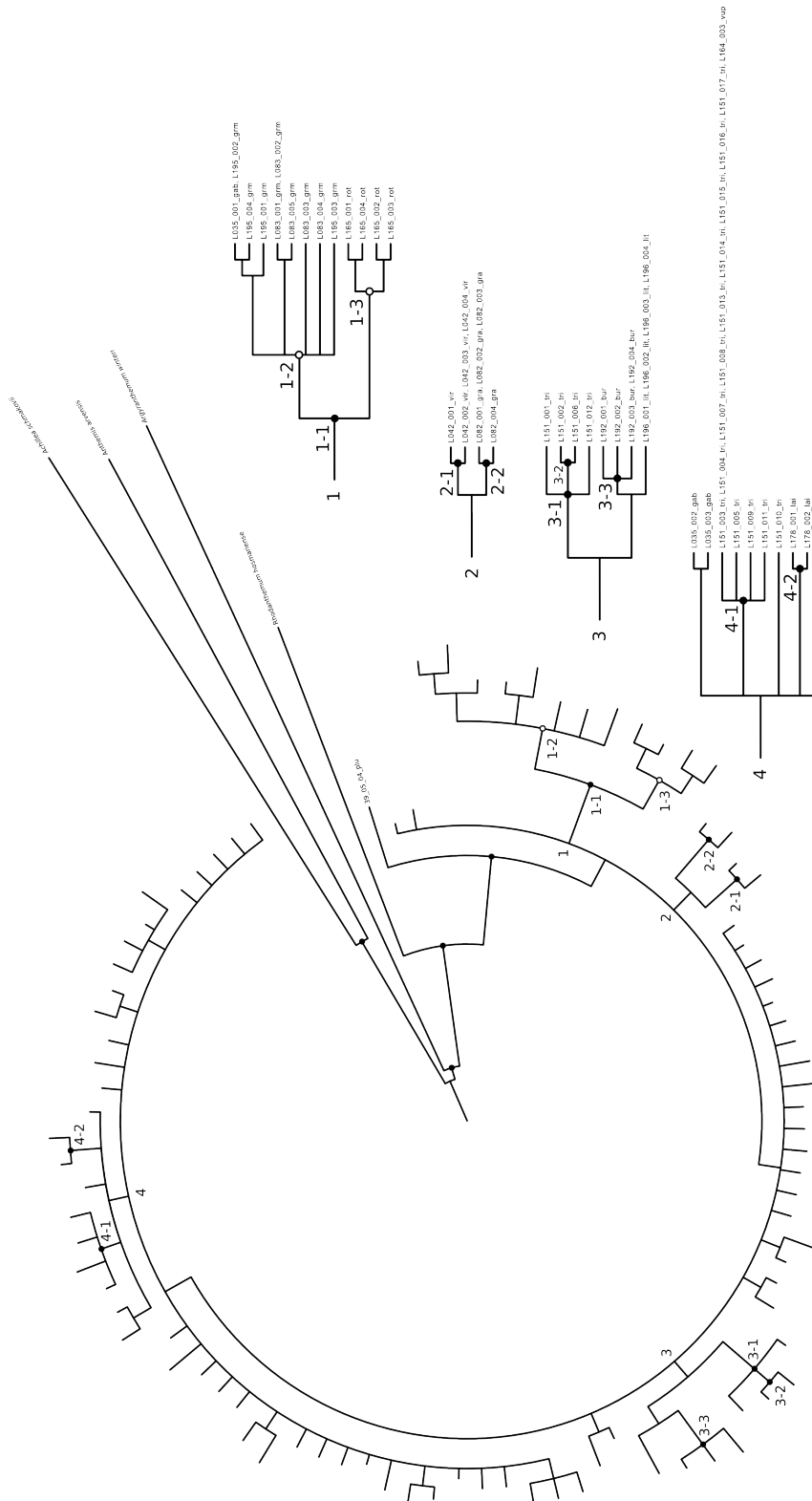


Figure 9: 50% majority rule consensus tree from the Bayesian analysis of the reduced dataset comprising 92 *Leucanthemum* ETS types and 4 outgroup sequences. Filled and open circles designate nodes with posterior probabilities  $\geq 0.95$  and  $\geq 0.99$ , respectively. Well supported clades of the main tree are shown in detail at the lower right of the figure. Taxon short names provided after sample numbers follow Table 3.



Figure 10: Splits graph diagram of the complete dataset comprising 303 *Leucanthemum* ETS types and 4 outgroup sequences (designated by asterisks). The two major subnetworks are shown in red and green, unassigned samples are colored in black. The number of clones in each subnetwork for each sample is given in Table 2.



Figure 11: Splits graph diagram of the reduced dataset comprising 92 *Leucanthemum* ETS types and 4 outgroup sequences (designated by asterisks). The two major subnetworks are shown in red and green, unassigned samples are colored in black. The number of clones in each subnetwork for each sample is given in Table 2.

*Results for diploids.* – Figure 12 summarizes the results of all three analyses for diploid accessions. Solid lines indicate ETS types shared by different taxa as identified in the sequence collapsing process. Dashed and dotted lines indicate shared membership of different taxa in supported and unsupported clades of the Bayesian analysis, respectively. Pie charts designate the fraction of ETS sequences belonging to subnetwork 1 (green) and subnetwork 2 (red) in the Neighbor-net analysis (c.f. Figure 10 and Figure 11).

The analysis of the sequence collapsing process demonstrates that many ETS types comprise sequences obtained from different taxa. *L. pluriflorum* shares a number of ETS types with *L. gaudinii* subsp. *cantabricum*, and both taxa are connected to several diploids. The Bayesian inference indicates a close relationship between *L. graminifolium* and *L. rotundifolium*. Finally, the Neighbor-net analysis shows that most of the 17 diploids either only contain ETS sequence types that belong to subnetwork 1 (green), which is considered to be the more ancestral one, or to subnetwork 2 (red). Yet, five of the investigated diploids – including *L. pluriflorum* – contain a mixture of the two major ETS types. While four of these diploid taxa (*L. pluriflorum*, *L. gaudinii* subsp. *cantabricum*, *L. vulgare* subsp. *eliasii*, and *L. gaudinii* subsp. *barrelieri*) are exclusively found on the Iberian Peninsula (Figure 13), *L. tridactylites* is endemic to Central Italy.

*Results for polyploids.* – Figure 14 summarizes the results from the phylogenetic analyses for *L. ircutianum* subsp. *pseudosylvaticum*, *L. sylvaticum*, and *L. merinoi*. Each of the three circular subdiagrams visualizes the phylogenetic relationships between one of the polyploids and the diploid *Leucanthemum* taxa. The triangular subdiagram in the center indicates phylogenetic relationships among polyploids. Solid lines indicate ETS types shared by different taxa as identified in the sequence collapsing process. Dashed and dotted lines indicate shared membership of different taxa in supported and unsupported clades of the Bayesian analysis,

respectively. Pie charts designate the fraction of ETS sequences belonging to subnetwork 1 (green) and subnetwork 2 (red) in the Neighbor-net analysis (c.f. Figure 10 and Figure 11).

The analysis of the sequence collapsing process demonstrates that both hexaploid species share ETS types with *L. gaudinii* subsp. *cantabricum* and with *L. pluriflorum*, although the connection is not very strong. By contrast, *L. ircutianum* subsp. *pseudosylvaticum* contains many ETS types that can also be found in *L. gaudinii* subsp. *cantabricum* and *L. pluriflorum*, indicating a close relationship of these three taxa. In addition, *L. ircutianum* subsp. *pseudosylvaticum* is connected to all diploids that share haplotypes with *L. pluriflorum*, except *L. halleri*. Furthermore, the polyploids are linked to each other by a number of ETS types. The results of the Bayesian analysis indicate a phylogenetic relationship between *L. merinoi* and *L. sylvaticum* (supported), as well as between *L. ircutianum* subsp. *pseudosylvaticum* and *L. gracilicaule* (unsupported). Finally, the Neighbor-net analysis demonstrates that all polyploids contain ETS sequences from both major subnetworks, with the more ancestral ETS type (green) being dominant especially in the hexaploids.

