Reconstructing reticulate relationships in the polyploid complex of *Leucanthemum* Mill. (Compositae, Anthemideae)

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I would like to dedicate that work to Agnieszka, Dobromiła and Radomir.

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1. GENERAL INTRODUCTION

1.1. Phylogenetic reconstruction

Phylogeny forms the hypotheses about the origin and relationships among species and tries to improve our understanding of evolution. Its history dates back to times of ancient philosophers but prominent development was reached after works of Darwin and Haeckel in XIX century. They laid the foundation of modern phylogeny by developing the theory of evolution (Darwin 1859) and principles regarding the representation of phylogeny on a phylogenetic tree (Haeckel 1866). Around the beginning and middle of XX century a new branch of phylogenetics appeared which transformed systematic biology – molecular phylogenetics. Initially it used information gained from sources such as serology (e.g. Nuttall 1902), chromatography (e.g. Pearson 1967) or electrophoresis of proteins (e.g. McCabe & Deutsch 1952). After the major breakthrough in molecular biology which was the discovery of the structure of DNA (Watson & Crick 1953) it started to develop very quickly and assimilated methods associated with nucleic acids. Firstly methods associated with DNA-DNA hybridization were dominating (e.g. De Ley et al. 1970), trough shearing of DNA with restriction enzymes, to finally reach the present stage of sequencing particular regions of DNA which will be likely exchanged with the sequencing of whole genomes in the future (Delsuc et al. 2005). Since the beginning, progress of that field was closely associated (and still is) with increasing usage of computers and development of statistical methods suited to analyze data in phylogenetic perspective – especially nucleotide sequences (Graur & Li 2000, Suárez-Díaz & Anaya-Muñoz 2008). Most widely used methods are parsimony, maximum likelihood, and Bayesian inference (Graur & Li 2000). Development of molecular phylogenetics is very important for the systematic biology because of its advantages over the classifications based only on morphological or chemical similarities. Most importantly in contrary to those, DNA sequencing can distinguish homology from analogy (Finch 1970). In its current form it presents a superior source of information regarding the relationships among organisms because it is more objective and may be used to estimate a molecular clock which is used not only to reveal the connections among organisms but also to put their evolution into a timeframe (Graur & Li 2000, Suárez-Díaz & Anaya-Muñoz 2008, Suárez-Díaz 2009). It presents a wide range of possible uses and influences other branches of biology such as biogeography, ecology, taxonomy, and evolutionary biology – or conversely it integrates all this disciplines. Many challenging projects arose such as those which aim to reconstruct the whole tree of life using molecular phylogeny (e.g. Tree of Life Web Project www.tolweb.org, see also Wolf

et al. 2002, Puigbò et al. 2009, House 2009, Pace 2009). Also of major importance to modern botany is the molecular-based flowering plant classification (APG 1998, APG 2003, APG 2009).

Starting with the DNA sequencing, classical work on molecular phylogeny at lower taxonomic levels involved working with one or few loci, in plant biology, typically nuclear internal transcribed spacer (ITS) and external transcribed spacer (ETS) with addition of some chloroplast markers (Appels & Dvořák 1982, Yokota et al. 1989, Baldwin 1992, Baldwin et al. 1995, Baldwin & Markos 1998). Although this combination revolutionized modern plant systematics it has several drawbacks. Among them are the multicopy nature of ITS and ETS, often low phylogenetic resolution and possible overestimation/underestimation of phylogenetic signal based on a single locus (Álvarez & Wendel 2003). Usually those traditional markers also fail to resolve relationships within evolutionary young groups which did not accumulate sufficient level of divergence, as it was the case with the genus under study (Hößl 2006). In addition, processes such as incomplete lineage sorting (ILS) and hybridization are difficult to distinguish with the usage of those markers.

The discordance between gene trees and species trees has been noticed already some time ago (e.g. Fitch 1970, Pamilo & Nei 1988). Still one has to agree that until recently, most of the phylogenies reconstructed gene trees rather than species trees, and claimed that gene tree represents the tree of species relationships (Degnan & Rosenberg 2009). The discordance between those two types of trees arises mainly because incomplete lineage sorting (ILS) which is a “failure of two or more lineages in a population to coalesce” (Degnan & Rosenberg 2009). To overcome those problems and transit from the gene tree reconstruction to the species tree reconstruction more than one locus is required. Ideal for that purpose are so called single-copy genes (sometimes referred as low-copy genes) distributed across the genome in orthologous positions. Incorporating them into phylogeny requires usage of new methods analyzing and summarizing trees obtained from multiple markers instead of examining single gene trees or trees based on concatenated data (Kubatko & Degnan 2007, Degnan & Rosenberg 2009). The species tree reconstruction furthermore needs to incorporate simultaneously incomplete lineage sorting among different genes as well as other processes causing incongruence among the gene trees. With regard to the plant biology, the most prominent processes may be hybridization and polyploidy. Hybridization has similar effects on phylogeny as ILS. It causes incongruence among gene trees by combining alleles in a way that some of them may be exchanged between distantly related species which induce the relationships that may not

fully represent the true/complete phylogeny (e.g. especially when there is one allele or an allele is missing). In the same manner as ILS, presence of hybridization in the dataset affects also the relationships between gene trees and species tree implying a network-like structure for the latter. Some approaches have been proposed to distinguish both processes (cf. Chapter 1). The second process of polyploidization complicates those relationships even further by duplication of loci leading to the situation that they eventually became paralologous. Hybridization and polyploidy, due to the frequent occurrence in *Leucanthemum*, are within main focus of this thesis.

1.2. Hybridization and homoploid hybrid speciation

Hybridization is an interbreeding of two genetically distinct individuals which produces progeny possessing a combination of heritable characteristics derived from both parents (Allendorf et al. 2001). Intermediacy between parents is present in the genome constitution and typically may be also observed on inherited traits. However, hybrids often have some novel features which are not present in the parents (sometimes observed as a hybrid vigor/heterosis) and may originate by processes such as for example novel gene expression patterns (e.g. Chelaifa et al. 2010). Hybridization may occur repeatedly especially in sympatric zones between two species or may occur by chance and very rarely with occasional migrants. Furthermore hybrids are either infertile, or fertile, which is decisive on their future and establishment. Another feature of hybrids in a polyploid complex is that they may be intermediate between two ploidies or retain ploidy level of the parents. In case when the ploidy level is maintained homoploid hybrid speciation may take place (typically this term is applied only to the hybrids on the diploid level). It is a variant of hybridization where after crossing of two taxa, their hybrid speciate into a new form and become isolated from their parental species to ultimately form a new taxon (Stebbins 1950, Grant 1971, Rieseberg 1997, Rieseberg & Willis 2007). This kind of hybridization requires that taxa are not so distant from each other because otherwise the difficulties in producing hybrid and problems with fertility increase (Buggs et al. 2008). The isolation from parental species can be achieved after several generations (cf. Grant 1966) or by spatial isolation (Buerkle et al. 2000). Incidence of homoploid hybrid speciation in the genus *Leucanthemum* has been suggested by Oberprieler et al. (2014) who found that majority of diploid species contained one of the two major ETS ribotypes while some species contained a mixture of both ribotypes (*L. pluriflorum*, *L. gaudinii* subsp. *cantabricum*, *L. gaudinii* subsp. *barrelieri*, *L. vulgare* subsp. *eliasii*, *L. tridactylites*). The authors

considered taxa which possessed two ribotypes as having putative hybridogenous origin (Oberprieler et al. 2014, Appendix B). Mainly because of this recent finding homoploid hybrid speciation within the genus *Leucanthemum* became one of the main focus of Chapter 1.

1.3. Polyploidy

Since the first mention of polyploidy by Strasburger (Strasburger 1910) and its definition (Winkler 1916), points of view considering the importance of this process changed. Hugo de Vries saw polyploidy as mutation leading to formation of new forms (de Vries, 1901). In more recent times probably most influential works came from Stebbins (e.g. Stebbins 1971, Stebbins 1985) which considered polyploidy itself to be a stabilizing and conservative evolutionary process. Besides points of view considering significance of this process, various studies estimated polyploidy frequency to account for 30% to 80% of current angiosperm species (reviewed in Soltis et al. 2009). With the discovery of multiple polyploidizations during evolution of all angiosperm lineages (Soltis et al. 2009, Jiao et al. 2011), polyploidy significance raised together with general interest in this phenomenon. While it may be responsible for the diversification of the whole families, in certain lineages it also plays an important role as a mode of speciation (Wood et al. 2009). In particular, it is very frequent within the Compositae family where numerous polyploid species are established and plants up to octotetracontaploid (48*x*) level are found (Semple & Watanabe, 2009). Commonness of this process especially in the plant kingdom highlights its importance and impact on evolution. But it is not only specific to plants – it is frequent in other organismal groups such as fungi or animals (Van de Peer et al. 2009, Albertin & Marullo 2012). Nowadays it is accepted that all major groups of vascular plants have gone through polyploid events at least once and in some families as in Compositae even more often during their evolutionary history (Soltis et al. 2009, Van de Peer et al. 2009). Many polyploid complexes were subjects of phylogenetic research and examples include: *Achillea* (Guo et al. 2004, Guo et al. 2005), *Silene* (Popp et al. 2005, Popp & Oxelman 2007), *Viola* (Marcussen et al. 2012), *Artemisia* (Pellicer et al. 2010, Garcia et al. 2011, Richardson et al. 2012), and *Melampodium* (Blöch et al. 2009, Rebernig et al. 2010). As majority of *Leucanthemum* taxa is polyploidy, it became focus of Chapter 2 which deals with phylogeny of polyploids, and Chapter 3 which gives insights into more specific question considering *L. glaucophyllum* and related taxa.

1.4. *Leucanthemineae* and *Leucanthemum*

All *Leucanthemum* taxa are perennial herbs with variable leaf morphology (entire, serrate, pinnatifid), alternate leaves, solitary or laxly corymbose capitula, receptacle convex or conical, without receptacular bracts (epaleate), white female ray florets (sometimes absent), disc corolla 5-lobed, ca. 10-ribbed cypselas with myxogenic cells and vascular strands between the ribs, possessing auriculate corona-like pappus (sometimes absent) (Bremer & Humphries 1993, Oberprieler et al. 2006). Its specific feature are anthocyanin red root tips, which are characteristic for that genus (Bremer & Humphries 1993). As treated in Euro+Med PlantBase (2006) and Oberprieler et al. (2009), *Leucanthemum* constitutes a morphologically homogenous genus.

The subtribe *Leucanthemineae* belongs to the Mediterranean clade of Eurasian grade of tribe Anthemideae and family Compositae (Oberprieler et al. 2009). The subtribe consists of eight genera and ca. 69 species, majority of which is concentrated within the genus *Leucanthemum* consisting of around 41 species (56 species and subspecies) (**Table 1**) while the other genera typically consist of one or few species. All unispecific *Leucanthemineae* genera also have somehow restricted endemic distributions in southern part of the Mediterranean basin (North Africa, Cyprus) in some cases also reaching similar regions in its northern part (southern Iberian Peninsula, southern Apennine Peninsula, Mediterranean islands; Oberprieler et al. 2009). *Leucanthemum* does not reach this part having its center of distribution rather in cooler regions of northern Mediterranean including the central and northern Iberian Peninsula, the Alps, the Apennine Peninsula, the Balkan Peninsula and reaching further north and east (Vogt 1991, Euro+Med PlantBase, 2006). Also noticeably most of the unispecific genera from southern taxa are diploids with hybrids being present but almost without records of polyploidy (Wilcox 1982, Oberprieler et al. 2011). In the northern lineage of *Leucanthemineae* i.e. *Leucanthemum*, polyploidy is a widespread phenomenon and from 41 species only 14 (19 taxa) are diploid whereas the others form the polyploid series from tetraploids ($2n = 4x = 36$) to dokosaploid ($2n = 22x = 198$). 19 diploid taxa distributed across the whole range of the genus have well defined distributions associated with their habitat requirements but in some cases they also overlap sympatrically. It has been shown that these ranges and overlaps changed significantly during the last ice ages leading undoubtedly to contact of some species which are separated today (Oberprieler et al. 2014, Appendix B). It has been also shown that some Iberian *Leucanthemum* taxa had even wider ranges during last glacial maximum compared with their present distribution (Oberprieler et al. 2014, Appendix B).

Leucanthemum represents an interesting model for studying polyploidy and hybridization but as illustrated in the various works done previously, relationships among taxa seem to be rather complex and often hard to resolve. This complexity partially originates from taxonomic classification and as noted by Pearson (1967): "survey of taxonomic literature on this species aggregate reveals that there is widespread confusion resulting from regional authors having created superfluous synonyms and giving inadequate plant descriptions" (Pearson 1967, p.92). In this thesis this problem is circumvented by usage of the taxonomic concept of Vogt (1991) which treated Iberian *Leucanthemum* plants as different species when they were different in ploidy, distributional range, and morphology. This concept in similar form was also adapted in Euro+Med treatment (Euro+Med PlantBase, 2006) which is a taxonomical backbone for the adapted classification. Available studies indicate that species barriers in the genus are not very strong and different taxa may hybridize when brought into contact (Villard 1971, Greiner & Oberprieler 2012). This creates a network of species relationships which needs detailed methods and sampling to resolve them. Usage of next generation sequencing coupled with low-copy nuclear and chloroplast markers seems to be a promising perspective in the investigation of polyploid origin. Inferring the origin of polyploid species represents the most difficult riddle in this complex genus but at the same time knowledge about it could be a key to understanding the evolution of this group.

Species	Ploidy	Distribution
Leucanthemum burnatii Briq. & Cavill.	2*x*	FR
Leucanthemum cf. monspeliense (L.) H. J. Coste	2*x*	FR
Leucanthemum gallaecicum Rodr. Oubiña & S. Ortiz	2*x*	ES
Leucanthemum gaudinii subsp. *barrelieri* (Dufour ex DC.) Vogt	2*x*	ES, FR
Leucanthemum gaudinii subsp. *cantabricum* (Font Quer & Guinea) Vogt	2*x*	ES
Leucanthemum gaudinii subsp. *gaudinii* Dalla Torre	2*x*	FR, IT, CH, AT, ME, CZ, PL, SK, DE, RO, UA
Leucanthemum gracilicaule (Dufour) Pau	2*x*	ES
Leucanthemum graminifolium (L.) Lam.	2*x*	FR
Leucanthemum halleri (Vitman) Ducommun	2*x*	AT, DE, CH, IT
Leucanthemum laciniatum Huter, Porta & Rigo	2*x*	IT
Leucanthemum ligusticum Marchetti , R. Bernardello , Melai & Peruzzi	2*x*	IT
Leucanthemum lithopolitanicum (E. Mayer) Polatschek	2*x*	AT, SI
Leucanthemum pluriflorum Pau	2*x*	ES
Leucanthemum rotundifolium (Willd.) DC.	2*x*	PL, SK, UA, RO, HU, BA
Leucanthemum tridactylites (Fiori) Bazzich.	2*x*	IT
Leucanthemum virgatum (Desr.) Clos	2*x*	FR, IT
Leucanthemum vulgare subsp. *eliasii* (Sennen & Pau) Sennen & Pau	2*x*	ES
Leucanthemum vulgare subsp. *parviceps* (Briq. & Cavill.) Vogt & Greuter	2*x*	FR
Leucanthemum vulgare subsp. *pujiulae* Sennen	2*x*	ES
Leucanthemum vulgare subsp. *vulgare* (Vaill.) Lam.	2*x*	widespread, $
Leucanthemum corsicum subsp. *corsicum* (Less.) DC.	4*x*	FR
Leucanthemum corsicum subsp. *fenzlii* Gamisans	4*x*	FR
Leucanthemum corunense Lago	4*x*	ES
Leucanthemum delarbrei Timb.-Lagr.	4*x*	FR
Leucanthemum ircutianum DC. subsp. *asperulum* (Terr.) Vogt	4*x*	IT
Leucanthemum ircutianum DC. subsp. *cantabricum* (Sennen) Vogt	4*x*	ES, FR
Leucanthemum ircutianum DC. subsp. *crassifolium* (Lange) Vogt	4*x*	ES, FR
Leucanthemum ircutianum DC. subsp. *ircutianum*	4*x*	widespread, $
Leucanthemum ircutianum DC. subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	4*x*	IT, BA, SI, HR, RS
Leucanthemum meridionale Legrand	4*x*	FR
Leucanthemum monspeliense (L.) Coste	4*x*	ES, FR
Leucanthemum pseudosylvaticum (Vogt) Vogt & Oberpr.	4*x*	ES, PT
Leucanthemum visianii (Gjurašin) Vogt & Greuter	4*x*	BA, ME, HR
Leucanthemum adustum (Koch) Gremli subsp. *adustum*	6*x*	AT, IT, FR, DE, CH
Leucanthemum adustum subsp. *margaritae* (Ják.) Holub	6*x*	AT, CZ, PL, SK, HU, UA, RO, BA, ME
Leucanthemum aligulatum Vogt	6*x*	ES
Leucanthemum atratum (Jacq.) DC. subsp. *atratum*	6*x*	AT
Leucanthemum chloroticum Kern. & Murb.	6*x*	BA, ME, HR, RS, GR
Leucanthemum coronopifolium subsp. *ceratophylloides* (All.) Vogt & Greuter	6*x*	FR, IT
Leucanthemum coronopifolium subsp. *tenuifolium* (Guss.) Vogt & Greuter	6*x*	IT
Leucanthemum coronopifolium Vill. subsp. *coronopifolium*	6*x*	FR, IT
Leucanthemum cuneifolium H. J. Coste	6*x*	FR, IT
Leucanthemum maestracense Vogt & Hellwig	6*x*	ES
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	PT, ES, FR, IT
Leucanthemum subglaucum De Laramb.	6*x*	FR
Leucanthemum silvaticum subsp. *merinoi* (Vogt & Castrov.) Vogt & Oberpr.	6*x*	ES, PT
Leucanthemum silvaticum subsp. *silvaticum* (Brot.) Nym.	6*x*	ES, PT
Leucanthemum cf. glaucophyllum (Briq. & Cavill.) Jahand. "esterellense"	8*x*	FR
Leucanthemum favargeri Vogt	8*x*	ES
Leucanthemum heterophyllum (Willd.) DC.	8*x*	AT, CH, IT, SI, HR, $
Leucanthemum illyricum (Horvatić) Vogt & Greuter	8*x*	BA, ME, HR
Leucanthemum platylepis Borb.	8*x*	HR, SI, RS, IT
Leucanthemum catalaunicum Vogt	10*x*	ES, FR
Leucanthemum glaucophyllum (Briq. & Cavill.) Jahand.	10*x*	IT
Leucanthemum montserratianum Vogt	10*x*	ES
Leucanthemum pachyphyllum Marchi & Illuminati	10*x*	IT
Leucanthemum maximum (Ramond) DC.	12*x*	ES, FR, $
Leucanthemum lacustre (Brot.) Samp.	22*x*	PT, $
Leucanthemum rohlenae (Horvatic) Vogt & Greuter	?	ME
Leucanthemum valentinum Pau (= *L. gracilicaule* ?)	?	ES

country codes according to ISO 3166-1, $ denotes cultivated species (according to Rutkowski 2004)

Table 1 - Species and taxa of *Leucanthemum* Mill. according to the Euro+Med PlantBase (2006). Country codes are according to ISO 3166-1, and $ denotes cultivated species.

1.5. Thesis outline

This thesis focuses on reconstructing the phylogeny of genus *Leucanthemum* which due to its intermediate size (ca. 41 species) and unbroken polyploid chain seem to be an ideal model for studying the history of polyploidy. Also as suggested by previous studies,

homoploid hybrid speciation could have played a role in the evolution of diploid species which provides the opportunity to examine that hypothesis in more detail.

The aim of Chapter 1 is to bring insights into the phylogeny of the diploids which form the foundation of the whole genus *Leucanthemum*. In order to establish relationships among taxa, nine low-copy nuclear genes are sequenced using 454 sequencing technology for all diploid taxa currently recognized with at least two accessions per taxon. Additionally, five markers from the chloroplast genome are sequenced with Sanger sequencing. This provides the possibility to construct a species tree and provides basic insights into the relationship with other *Leucanthemineae* genera. Furthermore this chapter is especially focused on the incidence of homoploid hybrid speciation and presents a novel method to evaluate it using a simulation study.

The aim of Chapter 2 is to explore relationships among all *Leucanthemum* taxa with inclusion of polyploids and special focus on them. As in the previous chapter it is based on low-copy nuclear and chloroplast markers. The results are presented as supernetwork generated from gene trees coded as a 0/1 matrix. Inspite of its reduced capacity of discrimination between actual hybridization and incomplete lineage sorting, it provides insights into the evolution of the whole genus and in particular sheds light on the relationships within several more specific groups.

The aim of Chapter 3 is to clarify relationships among several taxa classified earlier as *L. glaucophyllum* varieties (Briquet & Cavillier 1906) and *L. pallens* presumably related to them. It uses previous approaches coupled with more detailed sampling on the species level and additionally includes AFLP banding patterns. This sheds light onto the phylogeography of one of the most widespread *Leucanthemum* taxa in the Mediterranean – the hexaploid *L. pallens*. Insights into this intricate group of high polyploids related to *L. glaucopyllum* are presented with possible description of new species and several insights into evolution and processes occurring in the whole group and in particular taxa.

Any taxonomical novelties appearing in the present publication should not be regarded as an official statement, and have to be treated as a suggestion.

2. CHAPTER 1

Phylogeny of diploid *Leucanthemum* Mill. (Compositae , Anthemideae)

This chapter is designed for publication with following authorship: Kamil Konowalik, Florian Wagner, Salvatore Tomasello, Robert Vogt, Christoph Oberprieler. Author contributions are as follows: study conception and design: CO, KK, ST, FW; laboratory work: KK (AFLP: FW & KK); determination and collection of specimens: RV, CO, KK, ST; writing: KK; analysis of the data: KK, FW performed all calculations associated with hybrid index and prepared Figure 7.

2.1. Abstract:

The genus *Leucanthemum* Mill. is a species-rich polyploid complex of southern and central Europe, comprising ca. 41 species with ploidy levels ranging from 2*x* to 22*x*. In this chapter phylogenetic analysis of diploids is presented using species tree and network reconstructions. Previous studies have shown that some of the diploids may have originated trough homoploid hybrid speciation. In order to detect hybridization and pinpoint taxa with higher probabilities of being of hybrid origin compared to others, strategy based on the method for estimating the probability of a gene tree topology within a given phylogenetic network was used in the current study. Having inferred the species tree for 19 diploid *Leucanthemum* taxa and the outgroup taxa based on gene trees from nine nuclear and one plastidal loci, a "hybrid index" was calculated for each of the *Leucanthemum* taxa by (a) describing all possible triplet species trees, (b) pruning of gene trees to produce the corresponding triplet gene trees, (c) calculating the probabilities for hybrid vs. non-hybrid scenarios for a given triplet based on the simultaneous consideration of all ten triplet gene trees, and (d) summing up the frequency of its hybrid/non-hybrid interpretation for each diploid taxon. As a complement and to verify the results statistically, the hybrid index obtained from real data was compared to nineteen hybrid indices from simulated data which were influenced only by incomplete lineage sorting. The results indicated that hybridization was not involved in the formation of only five taxa while the other fourteen had significantly higher real hybrid indices than simulated values. This suggests that incomplete lineage sorting alone is not enough to explain incongruence among gene trees and that a hybridization likely played a role in the formation of these taxa. Furthermore results show that *Leucanthemum* diploids may be classified into two larger groups (named as 'Group 1' and 'Group 2') which are stable and found consistently by different analyses.

Keywords: ox-eye daisy, Asteraceae, homoploid hybrid speciation

2.2. Introduction

Hybridization is often stressed as one of the most important evolutionary processes. Especially within the plant kingdom around 25% of species may hybridize (Mallet 2005). Its exceptionality is highlighted by the rate of change induced by this process which at once merges two distinct genotypes into a novel combination. On the one hand it may be a

dead end for the species (despeciation and introgression) and on the other it may be a way to create new taxa (homoploid/polyploid hybrid speciation) (Mallet 2007, Abbott et al. 2013). Speciation via hybridization can occur in two ways – involving polyploidy and change in chromosome number or homoploid hybrid speciation where the hybrid have the same ploidy as parental taxa (Hegarty et al. 2005, Rieseberg & Willis 2007). The second process commonly thought to be less common is now attracting particular interest (Buerkle et al. 2000, Gross & Rieseberg 2005) and has been increasingly reported especially for plants(e.g. Howarth et al. 2005, James et al. 2005, Kelly et al. 2010, Peruzzi et al. 2011). Already Grant (1966) notes that homoploid hybrid speciation is an interesting process but at the same time rare in nature. But this rarity may be associated with the difficulties to detect it (reviewed in Kelly et al. 2010). Therefore the incidence of homoploid hybrid speciation is probably largely underestimated (Seehausen 2004, Mallet 2005, Kelly et al. 2010).

Incorporating hybridization into phylogenetic reconstruction was always a point of interest for many biologists. Within single gene phylogenies it involved comparison between chloroplast and nuclear markers (typically ITS or ETS) based on gene trees incongruence (e.g. Pirie et al. 2009). Although these methods are still in use, slowly the transition from single gene to multi gene reconstructions is observable. The advantage of the latter lies in the possibility of constructing not only a gene tree but also a species tree. However, incorporating hybridization into a species tree analysis is more challenging. Reconstructing species history needs to use more than a single locus, and typically it is based on single- or low-copy genes which are distributed across the genome in orthologous positions. Due to reticulate evolutionary processes they are often incongruent and species tree reconstruction method needs to take this into account (Maddison 1997). Most common source of reticulation is incomplete lineage sorting (ILS) and it is already widely addressed by majority of currently available methods. But apart from ILS, further species tree reconstruction needs to simultaneously incorporate hybridization which can cause incongruence among the gene trees as well.

Unfortunately due to computational difficulties hybridization was omitted or ignored in most of the available species tree reconstruction methods (e.g. Heled & Drummond 2010). Here attempts which tried to account for both processes, namely ILS and hybridization, are shortly reviewed. Sang and Zhong (2000) proposed a test to distinguish between hybridization and ILS based on differences in the divergence times in incongruent gene trees but their method was later criticized (Holder et al. 2001). Holland et al. (2008) used filtered supernetwork to distinguish ILS from hybridization but this

approach needed many gene trees which are congruent in large parts and in case when multiple hybridizations occurred, the phylogenetic inference was limited (Holland et al. 2008). Kelly et al. (2010) used supernetworks with combination of recombination tests and Lento plots to find hybrids. Joly et al. (2009) proposed a statistical test to detect hybridization but it relied on specified input species tree and may have failed to detect hybridization in cases when recombination was present within the dataset or when population sizes and divergence times were miscalculated (Joly et al. 2009). Kubatko (2009) developed a likelihood-based method later implemented in a software (Kubatko et al. 2009) but it was limited to one hybrid species per analysis which had to be chosen manually along with specification of the parents. Same limitation was also true for HybTree script of Gerard et al. (2011) and although both methods may be of interest in hypothesis testing, they are not suitable for computing phylogenetic trees and networks. Method of Maureira-Butler et al. (2008) extended by Blanco-Pastor et al. (2012) uses a simulation of ILS on obtained gene trees to create tree distances which are used for selection of taxa causing strongest incompatibility between gene trees. De Villiers et al. (2013) proposed an alternative method based on gene tree incongruence using supporting information obtained from morphology and genealogical sorting index, its main advantage lying in the possibility of detecting hybrids within coalescent stochasticity zone where ILS and hybridization signal is hard to distinguish. Significant progress has been made made continuously by L. K. Nakhleh group (CS Bioinformatics Group, Rice University, USA) who developed already several methods working with multiple alleles and multiple accessions which are typically too complex for previously mentioned attempts. All of them are also embedded in easy to use software which is continuously upgraded (Than et al. 2008, Than and Nakhleh 2009). Three currently available methods are able to infer hybridization in presence of incomplete lineage sorting. Yu et al. (2012) infers probability of parental contribution to the hybrid when hybrid and parents are already specified. Yu et al. (2013) proposed a method capable of allowing and inferring multiple hybrids in the species tree which creates then a species network based on the parsimony criteria. Sibling method but based on the maximum likelihood developed by Park & Nakhleh (2012) is also able to infer multiple hybrids within a species network. The drawback of these two methods is that they are relying on a number of reticulations suggested by the user although even this step may be optimized by usage of information criteria (AIC or BIC).

Besides those attempts incorporating hybridization into phylogenetic reconstructions is still accidental rather than an established procedure. The cause for this may be that all above methods have some limitations and typically require a lot of manual

work, expect difficult to format input files or they are computationally very intensive which prohibits larger analyses. One needs to bear in mind that ignorance of hybridization and favoring only ILS in the species tree analysis brings unreliability to phylogenetic reconstruction which is actually the case in majority of present phylogenetic studies. In fact, simulations of gene flow between species (which under some scenarios may be similar to hybridization) revealed that it may have a strong influence on accuracy of species tree reconstruction affecting correct topology, overestimating population sizes and underestimating divergence times (Leaché et al. 2013).

Processes like ILS and hybridization coupled with recent diversification were suggested to play an important role in the Mediterranean area (Blanco-Pastor et al. 2012 and references therein). In fact this region is spatially much differentiated climatically (with transitions from deserts to humid areas) and geologically (with alteration of mountains and plains). Moreover, the whole area is divided into many enclaves that harbor a rich endemic flora. Due to this mosaic and relatively warmer climate during Pleistocene (as compared to northern territories) some areas within Mediterranean acted as glacial refugia where many taxa survived to be then brought into contact and separated again during multiple warming-cooling episodes. These circumstances made the Mediterranean basin a biodiversity hotspot (or in Central part even "hotchpotch", Lo Presti & Oberprieler 2011) where all evolutionary processes could "spread their wings". This area was as well the ground of speciation within Mediterranean clade of *Anthemideae* and particularly the sub-tribe *Leucanthemineae* with its biggest genus *Leucanthemum* Mill.

Leucanthemum is part of the sub-tribe *Leucanthemineae* Bremer & Humphries which consist of 8 genera and around 71 species distributed in the Mediterranean and adjacent northern areas (Euro+Med PlantBase 2006, Oberprieler 2009). The subtribe can be divided into two provisional groups consisting of North African endemics which occupy the southern part of the Mediterranean basin and the other group of *Leucanthemum* distributed in the northern part. Most of the genera contain only few species contrary to the largest genus of *Leucanthemum* which contains around 41 species (Euro+Med PlantBase, 2006). *Leucanthemum* is widely known because of cultivation as an ornamental plant with the most famous max chrysanthemum (*L. maximum*), ox-eye daisy (*L. vulgare*) and including dozens of varieties and hybrids (e.g. shasta daisy, *Leucanthemum* × *superbum*). Many of the species enlarged their ranges in recent times with the spread of meadows and transportation as ornamentals. This leaded to the situation that *L. vulagre* and *L. ircutianum* that are native to Europe are treated as invasive plants in many areas of the world on all continents (Clements et al. 2004, Khuroo et al. 2010, DiTomaso 2012, Busso et al. 2013).

Previous works also documented that barriers between species are weak and most of them can cross with each other (Villard 1971, Greiner & Oberprieler 2012). These facts would suggest that hybrid formation (Piękoś 1970) and possibly homoploid hybrid speciation (Oberprieler et al. 2014) are present in the genus. The latter was suggested based on the existence of two groups of nrDNA ETS ribotypes in the genus where some of the taxa possessed either one of two types or both types (Oberprieler et al. 2014).

In this work, data obtained from 454 sequencing of nine low-copy nuclear markers along with traditionally sequenced five chloroplast markers and AFLP is provided for each currently recognized diploid taxon in the genus. Phylogenetic relationships within the subtribe are established as species tree and species network with special emphasis on diploids from the genus *Leucanthemum*. A modification of supertree method producing "supernetwork" is proposed. It is based on Matrix Representation with Parsimony (MRP) (Johnson et al. 2012 and references therein) and similar to the supertree method all trees are summarized into one matrix from which a network is constructed. Since *Leucanthemum* seems to be a good model for studying homoploid hybrid speciation existence of such hybrids is analyzed. Because no universal method which could handle obtained data is available a new method is proposed. Its core is located within method of Yu et al. (2012) which infers probability of parental contribution to specified hybrid taxon. Particular species receive their specific hybrid index which is then compared to the hybrid indexes obtained from the simulated gene trees influenced only by ILS. This provides an opportunity for statistical testing whether incongruence occurring among real gene trees can be explained solely by ILS. If it is not, other processes as hybridization must be invoked.

2.3. Material and methods

Sampling – In this study 39 specimens of diploid *Leucanthemum* species comprising 19 taxa were used (**Table 2, Figure 1**). All taxa were sampled with two accessions except of *L. rotundifolium* (3 accessions), *L. vulgare* (3 accessions) and *L. ligusticum* (1 accession) (as OTUs species and subspecies rank is used at an equal level). Only clearly determined specimens from two distinct populations were sampled. Furthermore, 10 species representing genera classified within or closely related to subtribe *Leucanthemineae* were added. As a more remote outgroup to all of them, *Artemisia vulgaris* was used. Preferentially material stored within silica gel was utilized but if it was not available herbarium specimens were used instead. The CTAB DNA extraction protocol

followed Doyle & Doyle (1987) with some minor modifications. All DNA extracts were diluted 10- or 100-fold prior to PCR reactions.

Species	Sample shortname	Internal sample name	Collection site	Coordinates	Collector	Herbarium	Voucher
Leucanthemum burnatii Briq. & Cavill.	bur1	90-6	FR, Provence-Alpes-Côte d'Azur, Grasse, 1235 m	43.76 N, 06.92 E	Vogt 16615, Oberprieler 10566 & Konowalik	B, VOGT	B 10 0464678
Leucanthemum burnatii Briq. & Cavill.	bur2	92-1	FR, Provence-Alpes-Côte d'Azur, Mgne Ste-Victoire, 650-750 m	43.55 N, 05.66 E	Vogt 16618, Oberprieler 10569 & Konowalik	B, REG	B 10 0464676
Leucanthemum cf. monspeliense (L.) H. J. Coste	mop1	131-20	FR, Languedoc-Roussillon, St.-André-de-Valborgne, 380 m	44.14 N, 03.73 E	Vogt 16716, Oberprieler 10671 & Konowalik	B, REG	B 10 0464615
Leucanthemum cf. monspeliense (L.) H. J. Coste	mop2	128-1	FR, Languedoc-Roussillon, l'Espérou, 750 m	44.09 N, 03.58 E	Vogt 16712, Oberprieler 10667 & Konowalik	B, REG	B 10 0464618
Leucanthemum gaudinii subsp. *barrelieri* (Dufour ex DC.) Vogt	gab1	L035	ES, Catalunya, Punta Brulle, 2350-2500m	42.58 N, 01.00 E	Vogt 5125 & Prem	VOGT	B 10 0216900
Leucanthemum gaudinii subsp. *barrelieri* (Dufour ex DC.) Vogt	gab2	266-1	ES, Aragon, Balneario de Panticosa, 2150 m	42.78 N, 00.23 W	Tomasello TS382	B, REG	-
Leucanthemum gaudinii subsp. *cantabricum* (Font Quer & Guinea) Vogt	gac1	L036	ES, Cantabria, Pozas de Lloroza, 1830 m	43.13 N, 04.75 W	Bayón 2132, Izuzquiza & Villanueva	VOGT	B 10 0420752
Leucanthemum gaudinii subsp. *cantabricum* (Font Quer & Guinea) Vogt	gac2	60-1	ES, Galicia, Piornedo, 1530 m	42.83 N, 06.86 W	Hößl 60	B	B 10 0413746
Leucanthemum gaudinii subsp. *gaudinii* Dalla Torre	gag1	L033	SK, Prešovský kraj, Siroké sedlo, 1700 m	49.25 N, 20.23 E	Knoph & Schrüfer s.n.	B, VOGT	B 10 0216898
Leucanthemum gaudinii subsp. *gaudinii* Dalla Torre	gag2	276-1	AT, Kärnten, Falkert, 2270 m	46.86 N, 13.82 E	Oberprieler 10866	B	B 10 0413015
Leucanthemum gallaecicum Rodr. Oubiña & Ortiz	gal1	159-11	ES, Galicia, Sierra de Basadre, 375 m	42.85 N, 07.99 W	Konowalik, Rodríguez Oubiña & Ortiz s.n.	B	B 10 0386789
Leucanthemum gallaecicum Rodr. Oubiña & Ortiz	gal2	L985	ES, Galicia, Paradela, 672 m	42.98 N, 07.92 W	Rodríguez Oubiña s.n.	-	-
Leucanthemum gracilicaule (Dufour) Pau	gra1	84-6	ES, Valencia, Benirrama, 296 m	38.84 N, 00.19 W	Konowalik KK20 & Ogrodowczyk	B, REG	B 10 0386704
Leucanthemum gracilicaule (Dufour) Pau	gra2	85-1	ES, Valencia, Altury, 337 m	39.31 N, 00.68 W	Konowalik KK25 & Ogrodowczyk	B, REG	B 10 0386702
Leucanthemum graminifolium (L.) Lam.	grm1	116-4	FR, Languedoc-Roussillon, Roqueredonde, 802 m	43.78 N, 03.24 E	Vogt 16693, Oberprieler 10648 & Konowalik	B, VOGT	B 10 0464684
Leucanthemum graminifolium (L.) Lam.	grm2	96-3	FR, Languedoc-Roussillon, Roc de L´Aigle, 560-600 m	43.15 N, 02.63 E	Vogt 16656, Oberprieler 10607 & Konowalik	B, VOGT	B 10 0464663
Leucanthemum halleri (Vitman) Ducommun	hal1	L1002	AT, Tirol, Tannheim, 1840 m	47.51 N, 10.60 E	Vogt 16874	B, VOGT	B 10 0420901
Leucanthemum halleri (Vitman) Ducommun	hal2	208-1	CH, Valais, Sion, 2320 m	46.33 N, 07.29 E	Tomasello TS65	B	B 10 0386672
Leucanthemum laciniatum Huter, Porta & Rigo	lai1	L179	IT, Basilicata, Castrovllari, 1900-2100 m	39.91 N, 16.19 E	Vogt 15614	VOGT	B 10 0420805
Leucanthemum laciniatum Huter, Porta & Rigo	lai2	280-1	IT, Calabria, Campo Tenese, 1481 m	39.90 N, 16.11 E	Tomasello TS420	B, REG	B 059-05-12-20
Leucanthemum ligusticum Marchetti, Bernardello, Melai & Peruzzi	lig1	258-1	IT, Liguria, Rochetta di Vara, 228 m	44.25 N, 09.76 E	Vogt 16944 & Oberprieler 10851	B	B 10 0420782
Leucanthemum lithopolitanicum (E. Mayer) Polatschek	lit1	L998	SI, Kamnik, Kamniška Bistrica, 1880-2120 m	46.35 N, 14.61 E	Hörandl, Hadaček, M. & jun.	W	W 1999-3533
Leucanthemum lithopolitanicum (E. Mayer) Polatschek	lit2	274-1	AT, Kärnten, Lesnik, 1999 m	46.38 N, 14.57 E	Oberprieler 10864	B	B 10 0413013
Leucanthemum pluriflorum Pau	plu1	40-6	ES, Galicia, Cabo Fisterra, 100 m	42.88 N, 09.27 W	Hößl 40	B	B 10 0413758
Leucanthemum pluriflorum Pau	plu2	55-1	ES, Galicia, Cangas de Foz, 10 m	43.63 N, 07.33 W	Hößl 55	B	B 10 0413749
Leucanthemum rotundifolium (Willd.) DC.	rot1	L990	RO, Prahova, Busteni, 1000-1500 m	45.42 N, 25.51 E	Hörandl 9063, Hadacek & Costea	W	W 1999-05366
Leucanthemum rotundifolium (Willd.) DC.	rot2	L989	BA, Fojnica, Paljike, 1800 m	43.95 N, 17.75 E	Horvat s.n.	ZA	-
Leucanthemum rotundifolium (Willd.) DC.	rot3	L992	PL, Podkarpackie, Zakopane, 1290 m	49.26 N, 19.92 E	Jasiewicz & Piękoś s.n.	W	W 1970-12192
Leucanthemum tridactylites (A. Kern. & Huter) Huter, Porta & Rigo	tri1	L151	IT, Abruzzo, Passo di San Leonardo, 1500-1800 m	42.08 N, 14.03 E	Vogt 14050 & Oberprieler 8355	VOGT	B 10 0420849
Leucanthemum tridactylites (A. Kern. & Huter) Huter, Porta & Rigo	tri2	278-1	IT, Abruzzo, Pennapiedimonte, 2065 m	42.14 N, 14.11 E	Tomasello TS417	B, REG	B 059-03-12-20
Leucanthemum virgatum (Desr.) Clos	vir1	L987	FR, Alpes Maritimes, Vésubie, 1013 m	43.98 N, 07.27 E	Saatkamp s.n.	-	-
Leucanthemum virgatum (Desr.) Clos	vir2	250-1	IT, Liguria, Pogli to Onzo, 215 m	44.06 N, 08.06 E	Vogt 16932 & Oberprieler 10839	B, VOGT	B 10 0350169
Leucanthemum vulgare subsp. *eliasii* (Sennen & Pau) Sennen & Pau	vel1	L996	ES, Burgos, San Pantaleón del Páramo, 973 m	42.56 N, 03.80 W	Cela 1433 & Lopez	VOGT	B 10 0420857
Leucanthemum vulgare subsp. *eliasii* (Sennen & Pau) Sennen & Pau	vel2	L162	ES, Burgos, Ubierna, 887 m	42.50 N, 03.70 W	Cela 465PG & Martin	VOGT	B 10 0420851
Leucanthemum vulgare subsp. *vulgare* (Vaill.) Lam.	vul1	94-1	FR, Languedoc-Roussillon, Montlaur, 160 m	43.13 N, 02.61 E	Vogt 16641, Oberprieler 10592 & Konowalik	B	B 10 0464674
Leucanthemum vulgare subsp. *vulgare* (Vaill.) Lam.	vul2	L046	DE, Bayern, Pittmannsdorf, 450 m	49.03 N, 11.88 E	Eder & Oberprieler s.n.	REG	-
Leucanthemum vulgare subsp. *vulgare* (Vaill.) Lam.	vul3	184-1	BA, Gacko, Ribari, 930 m	43.24 N, 18.34 E	Vogt 16806 & Prem-Vogt	B	B 10 0346626
Leucanthemum vulgare subsp. *pujiulae* Sennen	vup1	135-7	FR, Pyrénées-Orientales, La Vallée Heureuse, 410 m	42.50 N, 02.96 E	Konowalik KK42 & Ogrodowczyk	B, REG	B 10 0386712
Leucanthemum vulgare subsp. *pujiulae* Sennen	vup2	M60-1	ES, Castilla-La Mancha,, Salinas de Manzano, 1157 m	40.10 N, 01.52 W	Cordel s.n.	B	B 10 0345012
Chrysanthoglossum deserticola (Murb.) Wilcox, Bremer & Humphries	chd1	A791	TN, Tunisie du Sud, Tataouine, 450 m	32.50 N, 10.27 E	Vogt 13038 & Oberprieler 7343	REG	-
Chlamydophora tridentata (Del.) Less.	cht1	A795	CY, Larnaka, Meneou, 2 m	34.85 N, 33.61 E	Vogt 8120	VOGT	-
Coleostephus myconis (L.) Rchb.f.	com1	A792	IT, Calabria, Gambarrie, 551 m	38.17 N, 15.77 E	Vogt 13976 & Oberprieler 8281	VOGT	-
Glossopappus macrotus subsp. *hesperius* (Maire) Maire	glm1	A790	MA, Middle Atlas, Ifrane, 1090 m	33.80 N, 04.99 W	Vogt 12028	VOGT	-
Heteromera fuscata (Desf.) Pomel	hef1	A796	TN, Tozeur, Kariz, 65 m	34.05 N, 08.24 E	Vogt 16585, Oberprieler 10528 & Gstöttl	VOGT	B 10 0216212
Mauranthemum paludosum subsp. *ebusitanum* (Poir.) Vogt & Oberpr.	mae1	A799	ES, Ibiza, Atalaria de Sant Josep, 163 m	38.92 N, 01.26 E	Vogt	VOGT	-
Mauranthemum paludosum subsp. *paludosum* (Poir.) Vogt & Oberpr.	map1	A798	ES, Valencia, Xàbia, 210 m	38.80 N, 00.16 E	Konowalik KK9 & Ogrodowczyk	B, KONOWALIK	-
Plagius flosculosus (L.) Alavi & Heyw.	plf1	A793	IT, Sardinia, Sassari, 304 m	40.56 N, 08.66 E	Zedda s.n.	REG	-
Plagius maghrebinus Vogt & Greuter	plm1	A794	TN, Jendoube, Ain Draham, 950 m	36.79 N, 08.68 W	Vogt 13696 & Oberprieler 8001	VOGT	-
Rhodanthemum catananche (Ball) Wilcox, Bremer & Humphries	rhc1	A087	MA, Tadila-Azilal, Tizi N'Tichka, 2145 m	31.29 N, 07.38 W	Vogt 10332	VOGT	-
Artemisia vulgaris L.	Avul1	A838	DE, Bavaria, Regensburg, 335 m	49.02 N, 12.10 E	Konowalik s.n.	KONOWALIK	-

Table 2 - Taxa and accessions used in present study. Popuations ID's are specified, followed by internal sample number, collection site, geographical coordinates, collector, herbarium and voucher number. Herbarium names are according to Index Herbariorum except VOGT which denotes private collection of Robert Vogt and KONOWALIK which denotes private collection of Kamil Konowalik (since 2013 KONOWALIK became part of WRSL herbarium).

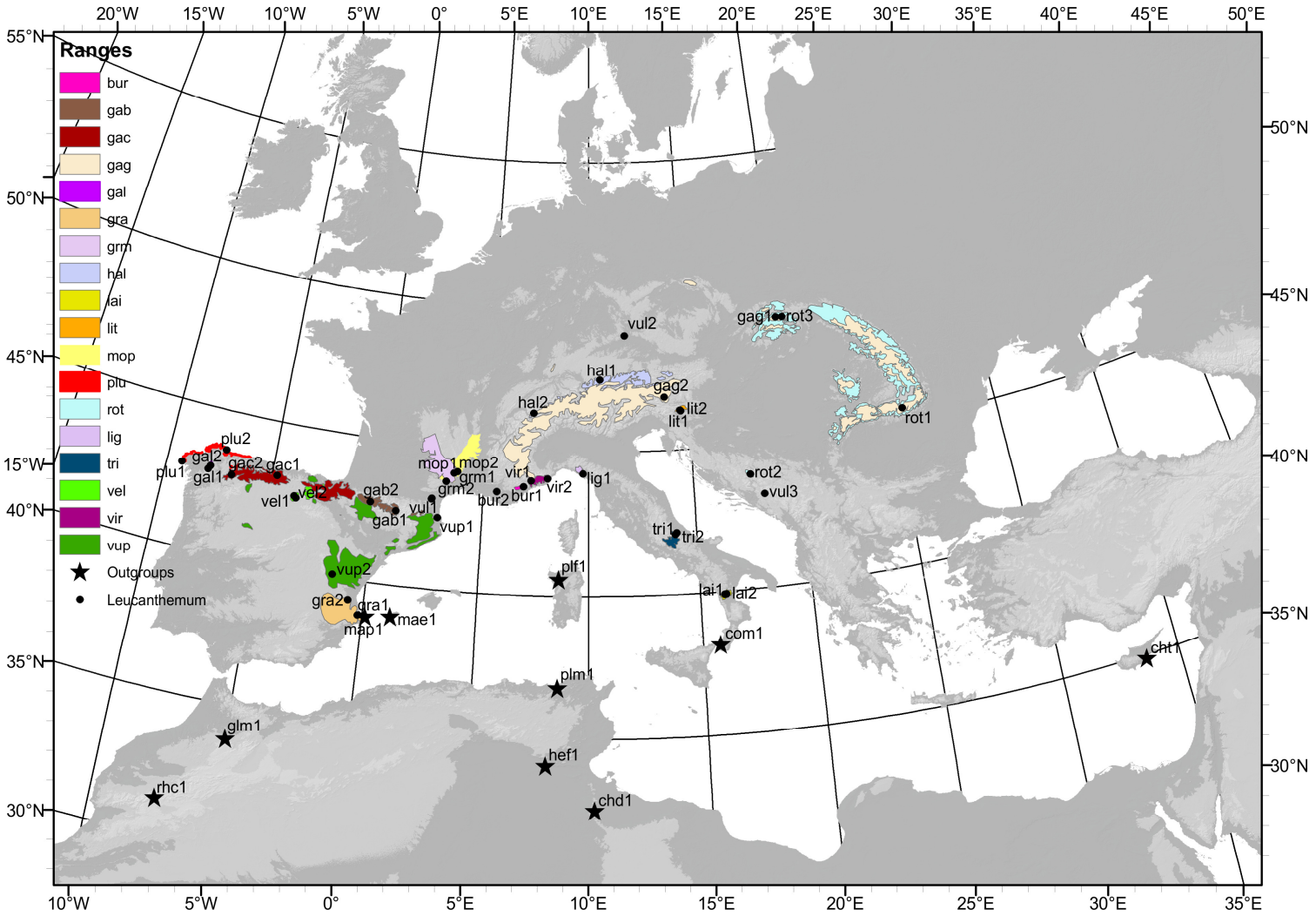

Figure 1 - Map showing sampling localities of all individuals used in this study together with ranges of all diploid *Leucanthemum* species. *L. vulgare* range is not shown since this species is common throughout whole Europe. More detailed information is available in Table 2.

Marker amplification – From universal single copy genes proposed for Compositae by Chapman et al. (2007), 67 which amplified in *Artemisia vulgaris* L. with single band were selected for screening. From them, 9 most variable and easily amplifiable were selected for this study. In order to obtain PCR products suitable for 454 sequencing the procedure consisted of two PCR steps. In the first PCR step, the target region was amplified using primers of 9 previously selected low copy nuclear genes (**Table 3**) (Chapman et al. 2007). Original primer sequences were modified by adding a M13 tail to the forward primer (5'-CACGACGTTGTAAAACGAC-original primer-3') and Titanium B sequencing primer motive to the reverse primer (5'-CTATGCGCCTTGCCAGCCCGCTCAG-primer-3'). For amplification following reagents were used in 10 µl volume: 2 µl KAPAHiFi Fidelity Buffer (5x), 0.3 µl dNTPs (10 mM each), 0.3 µl forward primer (10 mM), 0.3 µl reverse primer (10 mM), 0.1 µl KAPAHiFi DNA Polymerase (1 u/µl) (Peqlab, Germany) and 1.5 µl template DNA. The PCR was conducted on a thermal cycler (Mastercycler personal 5332, Eppendorf, Germany) and consisted of the following steps: 95ºC for 5 min; 5 touchdown cycles of 98º for 20 sec, 66ºC for 30 sec (-1ºC/cycle), 72º for 30 sec; 35 amplification cycles of 98º for 20 sec, 60º for 30 sec, 72º for 30 sec; 72º for 10 min and 8º for 5 min. In rare cases when amplification was unsuccessful, the PCR reaction was repeated and when the repeat failed it was modified using a combination of longer time for annealing and/or elongation, more cycles, lower annealing temperature or different DNA dilutions. Samples were purified with Agencourt Ampure magnetic beads solution (Agencourt Bioscience, The Netherlands) using a 1.5 ratio of beads (relatively to volume of PCR product) and dissolved in water to 15 µl. The second PCR was used to tag each sample with its accession-specific barcode. As forward primer, the Titanium A adaptor followed by a 4-letter-barcode and a M13-sequence was used (5'-CGTATCGCCTCCCTCGCGCCATCAG-barcode-M13-3') and as reverse primer Titanium B (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3') was used. The PCR mix was prepared in 15 µl and contained: 1.5 µl complete buffer (10x), 0.3 µl dNTPs (10 mM each), 0.3 µl forward primer (10 mM), 0.3 µl reverse primer (10 mM), 0.45 µl *Pwo* DNA Polymerase (1 u/µl) (Peqlab, Germany) and 0.5 µl of purified product from first PCR. The program was conducted on thermal cycler (Mastercycler ep gradient 5345, Eppendorf, Germany) and consisted of following steps: 95ºC for 3 min; 20 amplification cycles 95º for 20 sec, 68º for 60 sec; 72º for 5 min and 8º for 5 min. PCR products were purified with Agencourt Ampure and resuspended in 10 µl of water. Each sample was measured for DNA concentration in a Qubit 2.0 fluorometer (Life Sciences, USA) using the double stranded DNA high sensitivity assay kit. The length of each

fragment was then estimated on photographed agarose gels using GelAnalyzer2010a (Lazar Software, Hungary). After accomplishing all steps, the samples were multiplexed into a single mixture. In order to ensure equimolar mixing during final pipetting, the amount to pipette was calculated as follows: actual sample concentration was divided by mean concentration of all samples and then multiplied by length of sample divided by mean length of all samples. The mixed amplicons were sent to FLX 454 Genome Sequencer (Microsynth, Switzerland). The project was run in two parts on 1/16 plate and on 1/8 plate together with other projects. The chosen size of the plate was dependent on the number of species and desired coverage. Especially one of the goals behind choosing 454 sequencing was the possibility of recovering all alleles and variants present as it is very important to sample all of them for the sound phylogenetic reconstruction. To ensure that all alleles (for a diploid maximum 2 alleles were assumed) of a particular accession were sampled, minimum coverage to recover at least 10 reads of each allele with 0.99 probability was calculated. The formula used consisted of summarizing binominal distributions: $P = 1 - \sum_{k=0}^{10} P(k,n)$ where $P(k,n) = \left(\frac{n!}{k!(n-k)!} \right) \cdot p^k \cdot q^{n-k}$ and n is the number of all reads, k is the number of reads of one allele. According to the calculation for a diploid species minimum 33 reads of one marker are needed to recover each allele with at least 10 reads ($p>0.99$).

In case of one species and one marker (*L. virgatum* – C33), the PCR product exceeded the desirable length for next generation sequencing (by ca. 250 bp) and therefore it was cloned using NEB Turbo bacteria and pJet cloning kit (Fermentas, USA). 8 clones were sequenced using Sanger technique to ensure a 0.95 probability of sampling all alleles (Joly et al. 2006).

nrDNA primers	Sequence	Source
M13_A39_Leu350bp_f	CACGACGTTGTAAAACGACAATGGTGTTTCAATTGGTTTTC	this study, based on Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_A39_Leu350bp_r	CTATGCGCCTTGCCAGCCCGCTCAGCCAACTCCAACAAGTAGGAG	this study, based on Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_B12_f	CACGACGTTGTAAAACGACCAAGTGGCTGCAGCCATGGG	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_B12_Leu350bp_r	CTATGCGCCTTGCCAGCCCGCTCAGACGTAGTAGTTGATCAACTG	this study, based on Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_B20_f	CACGACGTTGTAAAACGACAGTGGWATYAGTGGKGCTAGTTACT	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_B20_r	CTATGCGCCTTGCCAGCCCGCTCAGCCACCACGHACAAGMAGCCAAAG	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_C12_f	CACGACGTTGTAAAACGACTCTTGCACCACCAACTGYTTGGC	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_C12_Leu350bp_r	CTATGCGCCTTGCCAGCCCGCTCAGGGGACAATGTTCAATGCTG	this study, based on Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_C20_f	CACGACGTTGTAAAACGACTTCTTCAATGCKKCTGCTTCTCA	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_C20_r	CTATGCGCCTTGCCAGCCCGCTCAGAGCCAGTTGAATGAYAGCTCA	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_C33_Leu350bp_f	CACGACGTTGTAAAACGACTCTACATCCAAAATACTACT	this study, based on Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_C33_Leu350bp_r	CTATGCGCCTTGCCAGCCCGCTCAGATTCCTGTTGACACATAAAC	this study, based on Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_D18_f	CACGACGTTGTAAAACGACGGAAGRCTHCTWAGATATGACCCWCC	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_D18_r	CTATGCGCCTTGCCAGCCCGCTCAGCTGCAACAATCAATWGCHACCCAA	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_D23_f	CACGACGTTGTAAAACGACAGAAGGGTGGAACAGARCATTTRGGGCT	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_D23_r	CTATGCGCCTTGCCAGCCCGCTCAGGGCATRATYCCRATCTTGCATTCWCCAGG	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
M13_D27_f	CACGACGTTGTAAAACGACATGATYAGTGAAAAGGAGCTYCT	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.
TitB_D27_r	CTATGCGCCTTGCCAGCCCGCTCAGGGWACAAAATGAGCMGTYACVACAGC	Chapman et al. (2007). TAG Theoretical and Applied Genetics 115, 747-755.

cpDNA primers	Sequence	Source
psbA	GTTATGCATGAACGTAATGCTC	Sang (1997). Am. J. Bot. 84, 1120-1137
trnHr	CGCGCATGGTGGATTCACAAATC	Sang (1997). Am. J. Bot. 84, 1120-1137
trnL2(e)	ATTTGAACTGGTGACACGAG	Taberlet (1991). Plant Molecular Biology 17, 1105-1109
trnFr(f)	ATTTGAACTGGTGACACGAG	Taberlet (1991). Plant Molecular Biology 17, 1105-1109
trnC	CCAGTTCAAATCTGGGTGTC	Demesure (1995). Mol. Ecol. 4: 129-131
petN1R	CCCAAGCAAGACTTACTATATCC	Lee (2004). Molecular Phylogenetics and Evolution 31, 894-903
petN1	GGATATAGTAAGTCTTGCTTGGG	Lee (2004). Molecular Phylogenetics and Evolution 31, 894-903
psbM2R	TTCTTGCATTTATTGCTACTGC	Lee (2004). Molecular Phylogenetics and Evolution 31, 894-903
trnQ2	GCGTGGCCAAGYGGTAAGGC	Shaw (2007). Am. J. Bot. 94, 275-288
rps16x1_leu	CAATCGAATTGTCAATGATGC	this study, based on Shaw (2007). Am. J. Bot. 94, 275-288

Barcodes	Sequence	Source
TitA_AAAG_M13	CGTATCGCCTCCCTCGCGCCATCAGAAAGCACGACGTTGTAAAACGAC	this study
TitA_AATG_M13	CGTATCGCCTCCCTCGCGCCATCAGAATGCACGACGTTGTAAAACGAC	this study
TitA_AACG_M13	CGTATCGCCTCCCTCGCGCCATCAGAACGCACGACGTTGTAAAACGAC	this study
TitA_AAGG_M13	CGTATCGCCTCCCTCGCGCCATCAGAAGGCACGACGTTGTAAAACGAC	this study
TitA_ATAG_M13	CGTATCGCCTCCCTCGCGCCATCAGATAGCACGACGTTGTAAAACGAC	this study
TitA_ATTG_M13	CGTATCGCCTCCCTCGCGCCATCAGATTGCACGACGTTGTAAAACGAC	this study
TitA_ATCG_M13	CGTATCGCCTCCCTCGCGCCATCAGATCGCACGACGTTGTAAAACGAC	this study
TitA_ATGG_M13	CGTATCGCCTCCCTCGCGCCATCAGATGGCACGACGTTGTAAAACGAC	this study
TitA_ACAG_M13	CGTATCGCCTCCCTCGCGCCATCAGACAGCACGACGTTGTAAAACGAC	this study
TitA_ACTG_M13	CGTATCGCCTCCCTCGCGCCATCAGACTGCACGACGTTGTAAAACGAC	this study
TitA_ACCG_M13	CGTATCGCCTCCCTCGCGCCATCAGACCGCACGACGTTGTAAAACGAC	this study
TitA_ACGG_M13	CGTATCGCCTCCCTCGCGCCATCAGACGGCACGACGTTGTAAAACGAC	this study
TitA_AGAG_M13	CGTATCGCCTCCCTCGCGCCATCAGAGAGCACGACGTTGTAAAACGAC	this study
TitA_AGTG_M13	CGTATCGCCTCCCTCGCGCCATCAGAGTGCACGACGTTGTAAAACGAC	this study
TitA_AGCG_M13	CGTATCGCCTCCCTCGCGCCATCAGAGCGCACGACGTTGTAAAACGAC	this study
TitA_AGGG_M13	CGTATCGCCTCCCTCGCGCCATCAGAGGGCACGACGTTGTAAAACGAC	this study
TitA_TAAG_M13	CGTATCGCCTCCCTCGCGCCATCAGTAAGCACGACGTTGTAAAACGAC	this study
TitA_TATG_M13	CGTATCGCCTCCCTCGCGCCATCAGTATGCACGACGTTGTAAAACGAC	this study
TitA_ATTC_M13	CGTATCGCCTCCCTCGCGCCATCAGATTCCACGACGTTGTAAAACGAC	this study
TitA_ATTT_M13	CGTATCGCCTCCCTCGCGCCATCAGATTTCACGACGTTGTAAAACGAC	this study
TitA_AACA_M13	CGTATCGCCTCCCTCGCGCCATCAGAACACACGACGTTGTAAAACGAC	this study
TitA_AAGA_M13	CGTATCGCCTCCCTCGCGCCATCAGAAGACACGACGTTGTAAAACGAC	this study
TitA_ATAT_M13	CGTATCGCCTCCCTCGCGCCATCAGATATCACGACGTTGTAAAACGAC	this study
TitA_ATTA_M13	CGTATCGCCTCCCTCGCGCCATCAGATTACACGACGTTGTAAAACGAC	this study
TitA_ATCA_M13	CGTATCGCCTCCCTCGCGCCATCAGATCACACGACGTTGTAAAACGAC	this study
TitA_ATGT_M13	CGTATCGCCTCCCTCGCGCCATCAGATGTCACGACGTTGTAAAACGAC	this study
TitA_ACAA_M13	CGTATCGCCTCCCTCGCGCCATCAGACAACACGACGTTGTAAAACGAC	this study
TitA_ACTA_M13	CGTATCGCCTCCCTCGCGCCATCAGACTACACGACGTTGTAAAACGAC	this study
TitA_ACCT_M13	CGTATCGCCTCCCTCGCGCCATCAGACCTCACGACGTTGTAAAACGAC	this study
TitA_ACGC_M13	CGTATCGCCTCCCTCGCGCCATCAGACGCCACGACGTTGTAAAACGAC	this study
TitA_AGAA_M13	CGTATCGCCTCCCTCGCGCCATCAGAGAACACGACGTTGTAAAACGAC	this study
TitA_AGTT_M13	CGTATCGCCTCCCTCGCGCCATCAGAGTTCACGACGTTGTAAAACGAC	this study
TitA_AGCC_M13	CGTATCGCCTCCCTCGCGCCATCAGAGCCCACGACGTTGTAAAACGAC	this study
TitA_AGGA_M13	CGTATCGCCTCCCTCGCGCCATCAGAGGACACGACGTTGTAAAACGAC	this study
TitA_TAAT_M13	CGTATCGCCTCCCTCGCGCCATCAGTAATCACGACGTTGTAAAACGAC	this study
TitA_TATA_M13	CGTATCGCCTCCCTCGCGCCATCAGTATACACGACGTTGTAAAACGAC	this study
TitA_TACG_M13	CGTATCGCCTCCCTCGCGCCATCAGTACGCACGACGTTGTAAAACGAC	this study
TitA_TAGG_M13	CGTATCGCCTCCCTCGCGCCATCAGTAGGCACGACGTTGTAAAACGAC	this study
TitA_TTAG_M13	CGTATCGCCTCCCTCGCGCCATCAGTTAGCACGACGTTGTAAAACGAC	this study
TitA_TTTG_M13	CGTATCGCCTCCCTCGCGCCATCAGTTTGCACGACGTTGTAAAACGAC	this study
TitA_TTCG_M13	CGTATCGCCTCCCTCGCGCCATCAGTTCGCACGACGTTGTAAAACGAC	this study
TitA_CCAG_M13	CGTATCGCCTCCCTCGCGCCATCAGCCAGCACGACGTTGTAAAACGAC	this study

***as reverse primer for all barcodes sequence of TitB adapter was used: CTATGCGCCTTGCCAGCCCGCTCAG**

Table 3 - Primers used in this study. The list contains primer name, sequence and citation of article where it was originally published.

454 sequncing – After retrievement of reads they were assigned to the species and marker using R (R Development Core Team 2008) and the Galaxy webportal (Giardine et al. 2005, Goecks et al. 2010) as described previously (Griffin et al. 2011). The barcode, M13 tail, forward and reverse primer sequences were removed using tools available in Galaxy webportal (Blankenberg et al. 2010). After removing primer sequences the quality of reads was assessed and they were filtered according to the following rule: if the phred quality score was equal or below 20 in more than 20% of the bases the read was discarded. After filtering, each marker in particular species was analyzed separately. To identify alleles, reads were aligned in mafft 6.833b (Katoh et al. 2002, Katoh and Toh 2008) and then analyzed in BAPS 5.2 using Bayesian clustering with linked loci (Corrander et al.

2006, Corrander et al. 2008, Cheng et al. 2011). The groups found by the program were considered as alleles and according to those results reads were manually grouped in BioEdit (Hall 1999). Additionally, alignment was inspected visually and if there was a variant recorded in more than 20% of reads it was retained as an additional allele. In some cases the BAPS analyses were unreliable in predicting the number of alleles (e.g. all sequences were regarded as one allele) – in that case the 20% rule was also applied to record all variants. BAPS was also used to aid discovering recombinants using "admixture based on mixture clustering" function on previously obtained groups (Corander and Marttinen 2006). Reads found by the program as a mixture between different clusters were deleted. Additionally to that, all reads which possessed 3'-end typical to the sequence of one allele and the 5'-end typical to a different allele were discarded as recombinants. Reads of one allele were collapsed to one consensus sequence and kept for further analyses. In analogy to the previous step if there was a variation within an allele present in more than 20% of the reads it was recorded as an UPAC base pair. The alignment of consensus sequences containing all alleles was done in mafft (Katoh et al. 2002, Katoh and Toh 2008) and inspected visually.

cpDNA markers – For chloroplast DNA markers (**Table 3**) the PCR reaction was carried with *Taq* RED Mix (Biomol, Germany) in a 12.5 µl reaction mix following manufacturers' protocol. For cycle sequencing the CEQ DTCS Quick Start Kit (Beckman-Coulter, Germany) was used after purification of PCR products. In case when sequence was not readable because of the length or poly(A) repeat, reverse primer was used. Electropherograms were manually checked for base call errors by the use of Chromas Lite version 2.0 (Technelysium, Australia). The alignment of sequences was done in mafft (Katoh et al. 2002, Katoh and Toh 2008) and inspected visually. After that all chloroplast markers were conceantated into one locus and from that point analyzed jointly.

Gene trees construction – Gapcoder (Young and Healy 2003) was used to code indels. For each gene, a Bayesian phylogenetic analysis was performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). For the nucleotide part, model from the best selection according to AIC implemented in jModelTest 0.1.1 (Posada 2008) (**Table 4**) was used. For the binary coded gaps, a Jukes-Cantor model (Jukes and Cantor 1969) was used. 15 000 000 generations were performed in two runs discarding the first 25% as the burnin fraction and sampling every 1000^{th} tree. Convergence of runs and Effective Sample Size (ESS) were checked in Tracer 1.5 (Rambaut and Drummond 2007). Majority-rule consensus trees from Bayesian analyses were collapsed to retain only nodes with support of at least 0.95 posterior probability.

marker	model	freqA	freqC	freqG	freqT	R(a) [AC]	(b) [AG]	R(c) [AT]	R(d) [CG]	R(e) [CT]	R(f) [GT]	n° states	rates	gamma shape	gamma categories	pin variation	kappa	ti/tv
A39	TVM+G	0.2897	0.1935	0.1473	0.3695	1.5344	2.8693	0.4444	1.8014	2.8693	1.0000	6	gamma	0.4540	4	0	-	-
B12	TPM2uf+G	0.3312	0.1839	0.1409	0.3439	0.7058	2.7471	0.7058	1.0000	2.7471	1.0000	6	gamma	0.3760	4	0	-	-
B20	HKY+G	0.2796	0.1765	0.2163	0.3276	-	-	-	-	-	-	2	gamma	0.5270	4	0	-	-
C12	TIM2+G	0.2987	0.1996	0.2022	0.2996	0.6281	1.7108	0.6281	1.0000	2.7081	1.0000	6	gamma	0.6210	4	0	-	-
C20	TPM3uf+G	0.3142	0.1399	0.1743	0.3716	1.6954	2.9112	1.0000	1.6954	2.9112	1.0000	6	gamma	1.5540	4	0	-	-
C33	TPM1uf+G	0.3162	0.1636	0.1888	0.3314	1.0000	2.3486	0.7340	0.7340	2.3486	1.0000	6	gamma	5.0170	4	0	-	-
D18	HKY+G	0.3143	0.1910	0.2031	0.2916	-	-	-	-	-	-	2	gamma	0.3410	4	0	3.0022	1.4372
D23	TPM1uf+G	0.2525	0.2010	0.1913	0.3553	1.0000	4.5295	1.4412	1.4412	4.5295	1.0000	6	gamma	0.3320	4	0	-	-
D27	HKY+G	0.2200	0.1753	0.1904	0.4143	-	-	-	-	-	-	2	gamma	1.9730	4	0	2.1619	1.0231
cpDNA	GTR+G	0.3361	0.1466	0.1486	0.3686	1.1236	1.9585	0.3593	0.9501	0.7753	1.0000	6	gamma	0.2560	4	0	-	-

Table 4 - Model chosen for Bayesian inference. All parameters listed by jModelTest and necessary for model specification in MrBayes are listed.

Supernetwork – The algorithm used for constructing the network followed Matrix Representation with Parsimony (MRP) method and standard Baum-Ragan coding (Johnson et al. 2012 and references therein). The principle is very similar to the one proposed by Doyle (1992). Prior to analysis all 10 gene trees (with nodes having 0.95 posterior probability) were transformed into multilabeled trees manually. In that procedure all accessions and alleles of particular taxon got the same label which allowed treating them later as one entity. Afterwards trees were coded as 0/1 matrices and merged together using a script of Johnson et al. (2012). In the resulting consensus matrix only information on the species level persisted since all alleles and accessions were summarized. The matrix was visualized in SplitsTree 4.11.3 (Huson & Bryant 2006) using the NeighborNet algorithm and Jaccard distances which take into account just presence in the node (1) while absence (0) is not considered as similarity. Although some methods were proposed previously to deal with MUL-trees or constructing networks (e.g. Huber & Moulton 2006, Holland et al. 2008) they appliance turned out to be limited and not compatible with our data so as a result only supernetwork as described here was computed.

Species tree reconstruction – To construct a species tree PhyloNet 3.5.1 (Than et al. 2008, Than and Nakhleh 2009, Yu et al. 2011) was used. Minimizing Deep Coalescences (MDC) method was employed allowing the final tree to be completely resolved.

AFLP – The AFLP protocol followed the original description of Vos et al. (1995) with modifications described in Oberprieler et al. (2011) and Greiner et al. (2013). In the first step, MseI and EcoRI restriction enzymes were used together with T4 DNA ligase and adaptors compatible with either of the two restriction sites. Restriction-ligation was carried out at 37°C for 2 h, after which the ligase was heat-inactivated. Pre-selective amplification used primers with one and two selective nucleotides (A for the EcoRI primer and CT for the MseI primer) while selective amplification used primers with further two selective nucleotides (CTAG for the Mse I primer). Fluorescently labelled EcoRI primers EcoRI-ACC, EcoRI-AGG and EcoRI-ACA were used within three separate reactions for each sample. The PCR products were united, precipitated and subsequently dissolved in a mixture of GenomeLab Sample Loading Solution and CEQ Size Standard 400 (Beckman Coulter, Germany). The fragment detection was performed on a CEQ8000 capillary sequencer (Beckman Coulter, Germany). To quantify AFLP genotyping errors, replicates were generated for randomly selected samples (39 samples, 9 replicates), representing 23% of the dataset.

A 0/1-matrix was constructed through automatic band scoring using GelCompar II (Applied Maths, Belgium) and 324 parameter combinations comprising different values for

minimal profiling (0.0; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0), minimal area (0.1; 0.2; 0.3; 0.4; 0.5) and matching tolerance (0.02; 0.06; 0.10; 0.14; 0.20; 0.28). In order to choose the best combination, Euclidean error, Jaccard distance, correctly paired individuals and resolution score were calculated by script developed by Holland et al. (2008). After calculation all results were standardized using z-transformation, brought to a positive number, multiplied by each other and subtracted from the combination with highest Euclidean error in order to allow comparison between them using a single value. Combination with highest score was chosen and used for band scoring for all individuals. Bands below 100 bp were discarded to omit high levels of homoplasy which could occur especially among short fragments.

To visualize the phenetic structure among individuals principal coordinate analysis (PCoA) was performed in MATLAB R2012b using Bray-Curtis coefficient. Network was constructed in Splits Tree 4.11.3 (Huson & Bryant 2006) using the NeighborNet algorithm and Nei-Li distances obtained from PAUP* 4.0b10 (Swofford 2003).

Bayesian clustering of populations was done with program Structure ver. 2.3.3 (Pritchard et al. 2000). Following the method described by Evanno et al. (2005) performed with Structure Harvester (Earl & von Holdt 2012), the inferred optimal number of clusters was 2. Allele frequencies were set to correlate and all individuals were assigned a diploid level. Burnin was set to 1 000 000 and chain length to 10 000 000. The analysis was simulated 10 times and the results were averaged between runs using clumpp 1.1.2 (Jakobsson & Rosenberg 2007). For visualization of the results program distruct 1.1 was used (Rosenberg 2004).

Detection of potential hybrids – In order to detect hybridization in the data set and identify taxa with higher probabilities of being of hybrid origin, a strategy based on the method described by Yu et al. (2012) for estimating the probability of a gene tree topology within a given phylogenetic network was used. In the first step of this method, a species network including reticulations is transferred into a multilabeled species tree (MUL-tree) by traversing the network from the leaves towards the root and substituting reticulation nodes by two copies of the subtree stemming from this node (child trees) positioned at the two parental branches. This is followed by the mapping of the alleles to the leaves of the MUL-tree in all possible combinations and computing the probability of the gene tree on the MUL-tree (Yu et al. 2012). In order to accomplish this calculation, two kinds of parameters are necessary besides the topology W of the species network and its derivate, the MUL-tree: (a) branch lengths for the species network (defined as a vector λ of branch lengths given in units of $2N$ generations where N is the effective population size of the branches concerned) are needed to describe the age of the hybridization events and the

ages of parental lineages and (b) for each hybridization event, a parameter γ (ranging from 0 to 1) needs to be given indicating for each allele in the hybrid population its probability of inheritance from each of the two parental populations.

A "Hybrid index" was calculated for each of the 19 *Leucanthemum* taxa as follows:

(1) Gene trees were pruned to contain only *Rhodanthemum* as an outgroup. While leaving the outgroup taxon unchanged, the 19 ingroup taxa were broken down to all 153 possible triplets that could be formed and 2907 triplet species networks were described in which each member of a triplet was considered being hybrid once and being a parental lineage in the other two cases.

(2) For each of the 2907 triplet species networks, 180 scenarios were formulated based on combinations of changes in the branch length vector λ (36 different values) and the hybridization parameter γ (5 different values). The total branch length vector λ was kept constant at 5. λ was constituted by a sum of three variables t_1, t_2 and t_3. The t_1 represents the time interval between split of the outgroup and two parental lineages, t_2 represents the time interval between formation of parental lineages and hybridization event and t_3 represents the time interval from the hybridization event to the present. Each of those three values was changed by 0.5 increments, in all possible combinations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4) which formed 36 scenarios. The length of λ was based on the equation $\lambda = \frac{t_g}{2 \cdot N_e}$, where *tg* is time in generations defined as $t_g = \frac{t_y}{3}$ where t_y is time in years since the split between outgroup and ingroup, and 3 is the assumed generation time in years for the short-lived perennials of *Leucanthemum* (personal observations). Calculations were based on a dated tree made from the chloroplast markers in *BEAST (Heled & Drummond 2010) where the split between *Rhodanthemum* and *Leucanthemum* equals $ty = 13.7 \cdot 10^6$ years. To calculate the average effective population size N_e first it was calculated for two species with available data using method described by Blanco-Pastor et al. (2012) and resulted in population sizes for *L. vulgare* (~995 000) and *L. pluriflorum* (~323 000). Based on those two values and distribution areals of all diploid species the regression analysis was made to calculate the mean N_e for *Leucanthemum* in general, which was found to be 465 692. Consequently the length of λ was $\frac{\left(\frac{13.7 \cdot 10^6}{3}\right)}{2 \cdot 465692} \approx 5$ in coalescent units. Additionally, the hybridization parameter γ was set to values of 0.0, 0.25, 0.5, 0.75, and 1.0, where the values of 0.0 and 1.0 indicate no hybridization (i.e., a tree scenario with either the one or the other parental lineage being the sister of the putative hybrid taxon) and the other three

scenarios translate into a network interpretation of the relationship among the three triplet accessions.

(3) From the 180 triplet species tree scenarios the one with the highest probability value was selected. Based on the gamma (γ) value the result indicated whether species was reconstructed as a hybrid or not. Index 0 meant no hybridization, index 0.5 indicated possible hybridization and index 1 indicated hybridization. The scenarios with gamma $\gamma = 0.25$ and $\gamma = 0.75$ resulted in hybrid index 0.5, with $\gamma = 0.5$ resulted in hybrid index 1 and with $\gamma = 0$ and $\gamma = 1$ resulted in hybrid index 0. Results within species were summarized and gave species specific "hybrid index".

(4) Hybrid index obtained from real data was compared to 19 hybrid indexes from simulated data in order to find whether it was significantly different from data influenced only by ILS. In total 190 gene trees were simulated using coalescence simulation available in Mesquite 2.75 (Maddison & Maddison 2011) to match real gene trees. Chloroplast gene tree used previously for population size estimation was treated as a species tree and therefore simulation assumed no influence of hybridization and only effect of ILS. 10 simulated gene trees were used to estimate species tree and then to calculate hybrid index as previously described. This step was repeated 19 times and allowed to assess statistical support pinpointing species with actual hybrid index higher than simulated hybrid indexes and thus indicating hybrid origin. Conversely, when actual hybrid index would be lower than simulated it could indicate that hybridization was not involved in formation of that taxon.

2.4. Results

454 Sequencing – Data retrieved from 454 Sequencing consisted from 31 857 reads. Reads were obtained for all submitted samples therefore giving us a dataset without any missing data. Equimolar mixing worked very well and reads within one species were proportional among all markers while higher variation was observed among different species or different markers (**Table 5**). In general shorter markers/sequences gave more reads than longer ones. Allele coverage was better than expected and on average 250% of the necessary reads per species were retained (range 131%-384%) in the raw data. Quality varied across markers and species with shorter sequences produced reads with better quality. Although primers were optimized in the beginning to give products of similar length, in such a study involving many diverse taxa and different markers it is unavoidable to have products with different sizes. After barcode assignment and quality control 25 038

(79%) of reads were kept (**Table 6**), which was further reduced to 22 974 (72% of the raw reads) by deleting potential recombinant sequences (**Table 7**). The average lengths are listed in tables 5, 6, and 7, while **Table 8** contains information about number of variable nucleotide positions.

					1	2	3	4	5	6	7	8	9	
N°	Outgroup	Code	Short	Population	A39	B12	B20	C12	C20	C33	D23	D27	D18	mean (SD)
1	*Chrysanthoglossum deserticola* (Murb.) Wilcox. & al.	ACAG	chd1	A791	49	67	50	59	53	39	46	31	103	55.2 (20.7)
2	*Chlamydophora tridentata* (Del.) Less.	ACTG	cht1	A795	52	69	69	74	76	23	42	63	81	61 (18.7)
3	*Coleostephus myconis* (L.) Rchb. f.	AATG	com1	A792	47	75	67	83	58	33	64	55	70	61.3 (15.1)
4	*Glossopappus macrotus* subsp. *hesperius* (Maire) Maire	ATGG	glm1	A790	38	58	37	67	54	63	39	45	43	49.3 (11.4)
5	*Heteromera fuscata* (Desf.) Pomel	AAAG	hef1	A796	28	36	204	43	0	32	25	45	56	33.1 (16.7)
6	*Mauranthemum paludosum* subsp. ebusitanum (Vogt) Vogt & Oberpr.	AAGG	mae1	A799	53	47	70	0	53	0	39	48	79	43.2 (27.3)
7	*Mauranthemum paludosum* subsp. *paludosum* (Poir.) Vogt & Oberpr.	AACG	map1	A798	54	73	62	0	55	17	45	51	66	47 (23.7)
8	*Plagius flosculosus* (L.) Alavi & Heyw.	ATCG	plf1	A793	55	67	77	81	59	57	63	58	18	59.4 (18)
9	*Plagius maghrebinus* Vogt & Greuter	ATTG	plm1	A794	61	1	68	80	53	5	47	60	34	45.4 (27.3)
10	*Rhodanthemum catananche* (Ball) Wilcox & al.	ATAG	rhc1	A087	49	38	82	71	51	73	67	63	66	62.2 (13.7)
11	*Leucanthemum burnatii*	AAAG	bur1	90-6	82	81	111	83	114	64	101	107	70	90.3 (18.4)
12	*Leucanthemum burnatii*	ACCG	bur2	92-1	58	67	43	57	58	47	66	61	75	59.1 (9.9)
13	*Leucanthemum cf. monspeliense*	AATG	mop1	131-20	60	106	134	86	85	77	108	114	116	98.4 (23)
14	*Leucanthemum cf. monspeliense*	ACGG	mop2	128-1	48	49	46	65	44	63	52	69	46	53.6 (9.5)
15	*Leucanthemum gallaecicum*	AACG	gal1	159-11	76	87	123	91	77	84	88	103	55	87.1 (18.8)
16	*Leucanthemum gallaecicum*	AGAG	gal2	L985	44	75	96	60	61	39	68	65	60	63.1 (16.7)
17	*Leucanthemum gaudinii* subsp. *barrelieri*	AAGG	gab1	L035	98	74	146	82	95	90	134	103	111	103.7 (23.5)
18	*Leucanthemum gaudinii* subsp. *barrelieri*	AGTG	gab2	266-1	44	71	82	57	31	64	52	70	55	58.4 (15.4)
19	*Leucanthemum gaudinii* subsp. *cantabricum*	ATAG	gac1	L036	106	102	139	164	89	75	125	112	87	111 (28)
20	*Leucanthemum gaudinii* subsp. *cantabricum*	AGCG	gac2	60-1	48	53	81	56	57	51	60	34	55	55 (12.3)
21	*Leucanthemum gaudinii* subsp. *gaudinii*	ATTG	gag1	L033	60	89	72	56	38	76	41	35	48	57.2 (18.7)
22	*Leucanthemum gaudinii* subsp. *gaudinii*	AGGG	gag2	276-1	47	75	64	66	46	43	59	65	68	59.2 (11.3)
23	*Leucanthemum gracilicaule*	ATCG	gra1	84-6	84	75	100	80	78	60	114	104	98	88.1 (17)
24	*Leucanthemum gracilicaule*	TATG	gra2	85-1	66	63	110	87	64	67	65	75	86	75.9 (15.8)
25	*Leucanthemum graminifolium*	ATGG	grm1	116-4	48	39	87	69	44	32	60	85	68	59.1 (19.7)
26	*Leucanthemum graminifolium*	ATTC	grm2	96-3	42	74	69	86	58	67	69	56	73	66 (12.6)
27	*Leucanthemum halleri*	ACAG	hal1	L1002	90	87	135	110	61	70	52	105	82	88 (25.9)
28	*Leucanthemum halleri*	ATTT	hal2	208-1	74	63	76	85	50	70	73	79	59	69.9 (10.8)
29	*Leucanthemum laciniatum*	ACTG	lai1	L179	63	88	141	121	85	88	84	88	106	96 (23.2)
30	*Leucanthemum laciniatum*	AACA	lai2	280-1	59	81	72	78	43	62	67	56	48	62.9 (12.9)
31	*Leucanthemum ligusticum*	ACGC	lig1	258-1	40	70	71	109	68	32	47	60	107	67.1 (26.9)
32	*Leucanthemum lithopolitanicum*	ACCG	lit1	L998	83	91	118	109	68	71	144	130	91	100.6 (26.3)
33	*Leucanthemum lithopolitanicum*	AAGA	lit2	274-1	51	91	67	91	61	48	58	50	95	68 (19.2)
34	*Leucanthemum pluriflorum*	AGAG	plu1	40-6	72	82	161	114	84	92	115	114	97	103.4 (26.6)
35	*Leucanthemum pluriflorum*	ATAT	plu2	55-1	48	89	93	63	68	41	60	59	76	66.3 (17.3)
36	*Leucanthemum rotundifolium*	AGTG	rot1	L990	87	71	117	84	77	62	98	114	120	92.2 (21.2)
37	*Leucanthemum rotundifolium*	ATTA	rot2	L989	81	57	53	85	36	73	58	59	90	65.8 (17.6)
38	*Leucanthemum rotundifolium*	ATCA	rot3	L992	61	88	85	106	50	90	75	48	84	76.3 (19.6)
39	*Leucanthemum tridactylites*	AGCG	tri1	L151	67	40	85	115	74	72	92	154	88	87.4 (32.2)
40	*Leucanthemum tridactylites*	ATGT	tri2	278-1	53	91	73	75	48	52	90	84	92	73.1 (17.9)
41	*Leucanthemum virgatum*	AGGG	vir1	L987	94	84	132	113	64	0	106	132	80	92.3 (33.9)
42	*Leucanthemum virgatum*	ACAA	vir2	250-1	56	80	55	84	56	0	65	57	75	58.7 (24.7)
43	*Leucanthemum vulgare*	TAAG	vul1	94-1	107	89	146	114	100	101	130	120	89	110.7 (19)
44	*Leucanthemum vulgare*	ACTA	vul2	L046	36	87	59	61	53	45	78	77	93	65.4 (19.4)
45	*Leucanthemum vulgare*	ACCT	vul3	184-1	64	109	50	63	49	79	82	87	81	73.8 (19.2)
46	*Leucanthemum vulgare* subsp. *eliasii*	TATG	vel1	L996	87	68	110	88	53	32	125	96	88	83 (28.4)
47	*Leucanthemum vulgare* subsp. *eliasii*	AGAA	vel2	L162	50	72	59	93	73	40	103	51	71	68 (20.5)
48	*Leucanthemum vulgare* subsp. *pujiulae*	ATTC	vup1	135-7	86	53	130	117	60	80	106	100	121	94.8 (27)
49	*Leucanthemum vulgare* subsp. *pujiulae*	AGTT	vup2	M60-1	46	75	78	93	67	47	70	72	92	71.1 (16.6)
				mean (SD):	62.3 (19.1)	71.8 (20)	88.6 (32.1)	81.1 (28.1)	61.2 (19.1)	55.4 (24.4)	75.9 (28.8)	76.9 (28.8)	77.8 (22.3)	
				length (SD):	330.9 (23.0)	371.7 (25.6)	330.4 (63.9)	358.6 (58.0)	241.4 (60.5)	340.5 (57.9)	343.6 (98.0)	274.9 (67.7)	285.6 (74.7)	

Table 5 - Summary of raw reads obtained per marker and species. The most right column summarizes reads per species (mean and standard deviation) and the bottom line summarizes reads per marker (mean and standard deviation). The average read length is given in the bottommost line.