The tetraploid *L. × corunnense*, which is considered to be a hybrid between *L. pluriflorum* and *L. merinoi*, exclusively contains ETS types also found in the four taxa of the *L. pluriflorum* group. The taxon is found in genotype 5 (*L. gaudinii* subsp. *gaudinii*, *L. gaudinii* subsp. *gaudinii*, *L. gallaecicum*, *L. ircutianum* subsp. *pseudosylvaticum*, *L. pluriflorum*, *L. vulgare*, *L. vulgare* subsp. *pujiulae*), genotype 16 (*L. ircutianum* subsp. *pseudosylvaticum*, *L. pluriflorum*), and genotype 203 (*L. ircutianum* subsp. *pseudosylvaticum*, *L. pluriflorum*). The Bayesian analysis identified one supported clade that contains *L. × corunnense* along with *L. ircutianum* subsp. *pseudosylvaticum*. The Neighbor-net analysis shows that *L. × corunnense* contains 20 % ETS sequences from subnetwork 1 (green), and 80 % from subnetwork 2 (red).

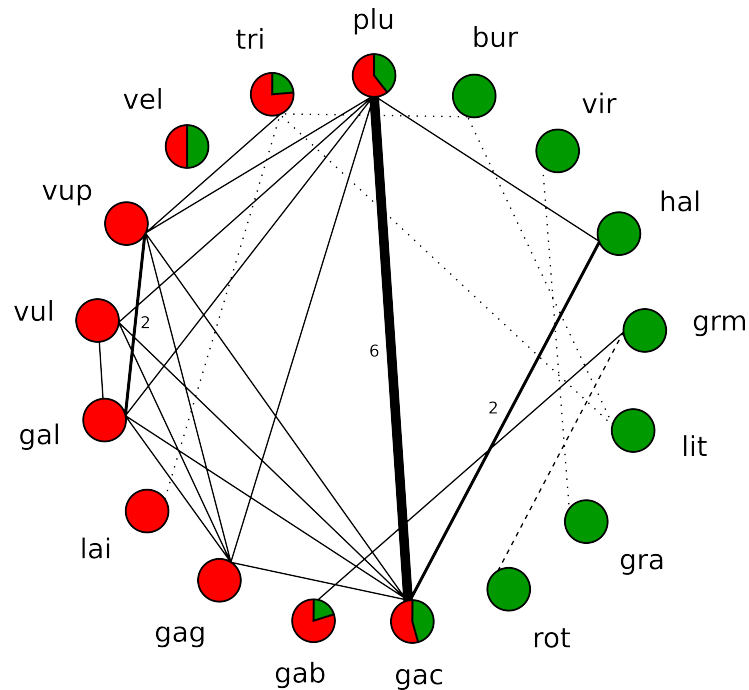


Figure 12: Results of the analysis of ETS diversity in diploid individuals. Taxon short names provided after sample numbers follow Table 3. Solid lines indicate ETS types shared by different taxa as identified in the sequence collapsing process. Dashed and dotted lines indicate shared membership of different taxa in supported and unsupported clades of the Bayesian analysis, respectively. Pie charts designate the fraction of ETS sequences belonging to subnetwork 1 (green) and subnetwork 2 (red) in the Neighbor-net analysis (c.f. Figure 10 and Figure 11).

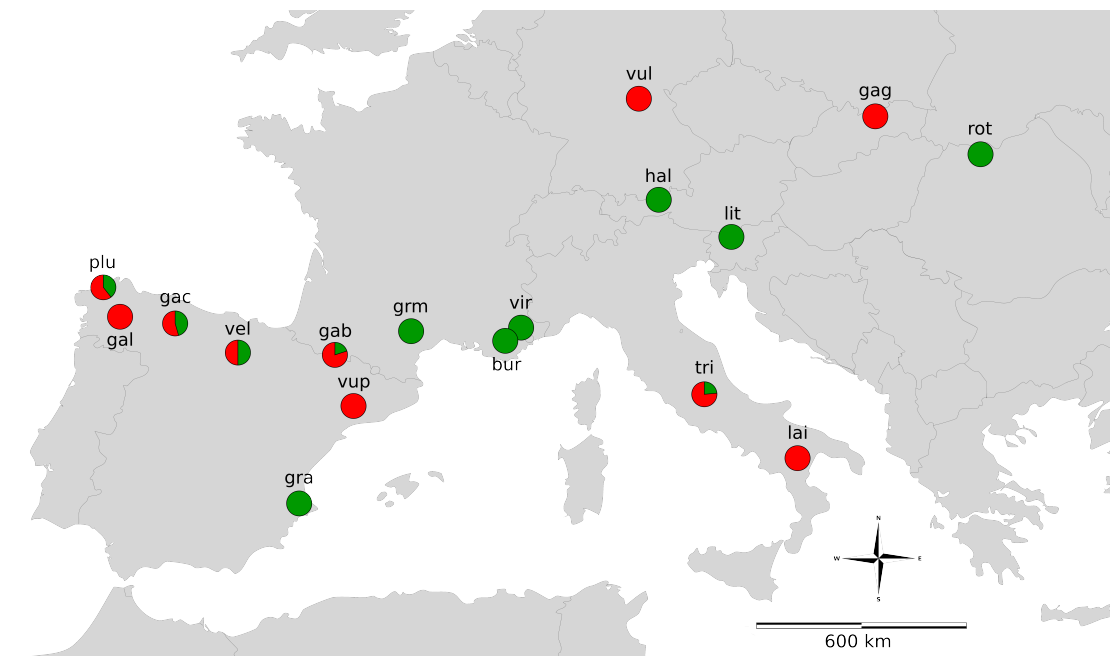


Figure 13: Map of diploid *Leucanthemum* taxa and their geographical localization. Taxon short names provided after sample numbers follow Table 3. More widespread species (*L. gaudinii* subsp. *gaudinii*, *L. gaudinii* subsp. *cantabricum*, *L. halleri*, *L. vulgare*) are represented by the locality used in this analysis. The red and green colored pie charts visualize the results of the Neighbor-net analysis (c.f. Figure 10 and Figure 11).

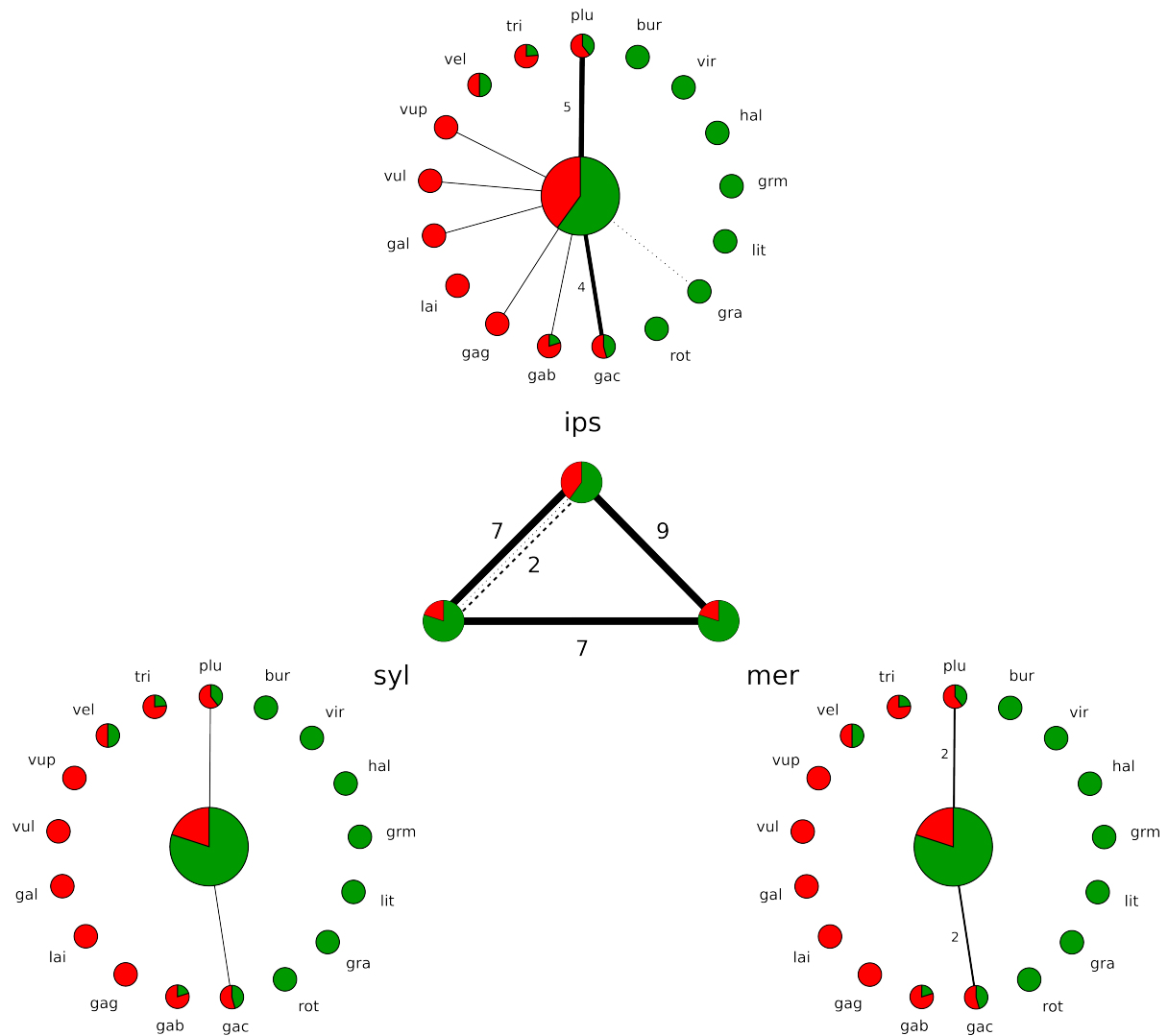


Figure 14: Results of the analysis of ETS diversity in polyploid individuals. Taxon short names provided after sample numbers follow Table 3. Each of the three circular subdiagrams visualizes the phylogenetic relationships between one of the polyploids and the diploid *Leucanthemum* taxa. The triangular subdiagram in the center indicates phylogenetic relationships among polyploids. Solid lines indicate ETS types shared by different taxa as identified in the sequence collapsing process. Dashed and dotted lines indicate shared membership of different taxa in supported and unsupported clades of the Bayesian analysis, respectively. Pie charts designate the fraction of ETS sequences belonging to subnetwork 1 (green) and subnetwork 2 (red) in the Neighbor-net analysis (c.f. Figure 10 and Figure 11).

## 2.5 Discussion

In the present study, extensive cloning of the nuclear ribosomal DNA (nrDNA) external transcribed spacer (ETS) was performed for three polyploid taxa from the genus *Leucanthemum* as well as for all presently known diploids in order to reconstruct the evolutionary history of the polyploids. In particular, an analysis of intra-individual and intra-specific polymorphisms of nrDNA is conducted to study evolution by whole genome duplication in *Leucanthemum*.

Basically, there are three processes that cause intra-individual and intra-specific DNA polymorphism: (i) the polymorphism already existed before the speciation event and has been preserved in some (or all) individuals (incomplete lineage sorting), (ii) new nrDNA types emerged by mutation from initially homogenous nrDNA pools after speciation, or (iii) a hybridization event combined two existing nrDNA types in a new, often polyploid genome. All of these processes are acting simultaneously, interactively and dynamically during evolution.

While sequence variation caused by the latter two processes can be used to evaluate evolutionary hypotheses, the presence of incomplete lineage sorting complicates phylogenetic analysis. Yet, to rule out incomplete lineage sorting one extensively has to investigate nrDNA diversity in individuals from the most basal species within the genus, as well as from all closely related outgroup species. This has not been done in the present analysis. However, although incomplete lineage sorting may be responsible for intra-individual and intra-specific polymorphisms in *Leucanthemum* by some degree, all taxa that combine the major ETS types seen in the Neighbor-net analyses are found in contact zones of 'green species' and 'red species' (Figure 13), a fact that strongly points towards hybridization as the main cause for the observed genetic pattern. In addition to this geographic consideration, ongoing investigations of non-nrDNA markers support the mosaic genetic



constitution of the potential hybrid taxa identified in the present study, and hence further back up the hybridization scenario.

*Monophyly of the genus Leucanthemum.* – The nrDNA ETS sequence based analyses presented here corroborate the monophyly of the genus *Leucanthemum* as defined on the basis of morphology (Vogt 1991, Brehmer & Humphries 1993, Vogt & Oberprieler 1995). All ETS sequences from *Leucanthemum* samples form a well-supported clade in the Bayesian analysis, with *Rhodanthemum* being the most closely related outgroup. These results are consistent with phylogenetic studies using *psbA/trnH* (Höfl 2006).

*Origin of L. pluriflorum.* – In the present study, hypotheses for the evolution of diploids can be put forward on the basis of (i) sequence collapsing, (ii) supported and unsupported monophyletic clades in the Bayesian analysis, and (iii) the structure seen in the Neighbor-net analysis.

While the results of the Bayesian analysis do not provide evidence on the evolutionary history of *L. pluriflorum*, the other two analyses indicate a homoploid hybridogenous origin of this diploid species. Unlike most other diploids, *L. pluriflorum* combines ETS types from the two major subnetworks of the Neighbor-net diagram. Through the sequence collapsing analysis, several potential 'red parents' for *L. pluriflorum* have been identified, including the geographically close *L. gallaecicum*, *L. gaudinii* subsp. *gaudinii* from the Carpathian Mountains, as well as the widely distributed *L. vulgare*. By contrast, only *L. halleri* can be considered to be the donor of the 'green' ETS type found in *L. pluriflorum*.

An identical situation is found for *L. gaudinii* subsp. *cantabricum*. This taxon from the Cantabrian Mountains shares ETS types with the same diploids as *L. pluriflorum*, and also the proportions of subnetwork 1 and subnetwork 2 ETS types are very similar to its close geographical neighbor. In addition, the two taxa are connected by

six ETS sequence types, four of which exclusively contain these diploids (Table 4). The close relationship indicated by the genetic analysis supports the initial hypothesis of a common origin of *L. pluriflorum* and *L. gaudinii* subsp. *cantabricum* based on the distinct morphological similarity of these two taxa. Particularly, *L. pluriflorum* and *L. gaudinii* subsp. *cantabricum* show strongly dissected leaves – a character which is unique among taxa on the Iberian Peninsula – and both of them produce a high number of flower heads. Further, their habitus is very similar, and *L. gaudinii* subsp. *cantabricum* can be considered to be an 'alpine midget version' of *L. pluriflorum*. Strikingly, investigation of chloroplast sequence information (chapter 2) identified one inland population of *L. pluriflorum* (population 64), containing the strongly derived cpDNA haplotype that is characteristic for the *L. pluriflorum* group. This population is located very close to a region where *L. gaudinii* subsp. *cantabricum* is frequent (Sierra de Ancares). This fact indicates that the coastal species *L. pluriflorum* may have originated from *L. gaudinii* subsp. *cantabricum* in the alpine habitats of Galicia, hence sharing the hybrid ETS constitution already seen in the ancestral taxon. In addition to this, analysis of AFLPs did not detect considerable differentiation of the two taxa (chapter 2), which perfectly fits into the picture that *L. pluriflorum* and *L. gaudinii* subsp. *cantabricum* have a common evolutionary history.

Although the present study provides ample evidence for a homoploid hybridogenous origin of *L. pluriflorum* and its sibling species *L. gaudinii* subsp. *cantabricum*, both the number of investigated individuals and the number of sequenced clones was quite low for most species. Hence, further analysis is needed to corroborate these hypotheses.