					1	2	3	4	5	6	7	8	9	
N°	**Outgroup**	**Code**	**Short**	**Population**	**A39**	**B12**	**B20**	**C12**	**C20**	**C33**	**D23**	**D27**	**D18**	**mean (SD)**
1	*Chrysanthoglossum deserticola* (Murb.) Wilcox. & al.	ACAG	chd1	A791	36	53	44	47	49	38	35	26	97	47.2 (20.4)
2	*Chlamydophora tridentata* (Del.) Less.	ACTG	cht1	A795	43	44	54	58	70	23	37	54	75	50.9 (16.2)
3	*Coleostephus myconis* (L.) Rchb. f.	AATG	com1	A792	39	59	56	74	52	27	53	43	69	52.4 (14.6)
4	*Glossopappus macrotus* subsp. *hesperius* (Maire) Maire	ATGG	glm1	A790	30	42	29	57	49	53	32	37	33	40.2 (10.5)
5	*Heteromera fuscata* (Desf.) Pomel	AAAG	hef1	A796	33	33	168	43	1	34	38	47	58	50.6 (46.7)
6	*Mauranthemum paludosum* subsp. ebusitanum (Vogt) Vogt & Oberpr.	AAGG	mae1	A799	41	39	65	0	49	0	29	44	76	38.1 (25.8)
7	*Mauranthemum paludosum* subsp. *paludosum* (Poir.) Vogt & Oberpr.	AACG	map1	A798	40	53	56	0	52	16	36	47	63	40.3 (20.4)
8	*Plagius flosculosus* (L.) Alavi & Heyw.	ATCG	plf1	A793	44	53	66	72	54	46	52	43	18	49.8 (15.4)
9	*Plagius maghrebinus* Vogt & Greuter	ATTG	plm1	A794	53	1	61	71	47	5	40	52	29	39.9 (24.1)
10	*Rhodanthemum catananche* (Ball) Wilcox & al.	ATAG	rhc1	A087	45	16	68	67	43	59	44	45	63	50 (16.4)
11	*Leucanthemum burnatii*	AAAG	bur1	90-6	53	49	79	69	97	27	60	89	58	64.6 (21.6)
12	*Leucanthemum burnatii*	ACCG	bur2	92-1	39	53	35	49	52	32	54	54	71	48.8 (12)
13	*Leucanthemum cf. monspeliense*	AATG	mop1	131-20	35	59	74	50	80	59	71	97	72	66.3 (18)
14	*Leucanthemum cf. monspeliense*	ACGG	mop2	128-1	41	40	41	53	40	58	47	65	42	47.4 (9.2)
15	*Leucanthemum gallaecicum*	AACG	gal1	159-11	48	55	82	62	72	71	62	88	44	64.9 (14.8)
16	*Leucanthemum gallaecicum*	AGAG	gal2	L985	30	53	72	47	59	37	59	59	57	52.6 (12.8)
17	*Leucanthemum gaudinii* subsp. *barrelieri*	AAGG	gab1	L035	65	50	81	48	90	72	83	84	63	70.7 (15.2)
18	*Leucanthemum gaudinii* subsp. *barrelieri*	AGTG	gab2	266-1	38	49	65	48	31	60	41	66	52	50 (12.1)
19	*Leucanthemum gaudinii* subsp. *cantabricum*	ATAG	gac1	L036	68	55	87	90	85	58	81	95	64	75.9 (14.8)
20	*Leucanthemum gaudinii* subsp. *cantabricum*	AGCG	gac2	60-1	41	45	64	36	57	46	46	31	51	46.3 (10.1)
21	*Leucanthemum gaudinii* subsp. *gaudinii*	ATTG	gag1	L033	37	56	43	28	36	56	30	25	41	39.1 (11.2)
22	*Leucanthemum gaudinii* subsp. *gaudinii*	AGGG	gag2	276-1	35	64	50	51	45	37	50	63	65	51.1 (11.2)
23	*Leucanthemum gracilicaule*	ATCG	gra1	84-6	57	53	72	62	68	47	74	88	51	63.6 (13.2)
24	*Leucanthemum gracilicaule*	TATG	gra2	85-1	54	47	85	72	56	64	47	68	84	64.1 (14.4)
25	*Leucanthemum graminifolium*	ATGG	grm1	116-4	32	20	49	51	39	22	37	63	55	40.9 (14.8)
26	*Leucanthemum graminifolium*	ATTC	grm2	96-3	31	58	52	72	53	45	60	51	71	54.8 (12.7)
27	*Leucanthemum halleri*	ACAG	hal1	L1002	65	42	84	64	55	29	29	89	70	58.6 (21.8)
28	*Leucanthemum halleri*	ATTT	hal2	208-1	61	50	67	75	45	52	56	69	59	59.3 (9.7)
29	*Leucanthemum laciniatum*	ACTG	lai1	L179	46	48	73	62	75	70	50	70	92	65.1 (15.1)
30	*Leucanthemum laciniatum*	AACA	lai2	280-1	42	59	52	63	39	59	58	55	47	52.7 (8.3)
31	*Leucanthemum ligusticum*	ACGC	lig1	258-1	34	54	63	87	64	30	41	54	100	58.6 (23.3)
32	*Leucanthemum lithopolitanicum*	ACCG	lit1	L998	60	57	87	76	56	63	83	111	72	73.9 (17.9)
33	*Leucanthemum lithopolitanicum*	AAGA	lit2	274-1	39	64	58	85	60	42	49	45	85	58.6 (17.2)
34	*Leucanthemum pluriflorum*	AGAG	plu1	40-6	54	51	109	81	83	70	71	90	80	76.6 (17.8)
35	*Leucanthemum pluriflorum*	ATAT	plu2	55-1	39	55	81	42	68	37	50	56	71	55.4 (15.3)
36	*Leucanthemum rotundifolium*	AGTG	rot1	L990	68	30	88	47	73	29	47	92	99	63.7 (26.6)
37	*Leucanthemum rotundifolium*	ATTA	rot2	L989	65	38	46	72	35	50	51	57	84	55.3 (16)
38	*Leucanthemum rotundifolium*	ATCA	rot3	L992	45	61	75	88	48	69	63	41	79	63.2 (16.2)
39	*Leucanthemum tridactylites*	AGCG	tri1	L151	49	31	54	85	64	39	46	130	57	61.7 (29.9)
40	*Leucanthemum tridactylites*	ATGT	tri2	278-1	38	66	57	65	48	47	66	74	90	61.2 (15.8)
41	*Leucanthemum virgatum*	AGGG	vir1	L987	66	49	72	82	57	0	59	107	68	62.2 (28.8)
42	*Leucanthemum virgatum*	ACAA	vir2	250-1	47	62	44	77	55	0	54	54	69	51.3 (21.9)
43	*Leucanthemum vulgare*	TAAG	vul1	94-1	68	52	100	80	98	82	100	97	70	83 (17.2)
44	*Leucanthemum vulgare*	ACTA	vul2	L046	28	66	50	52	52	42	66	66	87	56.6 (17)
45	*Leucanthemum vulgare*	ACCT	vul3	184-1	62	84	39	49	48	72	73	81	78	65.1 (16.3)
46	*Leucanthemum vulgare* subsp. *eliasii*	TATG	vel1	L996	62	37	57	44	53	25	70	73	50	52.3 (15.5)
47	*Leucanthemum vulgare* subsp. *eliasii*	AGAA	vel2	L162	38	52	45	76	71	34	90	42	70	57.6 (19.7)
48	*Leucanthemum vulgare* subsp. *pujiulae*	ATTC	vup1	135-7	54	36	84	66	58	64	77	80	87	67.3 (16.5)
49	*Leucanthemum vulgare* subsp. *pujiulae*	AGTT	vup2	M60-1	36	60	67	80	63	44	56	66	89	62.3 (16.3)
				mean (SD):	46.4 (12)	49 (14)	66.3 (22.8)	60.7 (19.7)	57 (17.5)	43.7 (20.3)	55.2 (16.6)	65.8 (23.2)	66.8 (18.2)	
				length (SD):	329.7 (17.1)	371.0 (18.7)	327.9 (64.5)	358.9 (56.3)	240.4 (59.2)	341.3 (63.6)	335.2 (103.9)	275.1 (63.0)	284.9 (73.0)	

Table 6 - Summary of obtained reads per marker and species after quality control. The rightmost column summarizes reads per species (mean and standard deviation) and the bottom line summarizes reads per marker (mean and standard deviation). The average read length is given in the bottommost line.

					1	2	3	4	5	6	7	8	9	
N°	**Outgroup**	**Code**	**Short**	**Population**	**A39**	**B12**	**B20**	**C12**	**C20**	**C33**	**D23**	**D27**	**D18**	**mean (SD)**
1	*Chrysanthoglossum deserticola* (Murb.) Wilcox. & al.	ACAG	chd1	A791	33	52	32	43	47	38	23	16	13	33 (13.6)
2	*Chlamydophora tridentata* (Del.) Less.	ACTG	cht1	A795	40	44	43	52	49	23	28	35	69	42.6 (13.6)
3	*Coleostephus myconis* (L.) Rchb. f.	AATG	com1	A792	38	55	47	51	46	31	46	37	47	44.2 (7.5)
4	*Glossopappus macrotus* subsp. *hesperius* (Maire) Maire	ATGG	glm1	A790	29	37	26	37	35	52	32	33	21	33.6 (8.7)
5	*Heteromera fuscata* (Desf.) Pomel	AAAG	hef1	A796	31	32	122	48	0	34	29	37	43	41.8 (32.9)
6	*Mauranthemum paludosum* subsp. ebusitanum (Vogt) Vogt & Oberpr.	AAGG	mae1	A799	44	37	54	0	48	0	26	39	56	33.8 (21.2)
7	*Mauranthemum paludosum* subsp. *paludosum* (Poir.) Vogt & Oberpr.	AACG	map1	A798	43	53	54	0	43	16	24	42	48	35.9 (18.5)
8	*Plagius flosculosus* (L.) Alavi & Heyw.	ATCG	plf1	A793	51	54	64	68	43	46	41	28	15	45.6 (16.6)
9	*Plagius maghrebinus* Vogt & Greuter	ATTG	plm1	A794	52	0	48	66	41	4	15	34	14	30.4 (23.2)
10	*Rhodanthemum catananche* (Ball) Wilcox & al.	ATAG	rhc1	A087	52	15	61	67	48	58	41	38	55	48.3 (15.5)
11	*Leucanthemum burnatii*	AAAG	bur1	90-6	53	49	79	69	97	0	60	89	58	61.6 (28.3)
12	*Leucanthemum burnatii*	ACCG	bur2	92-1	39	38	24	50	50	0	51	48	64	40.4 (18.8)
13	*Leucanthemum cf. monspeliense*	AATG	mop1	131-20	27	59	74	50	80	59	71	97	72	65.4 (19.9)
14	*Leucanthemum cf. monspeliense*	ACGG	mop2	128-1	29	40	40	50	32	57	32	58	33	41.2 (11.2)
15	*Leucanthemum gallaecicum*	AACG	gal1	159-11	41	55	82	62	72	71	62	88	44	64.1 (15.9)
16	*Leucanthemum gallaecicum*	AGAG	gal2	L985	32	53	59	46	53	36	54	48	36	46.3 (9.6)
17	*Leucanthemum gaudinii* subsp. *barrelieri*	AAGG	gab1	L035	57	50	81	53	90	72	83	84	63	70.3 (15)
18	*Leucanthemum gaudinii* subsp. *barrelieri*	AGTG	gab2	266-1	37	49	42	47	26	57	33	61	30	42.4 (12.1)
19	*Leucanthemum gaudinii* subsp. *cantabricum*	ATAG	gac1	L036	67	55	87	90	85	58	81	95	64	75.8 (14.9)
20	*Leucanthemum gaudinii* subsp. *cantabricum*	AGCG	gac2	60-1	41	40	60	35	56	36	43	23	20	39.3 (13.2)
21	*Leucanthemum gaudinii* subsp. *gaudinii*	ATTG	gag1	L033	36	56	43	28	36	56	30	25	41	39 (11.3)
22	*Leucanthemum gaudinii* subsp. *gaudinii*	AGGG	gag2	276-1	34	64	45	51	36	38	47	56	33	44.9 (10.7)
23	*Leucanthemum gracilicaule*	ATCG	gra1	84-6	56	53	72	62	68	47	74	88	51	63.4 (13.2)
24	*Leucanthemum gracilicaule*	TATG	gra2	85-1	55	46	64	49	52	52	34	57	28	48.6 (11.3)
25	*Leucanthemum graminifolium*	ATGG	grm1	116-4	29	20	49	51	39	22	37	63	55	40.6 (15)
26	*Leucanthemum graminifolium*	ATTC	grm2	96-3	46	58	51	72	42	44	56	49	54	52.4 (9.1)
27	*Leucanthemum halleri*	ACAG	hal1	L1002	62	42	84	64	55	33	29	89	70	58.7 (21.1)
28	*Leucanthemum halleri*	ATTT	hal2	208-1	62	50	65	75	46	67	53	67	36	57.9 (12.4)
29	*Leucanthemum laciniatum*	ACTG	lai1	L179	46	48	73	62	75	70	50	70	92	65.1 (15.1)
30	*Leucanthemum laciniatum*	AACA	lai2	280-1	42	42	57	49	36	59	48	49	46	47.6 (7.3)
31	*Leucanthemum ligusticum*	ACGC	lig1	258-1	34	53	46	87	62	28	35	50	58	50.3 (17.9)
32	*Leucanthemum lithopolitanicum*	ACCG	lit1	L998	53	57	87	76	56	63	83	111	72	73.1 (18.7)
33	*Leucanthemum lithopolitanicum*	AAGA	lit2	274-1	39	64	56	82	58	42	45	34	65	53.9 (15.3)
34	*Leucanthemum pluriflorum*	AGAG	plu1	40-6	43	51	109	81	83	70	71	90	80	75.3 (19.8)
35	*Leucanthemum pluriflorum*	ATAT	plu2	55-1	33	53	62	41	66	38	44	54	71	51.3 (13.2)
36	*Leucanthemum rotundifolium*	AGTG	rot1	L990	47	30	88	47	73	29	47	92	99	61.3 (27)
37	*Leucanthemum rotundifolium*	ATTA	rot2	L989	66	38	33	46	28	49	28	50	69	45.2 (15.1)
38	*Leucanthemum rotundifolium*	ATCA	rot3	L992	52	60	63	84	44	76	25	36	59	55.4 (18.6)
39	*Leucanthemum tridactylites*	AGCG	tri1	L151	41	31	54	85	64	39	46	130	57	60.8 (30.5)
40	*Leucanthemum tridactylites*	ATGT	tri2	278-1	37	65	41	64	46	34	63	71	86	56.3 (17.6)
41	*Leucanthemum virgatum*	AGGG	vir1	L987	51	49	72	82	57	0	59	107	68	60.6 (29)
42	*Leucanthemum virgatum*	ACAA	vir2	250-1	45	62	43	77	51	0	44	51	56	47.7 (20.8)
43	*Leucanthemum vulgare*	TAAG	vul1	94-1	59	52	100	80	98	82	100	97	70	82 (18.4)
44	*Leucanthemum vulgare*	ACTA	vul2	L046	29	65	44	32	49	42	57	61	57	48.4 (12.7)
45	*Leucanthemum vulgare*	ACCT	vul3	184-1	62	83	34	41	45	72	56	72	56	57.9 (16.1)
46	*Leucanthemum vulgare* subsp. *eliasii*	TATG	vel1	L996	57	37	57	44	53	27	70	73	50	52 (14.7)
47	*Leucanthemum vulgare* subsp. *eliasii*	AGAA	vel2	L162	36	52	27	40	66	35	46	35	57	43.8 (12.5)
48	*Leucanthemum vulgare* subsp. *pujiulae*	ATTC	vup1	135-7	49	36	84	66	58	64	77	80	87	66.8 (17.1)
49	*Leucanthemum vulgare* subsp. *pujiulae*	AGTT	vup2	M60-1	35	56	52	80	62	32	52	58	79	56.2 (16.6)
				mean (SD):	44.3 (10.9)	47.8 (14)	59.9 (21.6)	56.8 (20.1)	54 (18.8)	42 (22.1)	48.6 (18.7)	61.1 (26.2)	54.4 (20.3)	
				length (SD):	310.2 (6.5)	354.2 (9.6)	317.1 (15.4)	345.2 (27.2)	246.9 (43.5)	320.6 (77.2)	344.0 (15.7)	268.0 (9.3)	297.0 (18.5)	

Table 7 - Summary of obtained reads per marker and species after BAPS clustering and removal of chimeric sequences. The most right column summarizes reads per species (mean and standard deviation) and the bottommost line summarizes reads per marker (mean and standard deviation). The average read length is given in the most bottom line.

***Leucanthemum* & outgroups**

	Total number of		Constant characters		Variable (uninformative) characters		Parsimony informative characters	
Marker	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels
A39	357	21	255 (71%)	-	42 (12%)	11 (52%)	60 (17%)	10 (48%)
B12	437	50	286 (65%)	-	42 (10%)	22 (44%)	109 (25%)	28 (56%)
B20	363	19	233 (64%)	-	51 (14%)	12 (63%)	79 (22%)	7 (37%)
C12	439	33	271 (62%)	-	78 (18%)	21 (64%)	90 (21%)	12 (36%)
C20	492	19	407 (83%)	-	43 (9%)	9 (47%)	42 (9%)	10 (53%)
C33	679	40	474 (70%)	-	88 (13%)	27 (68%)	117 (17%)	13 (33%)
D18	526	47	374 (71%)	-	58 (11%)	25 (53%)	94 (18%)	22 (47%)
D23	391	30	275 (70%)	-	39 (10%)	19 (63%)	77 (20%)	11 (37%)
D27	302	12	219 (73%)	-	47 (16%)	7 (58%)	36 (12%)	5 (42%)
cpDNA	2566	102	2195 (86%)	-	264 (10%)	66 (65%)	107 (4%)	36 (35%)
psbA	595	46	505 (85%)	-	64 (11%)	29 (63%)	26 (4%)	17 (37%)
trnL2(e)	452	13	397 (88%)	-	41 (9%)	7 (54%)	14 (3%)	6 (46%)
trnC	576	14	479 (83%)	-	68 (12%)	9 (64%)	29 (5%)	5 (36%)
petN1	432	15	379 (88%)	-	35 (8%)	11 (73%)	18 (4%)	4 (27%)
trnQ2	511	14	435 (85%)	-	56 (11%)	10 (71%)	20 (4%)	4 (29%)

***Leucanthemum* only**

	Total number of		Constant characters		Variable (uninformative) characters		Parsimony informative characters	
Marker	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels
A39	357	21	313 (88%)	-	8 (2%)	15 (71%)	36 (10%)	6 (29%)
B12	437	16	353 (81%)	-	24 (5%)	4 (25%)	60 (14%)	12 (75%)
B20	363	7	288 (79%)	-	26 (7%)	3 (43%)	49 (13%)	4 (57%)
C12	439	10	384 (87%)	-	19 (4%)	5 (50%)	36 (8%)	5 (50%)
C20	492	6	466 (95%)	-	13 (3%)	3 (50%)	13 (3%)	3 (50%)
C33	679	18	568 (84%)	-	42 (6%)	12 (67%)	69 (10%)	6 (33%)
D18	526	19	470 (89%)	-	28 (5%)	10 (53%)	28 (5%)	9 (47%)
D23	391	9	351 (90%)	-	13 (3%)	5 (56%)	27 (7%)	4 (44%)
D27	302	2	288 (95%)	-	6 (2%)	0 (0%)	8 (3%)	2 (100%)
cpDNA	2566	25	2497 (97%)	-	32 (1%)	11 (44%)	37 (1%)	14 (56%)
psbA	595	11	576 (97%)	-	9 (2%)	6 (55%)	10 (2%)	5 (45%)
trnL2(e)	452	2	447 (99%)	-	2 (0%)	0 (0%)	3 (1%)	2 (100%)
trnC	576	4	559 (97%)	-	9 (2%)	0 (0%)	8 (1%)	4 (100%)
petN1	432	2	419 (97%)	-	6 (1%)	1 (50%)	7 (2%)	1 (50%)
trnQ2	511	6	496 (97%)	-	6 (1%)	4 (67%)	9 (2%)	2 (33%)

Table 8 - Summary on the number of characters and variable positions in the alignment. Parsimony informative and uninformative characters are calculated for *Leucanthemum* together with outgroups, and solely within *Leucanthemum*.

					1	2	3	4	5	6	7	8	9	
N°	**Outgroup**	**Code**	**Short**	**Population**	**A39**	**B12**	**B20**	**C12**	**C20**	**C33**	**D23**	**D27**	**D18**	**mean**
1	*Chrysanthoglossum deserticola* (Murb.) Wilcox. & al.	ACAG	chd1	A791	3	1	1	2	1	3	3	2	2	2.00
2	*Chlamydophora tridentata* (Del.) Less.	ACTG	cht1	A795	1	1	1	1	2	1	2	1	1	1.22
3	*Coleostephus myconis* (L.) Rchb. f.	AATG	com1	A792	3	4	2	2	2	1	2	2	1	2.11
4	*Glossopappus macrotus* subsp. *hesperius* (Maire) Maire	ATGG	glm1	A790	2	1	1	2	2	1	1	2	2	1.56
5	*Heteromera fuscata* (Desf.) Pomel	AAAG	hef1	A796	2	4	3	2	2	2	2	2	1	2.22
6	*Mauranthemum paludosum* subsp. ebusitanum (Vogt) Vogt & Oberpr.	AAGG	mae1	A799	1	2	2	2	1	1	1	1	2	1.44
7	*Mauranthemum paludosum* subsp. *paludosum* (Poir.) Vogt & Oberpr.	AACG	map1	A798	1	1	3	2	3	2	3	1	1	1.89
8	*Plagius flosculosus* (L.) Alavi & Heyw.	ATCG	plf1	A793	1	1	1	2	2	2	1	2	1	1.44
9	*Plagius maghrebinus* Vogt & Greuter	ATTG	plm1	A794	1	1	2	2	1	1	2	1	2	1.44
10	*Rhodanthemum catananche* (Ball) Wilcox & al.	ATAG	rhc1	A087	2	2	2	1	3	2	2	2	2	2.00
11	*Leucanthemum burnatii*	AAAG	bur1	90-6	2	2	2	2	2	1	2	1	2	1.78
12	*Leucanthemum burnatii*	ACCG	bur2	92-1	2	2	2	1	1	1	1	1	1	1.33
13	*Leucanthemum cf. monspeliense*	AATG	mop1	131-20	2	2	2	2	1	2	2	2	2	1.89
14	*Leucanthemum cf. monspeliense*	ACGG	mop2	128-1	2	2	1	1	2	1	2	1	1	1.44
15	*Leucanthemum gallaecicum*	AACG	gal1	159-11	2	1	2	2	2	1	2	1	2	1.67
16	*Leucanthemum gallaecicum*	AGAG	gal2	L985	1	2	1	1	1	1	2	1	1	1.22
17	*Leucanthemum gaudinii* subsp. *barrelieri*	AAGG	gab1	L035	2	2	2	2	2	1	1	2	1	1.67
18	*Leucanthemum gaudinii* subsp. *barrelieri*	AGTG	gab2	266-1	1	2	2	1	1	1	2	2	2	1.56
19	*Leucanthemum gaudinii* subsp. *cantabricum*	ATAG	gac1	L036	1	2	2	1	2	2	3	2	1	1.78
20	*Leucanthemum gaudinii* subsp. *cantabricum*	AGCG	gac2	60-1	1	2	2	2	1	2	2	2	1	1.67
21	*Leucanthemum gaudinii* subsp. *gaudinii*	ATTG	gag1	L033	1	1	2	2	1	2	2	1	2	1.56
22	*Leucanthemum gaudinii* subsp. *gaudinii*	AGGG	gag2	276-1	1	2	1	1	2	1	2	1	2	1.44
23	*Leucanthemum gracilicaule*	ATCG	gra1	84-6	2	1	2	1	1	2	2	1	1	1.44
24	*Leucanthemum gracilicaule*	TATG	gra2	85-1	1	1	2	2	1	1	2	1	3	1.56
25	*Leucanthemum graminifolium*	ATGG	grm1	116-4	2	1	1	2	1	1	1	1	1	1.22
26	*Leucanthemum graminifolium*	ATTC	grm2	96-3	1	1	1	2	2	2	1	1	2	1.44
27	*Leucanthemum halleri*	ACAG	hal1	L1002	2	1	1	2	1	1	2	1	1	1.33
28	*Leucanthemum halleri*	ATTT	hal2	208-1	1	1	1	1	1	2	2	1	2	1.33
29	*Leucanthemum laciniatum*	ACTG	lai1	L179	2	1	1	2	2	2	1	1	1	1.44
30	*Leucanthemum laciniatum*	AACA	lai2	280-1	2	2	1	2	1	1	1	1	1	1.33
31	*Leucanthemum ligusticum*	ACGC	lig1	258-1	2	2	2	1	2	2	2	2	2	1.89
32	*Leucanthemum lithopolitanicum*	ACCG	lit1	L998	1	1	1	2	1	2	1	1	2	1.33
33	*Leucanthemum lithopolitanicum*	AAGA	lit2	274-1	1	1	1	1	1	1	1	1	2	1.11
34	*Leucanthemum pluriflorum*	AGAG	plu1	40-6	1	1	2	2	1	1	2	1	2	1.44
35	*Leucanthemum pluriflorum*	ATAT	plu2	55-1	2	2	2	2	1	1	2	1	2	1.67
36	*Leucanthemum rotundifolium*	AGTG	rot1	L990	1	2	2	1	2	2	1	2	2	1.67
37	*Leucanthemum rotundifolium*	ATTA	rot2	L989	1	2	1	1	2	1	2	1	1	1.33
38	*Leucanthemum rotundifolium*	ATCA	rot3	L992	2	1	2	1	1	1	1	1	2	1.33
39	*Leucanthemum tridactylites*	AGCG	tri1	L151	2	2	1	2	1	2	1	1	2	1.56
40	*Leucanthemum tridactylites*	ATGT	tri2	278-1	2	2	2	1	1	1	1	1	2	1.44
41	*Leucanthemum virgatum*	AGGG	vir1	L987	2	1	2	1	1	1	1	2	2	1.44
42	*Leucanthemum virgatum*	ACAA	vir2	250-1	2	2	1	1	1	1	2	1	2	1.44
43	*Leucanthemum vulgare*	TAAG	vul1	94-1	1	2	2	2	2	2	2	1	2	1.78
44	*Leucanthemum vulgare*	ACTA	vul2	L046	1	2	2	2	2	1	2	1	2	1.67
45	*Leucanthemum vulgare*	ACCT	vul3	184-1	1	1	2	2	2	1	2	1	2	1.56
46	*Leucanthemum vulgare* subsp. *eliasii*	TATG	vel1	L996	1	2	2	2	2	1	1	1	2	1.56
47	*Leucanthemum vulgare* subsp. *eliasii*	AGAA	vel2	L162	2	2	2	2	2	1	2	2	2	1.89
48	*Leucanthemum vulgare* subsp. *pujiulae*	ATTC	vup1	135-7	1	1	2	1	1	2	1	2	2	1.44
49	*Leucanthemum vulgare* subsp. *pujiulae*	AGTT	vup2	M60-1	2	2	2	1	1	2	2	1	1	1.56

Table 9 - Number of alleles as achieved per species and marker. Last column is a mean number of alleles per species.

Gene trees – Retrieved gene trees varied in the support of resolved branches and topology (**appendix A**). High incongruence occurred among all of them (mean hardwired cluster distance 36.4 (min-max 25-49)). The number of alleles varied within one accession between different markers but typically diploids had either 1 or 2 alleles (**Table 9**). In many cases the analyses failed to resolve relationships with acceptable confidence (pp ≥ 0.95) and sometimes taxa sharing the same allele were grouped together but structure within the group was not visible. This applies mostly to *Leucanthemum* species while relationships among outgroup taxa were better resolved.

Species tree – The species tree reconstruction treats all reticulations as a product of incomplete lineage sorting and therefore its structure may be slightly different from the true structure. The monophyletic *Leucanthemum* clade is a sister clade to the clade containing all southern Mediterranean endemics and *Chlamydophora tridentata* is a basal species to both groups (**Figure 2**). The earliest diverging species within *Leucanthemum* are *L. rotundifolium* and *L. gracilicaule*. A further major split in *Leucanthemum* is dividing it into two large groups one consisting of *L. gracilicaule*, *L. graminifolium*, *L. halleri*, *L. laciniatum*, *L. lithopolitanicum*, *L. rotundifolium L. tridactylites* and *L. virgatum* ('Group 1') while the second group consists of *L. burnatii*, *L. gallaecicum*, *L. gaudinii* subsp. *barrelieri*, *L. gaudinii* subsp. *cantabricum*, *L. gaudinii* subsp. *gaudinii*, *L. ligusticum*, *L. monspeliense*, *L. pluriflorum*, *L. vulgare* subsp. *eliasii*, *L. vulgare* subsp. *pujiulae* and *L. vulgare* subsp. *vulgare* ('Group 2').

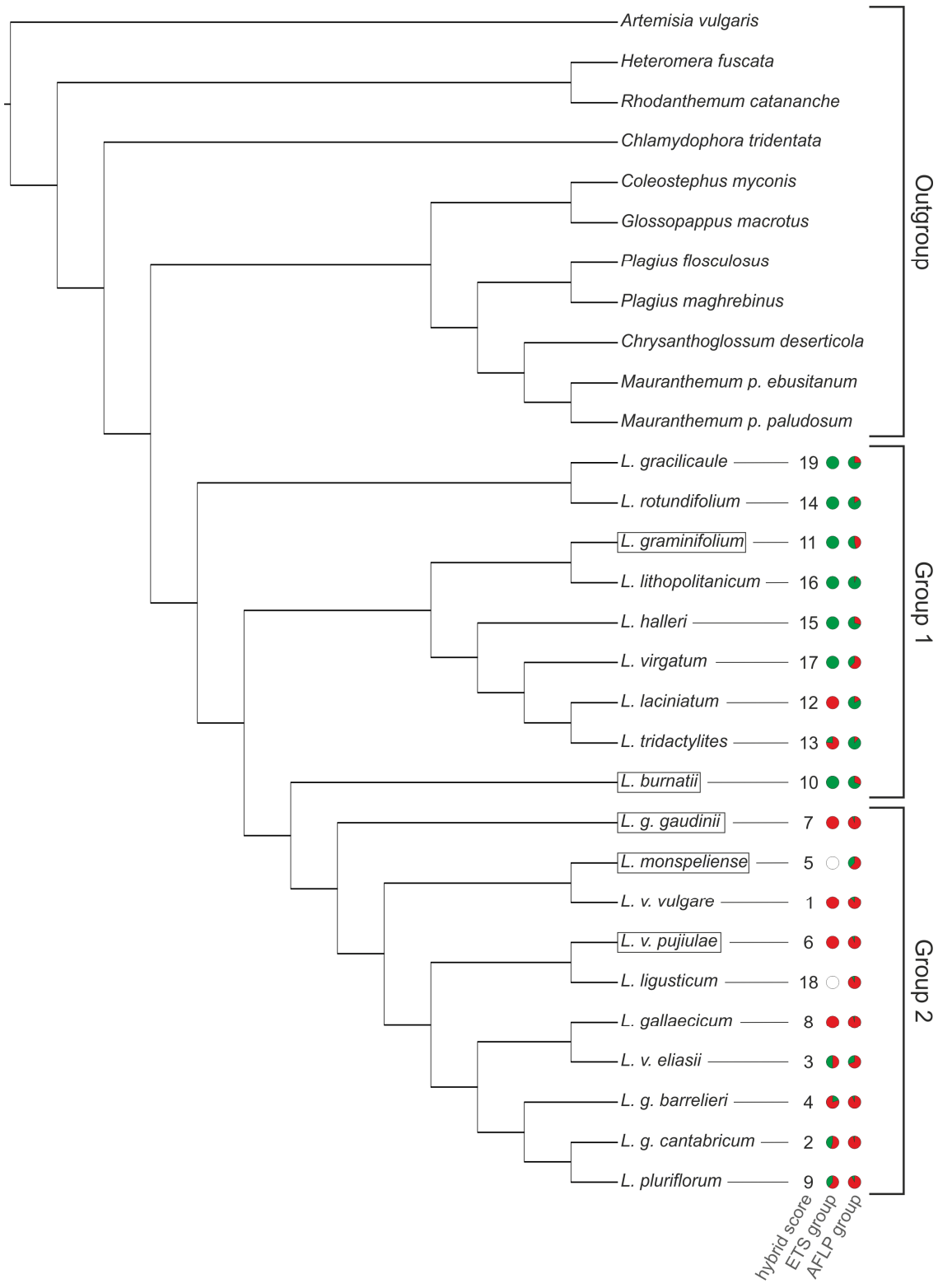

Figure 2 - Species tree constructed using minimizing deep coalescences (MDC) algorithm based on 10 gene trees. Species enclosed in a rectangle had significantly lower values of hybrid index obtained from real gene trees over hybrid indexes obtained from simulated trees, thus were specified as "non-hybrid" taxa. The number after species name (hybrid score) indicates position of the species in the ranking of hybrid indexes, where the highest hybrid index is 1st and the lowest 19th. The first coloured circle after species name indicates membership to one of the nrDNA ETS ribotype groups (green, red or mixed) from Oberprieler et al. (2014). *L. monspeliense* and *L. ligusticum* were not sampled by that study. The second coloured circle after species name indicates membership to one of the clusters found by Structure in AFLP data (green and red) summarized on species level by clumpp 1.1.2 (Jakobsson & Rosenberg 2007).

Supernetwork – The consensus network constructed from gene trees displays a high number of reticulations but some of them may arise due to the inability of distinguishing ILS from hybridization (in contrary to species tree analysis here all are treated as hybridization). Nevertheless its structure provides a good overview on the relationships among species. The genus *Leucanthmeum* is monophyletic and none of analyzed outgroups can be treated as its immediate sister genus (**Figure 3**). Although based on the structure of the network, probably the closest relative is *Chlamydophora tridentata* endemic to the Cyprus Island. The earliest diverging species in the genus is *L. rotundifolium* or *L. burnatii* and both are placed close to the outgroup taxa. Similar to the species tree analysis, two groups are visible with exactly the same species membership as previously mentioned except only *L. burnatii* which is placed in the 'Group 1'. Species which are members of the 'Group 2' seems to be more closely related to each other than species belonging to the 'Group 1' since the phylogenetic distances between them are smaller and display more reticulations.

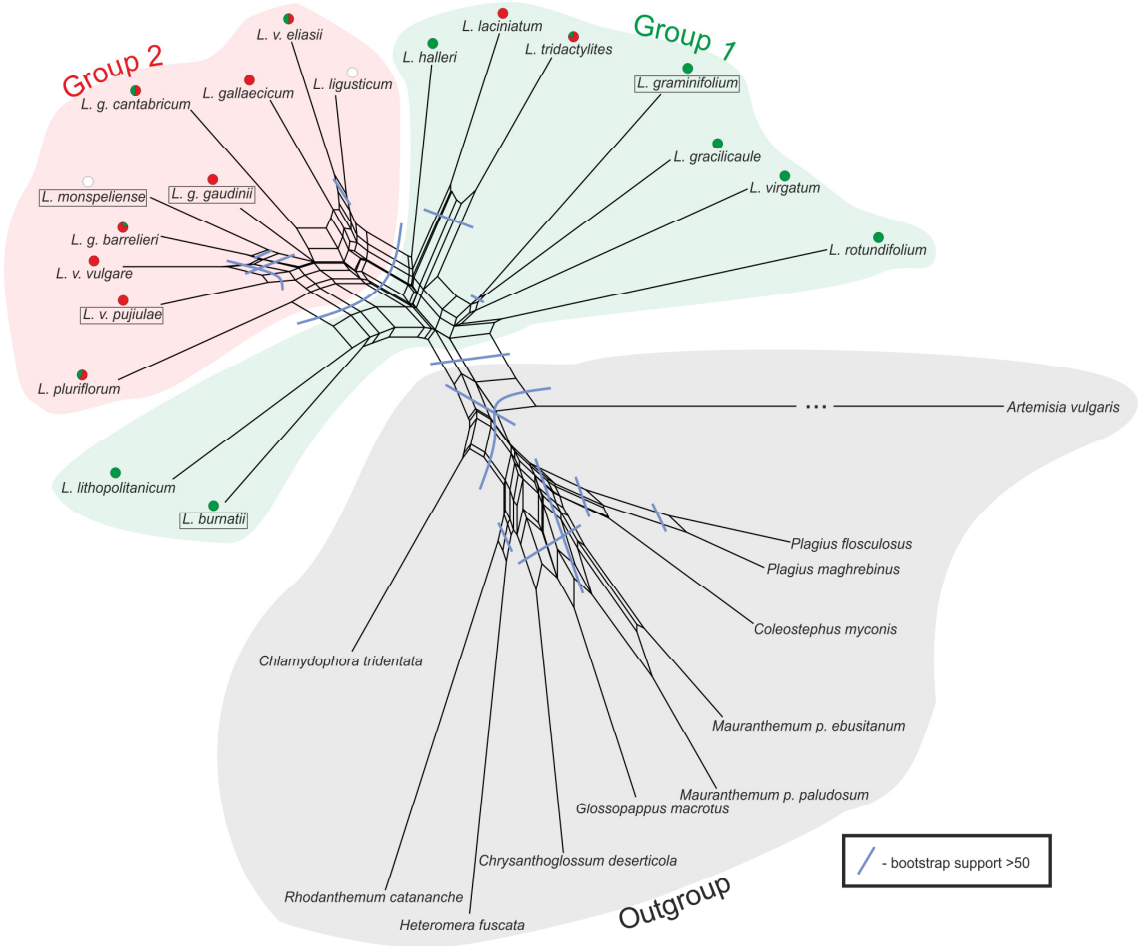

Figure 3 - Supernetwork as obtained from low copy nuclear genes and cpDNA markers. The blue lines represent splits with bootstrap support greater than 50. Species enclosed in a rectangle had significantly lower values of hybrid index obtained from real gene trees over hybrid indexes obtained from simulated trees, thus were specified as "non-hybrid" taxa. The coloured circle above species name indicates membership to one of the nrDNA ETS ribotype groups (green, red or mixed) from Oberprieler et al. (2014). *L. monspeliense* and *L. ligusticum* were not sampled by that study.

AFLP Network, PCoA and Structure – The total dataset included 610 loci (D2: 210, D3:183, D4: 217) with Euclidean error rate 9% and Jaccard error 41%. Resolution score was high and all replicates could be correctly paired. After discarding fragments below 100 bp, the dataset contained 469 bands (D2: 165, D3:138, D4:166) and the Euclidean error rate was 8% and Jaccard error 41%. Again, the main structure of the AFLP network is delimiting *Leucanthemum* into two groups just as the previous analyses (**Figure 4**). In difference to species tree analyses, accessions were not summarized into one species and treated as different entities. Most of the accessions of one taxon are grouped together but in some cases they failed to form a monophyletic groups. In the 'Group 1' that was the case of two taxa *L. tridactylites* and *L. rotundifolium*, while in the 'Group 2' it applied to *L. vulgare* subsp. *vulgare*, *L. vulgare* subsp. *eliasii*, *L. gaudinii* subsp. *gaudinii*, *L. gaudinii* subsp. *barrelieri* and *L. gaudinii* subsp. *cantabricum*. In the PCoA graph (**Figure 5**), the division between previously mentioned two groups (according to species tree and supernetwork) can be found as well. Additionally, in concordance to other results, the distances within the 'Group 2' and therefore the relationships among different taxa seems to be much closer when compared to relationships between taxa from the 'Group 1'. Clustering results of Structure which distinguished two groups within *Leucanthemum* are as well in agreement with previously mentioned groups (according to species tree and supernetwork) (**Figure 6**). Here, the border between two clusters is not strict and posterior probability for cluster membership never reaches 100%. The species/accessions which are belonging to the green cluster are in minority while most accessions have contribution of the red cluster which is increasing until reaching the maximum within the species forming the second group.

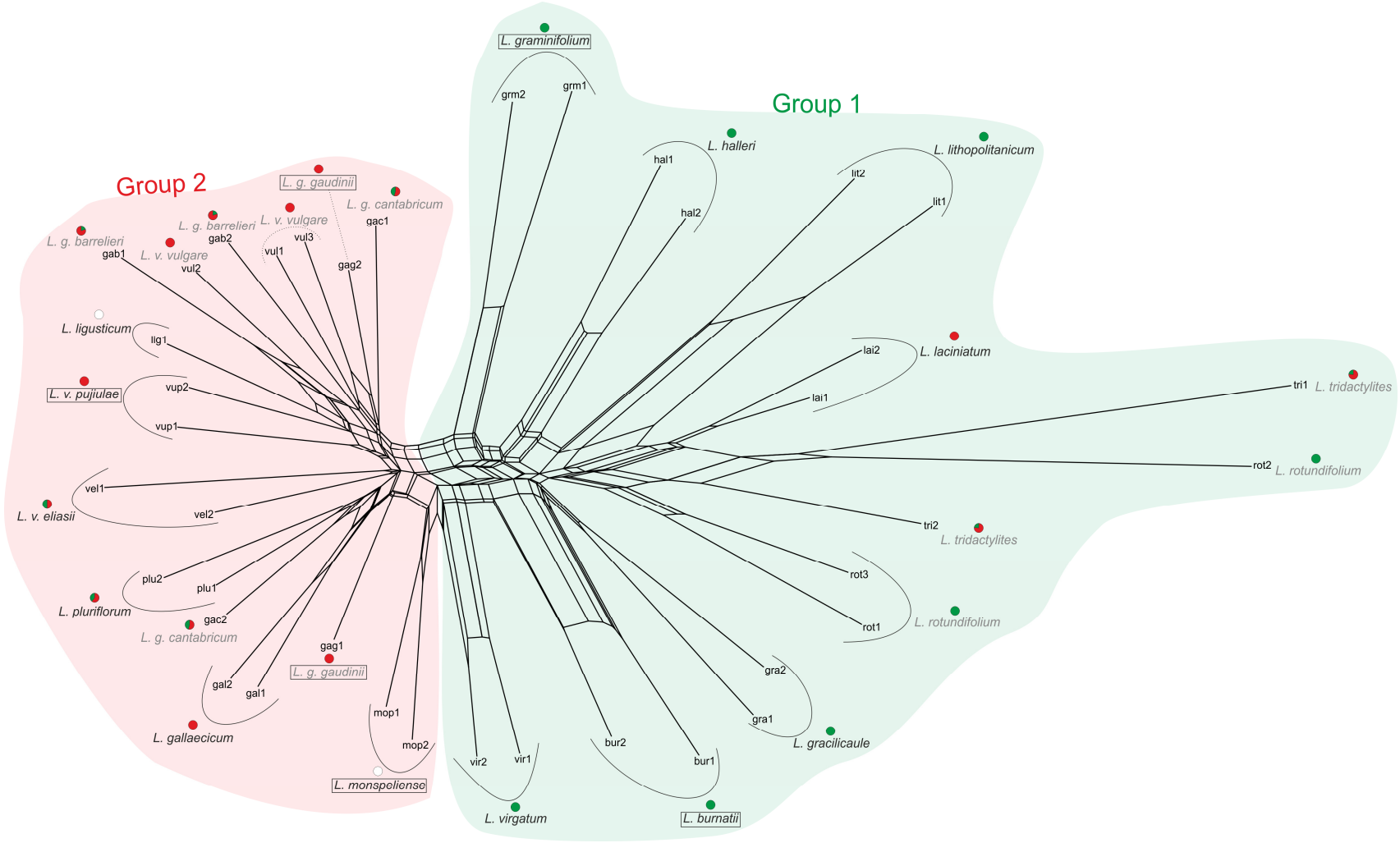

Figure 4 - Network obtained from AFLP data using Nei-Li distances. Species names in grey belong to taxa which failed to form a monophyletic group and their accessions are placed separately. Species enclosed in a rectangle had significantly lower values of hybrid index obtained from real gene trees over hybrid indexes obtained from simulated trees, thus were specified as "non-hybrid" taxa. The coloured circle above species name indicates membership to one of the nrDNA ETS ribotype groups (green, red or mixed) from Oberprieler et al. (2014). *L. monspeliense* and *L. ligusticum* were not sampled by that study.

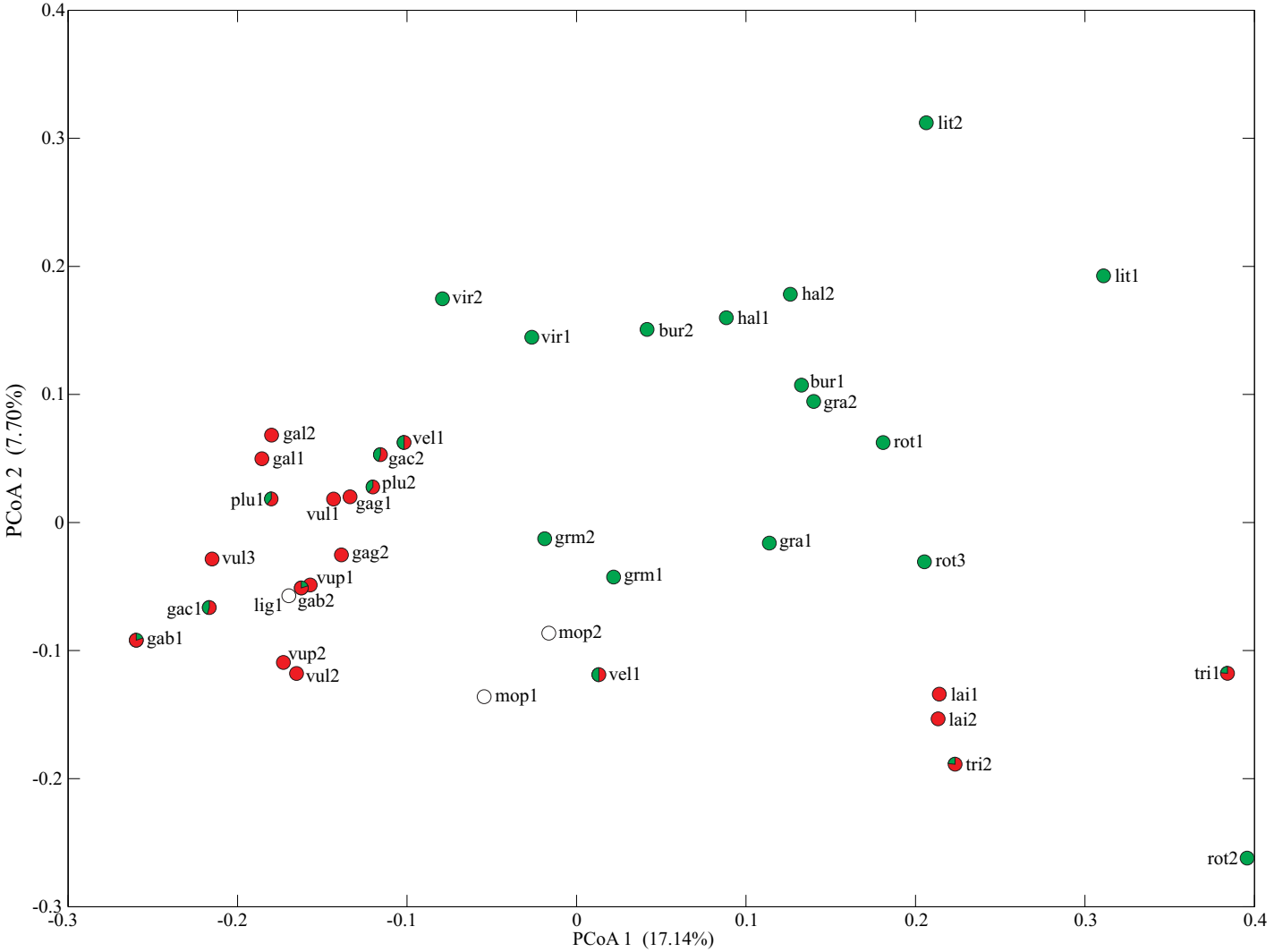

Figure 5 - PCoA graph using Bray-Curtis dissimilarity on the AFLP data. The coloured circle before species name indicates membership to one of the nrDNA ETS ribotype groups (green, red or mixed) from Oberprieler et al. (2014). *L. monspeliense* and *L. ligusticum* were not sampled by that study. The first axis explain 17.1% of variation and the second axis explain 7.7% of variation observable within the dataset.

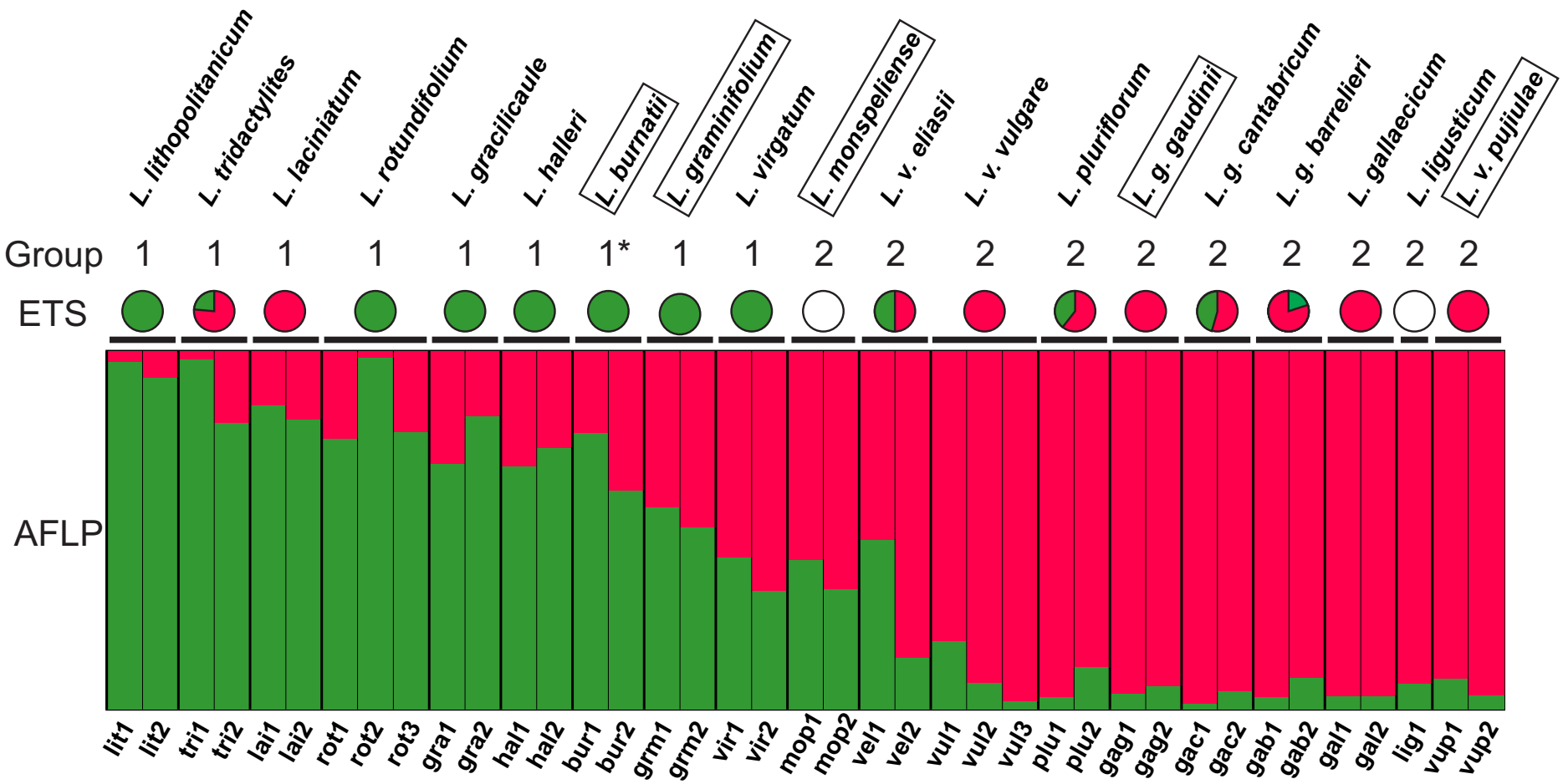

Figure 6 - Clustering performed by Structure on AFLP data. The bars in the bottom represent cluster membership inferred by Structure. Individuals are arranged species-wise according to their percentile cluster membership. Species enclosed in a rectangle had significantly lower values of hybrid index obtained from real gene trees over hybrid indexes obtained from simulated trees, thus were specified as "non-hybrid" taxa. The number below species name indicates membership to either first or the second group mentioned in this study (see discussion). The coloured circle below indicates membership to one of the nrDNA ETS ribotype groups (green, red or mixed) from Oberprieler et al. (2014). *L. monspeliense* and *L. ligusticum* were not sampled by that study.

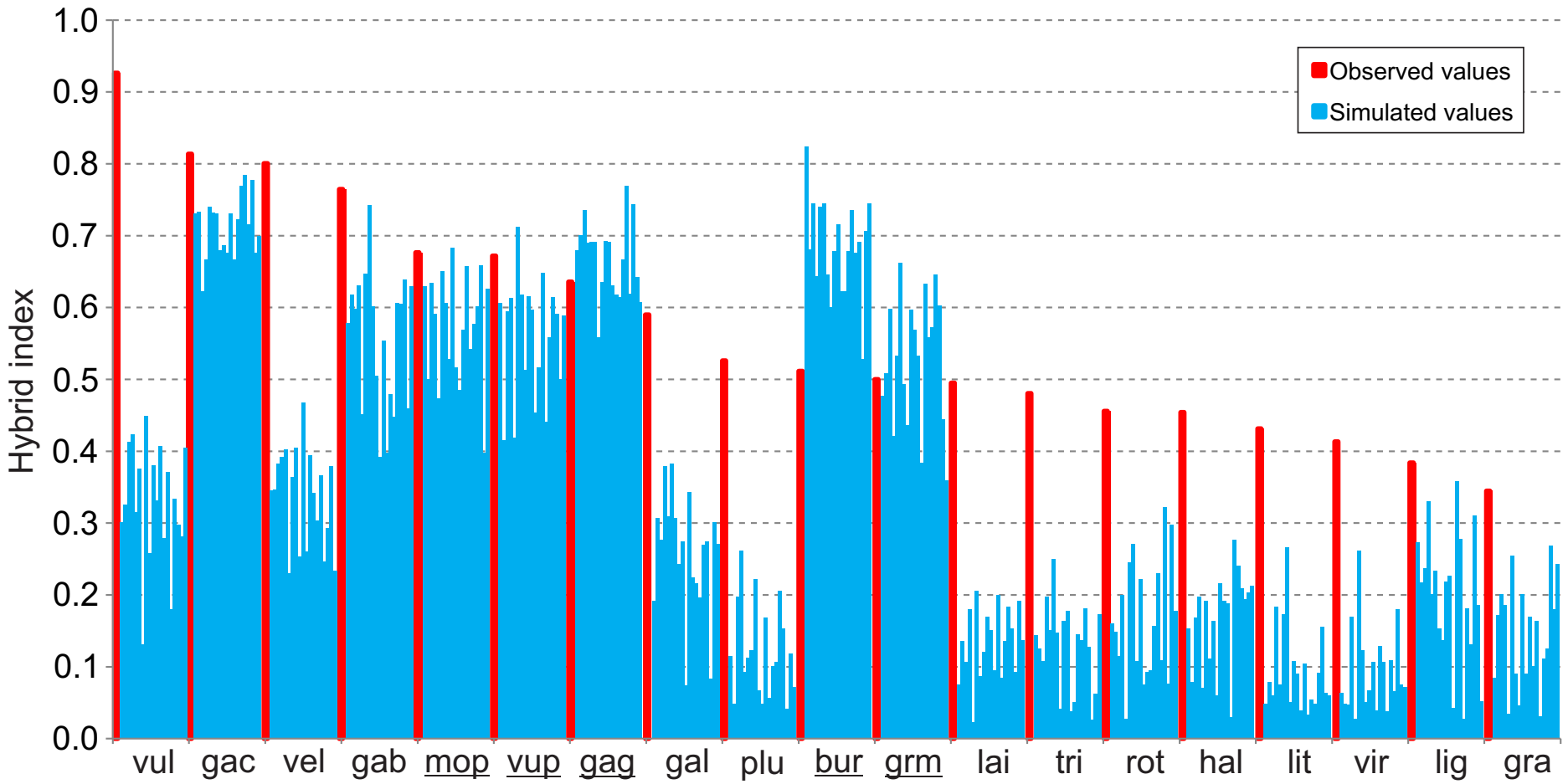

Figure 7 - Hybrid index values. The red bar is a hybrid index obtained from real gene trees and blue bars represent hybrid index values obtained from gene trees simulated using only incomplete lineage sorting (ILS) influence. The species in which hybrid index from real data is lower than hybrid index from simulated data are enclosed in a rectangle. Those five taxa are assumed to have a "non-hybrid" history contrary to the remaining fourteen taxa (see discussion).

Hybrid index – The results of the hybrid index computations are displayed in a graph showing the taxon specific hybrid index compared to 19 simulations based solely on incomplete lineage sorting simulated on the chloroplast tree (**Figure 7**). In 14 out of the 19 diploid *Leucanthemum* species the hybrid index value based on the true gene trees was found to be the highest among the 20 hybrid index values found. Only five taxa (i.e., *L. burnatii*, *L. monspeliense*, *L. gaudinii* subsp. *gaudinii*, *L. graminifolium*, and *L. vulgare* subsp. *pujiulae*) received hybrid index values comparable or lower to those from the simulated gene trees.

2.5. Discussion

Phylogeny of Leucanthemum *and homoploid hybrid speciation*

Present paper is the first attempt to analyze the genus *Leucanthemum* from phylogenetic point of view using species tree methods and complete sampling of all currently recognized diploid taxa. Single copy genes for Compositae (Chapman et al. 2007) as compared to other studies reporting universal single-copy genes (Strand et al. 1997, Steele et al. 2008) were superior by obtained quality (e.g. variability, length) and number of markers available for screening. Although as it turned out, even large screening could not find markers with divergence level sufficient to distinguish all species with significant posterior probability. Similar results were obtained by Oberprieler et al. (2014) who used nrDNA ETS and by Hößl (unpublished) which used nrDNA ITS, in both studies only few clades were supported and general resolution was low. Supposedly this case may be more general and characteristic for evolutionary young group possibly affected by such processes as hybridization. The most supported split occurs between so called here 'Group 1' and 'Group 2' ('Group 1' contains: *L. burnatii*[*], *L. gracilicaule*, *L. graminifolium*, *L. halleri*, *L. laciniatum*, *L. lithopolitanicum*, *L. rotundifolium L. tridactylites* and *L. virgatum*, 'Group 2' contains: *L. gallaecicum*, *L. gaudinii* subsp. *barrelieri*, *L. gaudinii* subsp. *cantabricum*, *L. gaudinii* subsp. *gaudinii*, *L. ligusticum*, *L. monspeliense*, *L. pluriflorum*, *L. vulgare* subsp. *eliasii*, *L. vulgare* subsp. *pujiulae* and *L. vulgare* subsp. *vulgare*). This split is visible in all analyses currently used, namely species tree and consensus network constructed from low copy nuclear genes as well as in the network, PCoA and clustering of AFLP loci. Membership in this groups is stable and always include the same set of taxa. Minor exception may be *L. burnatii* which in species tree reconstruction was placed in the 'Group 2' but even there it is located as a basal species and in this regard may be treated as intermediate between two groups. Additionally these

two groups correspond very well with the ETS types found by Oberprieler et al. (2014) where authors using cloning of nrDNA ETS reconstructed phylogenetic history of *Leucanthemum* with focus on NW-Iberian taxa. The result of that study suggested a split into two groups corresponding almost exactly to present results. Noticeable difference is treating *L. laciniatum* and *L. tridactylites* here as members of the 'Group 1' while in study of Oberprieler et al. (2014) they belonged to the red and the red/green ETS-ribotype which corresponds rather to the 'Group 2' in the present study. Based on this incongruence, the position of this two taxa is interesting because either: 1) red ETS-ribotype was independently formed two times in *L. laciniatum* and founder of the 'Group 2', 2) red ribotype appeared in *L. laciniatum* and trough hybridization with species possessing green ribotype gave rise to the whole 'Group 2' or 3) red ribotype was gained in *L. laciniatum* and *L. tridactylites* trough hybridization with a species from the 'Group 2' and this gain was followed by complete disappearance of green ribotype in *L. laciniatum* and partial disaperance in *L. tridactylites*. From those scenarios, the second solution would be the most parsimonious though it is unknown whether and how species endemic to the southern Apennine Peninsula could cross the whole peninsula and hybridize with other species later giving raise to many species occurring throughout the whole Europe. But it has to be noted as well that it is not completely irrelevant since its current endemic distribution is likely influenced by climatic oscillations and may have refugial character. If *L. tridactylites* is viewed as a hybrid between *L. laciniatum* (which always forms a monophyletic group with it) and another ("green", 'Group 1') species, it may suggest that range of *L. laciniatum* was indeed larger than presently.

View on different results gives impression that relationships among taxa are rather weakly supported and change depending on the method. It is likely a problem related to genetic markers used and samples itself which are under influence of hybridization and possibly recent speciation which are hard to analyze with presently available methods. AFLP network analysis which is based on unmerged populations (i.e. each accession is analyzed separately) further indicates that a few taxa failed to form monophyletic groups. These are *L. tridactylies*, *L. rotundifolium*, *L. gaudinii* subsp. *gaudinii*, *L. gaudinii* subsp. *barrelieri*, *L. gaudinii* subsp. *cantabricum*, *L. vulgare* subsp. *vulgare*. In case of *L. tridactylies* and *L. rotundifolium* this result may be artificial since populations tri2 (L151) and rot2 (L989) are poorly preserved herbarium specimens and seem to be outliers as distance from the other taxa is much bigger than compared to distances within the whole network. Removal of those two accessions changed neither the connections nor the structure of the network (results not shown) but after such deletion these two species would

become monophyletic. Stronger incongruence appears among other species and they are all placed within close relationship in the 'Group 2'. Here taxonomic error may be also applicable either by misclassification of individuals, over-estimation of species number or sampling cryptic species, although all would produce the same output. Another possible explanation would be the recent age or excessive hybridization among those taxa which confounds their independence as a separate entity (incomplete split and occurring gene flow or merging of the species trough hybridization). Taxonomy of this group should be refined anyway since the assignment to subspecies does not follow phylogenetic result especially in case of different subspecies in *L. vulgare* and *L. gaudinii*.

The excess of hybridization is highlighted in genus *Leucanthemum* since 14 out of 19 taxa (74%) are shown to have higher hybrid index in real gene trees than in those from simulations. As a consequence incomplete lineage sorting (ILS) alone is not enough to explain gene tree incongruence. This indicates that likely hybridization on the diploid level played an important role in the evolutionary history of the genus. However rather than independent multiple formation of hybrid taxa in recent time, this pattern could by influenced by homoploid hybrid origin of common ancestors of diploid taxon clades followed by preserving hybrid signal in their genome. The latter argument could be applicable especially to some of the closely related species forming clades placed within geographically close range as for example species from northern Iberian Peninsula (*L. gaudinii* subsp. *barrelieri*, *L. gaudinii* subsp. *cantabricum*, *L. pluriflorum*, *L. vulgare* subsp. *eliasii*). Except *L. vulgare* subsp. *eliasii* all this taxa are morphologically similar, their distribution is also somehow linear ranging from north-western coast of Iberian Peninsula through Cantabrian Mountains to central Pyrenees. In the species tree they cluster together with participance of one geographically close species (*L. gallaecicum*). In the study of Oberprieler et al. (2014) they also posses common characteristic of sharing mixed ETS ribotypes which is uncommon in the genus and occurs only in one more species (*L. tridactylites* from central Apennines). Similar situation occurred also in *Helianthus* when at least three species are of independent hybrid origin and share the same parental taxa (Rieseberg 1991). At least one of them (*H. deserticola*) was formed multiple times either by repeated hybridization or by backcrossing to parental species (Gross et al. 2003). If such situation also occurred in *Leucanthemum* it could produce similar image to the one from the present study. Finally, although morphological variation is common within many (if not all) plant species in some cases when it is extreme it may support hybrid origin of the species. Abbott et al. (2010) argued that morphological variability within homoploid hybrid *Senecio squalidus* is very high and reflects its recent

hybridogenous origin. Variability seen in many *Leucanthemum* species reflected in description of many subspecies and varieties (e.g. Briquet & Cavillier 1906, Piękoś 1971) could also indirectly support incidence of that process. The results suggesting the importance of hybridization within *Leucanthemum* are also congruent with the timeframe of its origin and diversification which seems to be influenced firstly by Pre-Pleistocene events (separation of *Leucanthemum* and sister genera ca. 7.9 – 4.0 Mya), then followed by Pleistocene events especially linked with range expansions and contractions during glaciations (divergence within *Leucanthemum* ca. 3.1 – 1.4 Mya) (Hößl 2006). In this background hybridization of many species would be an obvious consequence as it is assumed that species during multiple migrations were brought into contact and then separated. If hybridization occurs in such environment, it leads to gene flow which may be followed by homoploid hybrid speciation since this range changes and migrations coupled with local survival of a hybrid provide an excellent opportunity for the spatial isolation which highly influences successful establishment (Buerkle et al. 2000). Moreover climatic changes may provide opportunity for natural selection of new phenotypic traits in the newly arisen hybrid and help its establishment (Mallet 2007).

Furthermore, Oberprieler et al. (2014) hypothesized about homoploid hybrid speciation of taxa possessing two ribotypes of nrDNA ETS. In the present paper, the amount of homoploid hybrid species includes those five taxa but is even more drastic supposing hybrid origin or hybrid history of additional nine taxa, ranging to fourteen (out of nineteen) diploid species. Our results highlight the importance of hybrid speciation (and/or gene flow) on diploid level as it may be visible in majority of presently recognized species within *Leucanthemum*.

Five taxa received hybrid index values comparable or lower than those from the simulated gene trees (*L. burnatii*, *L. monspeliense*, *L. gaudinii* subsp. *gaudinii*, *L. graminifolium*, and *L. vulgare* subsp. *pujiulae*). They are therefore much less influenced by hybridization than remaining taxa and ILS is sufficient to explain their history. In this way they may be interpreted as descendants from ancestral species in which speciation was driven by mechanisms other than hybridization. They are however not reconstructed in the base of the *Leuanthemum* species tree which suggest that hybridization occurred since the genus came into existence.

It has to be noted that the method used in this study may also have some limitations as for example is not sure whether ILS and therefore the age of the species could contribute to the hybrid index. Sometimes it may be also hard to draw a line between speciation, gene flow and hybridization when one species is splitting into two with still occurring

occasional exchange of genetic material. Gene flow may be even more pronounced in such closely related taxa since it was shown that many *Leucanthemum* species are easy to cross with each other including crossings among diploids, diploids-polyploids and among polyploids on different ploidy levels (Villard 1971, Greiner & Oberprieler 2012). Potentially gene flow could also occur from polyploids to diploids. Although formation of interploidy crosses is rather rare in natural conditions in *Leucanthemum* (Greiner & Oberprieler 2012) and in general – especially when gene flow from triploids to diploids is considered (Petit et al. 1999), it can be assumed as a possible way since even low hybrid formation rates could be a source for the rapid spread of advantageous alleles. Lastly a hybrid index may be influenced not only by hybridization itself but by ancestor traces which may be still present in progeny even if they are a different entity from the parental species as discussed before in the example of Northern Iberian taxa. Another factor which could possibly affect the analysis is the taxonomical treatment used. As relationships between different accessions of one species are not always monophyletic but when considered separately it may suggest that some taxa need to be studied in more detail from the taxonomical point of view.

Software & method discussion

Remarkably although many studies with focus on homoploid hybrid speciation were done as shown in the introduction, almost none of them repeated methods used in previous ones and rather tried to find a new method for analyzing similar processes. This indicates that it is an active field of research but also that it is still in progress and no consensus and widely accepted method or software is available. The great advantage of presently used method is that it does not rely on user assumptions (as specification of potential hybrids or parental taxa) but takes into account only information provided in the data. Moreover, it has no restrictions considering unequal sampling or different allele number even within same species or accession. A step forward would be to analyze the dataset with the possibility of detecting not only hybrid index but also putative parental species of a hybrid so that a complete history could be inferred. But before such an attempt is made, it requires more computational and technical progress. Perhaps one of the emerging possibilities is usage of RAD sequencing which can contribute thousands of loci and has been recently used in the analysis of gene flow between closely related species (Eaton & Ree 2013).

Some of computational difficulties were associated with the nature of the data and especially with multiple alleles. This state of data (homozygosity and heterozygosity) in diploid organism is expected but most of the programs devoted to species tree analysis do

not allow such possibility. This leads to manipulation of the data (e.g. as in Blanco-Pastor et al. 2012 - deleting randomly one allele) or to excessive screening aiming to find single copy genes only in homozygotic phase alternatively amplifying only one of the alleles. To the best knowledge of authors, only PhyloNet used in this study and MP-EST (Liu et al. 2010, Shaw et al. 2013) have required abilities that enable user to analyze datasets with unequal number of samples or alleles among different gene trees. In the author's view some of those hindrances or difficulties associated with different software are not only computational but may have arisen because scientists are trying to solve problems without applying biological knowledge e.g. commonly used as an example 4 species phylogenetic tree is rarely addressed by any serious biological paper. Lastly, analyses like this one could benefit from the development of methods and software addressing recombination in the data. Although Lanier & Knowles (2012) concluded that this problem is of relatively small importance to species tree precision, it may still affect the accuracy and this influence may be presumably higher in reconstructions dealing with hybridization. Certainly other studies as Kelly et al. (2010) raised this problem already and even used recombination for discrimination of hybrid species within the dataset.

Conclusions and prospects

Among advantages of the new method are possibilities of analyzing large datasets without limiting factors such as number of considered species, number of accessions or number of alleles. Basing on the results it is apparent that influence of homoploid hybrid speciation had a huge impact on the history of *Leucanthemum*.

Importance of hybridization is especially highlighted in this paper since it seems to have a strong influence on the genus under study. Homoploid hybrid speciation was not reported in any other system in such abundance up to now but this may be likely linked to the emerging methods capable of analyzing this phenomenon along with other processes. As technology is continuously updated, future will likely show even more examples like this one and provide us the opportunity to accurately test the impact of hybridization on speciation and evolution.

3. CHAPTER 2

Phylogenetic patterns in *Leucanthemum* Mill. (Compositae, Anthemideae)

This chapter is designed for publication with following authorship: Kamil Konowalik, Robert Vogt, Christoph Oberprieler. Author contributions are as follows: study conception and design: CO, RV, KK; laboratory work: KK; determination and collection of specimens: RV, CO, KK; writing: KK; analysis of the data: KK.

3.1. Abstract:

The present Chapter addresses the phylogeny of the genus *Leucanthemum*, including diploids together with polyploids, and samples from almost the whole genus with the majority of species sampled from two accessions. The aim of the study is to resolve phylogenetic patterns in the genus using the network approach. These methods include amplification of low-copy nuclear genes, 454 sequencing and gene tree estimation with the Bayesian approach. As the reconstruction is challenging and complicated, mainly due to the lack of suitable analytical methods, the results are presented in the form of a supernetwork which is constructed from multi-labeled trees coded as a 0/1 matrix. In addition to the supernetwork, a pairwise similarity matrix is constructed from the same 0/1 matrix in order to present similarities among particular taxa more explicitly. The phylogenetic resolution of the supernetwork is low and presents many reticulations which hamper detailed analysis. However, it is apparent that many taxa cluster according to their geographical origin and sometimes also ploidy. This is, therefore, suggestive of the strong influence of geography on the evolution of *Leucanthemum* in general and on the emergence and evolution of polyploids in particular. Division of the genus into provisional species groups is proposed and groups which received bootstrap support are discussed in more detail.

3.2. Introduction

Phylogenetic reconstructions in polyploid complexes

Polyploids are included in reconstructions of phylogenies in many plant genera, but except for autopolyploids their origin cannot be straightforwardly reconstructed as a bifurcating tree due to their formation process, i.e. allopolyploids are formed by two distinct species inheriting two divergent genomes, and their history is best represented by a network linking parental species and their descendent species. Before methods constructing networks were available, attempts to infer the origin of polyploids were realized by comparing nuclear - plastidial phylogenies (e.g. Harris & Ingram 1992), and, with some success, this is also applied nowadays (e.g. Li et al. 2014). The next steps were not only to compare two trees, but also to try to form a network and propose a coherent scenario based on the set of initial trees. First inferences were probably made by phylogenetic reconstructions for the genera *Silene* (Popp et al. 2005) and *Cerastium* (Brysting et al. 2007). More recently, the polyploid phylogenies of *Viola* and *Hordeum* have been reconstructed using low-copy nuclear genes, cpDNA and ITS (Marcussen et al.

2011 or Brassac et al. 2012) but the weakness of these approaches is their inability to represent the true phylogeny because of such processes as the incomplete lineage sorting (ILS) visible after including several low-copy nuclear markers. To reconstruct a more reliable phylogeny in consideration of ILS more genes are necessary and the optimal number of genes correlates with diversification rates and population sizes – the younger the group and bigger the populations, the more intense ILS may be present within the dataset (Maddison 1997, Degnan & Rosenberg 2009). Approaches which include phylogenetic reconstructions for polyploids constructed with the use of at least a few single- or low-copy nuclear genes are still infrequent. This situation is linked to their availability – which may be solved by the use of published putative single copy genes (e.g. Wu et al. 2006, Chapman et al. 2007) or by obtaining them with next-generation sequencing (e.g. Lemmon & Lemmon 2012) – and more importantly by using methods devoted to the joint analysis of polyploidy and ILS which are still rare. This is not only because of the additivity of the genomes but also because of their merging (hybridization) which is a part of allopolyploidization. One recent approach based on Bayesian statistics is the method published by Jones et al. (2013), but it is currently limited to the tetraploid level. It is based on a popular tree inference program, BEAST (Drummond et al. 2012) and uses the calculation of multi-labeled species trees prior to the network reconstruction. The main difficulty in extending this method to higher ploidies is that it must simultaneously reconstruct putative polyploid hybrids (e.g. 4*x*, 6*x*) and then allow those taxa to hybridize with others in the dataset, i.e. "allowing hybrids to hybridize" (B. Oxelman, pers. comm.). Another two approaches proposed by Yu et al. (2013) and Park & Nakhleh (2012) (based on maximum parsimony and maximum likelihood, respectively) are limited to small systems due to the high memory requirements for computations; however, they have no restrictions associated with the ploidy.

One newly described method presented by Tomasello et al. (in prep.) has proved to be accurate and reliable in reconstructing polyploidy within the genus *Leucanthemopsis* which is closely related to the genus under study. This method utilizes information from gene trees to construct a species network. In the first step, different alleles of the same accession within one gene tree are assigned to the hypothetical parental genomes which are inferred as the species tree with the lowest parsimony score. The next step involves combining those alleles across gene trees, which is then used to infer a multi-labeled species tree from which a species network may be constructed (Tomasello et al. in prep.). Since it is based on a MDC algorithm which eliminates ILS noise from the dataset, this method is robust in detecting hybridization (polyploidization) and also discriminates

allopolyploidy from autopolyploidy. The limitation to the method is connected with those ploidies higher than hexaploid (6*x*) or even tetraploid (4*x*) which are computationally very intensive and their calculation, even on a supercomputer, could take several years (unpublished data). Apart from programs specifically devoted to dealing with the polyploids in phylogenetic reconstruction, numerous researchers have tried to infer polyploid history by employing combinations of multiple existing techniques and their own, often unique, ideas. Examples may include *Solanum* (Cai et al. 2012), *Artemisia* (Richardson et al. 2012), *Medicago* (Maureira-Butler et al. 2008), *Cortaderia* (Pirie et al. 2009) or *Polystachya* (Russell et al. 2010). Although these methods are interesting and present approaches based on original ideas, they usually lack any simulation test and their accuracy is not proved. Moreover, they frequently involve excessive amounts of manual work which could easily be conducted by a computer program. Because of the mentioned difficulties in the use of the specific programs and the manual methods, in the present contribution a supernetwork approach is used which, in comparison to the above methods, provides a relatively simple way to analyze polyploid history. In some respects, this method has already been used in studies on hybridization and/or polyploids (e.g. McBreen & Lockhart 2006, Holland et al. 2008, Russell et al. 2010, Chapter 1). It uses gene trees and coding of the presence/absence of the taxon in a certain node of the tree to construct a 0/1 matrix which is then the basis for the network construction (for a detailed description, see Chapter 1). Its main advantage when reconstructing the polyploid history is the treatment of multiple alleles coming from one species. Because the number of alleles for certain accessions varies between gene trees, this also affects the number of alleles for certain species. This method is convenient to use because it works on a species level which alleviates the problem of the assignment of different alleles to different parental genomes by summarizing all alleles and accessions into a consensus matrix which retains information only on the species level (**Figure 8**).

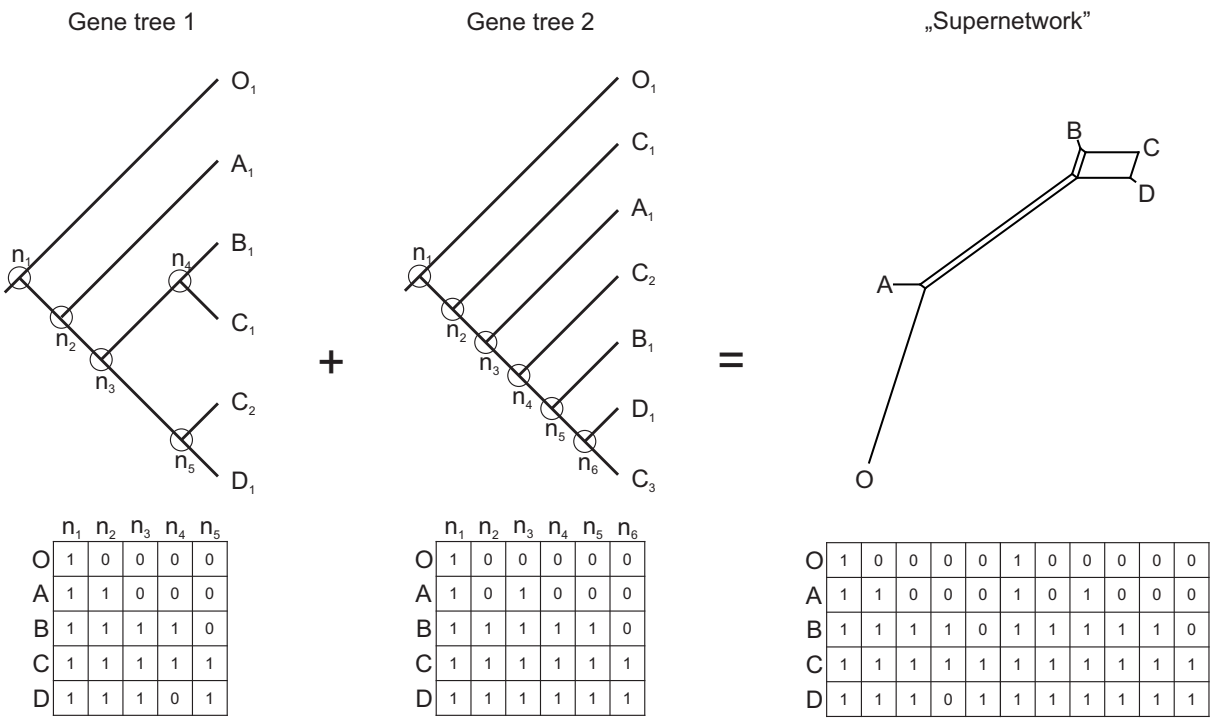

Figure 8 - Explanation of supernetwork construction. Letters "O", "A", "B", "C", "D" denote species, subscipt may denote allele or accession number, and n_x denote nodes in the gene trees. In this example two gene trees are coded into 0/1 matrix (corresponding matrix is located below the gene tree) – presence of species in the certain node is coded as 1, while absence is coded as 0. In the resulting matrix particular alleles or accessions are merged when they belong to one species. In the final step 0/1 matrices are merged into one supermatrix which is then used as an input for the network constructing algorithm in SplitsTree (distances are calculated using Jaccard coefficent distance, network is computed with Neighbour-Net network algorithm using ordinary least squares (Bryant & Moulton 2004) and drawn with equal angle algorithm (Dress & Huson 2004) and box-opening optimization (Gambette & Huson 2008); Huson & Bryant 2006).

Aims

The genus *Leucanthemum* Mill. is ideal for studying polyploidy, since it consists of an unbroken polyploid chain up to dokosaploid level ($2n = 22x = 198$). Study done on cpDNA (Greiner et al. 2012) has suggested that most of the polyploid species fall into one haplogroup and are related to each other via only one diploid species (*L. virgatum*) which is nowadays endemic to the Maritime Alps. In many of the previous studies *Leucanthemum* polyploids were assumed to originate via allopolyploidy (Faverger 1960, Pearson 1967, Vogt 1991, Oberprieler et al. 2011, Greiner 2011, Greiner et al. 2013, Oberprieler et al. 2014), but so far no phylogenetic study has been conducted on the whole genus. Available studies on the diploids (Chapter 1) indicate that the history of the genus on the diploid level may be shaped by such evolutionary processes as homoploid hybrid speciation which contributes significantly to the reticulate history of the whole genus. The robust division into the two groups is visible among diploids in different analyses (cf. Chapter 1), but their specific relationships are poorly resolved which hampers the phylogenetic inference for polyploids. As shown by Oberprieler et al. (2014), polyploid species may combine parents

from both groups either directly or by originating from species which already possessed traces of both groups (i.e. presumed hybrid species).

The sample set in this contribution covers nearly all currently accepted taxa of *Leucanthemum* with the aim of bringing new insights into the evolution of the genus with special emphasis on the polyploids. This is based on low-copy nuclear genes sequenced using 454 sequencing capturing all their alleles, which are then used to compute gene trees and finally a species network. Since the species network presents a high number of reticulations which confound its interpretation, the species specific pairwise similarity matrix is presented to view the obtained information more explicitly for particular taxa.

3.3. Material and methods

Sampling – Accessions of 36 taxa from the majority of the currently accepted polyploid species (Euro+Med PlantBase, 2006) were included (only *L. cuneifolium* from SW Alps and *L. rohlenae* from Montenegro are missing). In general, one to four samples per species were incorporated per taxon, which gave a total of 69 samples (**Table 10, Figure 9**). Preferably, recently collected material conserved in silica gel was used, but in cases when the collection of fresh material was not possible, material from herbarium specimens was included. The ploidy of all silica dried samples was confirmed with flow cytometry and, when possible, by chromosome counting (by Dr. Robert Vogt, Botanic Garden & Botanical Museum Berlin-Dahlem, Freie Universität Berlin). The CTAB DNA extraction protocol followed Doyle & Doyle (1987). All DNA extracts were diluted 1/10 or 1/100 prior to PCR reactions.