*Origin of the polyploids.* – As for diploids, information on the evolutionary history of the tetraploid *L. irtutianum* subsp. *pseudosylvaticum*, and of the two hexaploids *L. sylvaticum* and *L. merinoi*, as well as of the tetraploid hybrid species

*L. × corunnense* can be drawn from the sequence collapsing process, the Bayesian inference, and the Neighbor-net analysis. Altogether, the analyses strongly suggest that the polyploids formed by polyploidization of *L. pluriflorum*, as they all share the chloroplast haplotype of the Galician diploid *L. pluriflorum* (chapter 2), and they do not contain any ETS types that are not already present in this species. However, the ETS genotype of *L. halleri*, which represents one of the parent species of the potential homoploid hybrid *L. pluriflorum*, could not be found in the polyploids. It either got lost during evolution, or has not been sampled within the course of this study. The fact that the proportion of sequences from subnetwork 1 (green) increases from the diploid to the tetraploid to the hexaploids points towards recurrent backcrossing with *L. pluriflorum* or enrichment of 'green' ETS types during polyploidization. The results from the sequence collapsing process favors the latter hypothesis, as backcrossing with *L. pluriflorum* would not result in the loss of ETS types that can be observed in the polyploids.

Concerning the parentage of *L. × corunnense*, no conclusive evidence is provided by the present analyses. Although several ETS types that are also present in *L. pluriflorum* have been identified in *L. × corunnense*, ETS types from the other alleged parent *L. merinoi* are missing. However, the intermediate morphology of this species along with its tetraploid ploidy level represent a strong indication of a hybrid origin, and further data has to be acquired to shed light on the evolutionary history of *L. × corunnense*.

**Conclusion.** – The study at hand demonstrates the basic usability of nrDNA analysis to reconstruct phylogenetic reticulation by hybridization and genome duplication. ETS cloning and sequencing has revealed a hybrid origin of the diploid *L. pluriflorum* (along with its sibling taxon *L. gaudinii* subsp. *cantabricum*), and it further provides strong evidence that the investigated polyploid *Leucanthemum* taxa in NW Spain and Portugal formed by duplication of the *L. pluriflorum* genome. Yet, it has to be

considered that incomplete lineage sorting may produce similar genetic patterns like hybridization, and only combining genetic, morphological, cytological, and geographical data will result in robust evolutionary hypotheses. Additionally, far more extensive sampling is needed when studying polyploid complexes like *Leucanthemum*. Up to now, time and effort needed to clone and sequence an adequate number of samples represented the constraining factor in most studies, but hopefully upcoming massive parallel sequencing techniques will ease this crucial methodological issue.

## Chapter 3

### The Role of Inter-Ploidy Block for Reproductive Isolation of the Diploid *Leucanthemum pluriflorum* Pau (Compositae, Anthemideae) and its Tetra- and Hexaploid Relatives

#### 3.1 Abstract

Theory suggests that reproductive isolation of polyploids from their diploid progenitors is often caused by developmental disorder in the endosperm of hybrid seeds. Yet, this so-called triploid block is increasingly recognized to be less strong as initially assumed, indicating that other isolation mechanisms are needed to explain reproductive isolation of polyploids and diploids. For this study, the extent of inter-ploidy block was quantified based on crossing experiments between closely related diploid, tetraploid and hexaploid species from the genus *Leucanthemum* Mill. For these crosses, seed set and viability of seeds were measured and compared to fertilities following intra-cytype pollinations. Although inter-ploidy block was observed when diploids acted as pollen donors, the main observation was that all inter-ploidy crosses were basically capable of producing viable offspring. By contrast, flow cytometrical analysis of 233 individuals from natural populations point to a single locality where hybridization between different cytypes presumably occurred. Hence, the results of the present analysis demonstrate that inter-cytype mating may be rare even though inter-ploidy block is weak. Consequently, it can be assumed that pre-zygotic barriers and reduced fitness of inter-cytype hybrids play a decisive role in the reproductive isolation of polyploid *Leucanthemum* species.

## 3.2 Introduction

While genetic and genomic mutations give rise to evolutionary novelties, reproductive isolation of the carriers of these novelties is the single most important prerequisite for speciation. Classical concepts of speciation mainly consider geographical separation and subsequent gradual divergence to induce and maintain reproductive isolation between populations (Mayr 1942). By contrast, polyploidization (i.e., whole genome duplication) can immediately reduce or even eliminate geneflow, and therefore represents a feasible pathway for sympatric speciation, which is found to be common especially in plants (Masterson 1994, Otto & Whitton 2000). Despite the fact that polyploidy is increasingly accepted as a driving force in the evolution of angiosperms, few studies investigated the effects of polyploidization on reproductive isolation barriers. Historically, reproductive isolation by genome duplication was assumed to be caused by a distinct developmental disorder of inter-cytotype hybrids (Stebbins 1950). Besides low viability and fertility of inter-cytotype hybrids, embryo abortion by genomic disbalance in the endosperm (i.e., triploid block) is considered to be quite frequent after crossing of plants with different ploidy levels (Köhler et al. 2010). In addition to these post-zygotic mechanisms, theoretical models predict that the presence of pre-zygotic isolation barriers will promote polyploid speciation, as (i) parental cytotypes are preserved from the loss of fitness due to the production of unfit hybrids, and (ii) new cytotypes may overcome minority cytotype exclusion which strongly counteracts polyploid establishment, particularly in early generations following polyploid formation (Levin 1975). Indeed, recent studies of sympatric diploid and tetraploid *Chamerion angustifolium* found evidence that mechanisms like small-scale spatial distribution of cytotypes, flowering time asynchrony, and pollinator mediated assortative mating considerably contribute to isolation of cytotypes, and

that post-zygotic isolation between polyploids and their diploid ancestors can be quite weak (Husband & Sabara 2004). However, so far no other study tried to quantify relative contributions of pre- and post-zygotic barriers to overall reproductive isolation between diploids and tetraploids. Particularly, reproductive isolation barriers between different cytotypes have never been examined on ploidy levels higher than tetraploid.

Another unverified classical hypothesis states that polyploids have higher rates of self-fertilization than their diploid progenitors (Stebbins 1950). On the one hand, this again is justified on the basis of reduced reinforcement and increased chance to overcome minority cytotype exclusion when self-incompatibility (SI) is weak (Levin 1975; Felber 1991; Rodriguez 1996). On the other hand, whole genome duplication is expected to lower inbreeding depression, as fixed heterozygosity in polyploids decreases the probability of non-viable genotypes. However, there are several mechanisms of self-incompatibility (heteromorphic SI, gametophytic SI, sporophytic SI, late-acting SI), and although there is evidence for a tendency towards a break-down of self-incompatibility in polyploids with gametophytic SI systems (Miller & Venable 2000), it is still not clear whether this pattern can be generalized. Actually, Barrett (1988) consistently reported retention of self-incompatibility in families with sporophytic SI systems, and also put into perspective the idea of a stringent break-down of gametophytic self-incompatibility.

In this analysis, self-incompatibility within and inter-ploidy block between diploid, tetraploid and hexaploid cytotypes of four *Leucanthemum* taxa was studied. The genus *Leucanthemum* belongs to the Circum-Mediterranean clade of the Compositae-Anthemideae (Oberprieler 2005), and comprises 41 species (Euro+Med 2011) with ploidy levels ranging from diploid to dokosaploid (22-ploid). The four *Leucanthemum* taxa used in this analysis are all distributed in NW Spain and Portugal, and form a monophyletic group according to chloroplast sequence information (Höfl 2006). Crossing experiments between these taxa were used to

specifically address the following questions: (i) Does the rate of selfing decrease with rising ploidy level, (ii) do crosses between cytotypes suffer from an inter-ploidy block, and, if so, (iii) what is the basis of this inter-ploidy block in *Leucanthemum*?

### 3.3 Material & Methods

*Study organism.* – Plants from the genus *Leucanthemum* are small perennial herbs with flower heads that consist of yellow disk and white ray florets, except for three discoid species. They are characterized by simple or branched stems which grow from a rosette. The leaves are entire, toothed or pinnately lobed. The achenes are ribbed and show mucilage cells as well as resin ducts (Vogt 1991). *Leucanthemum* species can be found in as different habitats as calcareous dry grasslands, wet meadows and alpine communities – and even on serpentine derived soils or in brackish water. The highest diversity of the genus is found on the Iberian Peninsula, where it is represented by 16 species, nine of which are endemic to this geographical region (Vogt 1991). Recent genetic studies (Hößl 2006) using chloroplast sequence information identified a well differentiated monophyletic group that consists of the diploid *L. pluriflorum*, the tetraploid *L. ircutianum* subsp. *pseudosylvaticum*, and two hexaploid species (*L. sylvaticum* and *L. merinoi*). While both *L. pluriflorum* and *L. merinoi* are frequent along the coast of Galicia (NW Spain), *L. ircutianum* subsp. *pseudosylvaticum* and *L. sylvaticum* are distributed inland (Figure 2). Areas of sympatric distribution are reported for all combinations of taxa, except for the two hexaploid species (Vogt 1991). Due to the close phylogenetic relationship between members of the *L. pluriflorum* clade, and because of their overlapping areas of distribution, this group represents a well-suited model system to study inter-ploidy block in *Leucanthemum*.



*Plant material.* – During a field trip in 2007, 58 populations from *L. pluriflorum* group were sampled, representing 10, 18, 16 and 13 populations of *L. pluriflorum*, *L. irtutianum* subsp. *pseudosylvaticum*, *L. sylvaticum* and *L. merinoi*, respectively (Table 5). Furthermore, morphological characters indicate that one population was formed by hybridization between *L. merinoi* and *L. pluriflorum*, and therefore this population was classified as *L. × corunnense*. Herbarium specimens for each population are deposited in the private herbarium of the author. For all populations, leaf material was collected and dried in silica gel for subsequent ploidy level determination. For crossing experiments, seed material from two to nine populations of each taxon was germinated and grown in a greenhouse at the University of Regensburg. All seeds from each population were taken from a single capitulum.

*Flow cytometry.* – For each population used in this analysis, ploidy level of at least three individuals was determined by flow cytometry, either using fresh or silica dried leaf material. Specifically, all plants used in the crossing experiments were investigated. Measurements were performed by Plant Cytometry Services (Schijndel, NL) using DAPI as DNA stain and *Lactuca sativa* L. (6.38pg/2C) as internal standard.

*Crossing experiments.* – Plants started to develop flower heads about six months after germination. As soon as capitulum buds were visible they were covered with paper tea bags and sealed to avoid uncontrolled pollination. Crosses were performed by rubbing mature flower heads to one another, starting three days after the first flowers had opened. The procedure was repeated every five days, for a total of four treatments. Similarly, self-incompatibility was tested by rubbing over flower heads with clean tea bag tissue. In total, 163 cross-pollinations and 51 self-pollinations were completed, including all possible ploidy combinations. On average,

each population (i.e. seed family) was pollinated six times by randomly chosen pollinators.

*Data collection.* – Two months after the last treatment the number of fully developed (i.e. plump) achenes and the total number of flowers were counted for each capitulum. To test germination rate, up to 30 mature seeds from each flower head were sowed onto moist standard soil in a plastic pot and incubated at 20 °C in the greenhouse. After 15 days, the number of seedlings was counted. Seed maturation was expressed as the percentage of mature seeds in each flower head (referred to as seed index SI). Analogously, the germination rate of achenes from each capitulum was expressed as the percentage of successfully germinated seeds (referred to as germination index GI). For each cross type, overall post-zygotic fertility was calculated ( $FI = SI * GI$ ) and related to respective intra-cytotype fertilities  $FI'$  to quantify relative post-zygotic reproductive isolation  $rRI$ :

$$rRI = (1 - FI/FI') * 100$$

To exemplarily check ploidy levels of F1 hybrids, flow cytometry was conducted for 23 randomly chosen inter-cytotype crosses. For each of these crosses, five F1 individuals were investigated.

*Data analysis.* – As all representatives from one population were derived from a single seed family, self-incompatibility systems were likely to have strong influence when plants from the same population were crossed. In fact, among intra-cytotype crosses, a Mann-Whitney U test comparing seed index means of intra-population crosses and inter-population crosses showed significant differences in seed maturation. As a matter of fact, this self-incompatibility effect would be stronger in intra-cytotype than in inter-cytotype crosses, thereby disproportionally lowering

whithin-cytotype fertility. Thus, all intra-population crosses that did not produce any seeds were considered to be influenced by self-incompatibility and removed from the analysis.

To test for the effect of inter-cytotype pollination on seed maturation, germination and overall post-zygotic fertility, values of SI, GI and FI of each crosstype were compared to the values of respective intra-cytotype cross types. For the normally distributed data Student's *t*-test was used to identify significant differences. Otherwise, the Mann–Whitney U test was applied. Following the Bonferroni approximation (Abdi 2007), significance levels for both tests were lowered to 0.025 ( $\alpha = 0.05/2$ ).

Table 5: Taxa and populations included in chapter 3. Polyploid taxa are written in bold characters. For each accession, population ID, mean DNA content, number of measurements, geographical information and collectors are indicated. Asterisks designate populations which were used for crossing experiments. Collectors abbreviations are explained in Table 3.