Species	Ploidy	Sample shortname	Internal sample name	Collection site	Coordinates	Collector	Herbarium	Voucher
Leucanthemum adustum (Koch) Gremli subsp. *adustum*	6*x*	adu1	L044	DE, Bayern, Schönhofen, 390 m	49.00 N, 11.95 E	Eder & Oberprieler s.n.	REG	-
Leucanthemum adustum (Koch) Gremli subsp. *adustum*	6*x*	adu2	L057	DE, Bayern, Garmisch-Partenkirchen, 1369 m	47.42 N, 11.03 E	Eder & Oberprieler 10301	REG	-
Leucanthemum adustum subsp. *margaritae* (Jáv.) Holub	6*x*	ama1	L007	AT, Niederösterreich, Perchtoldsdorf, 280 m	48.12 N, 16.27 E	Schuhwerk 90/1108	B, VOGT	B 10 0416505
Leucanthemum adustum subsp. *margaritae* (Jáv.) Holub	6*x*	ama2	187-1	BA, Republika Srpska, Bojišta, 1341 m	43.29 N, 18.08 E	Vogt 16816 & Prem-Vogt	B, VOGT	B 10 0346610
Leucanthemum aligulatum Vogt	6*x*	ali1	78-1	ES, Aragón, Valle de Bardají, 782 m	42.46 N, 00.04 E	Höfl 78	B	B 10 0413728
Leucanthemum aligulatum Vogt	6*x*	ali2	M64-1	ES, Castilla-La Mancha, Riópar, 1100 m	38.48 N, 02.44 W	Cordel s.n.	B	B 10 0345018
Leucanthemum atratum (Jacq.) DC. subsp. *atratum*	6*x*	atr1	L1006	AT, Niederösterreich, Ybbs, 1500-1600 m	47.78 N, 14.81 E	Polatschek s.n.	W	W 1986-06926
Leucanthemum atratum (Jacq.) DC. subsp. *atratum*	6*x*	atr2	L1007	AT, Steiermark, Rax, 1500 m	47.69 N, 15.71 E	Ehrendorfer s.n.	W	W 1967-9780
Leucanthemum catalaunicum Vogt	10*x*	cat1	153-1	ES, Catalunya, Greixer, 1430 m	42.28 N, 01.88 E	Konowalik KK61 & Ogrodowczyk	B, REG, WRSL	B 10 0386726
Leucanthemum catalaunicum Vogt	10*x*	cat2	146-1	ES, Catalunya, Setcases, 2060 m	42.42 N, 02.28 E	Konowalik KK53 & Ogrodowczyk	B, REG, WRSL	B 10 0386709
Leucanthemum chloroticum Kern. & Murb.	6*x*	chl1	171-1	ME, Herceg Novi, Orjen, 1580-1590 m	42.56 N, 18.55 E	Vogt 16754 & Prem-Vogt	B	B 10 0346638
Leucanthemum chloroticum Kern. & Murb.	4*x*	chl2	189-1	BA, Republika Srpska, Bojišta, 1430-1460 m	43.29 N, 18.08 E	Vogt 16828 & Prem-Vogt	B, VOGT	B 10 0346609
Leucanthemum coronopifolium subsp. *ceratophylloides* (All.) Vogt & Greuter	6*x*	coc1	217-1	IT, Piemonte, Prov. Cuneo, Entrácque, 1544 m	44.18 N, 07.47 E	Vogt 16880 & Oberprieler 10790	B, VOGT	B 10 0411731
Leucanthemum coronopifolium subsp. *ceratophylloides* (All.) Vogt & Greuter	6*x*	coc2	238-1	IT, Liguria, Melosa, 1910 m	44.00 N, 07.67 E	Vogt 16915 & Oberprieler 10822	B, VOGT	B 10 0350153
Leucanthemum coronopifolium subsp. *tenuifolium* (Guss.) Vogt & Greuter	6*x*	cot1	279-1	IT, Abruzzo, Scanno, 1493 m	41.86 N, 13.91 E	Tomasello TS419	B	-
Leucanthemum coronopifolium subsp. *tenuifolium* (Guss.) Vogt & Greuter	6*x*	cot2	L1005	IT, Abruzzo, Scanno, 1200 m	41.88 N, 13.89 E	Pavesi, Zucconi & Millozza s.n.	VOGT	-
Leucanthemum coronopifolium Vill. subsp. *coronopifolium*	6*x*	cor1	207-1	IT, Piemonte, Ferrere, 2614 m	44.36 N, 06.90 E	Tomasello TS39	B	B 10 0386670
Leucanthemum coronopifolium Vill. subsp. *coronopifolium*	6*x*	cor2	204-1	IT, Piemonte, Usseaux, ca. 2000 m	45.07 N, 07.05 E	Tomasello TS9	B	B 10 0386674
Leucanthemum corsicum subsp. *corsicum* (Less.) DC.	4*x*	cos1	268-1	FR, Corsica, Pietra Niella, 1849 m	42.07 N, 09.14 E	Tomasello TS410	B	-
Leucanthemum corsicum subsp. *fenzlii* Gamisans	4*x*	cof1	269-1	FR, Corsica, La Foce, 1693 m	42.13 N, 09.09 E	Tomasello TS411	B	B 10 0458571
Leucanthemum delarbrei Timb.-Lagr.	4*x*	del1	L075	FR, Auvergne, Ferval, 1650-1750 m	45.05 N, 02.72 E	Lippert 24073 & Grenier	M, B	B 10 0420785
Leucanthemum delarbrei Timb.-Lagr.	4*x*	del2	L174	FR, Auvergne, Rombière, 1550-1640 m	45.08 N, 02.70 E	Lippert 23985 & Grenier	B, VOGT	B 10 0420852
Leucanthemum favargeri Vogt	8*x*	fav1	L076	ES, Navarra, Petilla de Aragón, ca. 900 m	42.45 N, 01.10 W	Aizpuru, Catalan & Pedrol 3872	B, VOGT	B 10 0416519
Leucanthemum favargeri Vogt	8*x*	fav2	74-1	ES, Aragón, Jaca, 1980 m	42.53 N, 00.56 W	Höfl 74 & Himmelreich	B	B 10 0413732
Leucanthemum glaucophyllum (Briq. & Cavill.) Jahand.	10*x*	gla1	253-1	IT, Liguria, Onzo, 1044 m	44.10 N, 08.06 E	Vogt 16935 & Oberprieler 10842	B, VOGT	B 10 0350175
Leucanthemum glaucophyllum (Briq. & Cavill.) Jahand. var. *esterellense* Briq. & Cavill.	8*x*	est1	227-1	FR, Provence-Alpes-Côte d´Azur, Agay, 18 m	43.45 N, 06.85 E	Vogt 16897 & Oberprieler 10807	B, VOGT	B 10 0411749
Leucanthemum heterophyllum (Willd.) DC.	8*x*	het1	L001	IT, Veneto, Passo Pordoi, 2211 m	46.48 N, 11.82 E	Vogt 6521, Hellwig, Oberprieler & Prem	B, VOGT	B 10 0416512
Leucanthemum heterophyllum (Willd.) DC.	8*x*	het2	L187	HR, Istria, Mala Učka, 1350 m	45.28 N, 14.20 E	Vogt 16068	B, VOGT	B 10 0420787
Leucanthemum illyricum (Horvatić) Vogt & Greuter	8*x*	ill1	200-1	ME, Žabljak, Durmitor, 1840 m	43.10 N, 19.05 E	Vogt 16861 & Prem-Vogt	B, VOGT	B 10 0346619
Leucanthemum illyricum (Horvatić) Vogt & Greuter	8*x*	ill2	318-1	BA, Federacija Bosne i Hercegovine, Mladeškovići, 890 m	43.57 N, 17.93 E	Vogt 17062 & Prem-Vogt	B, VOGT	B 10 0350205
Leucanthemum ircutianum DC. subsp. *asperulum* (Terr.) Vogt	4*x*	ias1	L183	IT, Calabria, Castrovillari, 800-1000 m	39.89 N, 16.16 E	Vogt 15588	B, VOGT	B 10 0420808
Leucanthemum ircutianum DC.	4*x*	irc1	L052	DE, Bayern, Regensburg, 380 m	48.98 N, 12.08 E	Eder s.n.	REG	-
Leucanthemum ircutianum DC.	4*x*	irc2	106-1	FR, Midi-Pyrénées, Mazamet, 410 m	43.48 N, 02.37 E	Vogt 16678, Oberprieler 10633 & Konowalik	B	B 10 0464641
Leucanthemum ircutianum DC.	4*x*	irc3	87-1	IT, Piemonte, Roccavione, 670 m	44.30 N, 07.51 E	Vogt 16611, Oberprieler 10561 & Konowalik	B, REG, VOGT	B 10 0464680
Leucanthemum ircutianum DC.	4*x*	irc4	177-1	ME, Cetinje, Bjeloši, 920 m	42.37 N, 18.89 E	Vogt 16794 & Prem-Vogt	B	B 10 0346630
Leucanthemum ircutianum DC. subsp. *cantabricum* (Sennen) Vogt	4*x*	ica1	L092	ES, Cantabria, Castro Urdiales, 50 m	43.38 N, 03.23 W	Vogt 4494	B, VOGT	B 10 0420793
Leucanthemum ircutianum DC. subsp. *cantabricum* (Sennen) Vogt	4*x*	ica2	L090	ES, Navarra, Ochagavia, 1300 m	42.97 N, 01.12 W	Vogt 5164 & Prem	B, VOGT	B 10 0420792
Leucanthemum ircutianum DC. subsp. *crassifolium* (Lange) Vogt	4*x*	icr1	L093	FR, Pyrénées Atlantiques, Guéthary, 10 m	43.45 N, 01.57 W	Vogt 4440	B, VOGT	B 10 0420794
Leucanthemum ircutianum DC. subsp. *crassifolium* (Lange) Vogt	4*x*	icr2	66-1	ES, Asturias, Ferrero, 60 m	43.66 N, 05.85 W	Höfl 66 & Himmelreich	B	B 10 0413740
Leucanthemum ircutianum DC. subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	4*x*	ile1	L190	HR, Primorje-Gorski Kotar, Osor, 69 m	44.72 N, 14.40 E	Vogt 16051	B, VOGT	B 10 0420796
Leucanthemum ircutianum DC. subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	4*x*	ile2	170-1	ME, Herceg Novi, Sutorina, 34 m	42.47 N, 18.47 E	Vogt 16724 & Prem-Vogt	B	B 10 0346645
Leucanthemum ircutianum DC. subsp. *pseudosylvaticum* Vogt	4*x*	ips1	4-1	ES, Castillia y León, San Martin de Castañeda, 1160 m	42.13 N, 06.71 W	Höfl 4 & Hutschenreuther	B	B 10 0413787
Leucanthemum ircutianum DC. subsp. *pseudosylvaticum* Vogt	4*x*	ips2	13-1	PT, Lisboa, Colares, 40 m	38.80 N, 09.44 W	Höfl 13 & Hutschenreuther	B	B 10 0413780
Leucanthemum lacustre (Brot.) Samp.	22*x*	lau1	L102	PT, Região do Centro, Nadadouro, 10 m	39.42 N, 09.17 W	Vogt 7219 & Prem	VOGT	B 10 0420898
Leucanthemum maestracense Vogt & Hellwig	6*x*	mas1	L103	ES, Valencia, Vistabella del Maestrazgo, 1000 m	40.30 N, 00.28 W	Hellwig & Matthies s.n.	VOGT	B 10 0216890
Leucanthemum maestracense Vogt & Hellwig	6*x*	mas2	M68-1	ES, Aragón, Chodos, 1061 m	40.28 N, 00.27 W	Cordel s.n.	B	B 10 0345032
Leucanthemum maximum (Ramond) DC.	12*x*	max1	79-1	ES, Midi-Pyrénées, Artigues, 1500 m	42.92 N, 00.21 E	Höfl 79	B	B 10 0413726
Leucanthemum maximum (Ramond) DC.	12*x*	max2	L105	ES, Euskadi, Zumaya, 150 m	43.27 N, 02.30 W	Vogt 4482	VOGT	B 10 0420900
Leucanthemum meridionale Legrand	4*x*	med1	L997	FR, Midi-Pyrénées, Ruau, 350 m	44.55 N, 02.30 E	Krendl s.n.	W	W-1974-7490
Leucanthemum merinoi Vogt & Castroviejo	6*x*	mer1	43-1	ES, Galicia, Vizus, 63 m	43.40 N, 08.21 W	Höfl 43	B	-
Leucanthemum merinoi Vogt & Castroviejo	6*x*	mer2	28-1	ES, Galicia, A Guarda, 60 m	41.91 N, 08.88 W	Höfl 28 & Greiner	B	B 10 0413767
Leucanthemum monspeliense (L.) Coste	4*x*	mop3	139-1	ES, Catalunya, Maçanet de Cabrenys, 1015 m	42.41 N, 02.75 E	Konowalik KK46 & Ogrodowczyk	B, REG, VOGT, WRSL	B 10 0386786
Leucanthemum monspeliense (L.) Coste	4*x*	mop4	101-1	FR, Languedoc-Roussillon, Lacombe, 193 m	43.33 N, 02.38 E	Vogt 16670, Oberprieler 10621 & Konowalik	B, REG, VOGT	B 10 0464658
Leucanthemum montserratianum Vogt	10*x*	mon1	142-1	ES, Catalunya, Santa Cecília, 711 m	41.61 N, 01.81 E	Konowalik KK49 & Ogrodowczyk	B, REG, WRSL	B 10 0386718
Leucanthemum pachyphyllum Marchi & Illuminati	10*x*	pac1	277-1	IT, Toscana, Gualchiera, 525 m	43.57 N, 12.01 E	Tomasello TS416	B	B 10 0464986
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	pal1	109-1	FR, Languedoc-Roussillon, Prades-sur-Vernazobre, 80 m	43.44 N, 02.99 E	Vogt 16684, Oberprieler 10639 & Konowalik	B, REG, VOGT	B 10 0464638
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	pal2	L121	ES, Castilla y León, San Rafael, 1200 m	40.73 N, 04.25 W	Vogt 3574 & Pedrol	VOGT	B 10 0420822
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	pal3	L114	IT, Emiliana Romagna, Rubbiano, 300 m	44.68 N, 10.08 E	Vogt 6350	VOGT	B 10 0420819
Leucanthemum pallens (Gay in Perreymond) DC.	4*x*	pal4	L128	IT, Toscana, Selvena, 733 m	42.77 N, 11.65 E	Bayón s.n.	VOGT	B 10 0420827
Leucanthemum pallens (Gay in Perreymond) DC.	4*x*	pal5	329-1	IT, Abruzzo, Piano d´Orta, 125 m	42.25 N, 13.97 E	Oberprieler 10870	OBERPRIELER	-
Leucanthemum platylepis Borb.	8*x*	pla1	L185	HR, Primorje-Gorski Kotar, Baška, 5 m	44.97 N, 14.75 E	Vogt 16052	B, VOGT	B 10 0420810
Leucanthemum platylepis Borb.	8*x*	pla2	L135	SI, Piran, Portorož, 5 m	45.52 N, 13.57 E	Schuhwerk 94/24	B, VOGT	B 10 0420895
Leucanthemum subglaucum De Laramb.	6*x*	sub1	114-1	FR, Languedoc-Roussillon, Laval de Nize, 430 m	43.71 N, 03.24 E	Vogt 16690, Oberprieler 10645 & Konowalik	B, REG, VOGT	B 10 0464681
Leucanthemum subglaucum De Laramb.	6*x*	sub2	133-1	FR, Languedoc-Roussillon, Anduze, 170 m	44.07 N, 03.97 E	Vogt 16721, Oberprieler 10676 & Konowalik	B, REG, VOGT	B 10 0464613
Leucanthemum sylvaticum (Brot.) Nym.	6*x*	syl1	27-1	PT, Região do Norte, Sistelo, 320 m	41.98 N, 08.36 W	Höfl 27 & Greiner	B	B 10 0413768
Leucanthemum sylvaticum (Brot.) Nym.	6*x*	syl2	11-1	PT, Guarda, Seixo Amarelo, 700 m	40.43 N, 07.35 W	Höfl 11 & Hutschenreuther	B	B 10 0413781
Leucanthemum visianii (Gjurašin) Vogt & Greuter	4*x*	vis1	285-1	HR, Lika-Senj, Sušanj Cesarički, 920 m	44.53 N, 15.14 E	Vogt 16962 & Prem-Vogt	B, VOGT	B 10 0350310
Leucanthemum visianii (Gjurašin) Vogt & Greuter	4*x*	vis2	281-1	HR, Zadar, Pag, 3 m	44.42 N, 15.07 E	Vogt 16955 & Prem-Vogt	B, VOGT	B 10 0350320

Table 10- Taxa and accessions used in the present study. Taxon ploidy is specified followed by popuation ID, internal sample number, collection site, geographical coordinates, collector, herbarium and voucher number. Herbarium names are according to Index Herbariorum except VOGT which denotes private collection of Robert Vogt and OBERPRIELER which denotes private collection of Christoph Oberprieler.

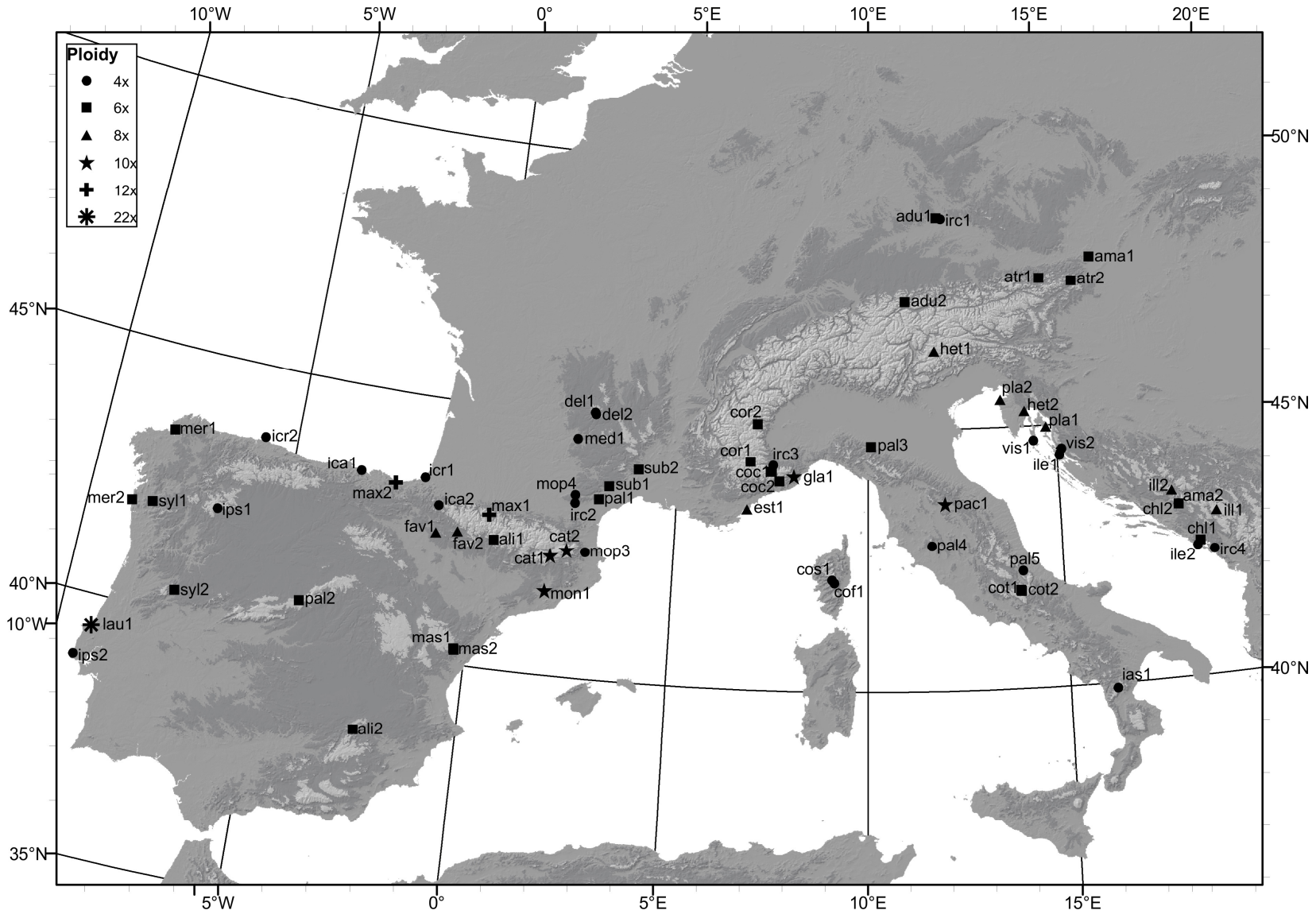

Figure 9 - Map showing sampling localities of all polyploid individuals used in this study. More detailed information is available in the corresponding table (10).

Low-copy nuclear genes – Amplification and 454 sequencing was performed in accordance with the methods described in Chapter 1. After PCR amplification and tagging of the PCR products with an accession-specific barcode, the samples were purified and pooled into a single mixture. In order to ensure equimolar mixing during final pipetting, the amount to pipette was calculated as follows:

$$\left(\frac{\text{actual sample concentration}}{\text{mean concentration of all samples}} \times \frac{\text{length of sample}}{\text{mean length of all samples}} \times \frac{\text{sample ploidy}}{\text{mean ploidy of all samples}}\right) \times 1\,\mu\text{l}$$

. The mixed amplicons were sent to an FLX 454 Genome Sequencer (Microsynth, Switzerland). The project was run on a ¼ plate together with other projects. To ensure that all alleles of a particular accession were sampled, the minimum coverage to recover at least 10 reads of each allele with a 0.99 probability was calculated. The formula used consisted of summarizing binominal distributions: $P = \prod_{i=0}^{m-1}\left(1 - \sum_{k=0}^{10} P(k,(n-10i))\right)$ where m is the maximum number of alleles expected which equals the ploidy level and $P(k,n) = \left(\frac{n!}{k!(n-k)!}\right) \cdot p^k \cdot q^{n-k}$ where n is the number of all reads and k is the number of reads of one allele. It is calculated in a way that the sampling of one allele is dependent on the number of reads obtained for other alleles; thus, the higher the number of possible alleles the higher the coverage that is needed to recover all variants. The calculated minimum number of reads required for a certain ploidy is: 102 for a tetraploid (4*x*), 155 for a hexaploid (6*x*), 235 for an octoploid (8*x*), 300 for a decaploid (10*x*), 367 for a dodecaploid (12*x*), and 702 for a dokosaploid (22*x*).

Data analysis – All steps after data retrieval, including the processing of raw reads, quality filtering and allele separation were conducted in accordance with the methods described in Chapter 1.

cpDNA markers – The number of chloroplast DNA markers was reduced in comparison to the diploid dataset. It included three pairs of primers: psbA (5'–GTTATGCATGAACGTAATGCTC–3') and trnHr (5'–CGCGCATGGTGGATTCACAAATC–3') (Sang 1997); petN1 (5'–GGATATAGTAAGTCTTGCTTGGG–3') and psbM2R (5'–TTCTTGCATTTATTGCTACTGC–3') (Lee 2004); trnQ2 (5'–GCGTGGCCAAGYGGTAAGGC–3') (Shaw 2007) and rps16x1_leu (5'–CAATCGAATTGTCAATGATGC–3') (this study, based on Shaw 2007). All subsequent steps, including PCR amplification and sequencing, were performed in accordance with the methods described in Chapter 1. Prior to analysis, the resultant sequences were merged with those obtained for diploid and outgroup taxa from Chapter 1. To compare obtained results with haplotype network obtained by Greiner et al. (2012) and

include into nework taxa which were not sampled by that study haplotype network was computed and visualized in the program TCS 1.21 (Clement et al. 2000). The haplotypes were grouped manually into the haplogroups according to the nesting rules of a nested clade analysis (Templeton et al. 1987, Templeton & Sing 1993).

Gene trees construction – Gene trees were constructed using the same methodology as described in Chapter 1. Prior to analysis, polyploid alleles were merged with the alleles obtained for diploid and outgroup taxa from Chapter 1. Prior to starting Bayesian inference a model of sequence evolution was chosen. For the nucleotide part, model from the best selection according to AIC implemented in jModelTest 0.1.1 (Posada 2008) (**Table 11**) was used. For the binary coded gaps, a Jukes-Cantor model (Jukes and Cantor 1969) was used.

Supernetwork – The construction of a supernetwork from gene trees was effected in accordance with the method described in Chapter 1. It included nine gene trees obtained from nuclear markers and one gene tree obtained from merged cpDNA sequences. The final matrix consisted of 382 characters which were coded presences/absences in the nodes of the 10 gene trees. Support for the splits was inferred by bootstrapping with 1000 replicates.

To visualize the phenetic structure among individuals, a principal coordinate analysis (PCoA) was performed using MVSP version 3.12f (Kovach, 1999) using the Bray-Curtis coefficient (Bray & Curtis 1957) on the 0/1 matrix used above.

The pairwise similarity indices were constructed by subtracting normalized Nei-Li distance values computed with PAUP* 4.0b10 (Swofford 2003) on the 0/1 matrix from unity. Prior to the calculation, the outgroup taxa were removed from the matrix together with 59 characters specific only to them. Also, 74 characters specific only to one of the taxa were removed, together with 16 characters which were specific to more than 19 taxa. The reduced matrix contained 233 characters. The visualization of the pairwise similarity indices was performed in R 3.0.2 (R Core Team, 2013) using the pheatmap package (Kolde, 2013). When the similarity is high, this may suggest a bond between the two taxa which could arise either because of their shared ancestry or because of the gene flow between them.

																		Nodes with support >0.95
marker	**model**	**freqA**	**freqC**	**freqG**	**freqT**	**R(a) [AC]**	**(b) [AG]**	**R(c) [AT]**	**R(d) [CG]**	**R(e) [CT]**	**R(f) [GT]**	**n° states**	**rates**	**gamma shape**	**gamma categories**	**pin variation**	**kappa**	**ti/tv**	**(divided by number of leaves)**
A39	GTR+G	0.2751	0.1948	0.1383	0.3918	1.5685	2.9647	0.5278	1.2543	1.8163	1.0000	6	gamma	0.5450	4	0	-	-	46 (0.13)
B12	TPM1uf+G	0.3276	0.1809	0.1530	0.3385	1.0000	3.0161	0.8268	0.8268	3.0161	1.0000	6	gamma	0.4480	4	0	-	-	63 (0.16)
B20	HKY+G	0.2760	0.1801	0.2120	0.3319	-	-	-	-	-	-	2	gamma	0.5440	4	0	-	1.4457	63 (0.15)
C12	TIM1+G	0.2863	0.1817	0.2040	0.3280	1.0000	1.6609	0.5655	0.5655	2.4322	1.0000	6	gamma	0.7200	4	0	-	-	43 (0.10)
C20	TIM3+G	0.3107	0.1439	0.1705	0.3750	1.4453	3.2646	1.0000	1.4453	2.3493	1.0000	6	gamma	1.1440	4	0	-	-	21 (0.06)
C33	HKY+G	0.3080	0.1607	0.1926	0.3387	-	-	-	-	-	-	2	gamma	3.3490	4	0	-	1.1997	39 (0.12)
D18	TIM3+G	0.3213	0.1592	0.2003	0.3192	1.5488	3.7174	1.0000	1.5488	5.1083	1.0000	6	gamma	0.3010	4	0	-	-	45 (0.12)
D23	TPM2uf+G	0.2322	0.2071	0.1928	0.3680	1.3984	4.2078	1.3984	1.0000	4.2078	1.0000	6	gamma	0.4210	4	0	-	-	35 (0.08)
D27	TPM3uf+G	0.2266	0.1599	0.1919	0.4216	1.6283	2.5793	1.0000	1.6283	2.5793	1.0000	6	gamma	2.7650	4	0	-	-	11 (0.04)
cpDNA	GTR+G	0.3470	0.1326	0.1301	0.3903	1.0654	2.2968	0.2983	1.0513	0.9496	1.0000	6	gamma	0.2710	4	0	-	-	14 (0.12)

Table 11 - Model chosen for Bayesian inference. All parameters listed by jModelTest and necessary for model specification in MrBayes are listed. The last column shows the number of nodes with the posterior probability above 0.95 together with the proportion between this number and the total number of leaves.

N°	Polyploid taxa	Code	Ploidy	Short	Population	1 A39	2 B12	3 B20	4 C12	5 C20	6 C33	7 D18	8 D23	9 D27	mean (SD)
1	*Leucanthemum chloroticum* A. Kern. & Murb.	TTCT	4x	chl2	189-1	168	123	111	164	1024	153	201	0	249	195.4 (38.4)
2	*Leucanthemum corsicum* subsp. *corsicum* (Less.) DC.	AAAG	4x	cos1	268-1	97	66	81	91	64	51	75	83	110	203.6 (66.1)
3	*Leucanthemum corsicum* subsp. *fenzlii* Gamisans	AATG	4x	cof1	269-1	109	94	132	205	129	54	97	60	144	222.7 (74.4)
4	*Leucanthemum delarbrei* Timb.-Lagr.	AACG	4x	del1	L075	101	69	76	62	59	42	84	45	136	125 (34.9)
5	*Leucanthemum delarbrei* Timb.-Lagr.	AAGG	4x	del2	L174	72	54	98	90	67	46	119	37	64	126.3 (36.4)
6	*Leucanthemum ircutianum* DC.	TACG	4x	irc3	87-1	194	208	240	246	199	171	197	163	198	195.8 (69.4)
7	*Leucanthemum ircutianum* DC.	TATA	4x	irc2	106-1	197	211	519	315	205	197	234	257	198	290 (99.5)
8	*Leucanthemum ircutianum* DC.	TAGG	4x	irc4	177-1	175	80	4	251	471	215	211	201	177	208 (60.6)
9	*Leucanthemum ircutianum* DC.	TAAT	4x	irc1	L052	154	247	213	276	251	197	245	215	225	321.3 (201.3)
10	*Leucanthemum ircutianum* subsp. *asperulum* (N. Terr.) Vogt	ATAG	4x	ias1	L183	89	156	180	18	118	73	120	77	151	107.6 (29.6)
11	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATCG	4x	ica2	L090	122	102	180	196	150	78	195	134	148	127.2 (53.6)
12	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATTG	4x	ica1	L092	128	113	196	71	106	88	63	85	110	97.1 (21.5)
13	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ACAG	4x	icr2	66-1	102	90	176	157	78	98	123	143	158	158.6 (44.6)
14	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ATGG	4x	icr1	L093	84	62	121	99	107	56	109	77	86	185 (54.3)
15	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACCG	4x	ile2	170-1	124	83	143	148	96	54	95	115	110	203.3 (52.4)
16	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACTG	4x	ile1	L190	116	74	170	148	135	91	126	95	182	200.3 (39.1)
17	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	AGAG	4x	ips2	13-1	123	106	155	125	215	42	100	86	193	219.2 (52.5)
18	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	ACGG	4x	ips1	4-1	85	82	128	115	93	59	113	87	112	195.9 (42.5)
19	*Leucanthemum meridionale* Legrand	ACGG	4x	med1	L997	80	101	87	108	59	83	93	101	121	236.3 (60.1)
20	*Leucanthemum monspeliense* (L.) H. J. Coste	AGGA	4x	mop4	101-1	206	208	211	242	204	193	273	225	223	302.6 (107.4)
21	*Leucanthemum monspeliense* (L.) H. J. Coste	AGCC	4x	mop3	139-1	206	214	207	200	180	191	312	186	179	243.7 (300.5)
22	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTTT	4x	pal5	329-1	121	113	134	156	138	89	74	116	121	221.3 (42.2)
23	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTAA	4x	pal4	L128	108	110	143	169	158	104	46	73	130	247.3 (66.5)
24	*Leucanthemum visianii* (Gjurašin) Vogt & Greuter	ACGC	4x	vis2	281-1	126	142	164	222	159	127	202	205	270	207.6 (105.7)
25	*Leucanthemum visianii* (Gjurašin) Vogt & Greuter	ACCT	4x	vis1	285-1	130	150	191	282	228	162	109	161	212	192.4 (137.2)
26	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	AGGG	6x	adu1	L044	185	187	252	280	183	164	178	200	174	207.2 (46)
27	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	TAAG	6x	adu2	L057	239	201	279	230	174	111	167	174	255	211.9 (59)
28	*Leucanthemum adustum* subsp. *margaritae* (Ják.) Holub	ATTC	6x	ama2	187-1	211	156	256	273	161	153	286	219	258	115.7 (39.5)
29	*Leucanthemum adustum* subsp. *margaritae* (Ják.) Holub	TATG	6x	ama1	L007	200	161	258	225	170	138	246	154	211	237.9 (78.8)
30	*Leucanthemum aligulatum* Vogt	ATTT	6x	ali1	78-1	158	159	200	249	197	133	215	212	236	118 (24.8)
31	*Leucanthemum aligulatum* Vogt	TACA	6x	ali2	M64-1	262	164	285	329	232	126	248	262	318	197.8 (41)
32	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TAGT	6x	atr1	L1006	172	199	231	261	249	153	278	202	247	200.2 (40.7)
33	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TTAA	6x	atr2	L1007	206	170	165	281	217	144	484	65	0	295.2 (116.9)
34	*Leucanthemum chloroticum* A. Kern. & Murb.	TTTA	6x	chl1	171-1	0	180	216	253	206	133	396	241	243	275 (73.8)
35	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TTGC	6x	coc1	217-1	334	198	306	521	276	151	370	241	326	371.8 (94.9)
36	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TCAA	6x	coc2	238-1	261	244	379	588	615	175	194	0	436	342.9 (85.1)
37	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TCGA	6x	cot1	279-1	191	139	301	259	185	131	229	164	273	246 (66.9)
38	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TGAT	6x	cot2	L1005	209	179	240	333	335	190	225	177	239	404.9 (92.9)
39	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCCC	6x	cor2	204-1	159	169	236	295	196	48	201	251	207	314.1 (345.1)
40	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCTT	6x	cor1	207-1	411	183	351	261	239	199	315	194	457	359.1 (176)
41	*Leucanthemum maestracense* Vogt & Hellwig	TGTC	6x	mas1	L103	192	220	106	238	161	155	342	194	224	396.1 (109.9)
42	*Leucanthemum maestracense* Vogt & Hellwig	TGCA	6x	mas2	M68-1	193	162	245	289	178	133	229	197	378	439.6 (92.5)
43	*Leucanthemum merinoi* Vogt & Castroviejo	TGGT	6x	mer1	43-1	264	187	234	210	149	148	277	214	293	301.2 (83.5)
44	*Leucanthemum merinoi* Vogt & Castroviejo	CAAA	6x	mer2	28-1	173	148	219	205	226	106	142	165	211	366.6 (101.1)
45	*Leucanthemum pallens* (Gay in Perreymond) DC.	CATA	6x	pal1	109-1	271	183	248	223	215	114	236	188	187	437.6 (173.9)
46	*Leucanthemum pallens* (Gay in Perreymond) DC.	CAGC	6x	pal3	L114	220	139	316	292	206	137	352	183	296	491.8 (87)
47	*Leucanthemum pallens* (Gay in Perreymond) DC.	CACT	6x	pal2	L121	271	175	280	260	144	139	230	151	257	462.7 (95.9)
48	*Leucanthemum subglaucum* De Laramb.	AGTG	6x	sub1	114-1	184	148	211	292	195	176	218	104	137	219.6 (52.6)
49	*Leucanthemum subglaucum* De Laramb.	AGCG	6x	sub2	133-1	158	159	71	230	157	142	131	200	179	177.2 (40.9)
50	*Leucanthemum sylvaticum* (Brot.) Nym.	CTCC	6x	syl1	27-1	179	189	250	239	176	134	193	167	253	1014.3 (187.9)
51	*Leucanthemum sylvaticum* (Brot.) Nym.	CTGA	6x	syl2	11-1	203	139	215	198	221	129	230	215	252	213.9 (52.5)
52	*Leucanthemum cf. glaucophyllum* (Briq. & Cavill.) Jahand.	AATT	8x	gla2	256-1	467	346	472	584	482	322	430	324	529	92.6 (18)
53	*Leucanthemum favargeri* Vogt	CCTC	8x	fav2	74-1	222	236	264	450	271	196	305	242	289	180.6 (53.6)
54	*Leucanthemum favargeri* Vogt	CCAT	8x	fav1	L076	438	352	119	387	347	221	241	145	407	210.9 (46.4)
55	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand. Cv. Esterel	AACA	8x	gla3	227-1	360	305	390	342	320	197	153	256	388	259.2 (104.9)
56	*Leucanthemum heterophyllum* (Willd.) DC.	CCCA	8x	het1	L001	322	300	431	448	531	223	433	320	338	179.7 (48.5)
57	*Leucanthemum heterophyllum* (Willd.) DC.	CCGT	8x	het2	L187	301	253	379	509	407	240	385	303	309	208.3 (40.8)
58	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACAA	8x	ill1	200-1	302	164	208	296	174	161	198	198	224	201.8 (27.4)
59	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACTA	8x	ill2	318-1	303	147	203	231	194	169	180	234	237	198.3 (127.6)
60	*Leucanthemum platylepis* Borb.	CGTA	8x	pla2	L135	181	201	62	1194	225	362	173	83	346	224.8 (35.8)
61	*Leucanthemum platylepis* Borb.	CGAC	8x	pla1	L185	339	207	337	414	306	179	331	331	788	220.6 (24.3)
62	*Leucanthemum catalaunicum* Vogt	CGCT	10x	cat1	153-1	247	188	259	355	262	165	159	256	323	79.8 (18.2)
63	*Leucanthemum catalaunicum* Vogt	CGGA	10x	cat2	146-1	346	337	459	501	440	263	445	315	538	113.8 (46)
64	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand.	AAAC	10x	gla1	253-1	339	281	568	564	426	291	405	294	397	74.9 (29.4)
65	*Leucanthemum montserratianum* Vogt	AAGA	10x	mon1	142-1	463	348	292	527	404	258	208	411	388	71.9 (26.3)
66	*Leucanthemum pachyphyllum* Marchi & Illuminati	ATAT	10x	pac1	277-1	438	349	455	534	473	236	257	376	820	109.1 (50.3)
67	*Leucanthemum maximum* (Ramond) DC.	ATTA	12x	max1	79-1	492	387	536	477	441	283	609	410	529	106.7 (39.5)
68	*Leucanthemum maximum* (Ramond) DC.	ATCA	12x	max2	L105	448	402	624	531	571	379	532	399	540	145 (40.8)
69	*Leucanthemum lacustre* (Brot.) Samp.	ATGT	22x	lau1	L102	968	862	1218	1182	972	640	1205	979	1103	89 (21.9)
					mean (SD):	227.5 (142.2)	192.2 (115.7)	256.3 (172.7)	304.3 (205.3)	255.1 (180.9)	161.8 (93.9)	244.6 (166.1)	197.6 (133.9)	276.0 (180.0)	
					length (SD):	329.8 (20.8)	373.9 (17.3)	342.2 (32.8)	356.4 (36.8)	247.9 (39.9)	351.6 (28.4)	301.5 (52.5)	373.7 (52.6)	287.3 (37.1)	

Table 12 - Summary of raw reads obtained per marker and species. The most right column summarizes reads per species (mean and standard deviation) and the bottom line summarizes reads per marker (mean and standard deviation). The average read length is given in the most bottom line.

N°	Polyploid taxa	Code	Ploidy	Short	Population	1 A39	2 B12	3 B20	4 C12	5 C20	6 C33	7 D18	8 D23	9 D27	mean (SD)
1	*Leucanthemum chloroticum* A. Kern. & Murb.	TTCT	4x	chl2	189-1	149	97	99	147	997	112	187	0	233	224.6 (296.9)
2	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TCGA	4x	cot1	279-1	161	110	278	228	177	101	212	141	248	184 (61.7)
3	*Leucanthemum corsicum* subsp. *corsicum* (Less.) DC.	AAAG	4x	cos1	268-1	88	54	69	74	62	42	69	73	101	70.2 (17.4)
4	*Leucanthemum corsicum* subsp. *fenzlii* Gamisans	AATG	4x	cof1	269-1	98	80	123	117	126	41	91	49	135	95.6 (33.7)
5	*Leucanthemum delarbrei* Timb.-Lagr.	AACG	4x	del1	L075	90	59	72	50	54	36	83	40	127	67.9 (28.8)
6	*Leucanthemum delarbrei* Timb.-Lagr.	AAGG	4x	del2	L174	64	49	85	73	66	37	106	32	59	63.4 (23.2)
7	*Leucanthemum ircutianum* DC.	TACG	4x	irc3	87-1	160	195	207	199	197	152	193	136	174	179.2 (24.8)
8	*Leucanthemum ircutianum* DC.	TATA	4x	irc2	106-1	154	167	394	262	200	137	138	177	175	200.4 (81.9)
9	*Leucanthemum ircutianum* DC.	TAGG	4x	irc4	177-1	156	66	0	194	465	196	203	169	159	178.7 (126.8)
10	*Leucanthemum ircutianum* DC.	TAAT	4x	irc1	L052	140	212	170	207	222	178	139	128	189	176.1 (34.6)
11	*Leucanthemum ircutianum* subsp. *asperulum* (N. Terr.) Vogt	ATAG	4x	ias1	L183	80	137	170	14	114	61	109	62	139	98.4 (48.5)
12	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATCG	4x	ica2	L090	106	87	164	169	148	60	184	108	138	129.3 (41.5)
13	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATTG	4x	ica1	L092	114	98	173	57	104	68	52	66	106	93.1 (37.9)
14	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ACAG	4x	icr2	66-1	90	76	159	127	77	76	113	130	143	110.1 (31.6)
15	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ATGG	4x	icr1	L093	73	52	107	74	106	43	99	70	79	78.1 (22.6)
16	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACCG	4x	ile2	170-1	109	72	125	134	94	46	91	98	106	97.2 (26.6)
17	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACTG	4x	ile1	L190	100	58	127	121	132	66	122	75	172	108.1 (36.8)
18	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	AGAG	4x	ips2	13-1	114	91	146	100	206	28	97	74	177	114.8 (54.2)
19	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	ACGG	4x	ips1	4-1	76	70	116	103	91	47	102	69	106	86.7 (22.4)
20	*Leucanthemum meridionale* Legrand	ACGG	4x	med1	L997	60	68	64	56	53	66	77	63	95	66.9 (12.6)
21	*Leucanthemum monspeliense* (L.) H. J. Coste	AGGA	4x	mop4	101-1	164	177	160	138	189	169	225	165	197	176 (25.1)
22	*Leucanthemum monspeliense* (L.) H. J. Coste	AGCC	4x	mop3	139-1	168	183	162	155	159	104	281	128	133	163.7 (50)
23	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTTT	4x	pal5	329-1	109	100	124	142	127	70	71	103	116	106.9 (24.3)
24	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTAA	4x	pal4	L128	94	99	131	158	156	82	43	64	122	105.4 (39.7)
25	*Leucanthemum visianii* (Gjurašin) Vogt & Greuter	ACGC	4x	vis2	281-1	112	121	155	188	150	96	188	169	244	158.1 (45.7)
26	*Leucanthemum visianii* (Gjurašin) Vogt & Greuter	ACCT	4x	vis1	285-1	111	124	173	256	201	124	99	140	195	158.1 (51.7)
27	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	AGGG	6x	adu1	L044	169	161	227	235	180	128	167	168	162	177.4 (33.5)
28	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	TAAG	6x	adu2	L057	208	175	253	181	171	79	157	154	238	179.6 (51.2)
29	*Leucanthemum adustum* subsp. *margaritae* (Ják.) Holub	ATTC	6x	ama2	187-1	188	134	240	221	152	115	265	187	242	193.8 (52.3)
30	*Leucanthemum adustum* subsp. *margaritae* (Ják.) Holub	TATG	6x	ama1	L007	174	142	229	184	159	107	235	137	196	173.7 (42.5)
31	*Leucanthemum aligulatum* Vogt	ATTT	6x	ali1	78-1	144	137	174	235	189	93	198	172	221	173.7 (44.1)
32	*Leucanthemum aligulatum* Vogt	TACA	6x	ali2	M64-1	233	133	259	278	225	100	236	246	303	223.7 (65.9)
33	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TAGT	6x	atr1	L1006	152	174	207	220	239	104	259	169	230	194.9 (49.1)
34	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TTAA	6x	atr2	L1007	182	140	138	231	186	108	425	58	0	163.1 (120.4)
35	*Leucanthemum chloroticum* A. Kern. & Murb.	TTTA	6x	chl1	171-1	0	149	192	217	200	97	368	181	222	180.7 (99.5)
36	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TTGC	6x	coc1	217-1	298	152	271	424	265	88	337	200	303	259.8 (100.7)
37	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TCAA	6x	coc2	238-1	221	199	345	500	545	95	177	0	390	274.7 (183)
38	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TGAT	6x	cot2	L1005	192	152	218	294	314	144	211	142	220	209.7 (62)
39	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCCC	6x	cor2	204-1	139	145	215	261	187	34	190	209	197	175.2 (64.3)
40	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCTT	6x	cor1	207-1	356	140	320	219	235	104	289	169	429	251.2 (106.6)
41	*Leucanthemum maestracense* Vogt & Hellwig	TGTC	6x	mas1	L103	176	179	89	208	135	123	315	160	211	177.3 (65.1)
42	*Leucanthemum maestracense* Vogt & Hellwig	TGCA	6x	mas2	M68-1	167	134	211	249	171	97	210	150	360	194.3 (76.9)
43	*Leucanthemum merinoi* Vogt & Castroviejo	TGGT	6x	mer1	43-1	231	158	208	176	143	119	253	187	275	194.4 (51.8)
44	*Leucanthemum merinoi* Vogt & Castroviejo	CAAA	6x	mer2	28-1	153	125	197	163	225	78	132	146	204	158.1 (45.4)
45	*Leucanthemum pallens* (Gay in Perreymond) DC.	CATA	6x	pal1	109-1	239	145	230	207	211	82	209	156	172	183.4 (49.8)
46	*Leucanthemum pallens* (Gay in Perreymond) DC.	CAGC	6x	pal3	L114	196	121	288	263	205	107	325	159	273	215.2 (76.8)
47	*Leucanthemum pallens* (Gay in Perreymond) DC.	CACT	6x	pal2	L121	237	143	245	232	141	100	207	132	240	186.3 (56.7)
48	*Leucanthemum subglaucum* De Laramb.	AGTG	6x	sub1	114-1	164	129	194	232	189	125	203	93	129	162 (45.6)
49	*Leucanthemum subglaucum* De Laramb.	AGCG	6x	sub2	133-1	137	134	63	194	155	95	112	170	166	136.2 (40.9)
50	*Leucanthemum sylvaticum* (Brot.) Nym.	CTCC	6x	syl1	27-1	154	169	234	201	176	106	184	146	244	179.3 (43.2)
51	*Leucanthemum sylvaticum* (Brot.) Nym.	CTGA	6x	syl2	11-1	177	121	195	151	213	101	216	182	230	176.2 (44.1)
52	*Leucanthemum cf. glaucophyllum* (Briq. & Cavill.) Jahand.	AATT	8x	gla2	256-1	410	274	429	524	469	257	403	269	500	392.8 (102.5)
53	*Leucanthemum favargeri* Vogt	CCTC	8x	fav2	74-1	189	199	231	400	258	144	281	196	269	240.8 (74.1)
54	*Leucanthemum favargeri* Vogt	CCAT	8x	fav1	L076	384	305	105	327	338	172	225	127	386	263.2 (108.7)
55	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand. Cv. Esterel	AACA	8x	gla3	227-1	310	265	361	303	308	158	135	221	360	269 (81.9)
56	*Leucanthemum heterophyllum* (Willd.) DC.	CCCA	8x	het1	L001	290	261	394	378	488	170	406	257	320	329.3 (96.5)
57	*Leucanthemum heterophyllum* (Willd.) DC.	CCGT	8x	het2	L187	268	206	350	459	293	186	359	263	291	297.2 (83.3)
58	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACAA	8x	ill1	200-1	267	137	193	272	174	126	177	166	206	190.9 (51)
59	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACTA	8x	ill2	318-1	271	125	184	208	191	132	165	197	216	187.7 (44.5)
60	*Leucanthemum platylepis* Borb.	CGTA	8x	pla2	L135	150	165	58	1080	191	279	156	64	325	274.2 (314.5)
61	*Leucanthemum platylepis* Borb.	CGAC	8x	pla1	L185	306	179	305	372	260	151	302	275	755	322.8 (175.6)
62	*Leucanthemum catalaunicum* Vogt	CGGA	10x	cat2	146-1	298	284	420	436	422	219	427	261	502	363.2 (98.2)
63	*Leucanthemum catalaunicum* Vogt	CGCT	10x	cat1	153-1	214	155	227	319	244	112	149	212	298	214.4 (68.2)
64	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand.	AAAC	10x	gla1	253-1	308	235	514	500	390	217	385	254	364	351.9 (108.6)
65	*Leucanthemum montserratianum* Vogt	AAGA	10x	mon1	142-1	423	306	264	475	356	206	193	360	366	327.7 (94.6)
66	*Leucanthemum pachyphyllum* Marchi & Illuminati	ATAT	10x	pac1	277-1	387	287	406	467	430	174	240	313	779	387 (174.9)
67	*Leucanthemum maximum* (Ramond) DC.	ATTA	12x	max1	79-1	443	328	465	387	404	192	565	355	491	403.3 (107.3)
68	*Leucanthemum maximum* (Ramond) DC.	ATCA	12x	max2	L105	397	337	559	460	548	256	514	325	501	433 (108.7)
69	*Leucanthemum lacustre* (Brot.) Samp.	ATGT	22x	lau1	L102	846	720	1089	998	916	467	1112	830	1028	889.6 (204.2)
					mean (SD):	199.8 (125.7)	161.8 (97.3)	228.2 (154.5)	259.2 (182.8)	240.1 (170.1)	121.5 (70.4)	223.9 (155)	164.4 (113.5)	256.3 (170.2)	
					length (SD):	328.9 (17.5)	372.6 (10.9)	341.0 (30.7)	355.1 (25.2)	246.1 (38.1)	348.2 (23.4)	301.0 (50.1)	371.1 (50.8)	287.0 (34.1)	

Table 13 - Summary of obtained reads per marker and species after quality control. The most right column summarizes reads per species (mean and standard deviation) and the bottom line summarizes reads per marker (mean and standard deviation). The average read length is given in the most bottom line.

N°	Polyploid taxa	Code	Ploidy	Short	Population	1 A39	2 B12	3 B20	4 C12	5 C20	6 C33	7 D18	8 D23	9 D27	mean (SD)
1	*Leucanthemum chloroticum* A. Kern. & Murb.	TTCT	4x	chl2	189-1	149	86	88	126	997	112	147	0	215	213.3 (299.6)
2	*Leucanthemum corsicum* subsp. *corsicum* (Less.) DC.	AAAG	4x	cos1	268-1	62	54	47	64	62	41	48	70	101	61.0 (17.7)
3	*Leucanthemum corsicum* subsp. *fenzlii* Gamisans	AATG	4x	cof1	269-1	98	80	106	95	126	41	80	41	135	89.1 (32.9)
4	*Leucanthemum delarbrei* Timb.-Lagr.	AACG	4x	del1	L075	89	44	67	50	54	36	79	38	118	63.9 (27.2)
5	*Leucanthemum delarbrei* Timb.-Lagr.	AAGG	4x	del2	L174	64	47	73	73	66	34	84	31	59	59.0 (18.2)
6	*Leucanthemum ircutianum* DC.	TACG	4x	irc3	87-1	160	187	201	145	188	143	101	123	163	156.8 (32.5)
7	*Leucanthemum ircutianum* DC.	TATA	4x	irc2	106-1	139	167	248	262	200	137	138	177	175	182.6 (46.3)
8	*Leucanthemum ircutianum* DC.	TAGG	4x	irc4	177-1	156	55	3	170	436	188	109	127	143	154.1 (120.6)
9	*Leucanthemum ircutianum* DC.	TAAT	4x	irc1	L052	140	212	170	207	222	178	139	128	189	176.1 (34.6)
10	*Leucanthemum ircutianum* subsp. *asperulum* (N. Terr.) Vogt	ATAG	4x	ias1	L183	57	137	170	11	114	61	78	57	139	91.6 (51.2)
11	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATCG	4x	ica2	L090	87	87	132	149	148	59	139	103	129	114.8 (31.9)
12	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATTG	4x	ica1	L092	114	91	172	52	104	68	22	62	105	87.8 (43.3)
13	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ACAG	4x	icr2	66-1	80	63	159	127	77	76	103	129	138	105.8 (33.7)
14	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ATGG	4x	icr1	L093	73	51	107	74	106	43	97	70	75	77.3 (22.5)
15	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACCG	4x	ile2	170-1	77	60	124	115	94	45	64	96	104	86.6 (26.7)
16	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACTG	4x	ile1	L190	82	57	127	119	132	76	117	72	169	105.7 (36.1)
17	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	AGAG	4x	ips2	13-1	73	86	145	99	203	24	32	59	169	98.9 (61.6)
18	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	ACGG	4x	ips1	4-1	64	54	105	90	67	47	80	69	106	75.8 (21.1)
19	*Leucanthemum meridionale* Legrand	ACGG	4x	med1	L997	58	68	64	56	53	66	77	63	95	66.7 (12.8)
20	*Leucanthemum monspeliense* (L.) H. J. Coste	AGGA	4x	mop4	101-1	117	172	160	138	189	169	225	165	197	170.2 (31.8)
21	*Leucanthemum monspeliense* (L.) H. J. Coste	AGCC	4x	mop3	139-1	168	178	162	155	159	104	281	128	133	163.1 (49.8)
22	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTTT	4x	pal5	329-1	62	73	120	138	127	70	22	99	113	91.6 (37.5)
23	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTAA	4x	pal4	L128	90	71	130	154	155	82	11	64	119	97.3 (46.9)
24	*Leucanthemum visianii* (Gjurašin) Vogt & Greuter	ACGC	4x	vis2	281-1	104	100	150	186	150	96	139	146	243	146.0 (46.7)
25	*Leucanthemum visianii* (Gjurašin) Vogt & Greuter	ACCT	4x	vis1	285-1	52	124	169	227	201	124	80	139	194	145.6 (57.7)
26	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	AGGG	6x	adu1	L044	101	132	225	212	180	128	167	166	162	163.7 (39.7)
27	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	TAAG	6x	adu2	L057	135	130	248	170	127	79	154	152	235	158.9 (53.3)
28	*Leucanthemum adustum* subsp. *margaritae* (Ját.) Holub	ATTC	6x	ama2	187-1	122	74	202	220	150	115	245	186	241	172.8 (60.5)
29	*Leucanthemum adustum* subsp. *margaritae* (Ját.) Holub	TATG	6x	ama1	L007	174	94	186	184	159	107	188	137	194	158.1 (37.2)
30	*Leucanthemum aligulatum* Vogt	ATTT	6x	ali1	78-1	144	125	156	235	188	93	188	168	217	168.2 (44.6)
31	*Leucanthemum aligulatum* Vogt	TACA	6x	ali2	M64-1	153	111	257	278	209	98	229	242	300	208.6 (72.4)
32	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TAGT	6x	atr1	L1006	94	132	202	220	227	104	245	166	223	179.2 (57.0)
33	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TTAA	6x	atr2	L1007	182	133	124	222	185	108	425	23	223	180.6 (111.2)
34	*Leucanthemum chloroticum* A. Kern. & Murb.	TTTA	6x	chl1	171-1	0	116	189	158	169	97	348	179	221	164.1 (94.7)
35	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TTGC	6x	coc1	217-1	294	152	259	311	228	88	332	172	302	237.6 (83.8)
36	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TCAA	6x	coc2	238-1	156	189	339	451	494	86	116	0	366	244.1 (173.5)
37	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TCGA	6x	cot1	279-1	126	103	232	188	163	101	210	120	246	165.4 (56.0)
38	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TGAT	6x	cot2	L1005	163	116	207	272	295	143	203	138	217	194.9 (61.1)
39	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCCC	6x	cor2	204-1	139	96	214	250	187	24	148	166	197	157.9 (67.3)
40	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCTT	6x	cor1	207-1	342	140	294	218	234	104	231	154	396	234.8 (96.1)
41	*Leucanthemum maestracense* Vogt & Hellwig	TGTC	6x	mas1	L103	173	144	81	159	133	121	314	159	209	165.9 (65.9)
42	*Leucanthemum maestracense* Vogt & Hellwig	TGCA	6x	mas2	M68-1	107	130	188	236	150	86	201	148	360	178.4 (82.6)
43	*Leucanthemum merinoi* Vogt & Castroviejo	TGGT	6x	mer1	43-1	158	158	196	122	143	119	205	187	273	173.4 (48.2)
44	*Leucanthemum merinoi* Vogt & Castroviejo	CAAA	6x	mer2	28-1	116	119	155	158	216	77	107	131	204	142.6 (45.4)
45	*Leucanthemum pallens* (Gay in Perreymond) DC.	CATA	6x	pal1	109-1	216	119	218	207	205	80	170	155	172	171.3 (47.3)
46	*Leucanthemum pallens* (Gay in Perreymond) DC.	CAGC	6x	pal3	L114	131	110	260	239	204	106	325	159	238	196.9 (75.4)
47	*Leucanthemum pallens* (Gay in Perreymond) DC.	CACT	6x	pal2	L121	141	113	174	222	141	98	154	115	240	155.3 (48.8)
48	*Leucanthemum subglaucum* De Laramb.	AGTG	6x	sub1	114-1	164	124	194	232	189	119	174	88	128	156.9 (45.4)
49	*Leucanthemum subglaucum* De Laramb.	AGCG	6x	sub2	133-1	106	103	53	166	155	94	103	165	166	123.4 (40.8)
50	*Leucanthemum sylvaticum* (Brot.) Nym.	CTCC	6x	syl1	27-1	112	160	198	184	176	105	80	145	241	155.7 (50.8)
51	*Leucanthemum sylvaticum* (Brot.) Nym.	CTGA	6x	syl2	11-1	103	118	174	151	213	101	188	181	224	161.4 (45.9)
52	*Leucanthemum cf. glaucophyllum* (Briq. & Cavill.) Jahand.	AATT	8x	gla2	256-1	504	250	414	330	439	256	326	245	496	362.2 (104.0)
53	*Leucanthemum favargeri* Vogt	CCTC	8x	fav2	74-1	185	175	227	370	257	144	209	157	268	221.3 (70.1)
54	*Leucanthemum favargeri* Vogt	CCAT	8x	fav1	L076	360	284	73	290	325	171	220	96	360	242.1 (108.5)
55	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand. Cv. Esterel	AACA	8x	gla3	227-1	298	265	340	288	303	158	98	220	346	257.3 (83.7)
56	*Leucanthemum heterophyllum* (Willd.) DC.	CCCA	8x	het1	L001	249	261	376	377	457	169	317	238	317	306.8 (87.8)
57	*Leucanthemum heterophyllum* (Willd.) DC.	CCGT	8x	het2	L187	186	190	249	417	266	186	347	245	288	263.8 (78.3)
58	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACAA	8x	ill1	200-1	205	126	192	257	169	126	172	166	205	179.8 (41.1)
59	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACTA	8x	ill2	318-1	216	113	162	207	191	132	74	152	216	162.6 (49.8)
60	*Leucanthemum platylepis* Borb.	CGTA	8x	pla2	L135	146	145	54	717	184	279	133	40	324	224.7 (206.5)
61	*Leucanthemum platylepis* Borb.	CGAC	8x	pla1	L185	227	130	296	354	224	151	182	271	752	287.4 (188.0)
62	*Leucanthemum catalaunicum* Vogt	CGGA	10x	cat2	146-1	136	215	412	319	386	213	338	261	502	309.1 (114.6)
63	*Leucanthemum catalaunicum* Vogt	CGCT	10x	cat1	153-1	190	123	216	319	224	103	141	212	296	202.7 (73.5)
64	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand.	AAAC	10x	gla1	253-1	305	215	499	434	364	216	252	242	310	315.2 (99.8)
65	*Leucanthemum montserratianum* Vogt	AAGA	10x	mon1	142-1	378	227	222	426	349	205	163	352	363	298.3 (93.7)
66	*Leucanthemum pachyphyllum* Marchi & Illuminati	ATAT	10x	pac1	277-1	385	225	368	453	406	174	158	303	779	361.2 (188.4)
67	*Leucanthemum maximum* (Ramond) DC.	ATTA	12x	max1	79-1	422	307	392	387	404	189	522	346	490	384.3 (98.5)
68	*Leucanthemum maximum* (Ramond) DC.	ATCA	12x	max2	L105	343	304	452	439	491	252	236	314	497	369.8 (101.5)
69	*Leucanthemum lacustre* (Brot.) Samp.	ATGT	22x	lau1	L102	829	696	778	898	822	461	955	821	1005	807.2 (159.4)
					mean (SD):	171.5 (127.0)	143.4 (92.8)	205.0 (123.1)	232.3 (147.8)	229.1 (159.7)	120.1 (69.9)	185.3 (137.5)	155.6 (112.5)	254.1 (164.8)	
					length (SD):	310.4 (4.9)	353.0 (3.7)	319.9 (2.3)	342.6 (22.9)	237.2 (36.7)	330.0 (10.0)	292.0 (1.2)	351.9 (12.7)	267.0 (0.3)	

Table 14 - Summary of obtained reads per marker and species after BAPS clustering and removal of chimeric sequences. The most right column summarizes reads per species (mean and standard deviation) and the most bottom line summarizes reads per marker (mean and standard deviation). The average read length is given in the most bottom line.

						1	2	3	4	5	6	7	8	9	
N°	Polyploid taxa	Code	Ploidy	Short	Population	A39	B12	B20	C12	C20	C33	D18	D23	D27	mean
1	*Leucanthemum chloroticum* A. Kern. & Murb.	TTCT	4x	chl2	189-1	4	3	3	4	3	2	1	4	3	3.0
2	*Leucanthemum corsicum* subsp. *corsicum* (Less.) DC.	AAAG	4x	cos1	268-1	4	6	3	6	3	5	4	5	2	4.2
3	*Leucanthemum corsicum* subsp. *fenzlii* Gamisans	AATG	4x	cof1	269-1	6	4	3	4	2	4	5	5	2	3.9
4	*Leucanthemum delarbrei* Timb.-Lagr.	AACG	4x	del1	L075	3	3	4	2	2	2	3	3	1	2.6
5	*Leucanthemum delarbrei* Timb.-Lagr.	AAGG	4x	del2	L174	3	1	4	2	2	2	2	3	2	2.3
6	*Leucanthemum ircutianum* DC.	TAAT	4x	irc1	L052	1	2	4	2	2	3	4	3	2	2.6
7	*Leucanthemum ircutianum* DC.	TATA	4x	irc2	106-1	4	3	4	4	3	3	2	4	3	3.3
8	*Leucanthemum ircutianum* DC.	TACG	4x	irc3	87-1	2	3	4	4	3	3	3	3	2	3.0
9	*Leucanthemum ircutianum* DC.	TAGG	4x	irc4	177-1	1	2	5	4	3	3	4	3	3	3.1
10	*Leucanthemum ircutianum* subsp. *asperulum* (N. Terr.) Vogt	ATAG	4x	ias1	L183	2	4	2	3	2	2	2	2	1	2.2
11	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATTG	4x	ica1	L092	2	3	4	3	2	2	1	4	3	2.7
12	*Leucanthemum ircutianum* subsp. *cantabricum* (Sennen) Vogt	ATCG	4x	ica2	L090	2	4	4	4	3	3	4	4	3	3.4
13	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ATGG	4x	icr1	L093	3	4	4	4	3	2	2	3	2	3.0
14	*Leucanthemum ircutianum* subsp. *crassifolium* (Lange) Vogt	ACAG	4x	icr2	66-1	3	3	3	4	3	2	2	4	3	3.0
15	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACTG	4x	ile1	L190	3	3	2	4	3	2	3	3	2	2.8
16	*Leucanthemum ircutianum* subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	ACCG	4x	ile2	170-1	2	4	3	3	1	2	2	4	2	2.6
17	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	ACGG	4x	ips1	4-1	2	4	2	2	3	3	3	4	2	2.8
18	*Leucanthemum ircutianum* subsp. *pseudosylvaticum* Vogt	AGAG	4x	ips2	13-1	3	2	2	3	4	3	2	4	4	3.0
19	*Leucanthemum meridionale* Legrand	ACGG	4x	med1	L997	2	4	4	2	2	4	1	3	1	2.6
20	*Leucanthemum monspeliense* (L.) H. J. Coste	AGCC	4x	mop3	139-1	2	4	4	4	3	3	4	3	2	3.2
21	*Leucanthemum monspeliense* (L.) H. J. Coste	AGGA	4x	mop4	101-1	3	3	3	3	2	4	2	3	4	3.0
22	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTAA	4x	pal4	L128	2	3	2	2	3	2	2	3	2	2.3
23	*Leucanthemum pallens* (Gay in Perreymond) DC.	CTTT	4x	pal5	329-1	3	3	2	2	2	3	2	4	1	2.4
24	Leucanthemum visianii (Gjurašin) Vogt & Greuter	ACCT	4x	vis1	285-1	5	4	5	5	4	2	5	6	1	4.1
25	Leucanthemum visianii (Gjurašin) Vogt & Greuter	ACGC	4x	vis2	281-1	5	6	5	5	5	4	3	5	2	4.4
26	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	AGGG	6x	adu1	L044	3	4	6	5	5	2	4	5	3	4.1
27	*Leucanthemum adustum* (W. D. J. Koch) Gremli subsp. *adustum*	TAAG	6x	adu2	L057	4	5	5	3	2	6	5	6	3	4.3
28	*Leucanthemum adustum* subsp. *margaritae* (Ják.) Holub	TATG	6x	ama1	L007	3	3	4	4	4	3	4	5	2	3.6
29	*Leucanthemum adustum* subsp. *margaritae* (Ják.) Holub	ATTC	6x	ama2	187-1	2	5	4	6	2	4	3	5	3	3.8
30	*Leucanthemum aligulatum* Vogt	ATTT	6x	ali1	78-1	1	6	6	6	5	3	3	5	4	4.3
31	*Leucanthemum aligulatum* Vogt	TACA	6x	ali2	M64-1	5	3	4	4	4	3	3	5	1	3.6
32	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TAGT	6x	atr1	L1006	2	3	4	4	6	6	4	5	4	4.2
33	*Leucanthemum atratum* (Jacq.) DC. subsp. *atratum*	TTAA	6x	atr2	L1007	3	4	3	3	8	5	4	3	1	3.8
34	*Leucanthemum chloroticum* A. Kern. & Murb.	TTTA	6x	chl1	171-1	6	4	6	5	3	3	4	6	3	4.4
35	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TTGC	6x	coc1	217-1	6	3	6	4	4	3	4	5	3	4.2
36	*Leucanthemum coronopifolium* subsp. *ceratophylloides* (All.) Vogt & Greuter	TCAA	6x	coc2	238-1	3	6	4	5	6	3	5	5	4	4.6
37	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TCGA	6x	cot1	279-1	4	3	4	5	3	6	3	5	4	4.1
38	*Leucanthemum coronopifolium* subsp. *tenuifolium* (Guss.) Vogt & Greuter	TGAT	6x	cot2	L1005	4	6	7	6	3	6	4	3	2	4.6
39	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCTT	6x	cor1	207-1	5	4	4	4	2	4	3	4	3	3.7
40	*Leucanthemum coronopifolium* Vill. subsp. *coronopifolium*	TCCC	6x	cor2	204-1	4	3	6	4	4	3	5	3	3	3.9
41	*Leucanthemum maestracense* Vogt & Hellwig	TGTC	6x	mas1	L103	4	4	6	5	3	3	4	5	4	4.2
42	*Leucanthemum maestracense* Vogt & Hellwig	TGCA	6x	mas2	M68-1	4	4	6	6	3	3	4	5	4	4.3
43	*Leucanthemum merinoi* Vogt & Castroviejo	TGGT	6x	mer1	43-1	3	4	3	4	5	2	5	6	1	3.7
44	*Leucanthemum merinoi* Vogt & Castroviejo	CAAA	6x	mer2	28-1	4	3	5	4	4	3	3	3	1	3.3
45	*Leucanthemum pallens* (Gay in Perreymond) DC.	CATA	6x	pal1	109-1	6	6	6	3	4	3	6	4	3	4.6
46	*Leucanthemum pallens* (Gay in Perreymond) DC.	CACT	6x	pal2	L121	4	5	5	6	3	4	3	5	2	4.1
47	*Leucanthemum pallens* (Gay in Perreymond) DC.	CAGC	6x	pal3	L114	6	6	4	4	3	3	2	5	2	3.9
48	*Leucanthemum subglaucum* De Laramb.	AGTG	6x	sub1	114-1	4	4	5	3	4	2	3	5	3	3.7
49	*Leucanthemum subglaucum* De Laramb.	AGCG	6x	sub2	133-1	4	4	1	6	3	4	5	6	2	3.9
50	*Leucanthemum sylvaticum* (Brot.) Nym.	CTCC	6x	syl1	27-1	3	4	3	6	4	4	4	3	3	3.8
51	*Leucanthemum sylvaticum* (Brot.) Nym.	CTGA	6x	syl2	11-1	4	3	5	3	4	3	6	5	1	3.8
52	*Leucanthemum favargeri* Vogt	CCAT	8x	fav1	L076	8	5	7	7	3	4	4	6	6	5.6
53	*Leucanthemum favargeri* Vogt	CCTC	8x	fav2	74-1	2	5	6	6	5	6	8	6	5	5.4
54	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand. Cv. Esterel	AACA	8x	gla3	227-1	7	8	8	6	5	2	8	8	3	6.1
55	*Leucanthemum heterophyllum* (Willd.) DC.	CCCA	8x	het1	L001	6	6	8	6	7	4	7	6	3	5.9
56	*Leucanthemum heterophyllum* (Willd.) DC.	CCGT	8x	het2	L187	2	4	3	6	5	7	1	6	4	4.2
57	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACAA	8x	ill1	200-1	4	3	6	5	6	5	8	8	4	5.4
58	*Leucanthemum illyricum* (Horvatić) Vogt & Greuter	ACTA	8x	ill2	318-1	5	6	6	4	4	5	6	5	4	5.0
59	*Leucanthemum platylepis* Borb.	CGAC	8x	pla1	L185	6	5	4	9	5	3	6	7	3	5.3
60	*Leucanthemum platylepis* Borb.	CGTA	8x	pla2	L135	4	8	4	8	6	7	3	4	2	5.1
61	*Leucanthemum catalaunicum* Vogt	CGCT	10x	cat1	153-1	7	7	10	9	6	7	7	6	5	7.1
62	*Leucanthemum catalaunicum* Vogt	CGGA	10x	cat2	146-1	4	8	10	7	7	8	7	9	3	7.0
63	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand.	AAAC	10x	gla1	253-1	6	7	10	10	7	5	5	9	2	6.8
64	*Leucanthemum glaucophyllum* (Briq. & Cavill.) Jahand.	AATT	10x	gla2	256-1	8	10	8	5	8	6	6	7	5	7.0
65	*Leucanthemum montserratianum* Vogt	AAGA	10x	mon1	142-1	12	6	7	8	8	4	5	8	3	6.8
66	*Leucanthemum pachyphyllum* Marchi & Illuminati	ATAT	10x	pac1	277-1	10	8	10	9	8	5	7	10	4	7.9
68	*Leucanthemum maximum* (Ramond) DC.	ATTA	12x	max1	79-1	3	10	7	8	11	8	8	9	6	7.8
69	*Leucanthemum maximum* (Ramond) DC.	ATCA	12x	max2	L105	7	7	10	8	8	5	6	10	3	7.1
67	*Leucanthemum lacustre* (Brot.) Samp.	ATGT	22x	lau1	L102	18	16	12	17	13	16	15	16	3	14.0
	Spearman rank correlations (ploidy vs. allele number)					0.79 (p<0.05)	0.86 (p<0.05)	0.87 (p<0.05)	0.90 (p<0.05)	0.86 (p<0.05)	0.85 (p<0.05)	0.79 (p<0.05)	0.93 (p<0.05)	0.62 (p<0.05)	

Table 15 - Number of alleles as achieved per species and marker. Last column is a mean number of alleles per species. In the row at the bottom correlation between allele numbers and ploidy is given.

Leucanthemum* diploids & polyploids								
	Total number of		Constant characters		Variable (uninformative) characters		Parsimony informative characters	
Marker	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels
A39	375	30	250 (67%)	-	51 (14%)	12 (40%)	74 (20%)	18 (60%)
B12	460	28	294 (64%)	-	44 (10%)	14 (50%)	122 (27%)	14 (50%)
B20	372	24	223 (60%)	-	48 (13%)	10 (42%)	101 (27%)	14 (58%)
C12	492	34	325 (66%)	-	63 (13%)	20 (59%)	104 (21%)	14 (41%)
C20	497	20	388 (78%)	-	62 (12%)	9 (45%)	47 (9%)	11 (55%)
C33	690	43	508 (74%)	-	85 (12%)	22 (51%)	97 (14%)	21 (49%)
D18	590	32	447 (76%)	-	45 (8%)	19 (59%)	98 (17%)	13 (41%)
D23	380	29	247 (65%)	-	56 (15%)	18 (62%)	77 (20%)	11 (38%)
D27	302	6	250 (83%)	-	35 (12%)	3 (50%)	17 (6%)	3 (50%)
cpDNA	1600	31	1528 (96%)	-	38 (2%)	22 (71%)	34 (2%)	9 (29%)
psbA	649	20	619 (95%)	-	18 (3%)	14 (70%)	12 (2%)	6 (30%)
petN1	436	4	417 (96%)	-	9 (2%)	3 (75%)	10 (2%)	1 (25%)
trnQ2	515	7	492 (96%)	-	11 (2%)	5 (71%)	12 (2%)	2 (29%)

*outgroups are excluded from calculation

Leucanthemum* polyploids only								
	Total number of		Constant characters		Variable (uninformative) characters		Parsimony informative characters	
Marker	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels	Nucleotide positions	Coded Indels
A39	375	28	256 (68%)	-	52 (14%)	12 (43%)	67 (18%)	16 (57%)
B12	460	27	308 (67%)	-	36 (8%)	15 (56%)	116 (25%)	12 (44%)
B20	372	23	229 (62%)	-	51 (14%)	9 (39%)	92 (25%)	14 (61%)
C12	492	30	334 (68%)	-	67 (14%)	17 (57%)	91 (18%)	13 (43%)
C20	497	17	394 (79%)	-	64 (13%)	6 (35%)	39 (8%)	11 (65%)
C33	690	29	548 (79%)	-	61 (9%)	10 (34%)	81 (12%)	19 (66%)
D18	590	23	457 (77%)	-	40 (7%)	12 (52%)	93 (16%)	11 (48%)
D23	380	27	256 (67%)	-	55 (14%)	18 (67%)	69 (18%)	9 (33%)
D27	302	4	255 (84%)	-	31 (10%)	3 (75%)	16 (5%)	1 (25%)
cpDNA	1600	20	1552 (97%)	-	29 (2%)	12 (60%)	19 (1%)	8 (40%)
psbA	649	14	629 (97%)	-	13 (2%)	8 (57%)	7 (1%)	6 (43%)
petN1	436	3	423 (97%)	-	8 (2%)	2 (67%)	5 (1%)	1 (33%)
trnQ2	515	3	502 (97%)	-	6 (1%)	2 (67%)	7 (1%)	1 (33%)

*outgroups are excluded from calculation

Table 16 - Summary on the number of characters and variable positions in the alignment. Parsimony informative and uninformative characters are calculated for *Leucanthemum* diploids and polyploids together, and solely within *Leucanthemum* polyploids. In both cases outgroup taxa are excluded.

3.4. Results

Sequencing – The overall number of reads obtained from 454 sequencing amounted to 133 316 which belonged to polyploid *Leucanthemum*. The numbers of reads obtained per species and marker are presented in **Table 12**. The read length on average was 368 bp and the length for particular markers is presented in **Table 12**. If the calculated minimum number of reads required to obtain at least 10 reads of each allele with 0.99 probability is treated as 100%, then the mean coverage across species was 145% (max. 268%) and most species received a higher number of reads than expected, except for five accessions which were below 100% (cat1 – 90%, cos1 – 83%, del1 – 81%, del2 – 80%, icr1 – 97%). After quality filtering, 128 008 reads (96%) were kept (**Table 13**) and these were further investigated for the presence of hybrid sequences and recombinants using BAPS and manual screening. Finally, 117 058 reads (88%) were kept (**Table 14**) and from these reads 2695 alleles were separated and the resulting number for particular species and markers is presented in **Table 15**. The number of alleles was significantly correlated with the ploidy (Spearman's rank correlation between different markers and ploidy r_S = 0.62-0.93, for all $p < 0.05$, **Table 15**) adding strength to the argument for considering selected markers as single- or low-copy genes the number of which follows on from the ploidy. The numbers of reads and alleles obtained for diploid *Leucanthemum* and outgroups are presented in Chapter 1.

Gene trees – Due to the high numbers of species and alleles, particular gene trees display a complex structure. The average number of alleles in the trees for 188 accessions (including diploids, polyploids and outgroup taxa) was 377 (min-max 258-437). The trees are presented in the appendix (**Appendix B**). The parsimony informative characters are presented in **Table 16**. Chloroplast haplotype network has the same structure as the one obtained by Greiner et al. (2012) and it is included in the appendix (**Appendix C Figure 61**).

Species network – As is visible in the network, the level of reticulation is high. *Leucanthemum* forms a monophyletic group well separated from the outgroup (**Figure 10**). Many taxa form groups irrespective of their ploidy, but often in accordance with the distribution area, so that closely occurring species are together. Groups which have a bootstrap support above 50 are named (**Figure 10, Table 17**). The first such group consists of *L. visianii* (4*x*), *L. chloroticum* (4*x*, 6*x*) and *L. illyricum* (8*x*) which all occur in the W Balkan Peninsula (I, *visianii*-group). Similarly, *L. pseudosylvaticum* (4*x*), *L. sylvaticum* (6*x*), and *L. merinoi* (6*x*), occurring in the NW Iberian Peninsula, are clustered together (II,

pseudosylvaticum-group). A cluster formed by *L. tridactylites* (2*x*) and *L. coronopifolium* subsp. *tenuifolium* (6*x*), which are both endemic to the central Apennine Peninsula, is also supported (IIIa, *tridactylites*-group). A group formed by *L. coronopifolium* (6*x*), *L. coronopifolium* subsp. *ceratophylloides* (6*x*), and *L. glaucophyllum* (10*x*), which are distributed in the SE Alps, is supported as well (IV, *coronopifolium*-group). A supported group is formed by *L. pallens* (6*x*) and *L. montserratianum* (10*x*), the ranges of which overlap in the eastern Iberian Peninsula where the few localities of the second taxon are located on Mount Montserrat (Catalan Pre-Coastal Range) and the former is widely distributed in the northern part of the Mediterranean region from the Iberian Peninsula to the northern Apennine Peninsula. Additionally, *L. catalaunicum* (10*x*) from SE Pyrenees (V, *pallens*-group) also belongs to this group. *L. maximum* (12*x*) and *L. lacustre* (22*x*) from the Iberian Peninsula are members of one cluster (VI, *maximum*-group). Some of the clusters are in accordance with both the ploidy and geographical distribution, such as the one formed by both subspecies of *L. corsicum* (4*x*): subsp. *corsicum* and subsp. *fenzlii* (VII, *corsicum*-group). *L. platylepis* (8*x*) and *L. heterophyllum* (8*x*), occurring in the SE Alps, Istria and NW Balkan Peninsula, also form a supported group (VIII, *heterophyllum*-group). *L. ircutianum* subsp. *asperulum* (4*x*) and tetraploid (4*x*) plants classified under *L. "pallens"*, which occur, respectively, in the southern and central Apennine Peninsula, form a supported group as well (IIIb, *tridactylites*-group). However, there are also some supported clusters which are more in accordance with the ploidy and show little geographic correspondence, such as the cluster composed from *L. pluriflorum* (2*x*), *L. gaudinii* subsp. *cantabricum* (2*x*), *L. gaudinii* (2*x*), and *L. delarbrei* (4*x*), where supported groups are formed by *L. pluriflorum* (2*x*) and *L. gaudinii* subsp. *cantabricum* (2*x*), then by *L. gaudinii* subsp. *cantabricum* (2*x*) and *L. gaudinii* (2*x*), and then by *L. gaudinii* (2*x*) and *L. delarbrei* (4*x*) (VIII, *gaudinii*-group). The last relationship may pinpoint the diploid *L. gaudinii* as the potential ancestor of *L. delarbrei*. A supported group is also formed by *L. ircutianum* (4*x*) and *L. adustum* (6*x*) (IXa, *ircutianum*-group), and it is located close to the supported group formed by *L. adustum* subsp. *margaritae* (6*x*) and *L. maestracense* (6*x*) (IXb, *ircutianum*-group). To illustrate other networks which contain less taxa and which may be easier to interpret, a series of reduced datasets was prepared and this included network just with diploids (2*x*) and tetraploids (4*x*) (**Appendix C, Figure 57**), then added hexaploids (6*x*) (**Appendix C, Figure 58**), and then added octoploids (8*x*) (**Appendix C, Figure 59**). If a stepwise increasing ploidy is assumed, this could present a hypothetical scenario which leads to the formation of higher polyploids. However, since this is not proved, the main reason to include these networks is that they show some splits that are

supported when higher polyploids are absent (e.g. division of the genus into two larger groups, in **Appendix C, Figure 57**). This may indicate that the complexity of the complete network and the lack of bootstrap support for larger groups within the genus (**Figure 10**) may result from the inclusion of high polyploids, because their origin can be traced to multiple ancestors from different parts of the network.

PCoA is included in the appendix (**Appendix C Figure 60**).

Species	Ploidy	Short	Diploid group*	Supernetwork group	cpDNA group*	cpDNA group*	Geographical area
Leucanthemum burnatii	2*x*	bur	Group 1	*burnatii* -group	II	H3-2-1	Southern France, SW Alps
Leucanthemum lithopolitanicum	2*x*	lit	Group 1	*burnatii* -group	unassigned	H2-1-1	SE Alps
Leucanthemum virgatum	2*x*	vir	Group 1	*gracilicaule* -group	I	H1-1-1	SW Alps
Leucanthemum graminifolium	2*x*	grm	Group 1	*gracilicaule* -group	II	H3-2-2	Southern France
Leucanthemum gracilicaule	2*x*	gra	Group 1	*gracilicaule* -group	unassigned	H2-1-1	Iberian Peninsula
Leucanthemum rotundifolium	2*x*	rot	Group 1	*gracilicaule* -group	unassigned	H1-1-2	Carpathians, Balkan Peninsula
Leucanthemum chloroticum (4*x*)	4*x*	chl	-	I, *visianii* -group	-	H1-1-1	Balkan Peninsula
Leucanthemum visianii	4*x*	vis	-	I, *visianii* -group	-	H1-1-1	Balkan Peninsula
Leucanthemum chloroticum (6*x*)	6*x*	chl	-	I, *visianii* -group	-	H1-1-1	Balkan Peninsula
Leucanthemum illyricum	8*x*	ill	-	I, *visianii* -group	-	H1-1-1	Balkan Peninsula
Leucanthemum pseudosylvaticum	4*x*	ips	-	II, *pseudosylvaticum* -group	III	H1-2-1	Iberian Peninsula
Leucanthemum merinoi	6*x*	mer	-	II, *pseudosylvaticum* -group	III	H1-2-1	Iberian Peninsula
Leucanthemum sylvaticum	6*x*	syl	-	II, *pseudosylvaticum* -group	III	H1-2-1	Iberian Peninsula
Leucanthemum coronopifolium subsp. *tenuifolium*	6*x*	cot	-	IIIa, *tridactylites* -group	-	H1-1-1	Apennine Peninsula
Leucanthemum tridactylites	2*x*	tri	Group 1	IIIa, *tridactylites* -group	unassigned	H2-1-1	Apennine Peninsula
Leucanthemum ircutianum subsp. *asperulum*	4*x*	ias	-	IIIa, *tridactylites* -group	I	H1-1-1	Apennine Peninsula
Leucanthemum "pallens" (4*x*)	4*x*	pal	-	IIIa, *tridactylites* -group	I, II	H1-1-1, H3-2-2	Apeninne Peninsula
Leucanthemum glaucophyllum	10*x*	gla	-	IV, *coronopifolium* -group	-	H1-1-1	SW Alps
Leucanthemum coronopifolium subsp. *coronopifolium*	6*x*	cor	-	IV, *coronopifolium* -group	-	H3-2-1	SW Alps
Leucanthemum coronopifolium subsp. *ceratophylloides*	6*x*	coc	-	IV, *coronopifolium* -group	II	H3-1-1, H3-2-1	SW Alps
Leucanthemum ircutianum	4*x*	irc	-	IXa, *ircutianum* -group	I	H1-1-1	widespread
Leucanthemum adustum subsp. *adustum*	6*x*	adu	-	IXa, *ircutianum* -group	I	H1-1-1	W Alps, Apeninne Peninsula
Leucanthemum adustum subsp. *margaritae*	6*x*	ama	-	IXb, *ircutianum* -group	I	H1-1-1	E Alps, Carpathians, Balkan Peninsula
Leucanthemum maestracense	6*x*	mas	-	IXb, *ircutianum* -group	I	H1-1-1	Iberian Peninsula
Leucanthemum catalaunicum	10*x*	cat	-	V, *pallens* -group	-	H1-1-1	Pyrenees
Leucanthemum montserratianum	10*x*	mon	-	V, *pallens* -group	I	H1-1-1	Iberian Peninsula
Leucanthemum pallens (6*x*)	6*x*	pal	-	V, *pallens* -group	I, II, unassigned	H1-1-1, H3-2-2	Mediterranean
Leucanthemum maximum	12*x*	max	-	VI, maximum-group	I	H1-1-1	Iberian Peninsula, Pyrenees
Leucanthemum lacustre	22*x*	lau	-	VI, maximum-group	I	H1-1-1	Iberian Peninsula
Leucanthemum corsicum subsp. *corsicum*	4*x*	cos	-	VII, *corsicum* -group	-	H1-1-1	Corsica
Leucanthemum corsicum subsp. *fenzlii*	4*x*	cof	-	VII, *corsicum* -group	-	H1-1-1	Corsica
Leucanthemum gaudinii subsp. *cantabricum*	2*x*	gac	Group 2	VIII, *gaudinii* -group	II	H3-2-1	Iberian Peninsula
Leucanthemum gaudinii subsp. *gaudinii*	2*x*	gag	Group 2	VIII, *gaudinii* -group	II	H3-1-1, H3-2-1	Alps, Carpathians
Leucanthemum delarbrei	4*x*	del	-	VIII, *gaudinii* -group	II, unassigned	H3-1-1, H3-2-2	Massif Central
Leucanthemum pluriflorum	2*x*	plu	Group 2	VIII, *gaudinii* -group	III	H1-2-2	Iberian Peninsula
Leucanthemum heterophyllum	8*x*	het	-	VIII, *heterophyllum* -group	I	H1-1-1	Eastern Alps, Balkan Peninsula
Leucanthemum platylepis	8*x*	pla	-	VIII, *heterophyllum* -group	I	H1-1-1	Balkan Peninsula
Leucanthemum cf. monspeliense	2*x*	ceb	Group 2	-	-	H3-2-2	Southern France
Leucanthemum ligusticum	2*x*	lig	Group 2	-	-	H3-2-1	Apennine Peninsula
Leucanthemum gaudinii subsp. *barrelieri*	2*x*	gab	Group 2	-	II	H3-2-1	Pyrenees
Leucanthemum vulgare	2*x*	vul	Group 2	-	II	H3-2-1, H3-2-2	widespread
Leucanthemum vulgare subsp. *eliasii*	2*x*	vel	Group 2	-	II	H3-2-1, H3-2-2	Iberian Peninsula
Leucanthemum vulgare subsp. *pujiulae*	2*x*	vup	Group 2	-	II	H3-2-2	Iberian Peninsula, Pyrenees
Leucanthemum gallaecicum	2*x*	gal	Group 2	-	unassigned	H3-2-1	Iberian Peninsula
Leucanthemum halleri	2*x*	hal	Group 1	-	unassigned	H3-1-1	NE Alps
Leucanthemum laciniatum	2*x*	lai	Group 1	-	unassigned	H2-1-1	Apennine Peninsula
Leucanthemum meridionale	4*x*	med	-	-	-	H1-1-1	Southern France
Leucanthemum ircutianum subsp. *crassifolium*	4*x*	icr	-	-	I	H1-1-1, H3-2-2	Iberian Peninsula
Leucanthemum ircutianum subsp. *cantabricum*	4*x*	ica	-	-	I, II	H3-2-1, H3-2-2	Iberian Peninsula
Leucanthemum ircutianum subsp. *leucolepis*	4*x*	ile	-	-	I, II	H1-1-1, H3-2-1	Apennine Peninsula, Balkan Peninsula
Leucanthemum monspeliense	4*x*	mop	-	-	II	H3-2-1	Southern France
Leucanthemum aligulatum	6*x*	ali	-	-	-	H1-1-1	Iberian Peninsula, Pyrenees
Leucanthemum subglaucum	6*x*	sub	-	-	-	H1-1-1	Southern France
Leucanthemum atratum subsp. *atratum*	6*x*	atr	-	-	II	H3-2-1	NE Alps
Leucanthemum glaucophyllum cv. "Esterel"	8*x*	est	-	-	-	H1-1-1	Southern France
Leucanthemum favargeri	8*x*	fav	-	-	I	H1-1-1	Iberian Peninsula, Pyrenees
Leucanthemum pachyphyllum	10*x*	pac	-	-	I	H1-1-1	Apennine Peninsula

*Chapter 1 *Greiner et al. 2012 *Appendix D, figure 61

Table 17 - Summary of assignment of *Leucanthemum* species to the groups as obtained from supernetwork analysis, cpDNA analysis (Greiner et al. 2012) and their geographical distribution.

F**igure 11** constructed on the 0/1 matrix displays pairwise similarities among all taxa from the genus *Leucanthemum*. Detailed inferences are often hampered by the fact that many taxa show similarities to more than one taxon. However, previously mentioned members of the supported clusters from the supernetwork analysis also generated high levels of similarity in this analysis. In addition to these, the following may be treated as potentially related: e.g. *L. atratum* (6*x*) to *L. halleri* (2*x*) and *L. coronopifolium* (6*x*); *L. ircutianum* subsp. *ircutianum*, subsp. *cantabricum*, subsp. *crassifolium* and members of the

'Group 2' of diploids (cf. Chapter 1). Many taxa on a hexaploid (6*x*) and higher ploidies show relatively high levels of similarity to each other.

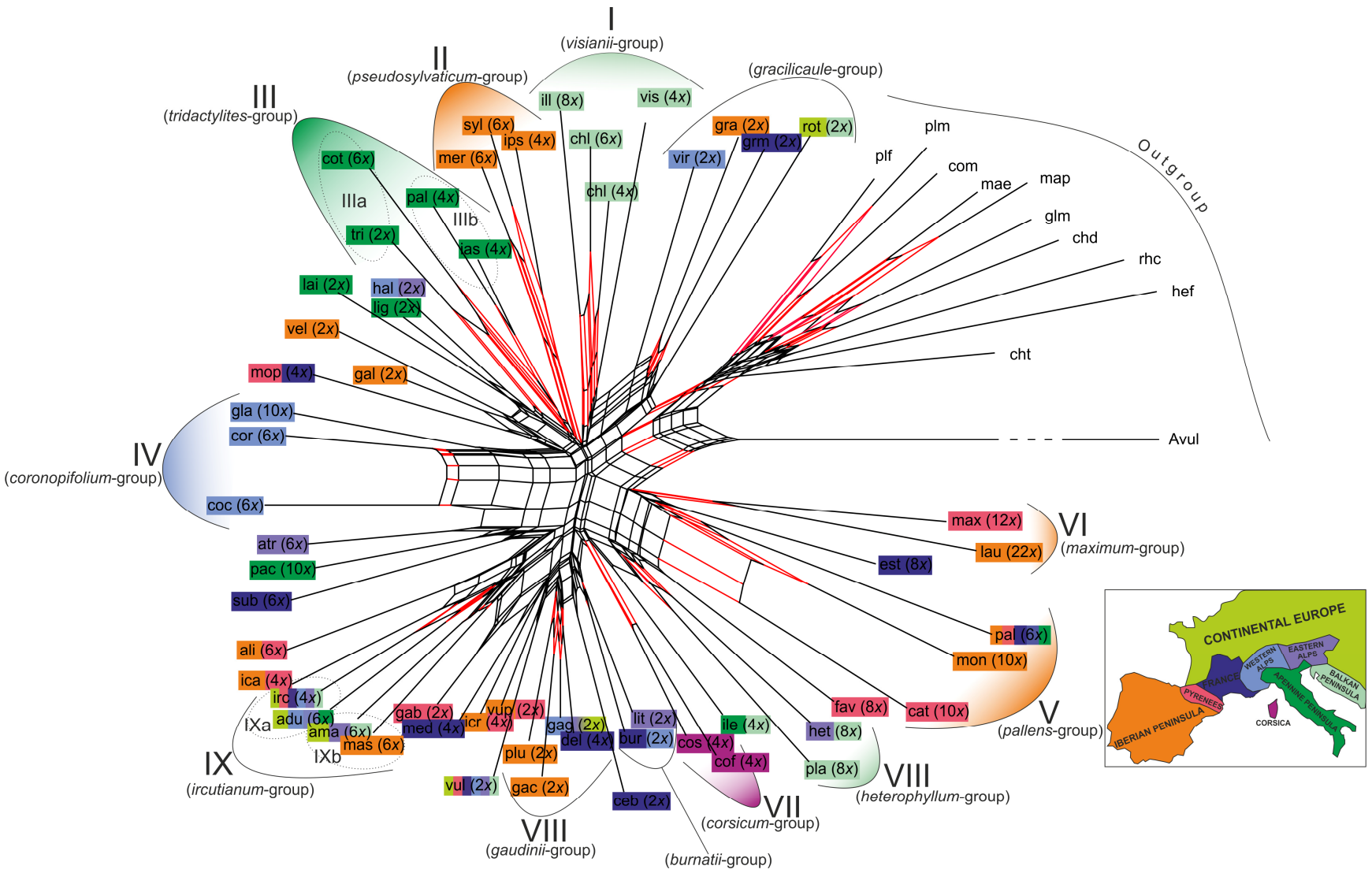

Figure 10 - Supernetwork as obtained from low copy nuclear genes and cpDNA. The red lines belong to the splits with bootstrap support greater than 50. Taxon shortcuts are followed by ploidy in brackets and are coloured according to the geographical origin (which is explained in the inset).

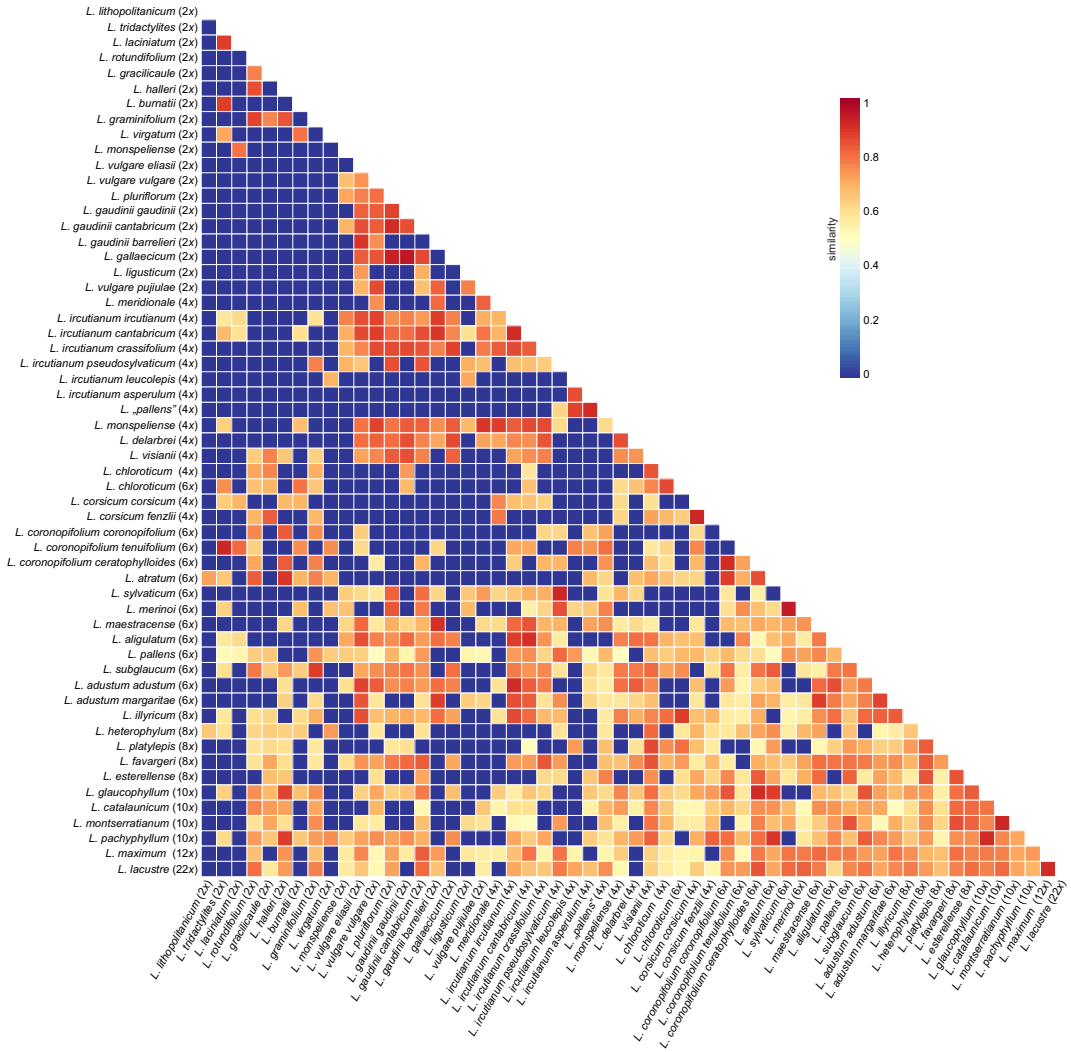

Figure 11 - The matrix displaying pairwise similarity indices constructed by subtracting from 1 normalized Nei-Li distance between two samples on 0/1 matrix. 0 (blue) indicates low similarity whereas 1 (red) indicates high similarity (similarity is proportional to the number of times two samples belonged to the same supported group in the gene trees).