Taxon	Population ID	Mean DNA content (number of measurements)	Country, province/district	Coordinates	Collectors
<i>Leucanthemum pluriflorum</i> Pau (2x)	39*	2.22 (4)	HS, A Coruña	42.9286, -9.2381	RH 39
	40*	2.23 (3)	HS, A Coruña	42.8838, -9.2726	RH 40
	42	2.18 (3)	HS, A Coruña	43.3069, -8.6186	RH 42
	46*	2.16 (3)	HS, A Coruña	43.5661, -8.3156	RH 46
	47*	2.27 (5)	HS, A Coruña	43.6418, -8.1225	RH 47
	48*	2.16 (3)	HS, A Coruña	43.7062, -8.0517	RH 48
	52	2.16 (3)	HS, Lugo	43.6842, -7.6153	RH 52
	54*	2.16 (5)	HS, Lugo	43.6876, -7.4125	RH 54
	55*	2.13 (3)	HS, Lugo	43.6309, -7.3330	RH 55
	64*	2.16 (3)	HS, Lugo	43.0797, -6.9559	RH 64
<i>Leucanthemum ircutianum</i> subsp. <i>pseudosylvaticum</i> Vogt (4x)	1	4.17 (3)	HS, Lugo	42.7267, -7.0258	RH 1 & AH
	2	4.29 (3)	HS, Lugo	42.8705, -7.0750	RH 2 & AH
	3	4.05 (3)	HS, Ourense	42.4634, -6.8950	RH 3 & AH
	4	3.96 (3)	HS, Zamora	42.1333, -6.7074	RH 4 & AH
	5	3.90 (3)	LU, Bragança	41.8498, -6.9456	RH 5 & AH
	6	3.90 (3)	LU, Bragança	41.7516, -6.7505	RH 6 & AH
	7	3.65 (1)	LU, Bragança	41.5582, -6.6754	RH 7 & AH
	8	3.78 (3)	HS, Salamanca	41.2843, -6.3787	RH 8 & AH
	12	3.92 (3)	LU, Lisbon	38.7924, -9.4257	RH 12 & AH
	13*	3.79 (8)	LU, Lisbon	38.7972, -9.4384	RH 13 & AH
	16	3.90 (3)	LU, Viseu	40.9372, -7.9255	RH 16 & NG
	18	3.76 (3)	LU, Porto	41.1768, -7.9443	RH 18 & NG
	19	3.89 (3)	LU, Porto	41.1803, -7.9344	RH 19 & NG
	45	3.62 (1)	HS, A Coruña	43.3972, -8.0959	RH 45
	59	4.14 (7)	HS, Lugo	42.8664, -7.1078	RH 59
61	4.18 (2)	HS, Lugo	42.8295, -6.8856	RH 61	
63*	4.01 (6)	HS, Lugo	43.0943, -7.0045	RH 63	
65	3.86 (2)	HS, Asturias	43.3935, -6.0980	RH 65	
<b><i>Leucanthemum x corunnense</i> Lago (4x)</b>	49	4.26 (3)	HS, A Coruña	43.7715, -7.8696	RH 49
<i>Leucanthemum sylvaticum</i> (Brot.) <i>Nyman</i> (6x)	9	5.49 (3)	LU, Guarda	40.3934, -7.5289	RH 9 & AH
	10	5.66 (3)	LU, Guarda	40.4434, -7.3463	RH 10 & AH
	11	5.48 (3)	LU, Guarda	40.4309, -7.3491	RH 11 & AH
	14*	5.49 (5)	LU, Lisbon	39.1859, -9.0581	RH 14 & AH
	15*	5.59 (3)	LU, Coimbra	40.2165, -7.9954	RH 15 & NG
	17*	5.28 (3)	LU, Viseu	41.0392, -7.8684	RH 17 & NG
	20*	5.54 (3)	LU, Braga	41.4953, -8.0370	RH 20 & NG
	21*	5.48 (4)	LU, Braga	41.4811, -8.0379	RH 21 & NG
	22	5.36 (3)	LU, Braga	41.7307, -8.1648	RH 22 & NG
	23*	5.62 (3)	LU, Braga	41.7057, -8.1673	RH 23 & NG
	24*	5.42 (3)	LU, Braga	41.7099, -8.2194	RH 24 & NG
	25*	5.49 (3)	LU, Braga	41.7260, -8.2165	RH 25 & NG
	26	5.49 (9)	LU, Braga	41.7659, -8.2437	RH 26 & NG
	27	5.65 (3)	LU, Viana do Castelo	41.9805, -8.3616	RH 27 & NG
	31	5.39 (3)	HS, Pontevedra	42.2756, -8.8038	RH 31 & NG
56*	5.53 (6)	HS, Lugo	43.5774, -7.3412	RH 56	
<i>Leucanthemum merinoi</i> Vogt & <i>Castroviejo</i> (6x)	28*	5.63 (10)	HS, Pontevedra	41.9132, -8.8760	RH 28 & NG
	29	5.58 (8)	HS, Pontevedra	42.0628, -8.8905	RH 29 & NG
	30*	5.48 (7)	HS, Pontevedra	42.2707, -8.8608	RH 30 & NG
	32*	5.44 (5)	HS, Pontevedra	42.2992, -8.8488	RH 32 & NG
	33*	5.47 (3)	HS, Pontevedra	42.3892, -8.7179	RH 33 & NG
	34*	5.68 (6)	HS, A Coruña	42.6013, -9.0563	RH 34
	36	5.62 (2)	HS, A Coruña	42.6977, -9.0237	RH 36
	37	5.63 (3)	HS, A Coruña	42.7835, -8.9231	RH 37
	38	5.85 (3)	HS, A Coruña	42.7605, -9.0649	RH 38
	41*	5.69 (3)	HS, A Coruña	43.0471, -9.2873	RH 41
43	5.67 (3)	HS, A Coruña	43.4020, -8.2093	RH 43	
44*	5.64 (21)	HS, A Coruña	43.3786, -8.2173	RH 44	

### 3.4 Results

*DNA contents.* - Both fresh leaves and silica-gel material was suitable for flow cytometry and produced good histograms (CVs < 5). All 233 individuals could be easily assigned to either diploid, tetraploid or hexaploid level, with mean relative DNA contents of 2.13-2.27, 3.62-4.29 and 5.28-5.85, respectively. None of the 58 investigated natural populations contained more than one cytotype, and neither triploids nor pentaploids were found (Table 5). By contrast, the majority of investigated inter-cytotype crosses (14 of 23 crosses) produced F<sub>1</sub> offspring with intermediate ploidy levels as expected.

*Autogamy and fertility in diploids, tetraploids and hexaploids.* – Only negligible selfing was observed for all cytotypes. Self-pollinated flower heads produced less than 1 % mature seeds, none of which were able to germinate under the chosen conditions (Table 6). By contrast, cross-pollinated flower heads produced 23-34 % mature achenes, which in turn showed germination rates of 38-76 % (Table 6). Overall post-zygotic fertility was relatively low in diploids (FI = 10 %) when compared to tetra- and hexaploids (FI = 23 % and 20 %, respectively). Both seed index, germination index and overall fertility of crosses between *L. sylvaticum* and *L. merinoi* did not significantly differ from crosses within the two species. Hence, the results of both species were pooled without consideration of taxonomic classification.

*Seed maturation after inter-cytotype crosses.* - Intra-cytotype crosses produced significantly more seeds than inter-cytotype crosses (31 % and 20 %, respectively;  $P = 0.000$ ,  $t$ -test). Most inter-cytotype crosses did not differ from the respective intra-cytotype crosses, but whenever diploids were used to pollinate polyploids, the seed index decreased significantly (Table 6).

*Germination rate after inter-cytotype crosses.* - Germination rates of intra-cytotype crosses were significantly higher than those of inter-cytotype crosses (62 % and 35 %, respectively;  $P = 0.000$ ,  $t$ -test). All inter-cytotype crosses produced considerable amounts of viable seeds, but germination indices of inter-cytotype crosses were significantly lowered when tetraploid plants acted as pollen recipients (Table 6).

*Overall post-zygotic fertility after inter-cytotype crosses.* - Overall fertility was significantly higher after intra-cytotype crosses than following inter-cytotype crosses (20 % and 10 %, respectively;  $P = 0.000$ , Mann-Whitney U-test). Specifically, fertility decreased significantly whenever tetraploids were used as pollen recipients, and post-zygotic reproductive isolation was nearly complete when diploid plants were used to pollinate polyploids (FI = 1 %, for both tetraploid-diploid and hexaploid-diploid crosses; Table 6).

Table 6: Genomic constitution of endosperm, endosperm genome ratio (EGR), seed index (SI), germination index (GI), overall fertility (FI) and relative post-zygotic reproductive isolation (rRI) for different cross types. <sup>1</sup>First and second ploidy levels refer to maternal and paternal parents, respectively. <sup>2</sup>Numbers represent percentage values. <sup>3</sup>Asterisks and circles designate values that are significantly different from values of respective intra-cytotype crosses, according to Student's  $t$ -test or Mann and Whitney's  $U$  test, respectively.

Cross type <sup>1</sup>	Genomic constitution of endosperm ( $n_{mat}/n_{pat}$ )	EGR	SI (standard error) <sup>2,3</sup>	GI (standard error) <sup>2,3</sup>	FI (standard error) <sup>2,3</sup>	rRI <sup>2</sup>
2x selfing	2/1	2	1 (0.6)	0 (n/a)	0 (n/a)	0
4x selfing	4/2	2	1 (0.4)	0 (n/a)	0 (n/a)	0
6x selfing	6/3	2	1 (0.7)	0 (n/a)	0 (n/a)	0
2x x 2x	2/1	2	23 (0.9)	38 (13.6)	10 (3.4)	0
2x x 4x	2/2	1	36 (9.0)	16 (8.3)	4 (1.9)	60
2x x 6x	2/3	0.67	17 (2.3)	29 (6.7)	5 (1.4)	50
4x x 4x	4/2	2	33 (2.5)	76 (12.3)	23 (4.2)	0
4x x 2x	4/1	4	11 (4.4)*	13 (7.2)*	1 (0.6)*	96
4x x 6x	4/3	1.33	33 (5.2)	35 (5.7)*	11 (2.7)*	52
6x x 6x	6/3	2	34 (0.3)	63 (2.7)	20 (1.6)	0
6x x 2x	6/1	6	3 (0.8)*	38 (17.9)	1 (0.4) <sup>o</sup>	95
6x x 4x	6/2	3	35 (4.5)	52 (4.3)	18 (2.5)	10

### 3.5 Discussion

The present study demonstrates that all four species from the *Leucanthemum pluriflorum* group are non-selfing, and capable of producing interspecific hybrids, even when different ploidy levels are crossed. Crosses between hexaploid species did not generate fewer or less viable seeds than intra-species crosses of hexaploids. This fact supports molecular evidence for a quite recent diversification within the *L. pluriflorum* group (HöbI 2006). Although some inter-cytotype crosses showed significantly reduced reproductive performance when compared to respective intra-cytotype crosses (especially when diploids acted as pollen donors), most inter-cytotype crosses produced considerable amounts of viable seeds, indicating that the inter-ploidy block does not generally eliminate geneflow between ploidy levels.