3.5. Discussion

Methodological aspects

It is clear that the present analysis does not present a complete answer to the question of the evolution of polyploidy in *Leucanthemum*. The biggest issue hampering a more detailed survey is that of data analysis. Those methods which will be able to analyze such datasets are likely to be developed, but at the present there are no published solutions or computer programs applicable for the analysis of the present data, which contain a high number of taxa at different levels of polyploidy. As the solution to this problem, a supernetwork approach is proposed here. Since this works more or less as a generalization, it is free from assumptions regarding allele treatment (i.e. assigning alleles to parental genomes across markers), the unavailability of which prevents other analyses. Unfortunately, it also has some drawbacks, such as the lack of the possibility to distinguish ILS and true hybridization. To test the impact of ILS on the "reticulateness" of the network and the correctness of the reconstructions, future studies should evaluate this method using simulations.

Specific considerations

As seen in this example, tracing the polyploid history in *Leucanthemum* is difficult. This is especially true if one intends to reconstruct a complete phylogeny, where each taxon has a supported position and estimated relationships with all other members of the genus. Theoretically, it is manageable but may require even more detailed sampling and/or finer methods than those employed in the present study. However, some insights into the smaller groups may also be gained from the present results. Those groups that received significant support from the bootstrap analysis and had been mentioned in previous studies are discussed below.

L. corsicum (4*x*) is interesting as it is the only species in the genus which colonized a Mediterranean island. Unfortunately, in the analysis it was reconstructed without any obvious connection to the remaining taxa. Horvatić (1928) supposed that *L. corsicum* (4*x*) is related to central Apennine species of *L. tridactylites* (2*x*) and *L. atratum* (6*x*) (since *L. atratum* do not occur there, likely he meant *L. adustum*). In the present analysis, the only trace of ancestry may be found in the shared split between *L. corsicum* (4*x*) and *L. burnatii* (2*x*), *L. litopolithanicum* (2*x*), *L. ircutianum* subsp. *leucolepis* (4*x*), *L. platylepis* (8*x*), and *L. heterophyllum* (8*x*) where the first three taxa could be treated as plausible candidates for the ancestors in terms of ploidy. Insular endemics are interesting objects to study, because their evolution is often shaped by genetic drift, small population sizes or single

colonization events (Suda et al. 2005, Woolfit & Bromham 2005), but those processes could also lead to the blurring of the relationships with other taxa. Therefore, more detailed study is needed to establish their relationships with other species.

Mirković (1966) proposed that *L. platylepis* (8*x*) originated by autopolyploidy of *L. visianii* (4*x*), but in the current study it is more closely related to *L. heterophyllum* (8*x*), which has the same ploidy. Furthermore, *L. platylepis* (8*x*) and *L. heterophyllum* (8*x*) occupy the same or close areas to those where *L. platylepis* (8*x*) is distributed on NW Balkan Peninsula and Istria and *L. heterophyllum* (8*x*) from SE Alps to Istria. Papeš (1971) noted some similarities between both taxa and stated that *L. platylepis* (8*x*) might have colonized the Balkan Peninsula from the Alps, which links both taxa. Papeš (1975) also included them in the same larger group of morphologically similar species (comprising *L. heterophyllum*, *L. illyricum*, *L. visianii*, *L. chloroticum*, and *L. platylepis*). Taking into consideration the above-mentioned studies and the results from this one, it seems that *L. heterophyllum* (8*x*) and *L. platylepis* (8*x*) are closely related to each other and may represent a vicariant species or a species with a disjunct areal. A possible close relative is located in the same cluster (but without bootstrap support), i.e. *L. ircutainum* subsp. *leucolepis* (4*x*) which occurs in the same geographical area. As the latter species is tetraploid and the previous ones are octoploids, there is a possibility that they originated by autopolyploidy. In the present study, another group of Balkan species constituted from *L. visianii* (4*x*), *L. chloroticum* (4*x*, 6*x*) and *L. illyricum* (8*x*) formed a group supported by bootstrap analysis. In general, species from this area show many common morphological characteristics: unbranched stems, smooth and leathery leaves and stems, narrow leaves pinnate or narrowly linear, and achenes with ligulate florets always with pappus (Papeš 1971). It is also interesting that on the Balkan Peninsula only two diploid species are found: widespread *L. vulgare* (2*x*) and *L. rotundifolium* (2*x*) (a Carpathian sub-endemic species), which is restricted to one mountain. Possibly for this reason, Papeš (Papeš 1971, Papeš 1975) proposed that some of the lower ploidies may have originated from higher ones by direct hybridization between different ploidy levels (e.g., 6*x* from a cross $4x \times 8x$). Taking into consideration all the data available up to now, this hypothesis is plausible and probably the most parsimonious way to explain their similarity and the absence of diploids.

Species from NW Iberian Peninsula - *L. pseudosylvaticum* (4*x*), *L. sylvaticum* (6*x*), and *L. merinoi* (6*x*) - form a group with bootstrap support. Their close relationships were previously reported by Greiner (Greiner 2011, Greiner & Oberprieler 2012, Greiner et al. 2013, Oberprieler et al. 2014). Notably, *L. pluriflorum* (2*x*) which is the presumed maternal parent is not placed together with them but instead joins a group formed by several other

diploid species. This is presumably the effect of stronger relationships between this taxon and other diploids than those that exist between polyploids and *L. pluriflorum* (2*x*). This is shown in the pairwise similarity matrix where the level of similarity between polyploids and *L. pluriflorum* (2*x*) is high and close to 1. This could be explained not only by the hypothetical allopolyploid origin of the polyploids from the *L. pluriflorum* clan, but also by the hybridization among diploids (Chapter 1) which would create stronger bonds between diploids, while the genetic exchange between diploid-polyploid populations could stop shortly after their origin. Based on eco-climatological niche modeling, Oberprieler et al. (2014) suggested that *L. gallaecicum* (2*x*) or *L. gaudinii* subsp. *cantabricum* (2*x*) may have acted as a paternal parent in the formation of polyploids from the *L. pluriflorum* clan. As depicted by the pairwise similarity matrix, *L. gallaecicum* (2*x*) does not show any similarity to the polyploids, which excludes this species as a potential ancestor. Interestingly, *L. gaudinii* subsp. *cantabricum* (2*x*) shows a remarkably high similarity in the pairwise similarity matrix to all polyploids from that group which rather argues that this species participated in their formation. It should also be noted that both *L. gallaecicum* (2*x*) and *L. gaudinii* subsp. *cantabricum* (2*x*) show a high similarity to *L. pluriflorum* (2*x*).

L. tridactylites (2*x*) and *L. coronopifolium* subsp. *tenuifolium* (6*x*), despite their different ploidy levels, show affinity to each other as a group with bootstrap support and in a similarity matrix, where the similarity between those two taxa is close to 1. They are both endemic to the central Apennine Peninsula, as are *L. ircutianum* subsp. *asperulum* (4*x*) and tetraploid (4*x*) "*L. pallens*" (which may be the same taxon as *L. ircutianum* subsp. *asperulum*), which are also members of this cluster. Therefore, in this case it could be hypothesized that *L. coronopifolium* subsp. *tenuifolium* (6*x*) originated as a hybrid between *L. tridactylites* (2*x*) and *L. ircutianum* subsp. *asperulum s. l.* (4*x*); however, this hypothesis should also be tested by different methods, which should also include morphological comparison.

L. coronopifolium subsp. *coronopifolium* (6*x*), *L. coronopifolium* subsp. *ceratophylloides* (6*x*), and *L. glaucophyllum* (10*x*) are grouped together and all these taxa occur in the SW Alps. In the cases of the first two taxa, the evidence argues for their close relationship, which is also expressed by classifying them into one species. With regards to *L. glaucophyllum* (10*x*), the origin similarity matrix furthermore indicates that *L. halleri* (2*x*) and *L. pachyphyllum* (10*x*) may also be related to it. Therefore, one could hypothesize that *L. glaucophyllum* (10*x*) originated via hybridization between *L. halleri* (2*x*) and one of the mentioned hexaploid species (or their common ancestor). Furthermore, *L. glaucophyllum* (10*x*) does not display any strong relationship with *L. glaucophyllum* var.

esterellense (=*L. "esterellense*") either in the network or similarity matrix, which further supports their separation as independent species. This is discussed in Chapter 3.

Two decaploid species from the NE Iberian Peninsula, *L. catalaunicum* (10*x*) and *L. montserratianum* (10*x*), are also classified in a group which received bootstrap support. On a morphological basis, they are easily distinguishable by the width of their involuclar bracts, the size of the capitula and individual florets, leaf shape and branching pattern (Vogt, 1991). They also inhabit different areas: *L. catalaunicum* (10*x*) occurs in a high mountain zone (1200-2200 m) and *L. monserratianum* (10*x*) is a narrow endemic inhabiting mainly the northern slopes of the Mount Montserrat at a height of 700 m to 800 m (Vogt 1991). Their common characteristic is the ploidy level which furthermore is unique to the whole Iberian Peninsula and adjacent areas to the north. Since they are different species but apparently related to each other, it may be postulated that their origin could involve the same parental species (or at least one), which moreover also occurred in the geographical proximity. One such species may be *L. pallens* (6*x*), which is grouped with them and could have played a role in their emergence. In particular, there is a close connection in the supernetwork between *L. pallens* (6*x*) and *L. montserratianum* (10*x*) which is also visible in the similarity matrix and could reflect a closer relationship between these two species than that which exists between *L. catalaunicum* (10*x*) and *L. pallens* (6*x*).

The grouping of *L. gaudinii* (2*x*) and *L. delarbrei* (4*x*) is also of interest. Their morphology may be regarded as similar (e.g. through *vulgare*-like leaves), but most importantly they also share an analogous habitat, both occur in a high mountain environment (mostly in sub-alpine and alpine zones). *L. gaudinii* (2*x*) does not occur in the Massif Central, but while it occurs in the Alps some of the taxa classified as its subspecies are found in the Pyrenees. Therefore, *L. gaudinii* (2*x*) could hypothetically be placed among the ancestral candidates of *L. delarbrei* (4*x*). Studies on cpDNA revealed that *L. delarbrei* (4*x*) shares a chloroplast type with *L. halleri* (2*x*) (Greiner et al. 2012), but this alpine taxon similarly does not occur presently in the Massif Central. Therefore, it may represent an interesting origin which presumably involved long-distance dispersal or vicariance, but more detailed studies are needed to clarify its phylogenetic position. Also, what should be noted is that it possesses some similarity in the pairwise similarity matrix to *L. monspeliense* (4*x*) distributed in adjacent areas (but with a different morphology and habitat). This again highlights the trend that geography played an important role in the emergence of polyploid species and/or it is still important to their contact and may facilitate gene flow.

Another group is formed by *L. ircutianum* (4*x*) and *L. adustum* (6*x*) – two species for which some previous studies are available. Dowrick (1952) and especially Pearson (1967) found difficulties in separating these two species and treated them as one taxon. However, with the adoption of ploidy-based species discrimination (Vogt 1991), and taking into account the fact that they also differ in their habitat requirements and morphology, there are clear arguments to support their separation into two different species. To a certain degree they still overlap geographically and form hybrids (Przywara 1974, Villard 1971), the artificial creation of which is very efficient as both taxa cross easily and produce fertile offspring (Villard 1971). Oberprieler et al. (2011) found a close relationship between those two species based on AFLP studies, and in particular *L. adustum* (6*x*) and *L. ircutianum* (4*x*) were more similar to each other than to *L. vulgare* (2*x*), which was treated as a hypothetical parental species based on morphological similarity. The conclusion of this 2011 paper was that both taxa are presumably allopolyploids. The present study still argues for their close relationship, but their presumed ancestors could not be specified. Further studies are needed and provisionally some similarities in the pairwise similarity matrix may only be noted between *L. adustum* (6*x*) and *L. vulgare* subsp. *eliasii* (2*x*), and *L. gaudinii* subsp. *barrelieri* (2*x*), while *L. ircutianum* (4*x*) shows many similarities with the taxa from 'Group 2' diploids (Chapter 1).

L. maximum (12*x*) and *L. lacustre* (22*x*), which are two taxa of the highest ploidy in the genus, are grouped together. Also, studies on cpDNA sequence variation by Greiner et al. (2012) showed that these two species (at least 2 accessions) share the same haplotype. It is a parsimonious solution to assume that such a high ploidy (22*x*) occurred in one step as an autopolyploid of the only dodecaploid (12*x*) in the genus: *L. maximum*. This is also supported by the similarity matrix, where these two taxa show a high similarity to each other. However, when the autopolyploidy of *L. maximum* occurs, it should be assumed that the resulting plant is 24*x* not 22*x*. Other considerations concerning the origin of that species suggest that it may be an artificial hybrid which escaped from cultivation. The reason for this hypothesis is the fact that this species resembles garden forms commonly grown throughout the Iberian Peninsula (Vogt 1991 and references therein). Since the long distance between natural *L. maximum* (12*x*) populations would almost certainly prevent the natural formation of *L. lacustre* by autopolyploidy and dispersal, human driven artificial formation could play a significant role. However, it is unclear if any other species was involved in the formation of that polyploid taxon; at least, there is no support for any in the current analysis. Forms resembling *L. lacustre* which have escaped from the gardens and developed wild populations which reproduce by seeds are quite common in the Iberian

Peninsula (personal observations). It may, therefore, be reasonable to think that *L. lacustre* represents an old escaper from the garden which through chance and suitable habitat was able to become established and naturalized. Still, its relatively long presence on the coast of Portugal should be sufficient to treat it as an independent species.

General considerations

When analyzing the network on the genus level a very interesting grouping is formed by four diploid species: *L. gracilicaule*, *L. rotundifolium*, *L. graminifolium*, and *L. virgatum* (*gracilicaule*-group, **Figure 10, Table 17**). Each of these species presents a unique morphology and has a well-defined range separated from the others. Also, as the results of Chapter 1 show (cf. **Figure 3**), these four species may be the core *Leucanthemum* diploid species which did not play a significant role in the formation of polyploids, but are related to all other species and may actually be the closest descendants of the founder species. Other species usually form groups of mixed ploidy, but often with geographical correspondence. There is a tendency that those groups are connected to the areas of hypothetical glacial refugia, i.e. Balkan Peninsula, SW Alps, Apennine Peninsula. This, therefore, links the evolution of *Leucanthemum* and more specifically polyploid formation with climatic changes occurring through the Quaternary.

The Ice Age may be one of the main factors which could have created favorable circumstances and facilitated polyploid formation in the genus. As early as 1975, Favarger (1975) suspected that the Ice Age played a role in polyploid formation in *Leucanthemum*. The idea that the climatic oscillations in the Pleistocene were important in the evolution of many genera that contain polyploids is often emphasized (e.g. Brochmann et al. 2004, Ståhlberg & Hedrén 2010, Casazza et al. 2012, Kolář et al. 2013). As Favarger (1975) concludes, *Leucanthemum* experienced range contractions in the glacial time and range expansion in interglacial periods. Evidence from *Leucanthemum* in the Iberian Peninsula (Oberprieler et al. 2014) furthermore suggest that some species may also have experienced the opposite effect – range expansion in the glacial periods followed by range contraction in the interglacial periods. This does not change the general picture, but it contributes to the complexity of changes occurring in the Quaternary. Such migrations and range contractions promote contact with other species or range breaking which leads to patchy distribution and species survival in a small populations followed by local extinction. In this way, any advantageous change will be promoted and such change may be a polyploidy. As he Iberian Peninsula presents the highest number of polyploid and diploid taxa, it may also be presumed that the higher heterogeneity of habitat there provides more space for different forms and that where raw material for polyploidization is abundant (i.e. many

taxa occur) the number of polyploids is higher because of the increased possibilities in creating combinations from the parental genomes. Similar findings have been reported for the genus *Draba* L. (Jordon-Thaden & Koch 2008) which showed that polyploid species were more frequent in areas with high species richness. The presence of many polyploids in the Iberian Peninsula as well as in the SW Alps, and Balkan and Apennine Peninsulas could also be related to glacial refugia, as mentioned above.

Since these "geographical clusters" are observed, it has to be clear that not only taxonomic and ploidy information should be used in the ancestry estimation but also locally abundant species which may play a key role in the formation of geographically close polyploids. This attempt requires much more detailed sampling using not collected taxa as a criteria but geographic coverage which covers regular intervals of sampled populations with special focus on areas with a high number of diverging habitats (i.e. mountains). Such a methodology provides an alternative view to often proposed scenarios of polyploid origin which consider only ploidy levels but tend to underestimate geography. For example, the hypothesis for the Balkan *L. illyricum* origin postulates that it may be a hybrid between central European *L. adustum* and south Italian species (Papeš 1973). However, as this study shows, it is rather more closely related to other polyploids occurring in proximity to it on the Balkan Peninsula. An extreme example of a "geographical concept" of polyploid taxonomy may be provided by the genus *Dupontia* R. Br. Which, after employing molecular methods (AFLP), showed tendencies to group according to the geographical origin of the samples instead of ploidy or morphology which led the authors to the conclusion that the genus should be treated as single polymorphic species instead of three taxa (Brysting et al. 2004). Similarly, a study conducted on *Centaurea* L. suggested that species defined on a morphological basis are highly incongruent with the genetic data and this suggested that extensive introgression is present among different taxa but showed that geographical patterns may found (Hilpold et al. 2014).

Previous studies on *Leucanthemum* assumed that allopolyploidy is the prevailing mode of polyploid formation in the genus, as well as in particular taxa that were studied in more detail (Faverger 1960, Vogt 1991, Oberprieler et al. 2011, Greiner 2011, Greiner et al. 2013, Oberprieler et al. 2014). Pearson (1967) argued that the *Leucanthemum* tetraploids (4*x*) he examined were not clear allopolyploids but segmental allopolyploids (i.e. containing two partially homologous chromosome sets, Stebbins 1950) and suggested that most of the polyploids in the genus could have originated in this way (but he was not aware that polyploids above hexaploid level exist). The results of the present study also

support the allopolyploid origin of most taxa; however, autopolyploidy cannot be ruled out in certain cases. Examples and hints may be found in the constructed network where most of the taxa are connected to at least two different taxa, some are grouped into assemblages following their taxonomical classification, and some are grouped together with species with lower ploidy levels (**Figure 10**).

Favarger (1975) proposed that the whole *Leucanthemum* polyploid complex evolved through the formation of a tetraploid *L. ircutianum* which then crossed with other diploid taxa producing hexa-, octo-, and higher poly-ploids. Indeed, it seems that many species with the higher ploidy show morphological similarities to each other. On a morphological basis, many of these species form an *L. vulgare* aggregate (Euro+Med PlantBase 2006) which argues for a high morphological similarity. Also, molecular studies on cpDNA (Greiner et al. 2012, Chapter 3) support the view that the majority of polyploid species belong to the same haplotype group which contains only one diploid species (*L. virgatum*). This may suggest that either this species is the ancestor of the majority of polyploid species or that relatively few other but closely related species were involved in polyploid formation (of the taxa forming group I, sensu Greiner et al. 2012). With the formation of the first polyploids which originated from that maternal lineage, they continued to be the maternal side in the subsequent hybridizations or those hybridizations only involved taxa from that lineage. There are studies indicating that the capability to produce unreduced gametes may be heritable (Bretagnolle & Thompson 1995, Ramsey & Schemske 1998 and references therein) and this could explain the recurrent polyploid formation in that lineage.

Some authors have tried to form detailed hypotheses about the polyploid formation in the genus under study (Papeš 1975, Villard 1971). They have often tended to give an almost complete picture by drawing a graph starting with diploids at the bottom which are connected to their polyploid descendants (Papeš 1975, Villard 1971). This is a rather simplified approach and involves a lot of assumptions, since these researchers had even less evidence to support their claims than we do now. The scenario advanced by Papeš (1975) proposing the origin of Yugoslavian *Leucanthemum* polyploids does not seem to be plausible in view of the modern treatment of *Leucanthemum* which no longer considers African diploids to be *Leucanthemum* (i.e. *Leucanthemum hosmariense* = *Rhodanthemum hosmariense*). In fact, her model also cannot be accepted due to new data on *Leucanthemum s. str*. Her theory is sometimes quite speculative suggesting, for example, that *L. graminifolium* (2*x*) and *L. burnatii* (2*x*) gave rise to *L. tridactylites* (4*x*) which then crossed with a hypothesized Asian *Leucanthemum* species (2*x*) to produce *L. chloroticum*

(6*x*). Another such scenario is that of Villard (1971) on the evolution of Swiss/Alpine polyploids. He also proposed a possible mode of polyploid formation up to decaploid (10*x*), but his scenario also looks quite speculative and does not provide too much detail (i.e. he proposes that the ancestral diploid produced an ancestral tetraploid which through possible hybridization with *L. graminifolium* (2*x*) gave rise to *L. adustum* (6*x*)). Although it may seem very attractive to provide an accurate scenario which completely explains how different species were formed with the inclusion of their ancestors and descendants, it may be illusive at the same time. Factors contributing to this situation are embedded in the nature of polyploids which include multiple formation, gene flow among the same and different ploidy levels, karyotypic rearrangements, and the retention of ancestral genes (Soltis & Soltis 1999, Arnold et al. 2012, Lipman et al 2013). Thus, the higher the ploidy the more complex the amalgamate genome the plant may contain. As a result, the complexity of the network grows with the ploidy and number of taxa – and in extreme cases even with each specimen. In the case of *Leucanthemum,* where many species above the hexaploid level seem to be related to each other, it is even more difficult because if one is not able to separate hypothetical ancestors then separating descendants is even more unlikely. Evolution is a dynamic process, as are polyploid species. So, it will be especially difficult to infer the history of species with large distribution areas, such as *L. ircutianum*, *L. pallens* or *L. adustum* which contain many deviating morphological types often restricted spatially. To solve the evolution of *Leucanthemum* and propose a detailed phylogenetic scenario, more precise methods will be needed, accompanied by even denser sampling.

4. CHAPTER 3

Puzzling phylogeography of high polyploid *Leucanthemum glaucophyllum* (Compositae, Anthemideae) and closely related taxa – including results from flow cytometry, AFLP and 454 sequencing

This chapter is designed for publication with following authorship: Kamil Konowalik, Andreas Fackelmann, Theodor Poettinger, Robert Vogt, Christoph Oberprieler. Author contributions are as follows: study conception and design: CO, RV, KK; laboratory work: KK (flow cytometry, 454 sequencing, cpDNA amplification) AF (AFLP), TP (AFLP initial optimization); determination and collection of specimens: RV, CO; writing: KK; analysis of the data: KK (flow cytometry, 454 sequencing, cpDNA, AFLP), AF (AFLP).

4.1. Abstract:

This study examines the relationships among few closely related high polyploid species of the genus *Leucanthemum* (Compositae, Anthemideae). Sampled species include *L. glaucophyllum* ($2n = 10x = 90$) distributed in Ligurian Alps, *L. subglaucum* ($2n = 6x = 54$) from Massif Central, *L. pallens* ($2n = 6x = 54$) distributed across north Mediterranean basin from Iberian Peninsula to Apennine Peninsula, and *L. ircutianum* subsp. *leucolepis* ($2n = 4x = 36$) occurring in Balkan and Apennine Peninsulas. 165 plants from 38 populations were used to infer relationships among these polyploid taxa. The methods included ploidy assessment with flow cytometry, AFLP which was further optimized with program AMARE (which aims at reducing the data matrix by deleting inconsistent bands), chloroplast haplotype network reconstruction followed by nested clade analysis, and species network based on low copy nuclear markers obtained from 454 sequencing. Ploidy screening revealed a new octoploid cytotype ($2n = 8x = 72$) in Esterel Massif which is treated here as „*L. esterellense*". Moreover, results indicate that the whole group has a reticulate structure and complex evolutionary history. Gene flow among different species and different ploidy levels is plausible as well as hybridization which is revealed in one of the studied populations.

Keywords: polyploidy, hybridization, biogeography, Mediterranean, *Leucanthemum*

4.2. Introduction

Examining the evolutionary history of polyploids is challenging. The key factors that contribute to this situation are large genomes and their origin. Typically a new polyploid plant evolves over a triploid bridge where unreduced pollen or egg cells are involved in the fertilization and leads to formation of a triploid. Other possibilities include formation through a homoploid bridge or somatic doubling (Tayalé & Parisod 2012). Those scenarios can be much more complicated at higher ploidy levels ($6x$, $8x$, $10x$, etc.) since they may combine parents with different ploidies including polyploids with different formation histories. Moreover, one polyploid "species" is usually formed multiple times which could also contribute significantly to its genetic diversity (Soltis & Soltis 1999). Nascent polyploids face many obstacles (e.g. minority cytotype exclusion) and their establishment is likely a matter of chance and availability of new niches (Weiss-Schneeweiss et al. 2013). Escaping into a new niche is crucial for newly formed polyploid

because they are facing the risk of backcrossing to the parental species which often results in producing sterile progeny or introgression.

The genus *Leucanthemum* Mill. (Compositae, Anthemideae) consists of around 41 species distributed in Europe with the highest diversity on Iberian Peninsula, Alps and Balkan Peninsula (Vogt 1991, Euro+Med PlantBase 2006). While most species occupy the northern part of the Mediterranean and European mountains, some are widely distributed and reach as far as Siberia (*L. vulgare*, and *L. ircutianum*). Because of human activity these two species occur nowadays all over the world and are treated as invasive (Clements et al. 2004, Khuroo et al. 2010, DiTomaso 2012, Busso et al. 2013). *Leucanthemum* is also well known as an ornamental plant with the most famous max chrysanthemum (*L. maximum*), ox-eye daisy (*L. vulgare*) and dozens of varieties and hybrids (e.g. shasta daisy, *Leucanthemum* × *superbum*). Noticeable feature of this genus is a high number of polyploid species. Of the accepted 41 species there are only 19 diploids while the others span the range from tetraploid ($2n = 4x = 36$) to dokosaploid ($2n = 22x = 198$).

This study focuses on a few polyploid species arranged by Briquet and Cavillier (1906) as varieties of *L. vulgare* subsp. *glaucophyllum*. This are *L. vulgare* subsp. *glaucophyllum* var. *eu-glaucophyllum*, *L. vulgare* subsp. *glaucophyllum* var. *subglaucum*, and *L. vulgare* subsp. *glaucophyllum* var. *esterellense* (**Table 18**). A joint morphological characteristic of those taxa is the possession of glaucous leaves which are relatively unique within the genus. The gross morphology of these species can be regarded as similar and particular taxa can be distinguished by minute characters. The main difference is the chromosome number and distributional area – var. *subglaucum* is hexaploid ($2n = 6x = 54$) and grows exclusively in Massif Central occupying dry habitats especially sunny slopes facing south, var. *esterellense* occurs solely in Esterel Massif in dry and sunny habitats on soil derived from volcanic rocks and var. *eu-glaucophyllum* is decaploid ($2n = 10x = 90$) and occurs in lower parts (from 500 to 1000 m a.s.l.) of central-eastern Ligurian Alps preferring shady habitats. Another presumably closely related species *L. pallens* included in that study is a hexaploid ($2n = 6x = 54$) and has sympatric or parapatric distribution with all above mentioned species. It is distributed across the north Mediterranean ranging from mid-northern Iberian Peninsula to northern Apennine Peninsula. Due to its wide distribution it is hypothesized that it is a relatively old polyploid species and could have played an important role in the formation of other high polyploids which often have sympatric distribution with it (Oberprieler et al. 2012). In particular some populations of *L. pallens* and *L. glaucophyllum* grow close to each other because of the human impact – *L. pallens* prefers open habitat and in places where forest was cut it infiltrates areas

previously occupied by *L. glaucophyllum*. In areas where their populations grow sympatrically, some anomalous ploidy levels have been detected (7*x*, 8*x*), suggesting that hybridization between them takes place (Marchi et al. 1983). As a part of clarification of *L. pallens* history, the tetraploid plants from Apennine Peninsula are included. Their morphological similarity led some authors to assign it to that species (Favarger 1975, Marchi 1982). In the present paper a hypothesis is tested that they may actually represent other similar taxon *L. ircutianum* subsp. *leucolepis* ($2n = 4x = 36$), occurring on Balkan Peninsula.

Taxonomic placement of considered *Leucanthemum* species

this study	Ploidy	Distribution	Briquet & Cavillier, 1906	Marchi, 1982	Favarger, 1975
L. glaucophyllum (Briq. & Cavill.) Jahand.	$2n = 10x = 90$	Ligurian Alps	*L. vulgare* subsp. *glaucophyllum* var. *eu-glaucophyllum* Briq. & Cavill.	*L. subglaucum* De Laramb.	*L. vulgare* subsp. *glaucophyllum* Briq. & Cavill. var. *glaucophyllum*
L. subglaucum De Laramb.	$2n = 6x = 54$	Massif Central	*L. vulgare* subsp. *glaucophyllum* var. *subglaucum* Rouy	-	*L. vulgare* subsp. *glaucophyllum* var. *subglaucum* Rouy**
L. "esterellense"	$2n = 8x = 72$	Esterel Massif	*L. vulgare* subsp. *glaucophyllum* var. *esterellense* Briq. & Cavill.	*L. subglaucum* De Laramb.(?)*	-
L. pallens (Perreym.) DC.	$2n = 6x = 54$	from Iberian Peninsula to Apennine Peninsula	*L. vulgare* subsp. *pallens* var. *pallens* Gay	*L. pallens* (Perreym.) DC.	*L. pallens* subsp. *pallens* Gay (DC.)
L. ircutianum subsp. *leucolepis* (Briq. & Cavill.) Vogt & Greuter	$2n = 4x = 36$	Balkan and Apennine Peninsula	*L. vulgare* subsp. *leucolepis* var. *pallidum* Fiori & Paol.	*L. pallens* (Perreym.) DC.	*L. pallens* subsp. *leucolepis* (Briq. & Cavill.) Faverger

*he could mean also *L.* x *marchii* **he proposed to synonimize it with *L. adustum*

Table 18 - Taxonomic placement of considered species and their selected characteristics.

In the present study cytometry, AFLP, chloroplast haplotype network reconstruction and species network reconstruction using next generation sequencing (454 sequencing) were chosen to investigate the ploidy, structure and the origin of the studied taxa. Cytometry has been shown to be a valuable tool in studies on polyploidy allowing examining ploidy of numerous plants after preserving them in the field (Suda et al. 2007). AFLP has been used extensively in the reconstruction of the polyploid history of many plant genera as well as within *Leucanthemum* (Oberprieler et al. 2011, Greiner et al. 2013). It allows investigating many anonymous loci across the whole genome which is advantageous in non-model organisms (Vos et al. 1995). In comparison with AFLP fingerprinting which includes information mainly from the nuclear genome as a complementary technique chloroplast haplotype networks are often used (Greiner et al. 2013). It allows inferring solely the history of maternal lineage and in connection with other techniques it may reveal parental combination which gave rise to a hybrid or polyploid plant. Another promising technique for the reconstruction of the phylogeny are single or low copy nuclear genes which in connection with next generation sequencing (here 454 sequencing) offers cost-effective possibilities of sequencing multiple markers and has been successfully applied to some plant groups containing polyploids (Griffin et al. 2011, Richardson et al. 2012), including *Leucanthemum* (Chapter 2). In polyploids low-copy gene may be present in many copies since they are composed of at least two genomes. Therefore, deep sequencing by e.g. 454 sequencing allows retaining all possible alleles of a certain gene (Chapter 2). In combination with reconstruction methods devoted especially to analyze polyploidy it provides an unprecedented source of information concerning parental origin (Tomasello et al. unpublished).

In the present paper special focus is devoted to unravel the relationships among the three morphologically close taxa classified by Briquet & Cavillier (1906) as varieties of *L. vulgare* subsp. *glaucophyllum*. Additionally, the rangewide sampling of *L. pallens* populations provides insights into its biogeography and relationships to other taxa. Possibility of hybridization between *L. pallens* and *L. glaucophyllum* in areas where these two species are co-occurring is examined. Furthermore tetraploid plants on Apennine Peninsula were studied with the aim of clarifying its relationship with other species included in that study.

4.3. Materials and methods

Plant material and DNA extraction – Silica dried leaf material from 165 individuals collected from 38 populations was used (**Table 19, Figure 12**). The following taxa were included in the analysis: *L. ircutianum* subsp. *leucolepis*, *L. pallens*, and those classified by Briquet and Cavillier (1906) as varieties of *L. vulgare* subsp. *glaucophyllum* (*L. vulgare* subsp. *glaucophyllum* var. *subglaucum*, var. *esterellense*, and var. *eu-glaucophyllum*).

Additionally, for chloroplast network analysis more accessions from herbarium specimens were included together with 19 representatives from all currently known diploid species within the genus (**Table 19**). For the reconstruction of a haplotype network all accessions were used with one representative per population whereas for AFLP fingerprinting diploid taxa and herbarium material were excluded. For the reconstruction of species network based on 454 sequencing, data from Chapter 2 was used additionally including an alleged hybrid plant between *L. pallens* and *L. glaucophyllum* from population 256. Extraction of total genomic DNA was done with CTAB extraction protocol (Doyle & Doyle 1987). The DNA amount of all extracts used in AFLP was measured on a Nanodrop spectrophotometer (Peqlab, Germany) and then dilutions of 12.5 ng/µl were prepared.

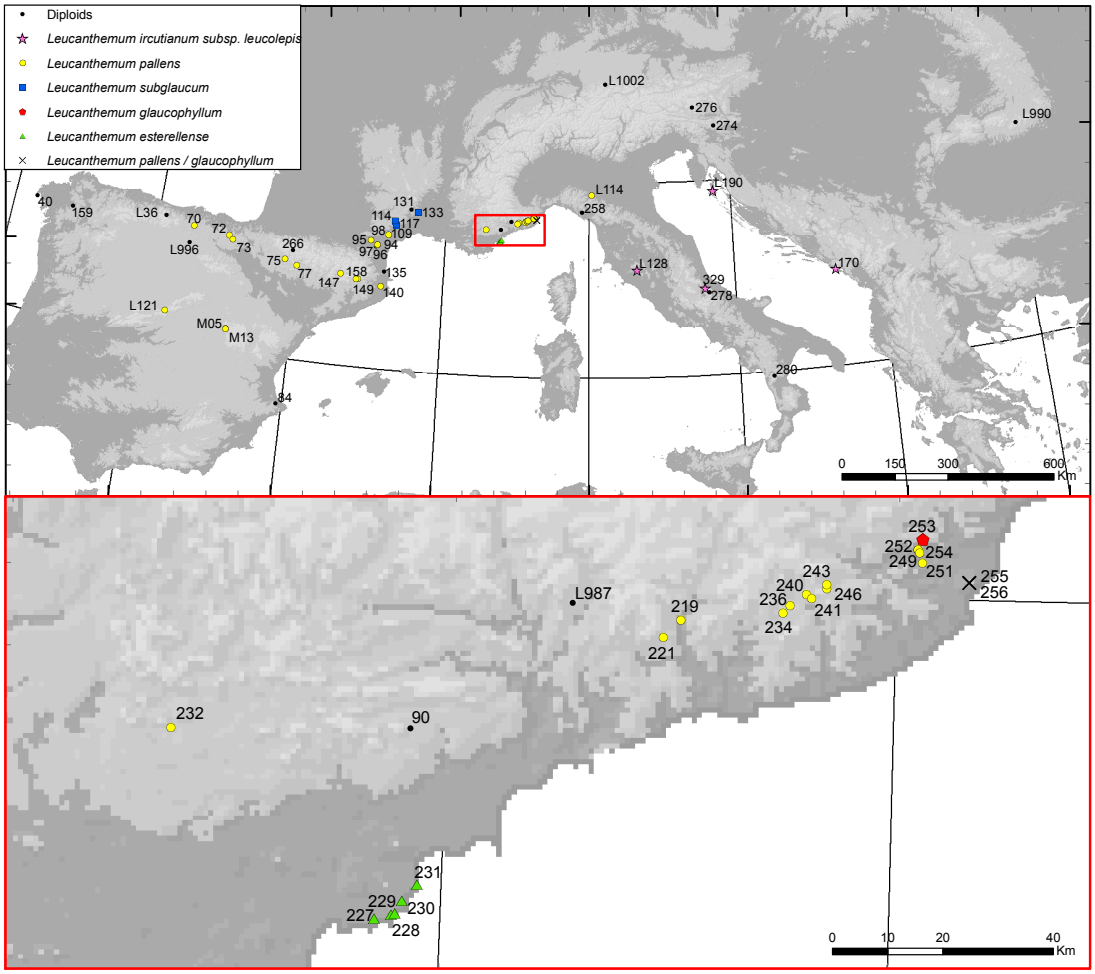

Figure 12 - Map showing sampling localities of all individuals used in this study. More detailed information is available in table with localities (Table 19). The location of lower panel is marked by the red rectangle on the upper panel.