*Autogamy in diploids and polyploids.* - Stebbins (1950) stated that newly arisen polyploids may benefit from a break-down of self-incompatibility that comes along with genome duplication. This gain of fitness is suggested to be caused by (i) the loss of problems associated with availability of mating partners of the same ploidy level (Stebbins 1950; Levin 1975, Felber 1991; Rodriguez 1996; Miller & Venable 2000), and by (ii) lowered inbreeding depression in polyploids due to fixation of heterozygosity (Schemske & Lande 1985; Hedrick 1987; Ronfort 1999). However, although increased rates of selfing have been observed in some tetraploid angiosperms (Husband & Schemske 1997; Cook & Soltis 2000), there is also evidence that SI systems do not necessarily break down in polyploids (Busbice & Wilsie 1966). Strikingly, the results of the present analysis show that SI mechanisms take effect in all investigated *Leucanthemum* species, regardless of ploidy level. Yet, because selfing extenuates frequency-dependent mating disadvantage of newly formed cytotypes, evolutionary benefit of SI break-down is most distinct in early

stages of polyploid establishment (Levin 1975; Husband 2000; Baack 2005). Hence, when the number of mates with the same ploidy level is rising and, consequently, selection against rare cytotypes is decreasing, re-establishment of SI systems is facilitated. Studies in *Arabidopsis* and *Capsella* support this idea, as they show that expression of sporophytic SI, which is also present in the Asteraceae, is controlled epigenetically and thus highly 'flexible' (Nasrallah et al. 2007). Brennan & Hiscock's (2010) investigations on selfing in the allohexaploid species *Senecio cambrensis* further illustrate the evolutionary potential of sporophytic self-incompatibility. While natural populations of *S. cambrensis* showed a high frequency of self-incompatible plants, synthetic neo-polyploids were all selfing in the F<sub>1</sub> generation. However, self-incompatible neo-polyploids were frequently encountered as early as in the F<sub>2</sub> generation, thereby resembling their natural counterparts. These findings indicate that commonly accepted ideas of the evolutionary consequences of whole genome duplication, e.g. a general break-down of self-incompatibility in polyploids, may be obsolete and have to be revised thoroughly. For this purpose, the analysis of selfing in synthetic polyploids has proven to be useful to supplement the results from studies in natural polyploids.

*Inter-ploidy block between cytotypes.* - Several pre- and post-zygotic processes may contribute to reproductive isolation of sympatric cytotypes. Inter-cytotype fertilization can be impeded by the populations spatial structure (Sabara 2009), flowering time asynchrony between cytotypes (Pires et al. 2004) or by pollinator-mediated assortative mating (Kennedy et al. 2006). In addition, prepotency of domestic over foreign cytotype pollen (i.e. pollen with the same or a different cytotype than the egg cell, respectively) has been described repeatedly (Smith 1968). Post-zygotic isolation of cytotypes is caused by failure of endosperm development in hybrid seeds (triploid block: Köhler et al. 2010), as well as by decreased germination and survival rates, low pollen fertility, and increased



inbreeding depression through selfing in newly formed hybrids. While former publications on seed formation in crosses between diploids and tetraploids suggested that the triploid block is the main bottleneck for inter-cytotype mating (Ramsey & Schemske 1998), recent studies in *Chamerion angustifolium* demonstrate that the triploid block may be rather weak (Husband & Sabara 2004). As the realized reproductive isolation between diploid and tetraploid *C. angustifolium* (87 %; estimated from the number of triploids in mixed-ploidy populations) was much higher than expected from the extent of the triploid block (45 %), the authors suggested pre-zygotic isolation to play a decisive role for isolation of cytotypes in *Chamerion*. The picture is similar for the *Leucanthemum* taxa investigated in this study. Both seed maturation and germination are largely normal after most inter-cytotype crosses when compared to respective intra-ploidy pollinations, and relative post-zygotic reproductive isolation is only strong when diploids act as pollen donor ( $rRI \geq 95\%$ ). For all other cross types,  $rRI$  was not greater than 52 % (Table 6). Hence, under the assumptions of random mating, the formation of inter-cytotype hybrids in mixed-ploidy populations of the studied *Leucanthemum* species should be very frequent, especially in areas where tetraploid and hexaploid cytotypes co-occur. Although there were no mixed-ploidy populations included in this study, distances between many populations are rather small (< 5 km). As *Leucanthemum* is mostly pollinated by insects which easily overcome such small distances (syrphid flies and solitary bees; personal observations), frequent hybridization is likely to happen – even if small scale spatial separation of cytotypes lowers its probability. Yet, within the 233 investigated individuals neither triploids nor pentaploids were observed. Since the interploidy block is weak and inter-cytotype hybrids are vigorous, but no plants with intermediate ploidy levels could be found in natural populations, pre-zygotic isolation barriers might impede hybrid formation between cytotypes within the *L. pluriflorum* group. Further support for this hypothesis was found by Vogt (1991).

His comprehensive morphological and cytological studies on *Leucanthemum* on the Iberian Peninsula did not observe any triploid or pentaploid individuals. He merely found a single population where hybridization between *L. pluriflorum* and *L. merinoi* presumably had occurred. This population was characterized by intermediate morphology (fleshy, slightly dissected leaves) and ploidy level (4x) compared to its presumptive diploid and hexaploid parental species. In 1990, Lago described the hybrid species *Leucanthemum* × *corunnense* using a plant from this locality as *typus*. It was possible to resample this population for the present study, and flow cytometry confirmed its intermediate ploidy level. However, genetic studies using ETS sequence data could not detect any evidence for a hybrid origin of *L. × corunnense* from *L. pluriflorum* and *L. merinoi*, consequently challenging the taxonomic status of this species (chapter 2).

*Mechanism of inter-ploidy block.* - The results of the present analysis corroborate the hypothesis of an endosperm dosage effect as major mechanism of an inter-ploidy block in *Leucanthemum*. According to this hypothesis, deviations from the normal ratio of two maternal ( $n_{\text{mat}} = 2$ ) to one paternal ( $n_{\text{pat}} = 1$ ) genome in the endosperm lead to regulatory imbalances in the endosperm, either because of cytoplasmatic effects or due to genomic imprinting (Köhler et al. 2010). Further, the inter-ploidy block is suggested to become stronger with increasing deviation from the normal endosperm genome ratio. Indeed, this mechanism is well-supported by the results of the present analysis. Within the *L. pluriflorum* group, reproductive isolation is clearly asymmetric in crosses between diploids and polyploids. While rRI values are high when polyploids receive pollen from diploid plants ( $\geq 95\%$ ), they are rather low when polyploids act as pollen donor for diploids ( $\leq 60\%$ ). This clearly reflects the corresponding endosperm genome ratios, which are 4 and 6 in the former case, but only 1 and 0.67 in the latter (Table 6).

By contrast, the expected asymmetry of the inter-ploidy block was not evident in crosses between tetra- and hexaploids. Here, the deviation from the normal endosperm genome ratio is lower when tetraploids act as pollen recipients (EGR = 1.33, compared to EGR = 3 when tetraploids pollinate hexaploids), but the inter-ploidy block is weaker when tetraploid plants were used as pollen donor (10 % rRI, compared to 52 % when hexaploids pollinate tetraploids). However, the differences in deviations from the normal genomic constitution of the endosperm are quite similar, indicating that additional factors are crucial for the inter-ploidy block in these two cross types. Strikingly, while relative overexpression of paternal genes in the endosperm is known to cause overproliferation of endosperm tissue and, eventually, embryo abortion, increased expression levels of maternal genes merely lead to slightly reduced endosperm mass, but often viable embryos (Scott et al. 1998). This perfectly explains why pollinations of tetraploids by hexaploids are relatively sterile in *Leucanthemum* (dominance of paternal genomes), while inverse crosses produce a considerable amount of viable seeds (dominance of maternal genomes).

Further evidence for a developmental disorder of the endosperm as cause for the inter-ploidy block in *Leucanthemum* is provided by the fact that flow-cytometrical analysis of  $F_1$  hybrids showed that crosses between diploids and polyploids frequently produced offspring with unexpected ploidy level. This was most obvious in the case of the diploid plant 54\_01\_01: nine of ten  $F_1$  investigated individuals from crosses of this plant with two different hexaploids were pentaploid, and only a single individual was tetraploid. Also, pollination of this plant by a tetraploid did not produce plants with intermediate chromosome number ( $3x$ ), but consistently resulted in tetraploid offspring. By contrast, when 54\_01\_01 was used as pollen donor for a tetraploid plant, all five tested individuals showed the expected ploidy levels ( $3x$ ). Similar irregularities of offspring ploidy level were observed for three other diploid-polyploid crosses. Most likely, non-reduction during meiosis of

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megaspore mother cells produces 2x embryo sacs from 2x mother plants, thereby lowering or even eliminating endosperm disbalance and restoring embryo viability. If so, the reproductive success of diploid-polyploid crosses would considerably depend on the rate of meiotic non-reduction during female gametogenesis.

*Taxonomical implications.* - Within the past decades, ploidy levels have increasingly been considered in the taxonomical classification of many taxa, as it is the case with *Leucanthemum* (Vogt 1991). This development is deeply rooted on the assumption that polyploidization is accompanied by the establishment of strong post-zygotic reproductive isolation barriers between newly formed cytotypes, thereby generating biological species (Grant 1971). Yet, more recent investigations – including the present analysis – show that different cytotypes within a species or a genus are often capable of producing viable offspring (Ramsey & Schemske 1998; Husband & Sabara 2004). Apparently, whole genome duplication does not necessarily eliminate gene flow, and therefore, polyploidy itself should not be used as a single criterion for the description of new species on the basis of a biological species concept. Nevertheless, as pre-zygotic isolation mechanisms often lead to almost complete reproductive isolation, taxonomical classification of cytotypes as species is often reasonable, even if the break-down of gene flow between species does not directly result from inter-cytotype sterility.

## Synopsis

The thesis at hand investigates speciation by polyploidy in the genus *Leucanthemum*. In general, speciation is characterized by the formation of reproductive isolation barriers between previously interbreeding populations (Mayr 1942), and whole genome duplication is commonly considered to lead to immediate reproductive isolation between newly formed polyploids and their diploid progenitors (Stebbins 1950, Linder & Rieseberg 2004, Mallet 2007). It often triggers gametic incompatibility, as well as a number of morphological, phenological, and ecological changes that may impede genetic exchange between polyploids and their diploid progenitors (Grant 1971).

Polyploid speciation can be studied by two fundamentally different approaches. On the one hand, molecular phylogenetics may be used to reconstruct the evolutionary history of a polyploid taxon, and thus speciation events become uncovered and can be discussed in a long-term evolutionary context. On the other hand, various experimental settings make it possible to identify the mechanisms that cause speciation after whole genome duplication. However, only a combination of these two methodologies can provide a comprehensive picture of how evolution by polyploidization works. Consequently, both approaches are used in the present thesis.

The first two chapters try to trace past hybridization and polyploidization events that gave rise to the three polyploid *Leucanthemum* taxa investigated here. Both information from the chloroplast and from the nuclear genome is analyzed to obtain robust hypotheses, and several statements can be made on the basis of this phylogenetic investigations.

The cpDNA sequence analysis aims at testing if all members of the *L. pluriflorum* group share a common chloroplast haplotype as indicated by previous phylogenetic

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investigations of the whole genus *Leucanthemum* (Höfl 2006). The present data corroborate the initial hypothesis that all populations of the diploid *L. pluriflorum* and of the three polyploids *L. irtutianum* subsp. *pseudosylvaticum*, *L. sylvaticum* and *L. merinoi* are characterized by a chloroplast haplotype that features a number of apomorphic characters. Thus, concerning the chloroplast genome, these taxa are clearly differentiated from the other species of the genus *Leucanthemum* and form a monophyletic group.

Furthermore, the cpDNA sequence analysis suggests that there were at least three independent genome duplication events where *L. pluriflorum* acted as maternal parent, resulting in three evolutionary lineages of polyploids. Recurrent formation of polyploids is a common phenomenon that can be found in many plant species, e.g. *Tragopogon* (Soltis et al. 2004b), *Draba* (Brochmann et al. 1992b), *Arabis* (Sharbel & Mitchell-Olds 2001), and *Saxifraga* (Brochmann et al. 1998), thereby impressively illustrating one of the most specific characteristics of polyploid speciation: While species that were formed by geographical speciation are considered to have a single evolutionary origin, whole genome duplication may repeatedly give rise to morphologically (and sometimes even genetically) identical evolutionary lineages. This fact strongly conflicts with the traditional view of species as reproductive communities with a common phylogenetic origin.

While the cpDNA sequences clearly support monophyly of the *L. pluriflorum* group as well as recurrent polyploidization, the question whether other *Leucanthemum* species played a role in the evolution of the study group remains unclear. On the one hand, the AFLP fingerprinting in chapter 1 shows that the polyploids are genetically quite different from *L. pluriflorum*, and therefore suggests that they did not form by strict autopolyploidization of the Galician diploid. On the other hand, the ETS sequence data analysis in chapter 3 does not provide evidence for any species other than *L. pluriflorum* being involved in the polyploid formation. It rather indicates that the diploid itself, along with its sibling species

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*L. gaudinii* subsp. *cantabricum*, formed by hybridization of a *L. halleri*-like species and an elusive second diploid, which subsequently gave rise to the polyploids by duplication of its chimeric genome. However, to definitely settle this matter, more genetic data and sophisticated analyses are needed.

The third chapter finally aims at identifying present reproductive isolation barriers between the different cytotypes realized in the *L. pluriflorum* group. As already mentioned before, whole genome duplication often causes strong post-zygotic isolation barriers between polyploids and their diploid progenitors (Stebbins 1950, Linder & Rieseberg 2004, Mallet 2007), but this is not necessarily the case. For example, Slotte et al. (2008) showed that whole genome duplication in *Capsella* did not result in immediate and complete reproductive isolation, and that post-polyploidization hybridization and introgression is possible and frequent. Further, Husband & Sabara (2004) quantified that pre-zygotic isolation barriers between diploid and autotetraploid *Chamerion angustifolium* accounted for 97.6 % of the total reproductive isolation, thereby demonstrating the limited role of gametic incompatibility for polyploid speciation in this species. A similar picture results from the crossing experiments conducted within the course of the present study. Although reduced relative reproduction rates were observed when pollen of *L. pluriflorum* was transferred to any of its polyploid relatives, the central observation was that, basically, all inter-ploidy crosses were capable of producing viable offspring. Consequently, reproductive isolation between the members of the study group is not exclusively based on gametic incompatibility. In fact, pre-zygotic isolation barriers are likely to play an important role for speciation by whole genome duplication in the *L. pluriflorum* group. This assumption is confirmed by the finding that extensive ploidy level determination did not reveal any intermediate cytotypes, not even in regions where two different cytotypes co-occur. Yet, this would be the case if inter-cytotype pollination was frequent.

In conclusion, the thesis at hand illustrates the multiplicity of facts that have to be considered when studying evolution by whole genome duplication. Processes like recurrent formation of polyploids, pre-polyploidization hybridization, introgression, and inter-cytotype geneflow generate a complex pattern of reticulation that challenges the current methods of evolutionary biology. Yet, upcoming methods such as massive parallel sequencing and high-throughput cytotype determination are about to initiate a huge leap forward, and may enable us to better understand the mechanisms underlying polyploid evolution.



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