Species	Ploidy	Internal sample name	AFLP	NGS	cpDNA	Flow cytometry	Coordinates	Collection site	Collector	Herbarium	Voucher
Leucanthemum burnatii Briq. & Cavill.	2*x*	90-06	-	+	+	-	43.76 N, 06.92 E	FR, Provence-Alpes-Côte d'Azur, Grasse, 1235 m	Vogt 16615, Oberprieler 10566 & Konowalik	B, VOGT	B 10 0464678
Leucanthemum "esterellense"	8*x*	227-01	+	+	+	+	43.45 N, 06.85 E	FR, Provence-Alpes-Côte d´Azur, Agay, 18 m	Vogt 16897 & Oberprieler 10807	B, VOGT	B 10 0411749
Leucanthemum "esterellense"	8*x*	228-01	+	-	+	+	43.46 N, 06.89 E	FR, Provence-Alpes-Côte d´Azur, Agay, 193 m	Vogt 16898 & Oberprieler 10808	B, VOGT	B 10 0350138
Leucanthemum "esterellense"	8*x*	229-01	+	-	+	+	43.48 N, 06.91 E	FR, Provence-Alpes-Côte d´Azur, Le Trayas Supérieur, 317 m	Vogt 16901 & Oberprieler 10811	B, VOGT	B 10 0350141
Leucanthemum "esterellense"	8*x*	230-01	+	-	+	+	43.46 N, 06.90 E	FR, Provence-Alpes-Côte d´Azur, Sainte Baume, 161 m	Vogt 16902 & Oberprieler 10812	B, VOGT	B 10 0350142
Leucanthemum "esterellense"	8*x*	231-01	+	-	+	+	43.51 N, 06.94 E	FR, Provence-Alpes-Côte d´Azur, Théoule-sur-Mer, 50 m	Vogt 16903 & Oberprieler 10813	B, VOGT	B 10 0350143
Leucanthemum gallaecicum Rodr. Oubiña & Ortiz	2*x*	159-11	-	+	+	-	42.85 N, 07.99 W	ES, Galicia, Sierra de Basadre, 375 m	Konowalik, Rodríguez Oubiña & Ortiz s.n.	B	B 10 0386789
Leucanthemum gaudinii Dalla Torre	2*x*	276-01	-	+	+	-	46.86 N, 13.82 E	AT, Kärnten, Falkert, 2270 m	Oberprieler 10866	B	B 10 0413015
Leucanthemum gaudinii subsp. *barrelieri* (Dufour ex DC.) Vogt	2*x*	266-01	-	+	+	-	42.78 N, 00.23 W	ES, Aragon, Balneario de Panticosa, 2150 m	Tomasello TS382	B, REG	-
Leucanthemum gaudinii subsp. *cantabricum* (Font Quer & Guinea) Vogt	2*x*	L36	-	+	+	-	43.13 N, 04.75 W	ES, Cantabria, Pozas de Lloroza, 1830 m	Bayón 2132, Izuzquiza & Villanueva	VOGT	B 10 0420752
Leucanthemum glaucophyllum (Briq. & Cavill.) Jahand.	10*x*	253-01	+	+	+	+	44.10 N, 08.06 E	IT, Liguria, Onzo, 1044 m	Vogt 16935 & Oberprieler 10842	B, VOGT	B 10 0350175
Leucanthemum gracilicaule (Dufour) Pau	2*x*	84-06	-	+	+	-	38.84 N, 00.19 W	ES, Valencia, Benirrama, 296 m	Konowalik KK20 & Ogrodowczyk	B, REG, WRSL	B 10 0386704
Leucanthemum graminifolium (L.) Lam.	2*x*	96-03	-	+	+	-	43.15 N, 02.63 E	FR, Languedoc-Roussillon, Roc de L´Aigle, 560-600 m	Vogt 16656, Oberprieler 10607 & Konowalik	B, VOGT	B 10 0464663
Leucanthemum halleri (Vitman) Ducommun	2*x*	L1002	-	+	+	-	47.51 N, 10.60 E	AT, Tirol, Tannheim, 1840 m	Vogt 16874	B, VOGT	B 10 0420901
Leucanthemum ircutianum DC. subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	4*x*	L128	-	+	+	-	42.77 N, 11.65 E	IT, Toscana, Selvena, 725 m	Bayón s.n.	VOGT	B 10 0420827
Leucanthemum ircutianum DC. subsp. *leucolepis* (Briq. & Cav.) Vogt & Greuter	4*x*	170-01	+	+	+	+	42.47 N, 18.47 E	ME, Herceg Novi, Sutorina, 34 m	Vogt 16724 & Prem-Vogt	B	B 10 0346645
Leucanthemum laciniatum Huter, Porta & Rigo	2*x*	280-01	-	+	+	-	39.90 N, 16.11 E	IT, Calabria, Campo Tenese, 1481 m	Tomasello TS420	B, REG	B 059-05-12-20
Leucanthemum ligusticum Marchetti, Bernardello, Melai & Peruzzi	2*x*	258-01	-	+	+	-	44.25 N, 09.76 E	IT, Liguria, Rochetta di Vara, 228 m	Vogt 16944 & Oberprieler 10851	B	B 10 0420782
Leucanthemum lithopolitanicum (E.Mayer) Polatschek	2*x*	274-01	-	+	+	-	46.38 N, 14.57 E	AT, Kärnten, Lesnik, 1999 m	Oberprieler 10864	B	B 10 0413013
Leucanthemum monspeliense (L.) Coste	2*x*	131-20	-	+	+	-	44.14 N, 03.73 E	FR, Languedoc-Roussillon, St.-André-de-Valborgne, 380 m	Vogt 16716, Oberprieler 10671 & Konowalik	B, REG	B 10 0464615
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	70-01	+	-	+	+	42.99 N, 03.75 W	ES, Castilla y León, Argomedo, 760 m	Höfl 70 & Himmelreich	B	B 10 0413736
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	72-01	+	-	+	+	42.91 N, 02.49 W	ES, País Vasco, Ozaeta, 610 m	Höfl 72 & Himmelreich	B	B 10 0413734
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	73-01	+	-	+	+	42.82 N, 02.35 W	ES, País Vasco, Salvatierra, 760 m	Höfl 73 & Himmelreich	B	B 10 0413733
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	75-01	+	-	+	+	42.53 N, 00.48 W	ES, Aragón, Narvasa, 1012 m	Höfl 75 & Himmelreich	B	B 10 0413731
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	77-01	+	-	+	+	42.40 N, 00.06 W	ES, Aragón, Boltaña, 1240 m	Höfl 77	B	B 10 0413729
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	95-01	+	-	+	+	43.16 N, 02.63 E	FR, Languedoc-Roussillon, Moux, 250 m	Vogt 16644, Oberprieler 10595 & Konowalik	B	B 10 0464673
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	97-01	+	-	+	+	43.17 N, 02.64 E	FR, Languedoc-Roussillon, Moux, 237 m	Vogt 16666, Oberprieler 10617 & Konowalik	B, REG, VOGT	B 10 0464662
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	98-01	+	-	+	+	43.27 N, 02.40 E	FR, Languedoc-Roussillon, Conques-sur-Orbiel, 123 m	Vogt 16667, Oberprieler 10618 & Konowalik	B, REG, VOGT	B 10 0464661
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	109-01	+	+	+	+	43.44 N, 02.99 E	FR, Languedoc-Roussillon, Prades-sur-Vernazobre, 80 m	Vogt 16684, Oberprieler 10639 & Konowalik	B, REG, VOGT	B 10 0464638
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	140-01	+	-	+	+	42.12 N, 02.88 E	ES, Catalunya, Sant Esteve de Guialbes, 114 m	Konowalik KK47 & Ogrodowczyk	B, REG, WRSL	B 10 0386723
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	147-01	+	-	+	+	42.25 N, 02.08 E	ES, Catalunya, Gombrèn, 972 m	Konowalik KK55 & Ogrodowczyk	B, REG, WRSL	B 10 0386708
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	149-01	+	-	+	+	42.24 N, 02.01 E	ES, Catalunya, Pobla de Lillet, 995 m	Konowalik KK57 & Ogrodowczyk	B, REG, WRSL	B 10 0386707
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	158-01	+	-	+	+	42.34 N, 01.47 E	ES, Catalunya, La Seu d'Urgell, 827 m	Konowalik KK66 & Ogrodowczyk	B, REG, WRSL	B 10 0386724
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	219-01	+	-	+	+	43.95 N, 07.52 E	FR, Provence-Alpes-Côte d´Azur, La Giandola, 366 m	Vogt 16882 & Oberprieler 10792	B, VOGT	B 10 0411733
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	221-01	+	-	+	+	43.92 N, 07.48 E	FR, Provence-Alpes-Côte d´Azur, Brail-Sur-Roya, 883 m	Vogt 16884 & Oberprieler 10794	B	B 10 0411736
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	232-01	+	-	+	+	43.74 N, 06.38 E	FR, Provence-Alpes-Côte d´Azur, Carpre, 309 m	Vogt 16904 & Oberprieler 10814	B, VOGT	B 10 0350144
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	234-01	+	-	+	+	43.98 N, 07.76 E	IT, Liguria, Molini di Triora, 691 m	Vogt 16910 & Oberprieler 10817	B	B 10 0350147
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	236-01	+	-	+	+	43.97 N, 07.74 E	IT, Liguria, Cetta, 1030 m	Vogt 16912 & Oberprieler 10819	B	B 10 0350149
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	240-01	+	-	+	+	44.00 N, 07.80 E	IT, Liguria, Andagna, 923 m	Vogt 16917 & Oberprieler 10824	B	B 10 0350155
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	241-01	+	-	+	+	44.00 N, 07.81 E	IT, Liguria, Grattino, 1088 m	Vogt 16920 & Oberprieler 10827	B, VOGT	B 10 0350158
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	243-01	+	-	+	+	44.01 N, 07.84 E	IT, Liguria, Rezzo, 897 m	Vogt 16922 & Oberprieler 10829	B	B 10 0350160
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	246-01	+	-	+	+	44.02 N, 07.84 E	IT, Liguria, Rezzo, 738 m	Vogt 16926 & Oberprieler 10833	B	B 10 0350164
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	249-01	+	-	+	+	44.06 N, 08.06 E	IT, Liguria, Pogli, 170 m	Vogt 16931 & Oberprieler 10838	-	-
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	251-01	+	-	+	+	44.08 N, 08.05 E	IT, Liguria, Menezzo, 495 m	Vogt 16933 & Oberprieler 10840	B	B 10 0350173
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	252-01	+	-	+	+	44.08 N, 08.05 E	IT, Liguria, Menezzo, 495 m	Vogt 16934 & Oberprieler 10841	B, VOGT	B 10 0350174
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	254-01	+	-	+	+	44.07 N, 08.05 E	IT, Liguria, Castell´Ermo, 532 m	Vogt 16939 & Oberprieler 10846	B	B 10 0350179
Leucanthemum pallens (Gay in Perreymond) DC.	4*x*	329-01	+	+	+	+	42.24 N, 13.97 E	IT, Abruzzo, Piano d´Orta, 125 m	Oberprieler 10870	OBERPRIELER	-
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	L114	+	+	+	-	44.68 N, 10.08 E	IT, Emiliana Romagna, Rubbiano, 300 m	Vogt 6350	VOGT	B 10 0420819
Leucanthemum pallens (Gay in Perreymond) DC.	6*x*	L121	+	+	+	-	40.73 N, 04.25 W	ES, Castilla y León, San Rafael, 1200 m	Vogt 3574 & Pedrol	VOGT	B 10 0420822
Leucanthemum pallens (Gay in Perrymond) DC.	6*x*	M05-08	-	-	+	+	40.54 N, 02.15 W	ES, Castilla-La Mancha, Puente de Vadillos, 1057 m	Cordel s.n.	B	B 10 0297954
Leucanthemum pallens (Gay in Perrymond) DC.	6*x*	M13-09	-	-	+	+	40.54 N, 02.15 W	ES, Castilla-La Mancha, Puente de Vadillos, 957 m	Cordel s.n.	B	B 10 0297962
Leucanthemum pallens (Gay in Perrymond) DC.	6*x*	255-01	+	-	+	+	44.03 N, 08.16 E	IT, Liguria, Vegliasco, 580 m	Vogt 16941 & Oberprieler 10848	B, VOGT	B 10 0350181
Leucanthemum glaucophyllum / *pallens* x *glaucophyllum*	8*x*-10*x*	256-01	+	+	+	+	44.03 N, 08.16 E	IT, Liguria, Vegliasco, 580 m	Vogt 16942 & Oberprieler 10849	B, VOGT	B 10 0350183
Leucanthemum pluriflorum Pau	2*x*	040-06	-	+	+	-	42.88 N, 09.27 W	ES, Galicia, Cabo Fisterra, 100 m	Höfl 40	B	B 10 0413758
Leucanthemum rotundifolium (Willd.) DC.	2*x*	L990	-	+	+	-	45.42 N, 25.51 E	RO, Prahova, Busteni, 1000-1500 m	Hörandl 9063, Hadacek & Costea	W	W 1999-05366
Leucanthemum subglaucum De Laramb.	6*x*	114-01	+	+	+	+	43.71 N, 03.24 E	FR, Languedoc-Roussillon, Laval de Nize, 430 m	Vogt 16690, Oberprieler 10645 & Konowalik	B, REG, VOGT	B 10 0464681
Leucanthemum subglaucum De Laramb.	6*x*	117-03	+	-	+	+	43.82 N, 03.17 E	FR, Languedoc-Roussillon, Caussareilles, 537 m	Vogt 16695, Oberprieler 10650 & Konowalik	B, REG, VOGT	B 10 0464630
Leucanthemum subglaucum De Laramb.	6*x*	133-01	+	+	+	+	44.07 N, 03.97 E	FR, Languedoc-Roussillon, Anduze, 170 m	Vogt 16721, Oberprieler 10676 & Konowalik	B, REG, VOGT	B 10 0464613
Leucanthemum tridactylites (A. Kern. & Huter) Huter, Porta & Rigo	2*x*	278-01	-	+	+	-	42.14 N, 14.11 E	IT, Abruzzo, Pennapiedimonte, 2065 m	Tomasello TS417	B, REG	B 059-03-12-20
Leucanthemum virgatum (Desr.) Clos	2*x*	L987	-	+	+	-	43.98 N, 07.27 E	FR, Alpes Maritimes, Vésubie, 1013 m	Saatkamp s.n.	-	-
Leucanthemum vulgare (Vaill.) Lam	2*x*	94-01	-	+	+	-	43.13 N, 02.61 E	FR, Languedoc-Roussillon, Montlaur, 160 m	Vogt 16641, Oberprieler 10592 & Konowalik	B	B 10 0464674
Leucanthemum vulgare subsp. *eliasii* (Sennen & Pau) Sennen & Pau	2*x*	L996	-	+	+	-	42.56 N, 03.80 W	ES, Burgos, San Pantaleón del Páramo, 973 m	Cela 1433 & Lopez	VOGT	B 10 0420857
Leucanthemum vulgare subsp. pujiulae Sennen	2*x*	135-07	-	+	+	-	42.50 N, 02.96 E	FR, Pyrénées-Orientales, La Vallée Heureuse, 410 m	Konowalik KK42 & Ogrodowczyk	B, REG, WRSL	B 10 0386712

Table 19 - Taxa and accessions used in the present study. Table indicates whether sample was used for AFLP, Next Generation Sequencing (NGS), chloroplast network reconstruction (cpDNA) and flow cytometry. Moreover popuations ID's are specified (internal sample name), geographical coordinates, collection site, collector, herbarium and voucher number. Herbarium names are according to Index Herbariorum except VOGT which denotes private collection of Robert Vogt and OBERPRIELER which denotes private collection of Christoph Oberprieler.

Flow cytometry – For flow cytometry, a two step protocol was used (Doležel et al. 2007) with diploid *L. pluriflorum* as a standard. In order to minimize the variation among measurements, leaves from the same standard plant were taken for all analyses. The amount of sample leaf (about 30 mm^2) was approximately threefold of the internal standard. Leaf fragments were chopped with a razor blade in Otto I buffer (Otto et al. 1981). The suspension of nuclei was filtered through a mesh with a pore size of 50 µm and kept on ice. Afterwards centrifugation was performed for 5 min at 150 g in 4˚C. The isolation buffer was removed leaving ca. 50 µl, and the pellet was dissolved in ice-cold LB01 buffer (Doležel et al. 1989) with 4 mg/l of DAPI (Carl Roth, Germany). Excitation of the sample was done using a UV laser (365 nm; 16 mW) with an accompanying bandpass filter 455/50 nm on a CyFlow Space cytometer (Partec, Germany). Acquisition was automatically stopped at 8000 measured nuclei. The relative ploidy was calculated by multiplying the known ploidy in *L. pluriflorum* (2*x*) by the quotient between the 2C peak positions of the target species and the internal standard in the histogram of fluorescence (Doležel et al. 2007). Since the relationship was not linear, relative ploidy values were obtained by comparison to values obtained from measurements of reference samples with known chromosome numbers from the study group. Those ratios were: 3.3 for a tetraploid (4*x*), 4.0 for a hexaploid (6*x*), 5.1 for an octoploid (8*x*), 6.1 for a decaploid (10*x*).

AFLP – The AFLP protocol followed original description of Vos et al. (1995) with modifications described in Oberprieler et al. (2011) and Greiner et al. (2013). Briefly, the method proceeded as follows. In the first step, MseI and EcoRI restriction enzymes were used together with T4 DNA ligase and adaptors compatible with either of the two restriction sites. Restriction-ligation was carried out at 37°C for 2 h, after which the ligase was heat-inactivated. Pre-selective amplification used primers with one and two selective nucleotides (A for the EcoRI primer and CT for the MseI primer) while selective amplification used primers with further two selective nucleotides (CTAG for the MseI primer). Fluorescently labelled EcoRI primers EcoRI-ACC, EcoRI-AGG and EcoRI-ACA were used in three independent reaction mixes for each sample. The PCR products were united, precipitated and subsequently dissolved in a mixture of GenomeLab Sample Loading Solution and CEQ Size Standard 400 (Beckman Coulter, Germany). The fragment detection was performed on a CEQ8000 capillary sequencer (Beckman Coulter, Germany). To quantify AFLP genotyping errors, replicates were generated for 11 randomly selected samples, representing 7.8% of the total sample number.

A 0/1 matrix was generated by automatic band scoring using GelCompar II (Applied Maths) and screening through 84 parameter combinations comprising different

combinations of values for peak minimal profiling (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0), peak minimal area (0.1, 0.3 and 0.5) and band matching tolerance (0.02, 0.06, 0.10 and 0.14). In order to chose the most reliable combination, the Euclidean error, the Jaccard distance, the number of correctly paired individuals and the phylogenetic resolution score were calculated using a Python script developed by Holland et al. (2008). Afterwards all results were standardized using z-transformation, brought to a positive number, multiplied by each other and subtracted from the combination with highest Euclidean error in order to allow comparison among them using a single value. Combination with the highest score was chosen and used for band scoring for all individuals. To reduce the noise in the dataset and to improve the signal, the program Amare (Kück et al. 2012) was used. It aims at reducing the data matrix by deleting inconsistent bands by searching for most reliable band combinations among replicates. The procedure is divided into three steps which calculate replicate reliability (RR), bin reliability (BR), and minimum bin distance (BD). The last step was omitted because it is equivalent to matching tolerance used by GelCompar. Program performs automatic band masking and proceeds until the best result is achieved by modifying settings automatically. The matrix in which no individual was deleted was selected and all the following analyses are using the matrix obtained from that program.

To visualize the phenetic structure among individuals, a principal coordinate analysis (PCoA) was performed using MVSP version 3.12f (Kovach, 1999) using the Bray-Curtis coefficient (Bray & Curtis 1957). A Bayesian clustering of individuals was done with the program Structure ver. 2.3.3 (Pritchard et al. 2000). For the estimation of optimal cluster number, the method of Evanno et al. (2005) was used. Allele frequencies were set to correlate, all individuals were assigned to haploid level to account for the dominant marker system and mixed ploidy levels in the dataset. Sampling location information (population number) was included as part of the prior (Hubisz et al. 2009). The burnin was set to 250’000 generations and chain length to 1’000’000 generations. The analysis was simulated 10 times and results were averaged using clumpp 1.1.2 (Jakobsson & Rosenberg 2007). For visualization of the results, the program distruct 1.1 was used (Rosenberg 2004).

cpDNA haplotype network – For the amplification of chloroplast DNA markers the primers psbAf (5’-GTTATGCATGAACGTAATGCTC-3’; Sang et al. 1997), trnHr (5’-TTTGTTCTACGTCTCCGAGC-3’; Hamilton 1999), rpL16F71 (5’-GCTATGCTTAGTGTGTGACTCGTTG-3’; Jordan et al. 1996), and rpL16R1516 (5’-CCCTTCATTCTTCCTCTATGTTG-3’; Kelchner & Clark 1997) were used. The PCR was carried out with RedMix (Biomol, Germany) using the manufacturer’s protocol. For

cycle sequencing, the CEQ DTCS Quick Start Kit (Beckman Coulter, Germany) was used. Electropherograms were manually checked for base call errors by using of the editor program Chromas Lite version 2.0 (Technelysium, Australia). The alignment was performed with mafft 6.833b (Katoh et al. 2002, Katoh and Toh 2008) and inspected visually. After the alignment, Gapcoder (Young and Healy 2003) was used to code gaps. To compute and visualize the haplotype network, the program TCS 1.21 (Clement et al. 2000) was used. Based on visualization of the network in places with parallel characters producing loops it was judged whether they were correctly and unambiguously aligned. After this check gaps in positions: 297-301 and 1113-1149 were coded as missing data and excluded from the analysis because there was more than one aligning possibility. Similarly, positions 267-279 were excluded from the analysis because of a tandem repeat with more than one alignment solution. The haplotypes were grouped manually into the haplogroups according to the nesting rules of a nested clade analysis (Templeton et al. 1987, Templeton & Sing 1993).

454 sequencing – Amplification and sequencing of low copy nuclear markers was done by 454 sequencing as described in Chapter 1. The data from the Chapter 1 on *Leucanthemum* diploids was included and additionally sequences from four representative individuals of *L. ircutianum* subsp. *leucolepis*, three of *L. pallens*, two of *L. subglaucum*, one of "*L. esterellense*" and one of *L. glaucophyllum* were incorporated. Additionally, one individual was included (256_01) which according to cytometry was a hybrid *L. pallens* × *glaucophyllum* and came from the previously mentioned mixed population. Briefly, the method was conducted as follows. Modified markers from Chapman et al. (2007) selected in Chapter 1 were amplified using a two step PCR protocol. Each sample received a specific barcode and was included in a equimolar mixture submitted for sequencing to an external company (Microsynth AG, Switzerland). To allow assessment of all alleles, the final concentration of sample was adjusted to reach at least 10 reads per allele (Chapter 2). Reads were filtered according to quality by discarding reads where phred quality score was equal or below 20 in more than 20% of the bases, and then grouped into species and markers. To identify alleles, reads were aligned in mafft 6.833b (Katoh et al. 2002, Katoh and Toh 2008) and then analyzed in BAPS 5.2 using Bayesian clustering with linked loci (Corrander et al. 2006, Corrander et al. 2008, Cheng et al. 2011). The groups found by the program were considered as alleles and according to those results reads were manually grouped in BioEdit (Hall 1999). Additionally alignment was inspected visually and if there was a variant recorded in more than 20% of reads it was retained as an additional allele. In some cases the BAPS analyses were unreliable in predicting the number of alleles (e.g. all

sequences were regarded as one allele) – in that case the 20% rule was also applied to record all variants. The consensus sequences representing particular alleles were aligned using the program mafft 6.833b (Katoh et al. 2002, Katoh & Toh 2008) and the alignment was checked visually. Gaps were coded using the program Gapcoder (Young and Healy 2003). Gene trees were produced using MrBayes 3.2.1 (Ronquist et al. 2012) with model parameters suggested by highest AIC score in jModelTest 2.1.1 (Darriba et al. 2012).

Supernetwork – The method used for constructing the network is the same as described in Chapter 1. As the input the 9 gene trees from low copy nuclear genes and the tree constructed on concatenated chloroplast loci were used. Chloroplast loci consisted of markers used in Chapter 2 and additionally included rpL16F71-rpL16R1516. All gene trees were modified to retain only nodes with posterior probability equal or higher than 0.95. In the present study, individuals of the same taxon were not merged and analyzed separately. The matrix was visualized in SplitsTree 4.11.3 (Huson & Bryant 2006) using the NeighborNet algorithm and Jaccard distances which take into account just presence in the node (1) while absence (0) is not considered as similarity.

4.4. Results

Cytometry – Results indicate that *L. subglaucum* is entirely hexaploid as well as all *L. pallens* from its whole range (**Table 21**). Plants of *L. ircutianum* subsp. *leucolepis* including Balkan and Apeninne populations were tetraploid. "*L. esterellense*" was an octoploid ($2n = 8x = 72$) trough all samples and all plants of *L. glaucophyllum* were decaploid. In one of the locations (Monte Bignone near Alassio, Italy) where populations of *L. pallens* (255) and *L. glaucophyllum* (256) were growing together, individuals with intermediate ploidy levels were found (octoploids, nanoploids). Since the morphology of those plants was also intermediate they are apparently representing a hybrids *L. pallens* × *L. glaucophyllum*.

Species	Sample	Mean of sample	CV of sample	Mean of Standard	CV of standatd	Diploid/Sample ratio	Estimated ploidy
L. pallens	070_02	8.97	8.42	4.61	5.15	3.89	6x
L. pallens	072_01	10.28	6.83	4.77	6.06	4.31	6x
L. pallens	073_01	7.42	9.17	3.91	5.8	3.80	6x
L. pallens	075_02	8.33	8.45	4.36	5.37	3.82	6x
L. pallens	077_01	7.3	10.78	4.17	7.87	3.50	6x
L. pallens	095_02	8.12	8.05	4.23	6.18	3.84	6x
L. pallens	097_01	8.66	7.09	4.23	6.3	4.09	6x
L. pallens	098_01	8.92	5.66	4.26	6.24	4.19	6x
L. subglaucum	114_03	7.87	6.72	3.94	6.09	3.99	6x
L. subglaucum	117_01	7.36	10.05	3.96	6.15	3.72	6x
L. subglaucum	133_01	6.26	7.01	3.61	6.69	3.47	6x
L. pallens	140_01	8.31	8.72	4.16	6.41	4.00	6x
L. pallens	147_01	8.29	9.51	4	6.85	4.15	6x
L. pallens	149_01	7.78	8.57	4.02	5.57	3.87	6x
L. pallens	158_01	8.44	7.32	4.15	5.99	4.07	6x
L. ircutianum subsp. *leucolepis*	170_03	8.02	6.94	4.86	5.13	3.30	4x
L. pallens	219_02	8.28	6.96	4.07	5.91	4.07	6x
L. pallens	221_01	6.12	10.81	3.66	9.12	3.34	4x
L. esterellense	227_02	9.14	9.5	3.8	7.88	4.81	8x
L. esterellense	227_03	6.65	9.41	3.07	6.69	4.33	8x
L. esterellense	227_04	10.11	6.99	4.3	7.64	4.70	8x
L. esterellense	227_06	11.79	7.26	4.6	5.75	5.13	8x
L. esterellense	227_07	10.01	9.05	4.28	6.17	4.68	8x
L. esterellense	227_08	9.89	7.82	3.91	6.7	5.06	8x
L. esterellense	228_01	9.2	7.97	3.66	7.61	5.03	8x
L. esterellense	228_02	8.11	7.14	3.36	6.99	4.83	8x
L. esterellense	228_03	9.43	7.33	3.57	7.43	5.28	8x
L. esterellense	229_01	8.71	5.57	3.54	7.61	4.92	8x
L. esterellense	229_02	9.6	4.39	3.61	7.66	5.32	8x
L. esterellense	229_03	9.55	7.6	3.94	8.55	4.85	8x
L. esterellense	229_04	9.77	9.25	3.91	7.74	5.00	8x
L. esterellense	229_06	8.96	10.34	3.8	8.44	4.72	8x
L. esterellense	229_07	9.21	9.97	3.94	7.22	4.68	8x
L. esterellense	229_08	9.33	8.56	3.72	7.41	5.02	8x
L. esterellense	230_00	9.42	11.83	3.83	8.23	4.92	8x
L. esterellense	231_01	9.39	9.12	3.9	7.28	4.82	8x
L. esterellense	231_02	8.4	11.57	3.49	10.72	4.81	8x
L. esterellense	231_03	7.87	11.88	3.39	11.08	4.64	8x
L. esterellense	231_04	8.22	6.94	3.46	8.53	4.75	8x
L. esterellense	231_05	8.55	11.49	3.4	10.8	5.03	8x
L. pallens	232_01	6.82	8.48	3.81	5.91	3.58	6x
L. pallens	234_01	7.39	10.73	3.67	7.33	4.03	6x
L. pallens	236_01	7.85	7.91	3.97	7.5	3.95	6x
L. pallens	240_00	6.73	11.55	3.52	8.22	3.82	6x
L. pallens	241_01	7.57	9.05	3.66	7.75	4.14	6x
L. pallens	243_01	7.28	10.48	3.62	8.43	4.02	6x
L. pallens	246_01	7.65	10.4	3.8	9.02	4.03	6x
L. pallens	249_00	7.57	11.3	3.74	8.05	4.05	6x
L. pallens	251_00	7.97	9.03	3.78	7.3	4.22	6x
L. pallens	252_00	6.83	10.6	3.5	7.96	3.90	6x
L. glaucophyllum	253_02	10.53	7.67	3.64	6.67	5.79	10x
L. glaucophyllum	253_03	11.95	7.8	3.83	7.18	6.24	10x
L. glaucophyllum	253_04	12.08	8.44	3.91	7.57	6.18	10x
L. glaucophyllum	253_05	11.39	8.98	3.76	6.07	6.06	10x
L. glaucophyllum	253_07	13.3	9.1	4.52	6.44	5.88	10x
L. glaucophyllum	253_08	10.31	8.93	3.39	7.95	6.08	10x
L. glaucophyllum	253_09	9.69	9.21	3.51	7.53	5.52	10x
L. glaucophyllum	253_10	10.96	7.89	3.54	7.57	6.19	10x
L. glaucophyllum	253_11	9.81	9.08	3.24	7.93	6.06	10x
L. glaucophyllum	253_12	9.05	7.12	3.14	9.11	5.76	10x
L. glaucophyllum	253_13	9.7	9.02	3.12	7.94	6.22	10x
L. glaucophyllum	253_14	9.96	8.85	3.36	8.45	5.93	10x
L. glaucophyllum	253_15	10.71	10.58	3.64	8.85	5.88	10x
L. pallens	254_00	7.28	9.64	3.65	7.22	3.99	6x
L. pallens	255_01	7	8.45	3.53	7	3.97	6x
L. pallens	255_02	7.15	8.32	3.82	7.12	3.74	6x
L. pallens	255_03	7.46	8.87	3.75	7.62	3.98	6x
L. pallens	255_04	7.58	8.4	3.94	7.71	3.85	6x
L. pallens	255_06	7.19	9.35	3.75	6.99	3.83	6x
L. pallens	255_07	7.31	8.46	3.77	7.18	3.88	6x
L. pallens	255_08	6.78	9.65	3.52	7.39	3.85	6x
L. pallens	255_09	7.55	8.95	3.82	6.7	3.95	6x
L. pallens	255_11	6.94	9.61	3.83	7.93	3.62	6x
L. pallens	255_12	6.86	8.06	3.52	7.84	3.90	6x
L. pallens	255_13	7.14	8.31	3.62	6.77	3.94	6x
L. pallens	255_15	6.87	8.19	3.42	7.52	4.02	6x
L. pallens	255_16	6.97	9.2	3.59	7.77	3.88	6x
L. pallens	255_17	6.25	9.17	3.21	7.64	3.89	6x
L. pallens	255_18	6.3	8.49	3.25	10.17	3.88	6x
L. pallens	255_19	6.9	9.6	3.61	7.58	3.82	6x
L. pallens	255_20	6.56	7.35	3.26	8.77	4.02	6x
L. pallens	255_21	6.42	8.56	3.15	8.77	4.08	6x
L. glaucophyllum x pallens	256_01	8.61	7.16	3.65	6.18	4.72	8x
L. glaucophyllum	256_02	11.32	7.93	3.89	6.29	5.82	10x
L. glaucophyllum	256_03	9.94	8.94	3.43	7.05	5.80	10x
L. glaucophyllum	256_04	11.35	7.85	3.71	7.15	6.12	10x
L. glaucophyllum	256_05	11.04	8.31	3.77	7.81	5.86	10x
L. glaucophyllum x pallens	256_06	9.39	8.46	3.62	6.58	5.19	8x
L. glaucophyllum x pallens ?	256_07	9.06	9.57	3.29	8.68	5.51	9x/10x
L. glaucophyllum	256_08	10.31	8.57	3.5	8.02	5.89	10x
L. glaucophyllum x pallens ?	256_09	10.54	9.43	3.77	6.55	5.59	9x/10x
L. glaucophyllum x pallens ?	256_10	10.14	9.58	3.68	8.28	5.51	9x/10x
L. glaucophyllum x pallens	256_11	7.71	10.71	3.11	9.54	4.96	8x
L. glaucophyllum	256_12	11.09	10.88	3.76	7.26	5.90	10x
L. glaucophyllum x pallens	256_13	8.72	7.05	3.34	8.01	5.22	8x
L. glaucophyllum	256_14	9.64	8.25	3.17	8.78	6.08	10x
L. glaucophyllum	256_15	9.62	9.82	3.37	8.29	5.71	10x
L. glaucophyllum x pallens	256_16	7.84	12.11	3.13	8.81	5.01	8x
L. glaucophyllum x pallens ?	256_17	12.88	9.88	4.58	6.55	5.62	9x/10x
L. glaucophyllum	256_18	10.25	10.21	3.94	7.48	5.20	8x
L. glaucophyllum	256_19	10.53	7.41	3.52	6.6	5.98	10x
L. glaucophyllum	256_20	9.52	10.13	3.3	7.39	5.77	10x
L. glaucophyllum	256_21	9.86	9.85	3.33	8.47	5.92	10x
L. glaucophyllum x pallens ?	256_22	9.26	12.69	3.27	10.12	5.66	9x/10x
L. glaucophyllum	256_23	8.67	12.51	2.92	9.03	5.94	10x
L. glaucophyllum	256_24	9.11	10.31	3.01	8.33	6.05	10x
L. pallens	329_01	5.23	9.09	3.76	6.76	2.78	4x
L. pallens	329_02	5.2	8.17	3.69	6.33	2.82	4x
		mean	8.91	mean	7.50		
		SD	1.50	SD	1.17		
		max	12.69	max	11.08		
		min	4.39	min	5.13		

Table 20 - Table summarizing the results of flow cytometry. The columns contain information about relative fluorescence of sample (mean of sample), its covariance (CV of sample), relative fluorescence of standard (mean of standard), its covariance (CV of standard), ratio between relative fluorescence of sample and standard, and estimated ploidy. The mean covariance of samples was 8.91 (± 1.50) and mean covariance of standard was 7.50 (± 1.17).

AFLP – Automatic band scoring yielded 824 bands with Euclidean error rate 10.42% and Jaccard error rate 64%. Generally resolution score was low (8%) and maximally 4 out of 11 replicates could be paired. The final matrix from Amare consisted of 134 bands, replicate reliability was 80%, bin reliability 82%, and Euclidean error was lowered to 8.74%.

The first two axes of PCoA analysis explained 18.83% of the total variation (10.86% and 7.98%). The clearest separation is visible between the decaploid *L. glaucophyllum* on the one hand and the other taxa on the other (**Figure 13**). Other groups intermingled with each other and were rather hard to notify.

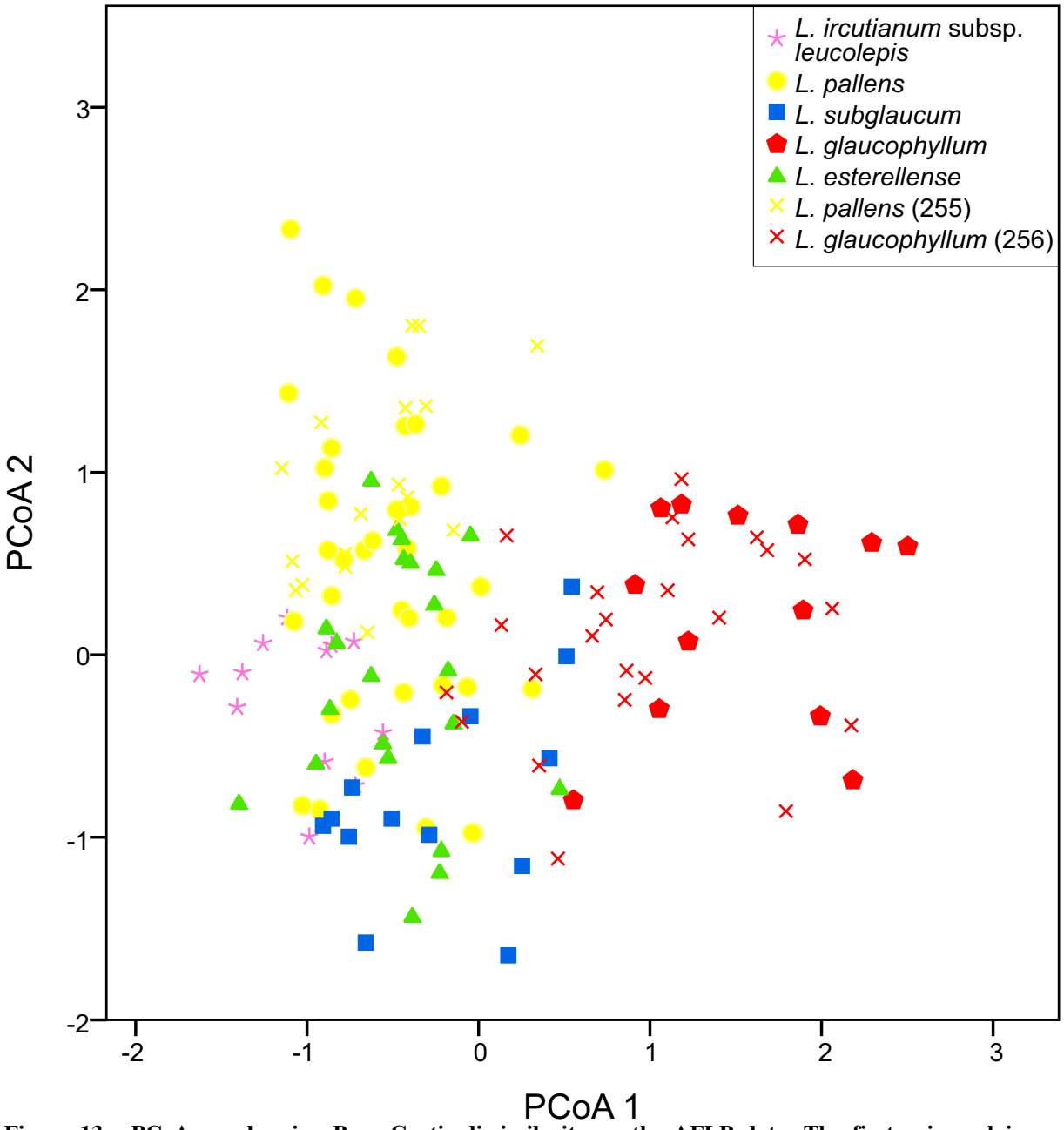

Figure 13 - PCoA graph using Bray-Curtis dissimilarity on the AFLP data. The first axis explain 10.86% of variation and the second axis explain 7.98% of variation observable within the dataset.

Following the method of Evanno et al. (2005), the optimal number of clusters was inferred to be five. The clusters differentiated by the Bayesian cluster analysis with the program Structure were in some extent attributable to the particular species and geography (**Figure 14**) and *L. pallens* individuals were predominantly assigned to green and yellow cluster, *L. subglaucum* to the blue cluster, *L. glaucophyllum* to the red cluster, *L. ircutianum* subsp. *leucolepis* to the pink cluster and octoploid individuals from Esterel Massif were not well assigned into either cluster. a) *L. pallens* individuals coming from the eastern part of the distribution (Maritime Alps and adjacent areas) had higher posterior probability of being members of the green cluster while individuals coming from the western part of the distribution (Iberian Peninsula, Pyrenees and adjacent areas) had higher posterior probability of being members of the yellow cluster. Some exceptions existed as population 241 from the eastern part of the distribution which was placed in the yellow cluster or few populations from the eastern Pyrenees (149, 158) where individuals received high posterior probability of being members of the green cluster. It has to be noted as well that the border between those two clusters was not strict and the green cluster dominated on the eastern part and diminished gradually towards the western part in exchange for yellow cluster. Furthermore many specimens of *L. pallens* received relatively high posterior probability of being members of the blue cluster. b) Individuals of *L. subglaucum* received a high posterior probability of being members of the blue cluster. This was the case especially in the populations 114 and 117, but individuals from the third available population (133) additionally showed relatively high posterior probability of being members of the red cluster. c) Individuals of *L. glaucophyllum* showed highly supported membership to the red cluster. Plants coming from the mixed stand of *L. pallens* and *L. glaucphyllum* (255, 256) showed membership to the green cluster in population 255 (*L. pallens*-like plants) and varying membership to the red, yellow, and green clusters in population 256 (*L. glaucophyllum*-like plants and hybrids). d) *L. ircutianum* subsp. *leucolepis* received a high posterior probability of belonging to the pink cluster. This was the case especially for individuals coming from the Balkan Peninsula (170). The population from the Apennine Peninsula showed high posterior probability of belonging to the pink cluster but also relatively high posterior probability of belonging to the green and yellow cluster. e) Octoploid individuals from Esterel Massif were not assigned to any specific cluster. Only population 231 occurring outside the Esterel Massif in anthropogenic habitat showed higher posterior probability of membership to the blue cluster than other populations.

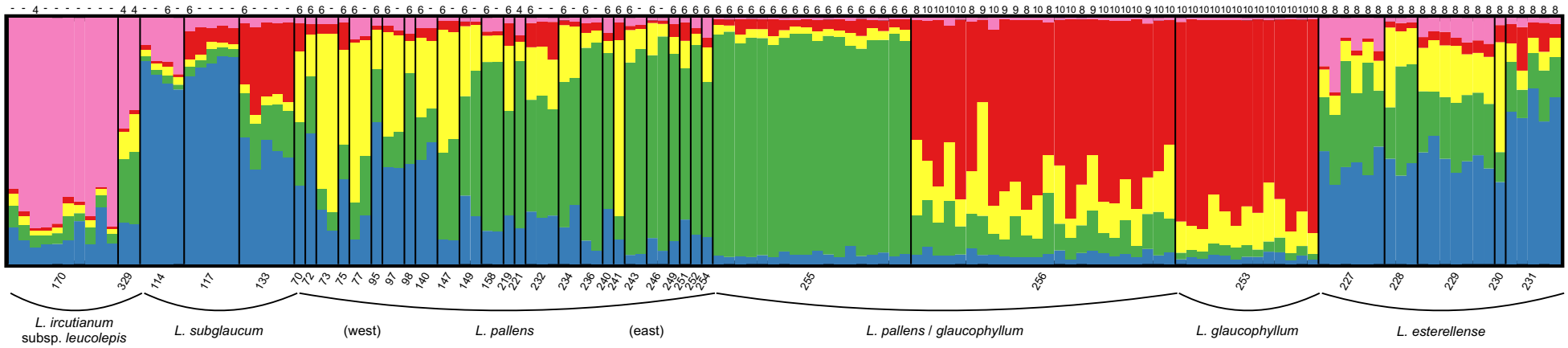

Figure 14 - Clustering performed by Structure on AFLP data. The bars represent cluster membership inferred by Structure. Individuals are arranged species-wise and by population number. Within a population individuals are arranged increasingly from left to right but for clarity they are not numbered and only population number is shown below the bar. The number above bar indicates ploidy of the individual as inferred from flow cytometry. In case that measurement was not performed it is denoted by "-".

cpDNA nested clade analysis – There is a clear pattern in the network concerning the diploids (cf. Greiner & al. 2012) as all but one fall into one haplogroup and *L. virgatum* is well separated. *L. virgatum* placement also suggest that it played an important role in the formation of polyploids as it was placed among them. Because of increased sampling group I from Greiner et al. (2012) was much more differentiated and subdivided. Furthermore, the network was partitioned into four main haplogroups (I, II, III, IV), where one is specific to diploids (IV) and the other three for polyploids (**Figure 15**). Haplogroup I was composed from plants occurring in region adjacent to Maritime Alps or on Apennine Peninsula. The majority of them belongs to *L. pallens* accompanied with one individual of "*L. esterellense*" (231_01) and one individual of *L. ircutianum* subsp. *leucolepis* (329_01). Haplogroup II consisted of single accession of a diploid *L. virgatum*. Within this group two individuals of *L. pallens* from the central Pyrenees may be found (75_01, 77_01), one "*L. esterellense*" individual (230_01) and one individual of the alleged hybrid *L. pallens* × *L. glaucophyllum* (256_01). Haplogroup III consist of all accessions of *L. pallens* coming from the western part of its distribution. These haplogroup also contains individuals from *L. glaucophyllum*, *L. subglaucum*, "*L. esterellense*" and one of *L. ircutianum* subsp. *leucolepis* (170_01). Apparently none of the latter taxa formed a monophyletic clade. Haplogroup IV was composed from all different diploid species (except *L. virgatum*) with few polyploids. These polyploid accessions were: one individual of *L. ircutianum* subsp. *leucolepis* (L128), and two *L. pallens* individuals (70_01 and L121) which were placed closely to the group formed by *L. graminifolium*, *L. gallaecicum*, and *L. vulgare* subsp. *pujiulae*. Another *L. pallens* individual (M13_09) was placed closely to *L. gaudinii* and *L. halleri*.

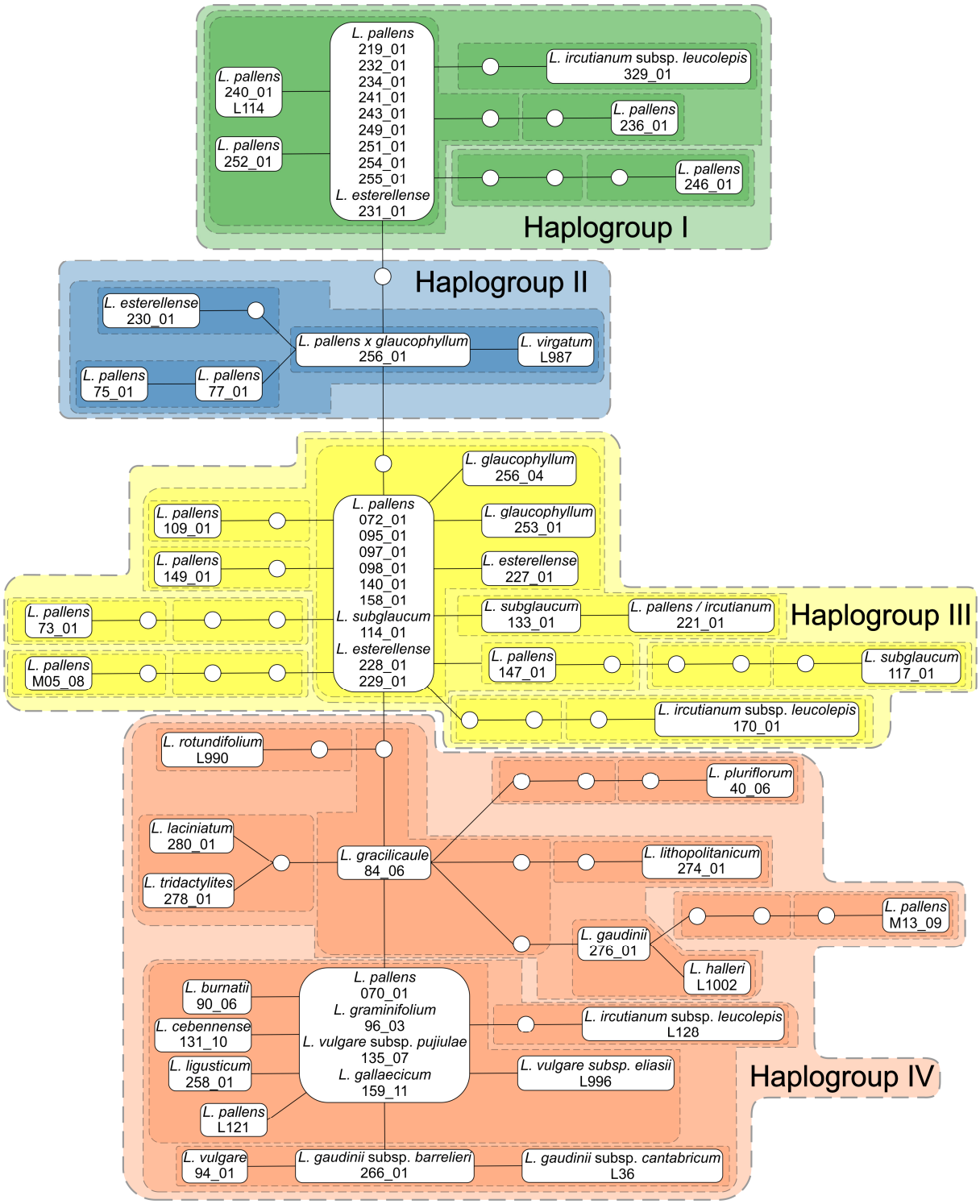

Figure 15 - Chloroplast haplotype network with samples grouped according to the nested clade analysis. Empty circles represent missing haplotypes.

Supernetwork – Supernetwork suggests that level of reticulation is very high within *Leucanthemum* because as noted previously this approach cannot distinguish actual hybridization from incomplete lineage sorting. Polyploids seem to form a group of related species since they are more connected to each other than to any diploid (**Figure 16**). It is consistent with the results of cpDNA haplotype network (see above). The exception from

this pattern is *L. pallens* from central Iberian Peninsula (L121) placed closely to *L. vulgare* subsp. *eliasii*. Also plants of *L. ircutianum* subsp. *leucolepis* (L190, Croatia and L128, Northern Apennine Peninsula) were placed closely to the diploid *L. ligusticum*. The other two accessions of *L. ircutianum* subsp. *leucolepis* (L170, Montenegro and 329_01, Central Apennine Peninsula) were connected rather to *L. pallens*. Two accessions of *L. subglaucum* were placed in one cluster. "*L. esterellense*" was placed closely to *L. glaucophyllum* in position between it and *L. subglaucum* on the other side. The hybrid *L. pallens* × *glaucophyllum* was placed between *L. pallens* coming from eastern clade and *L. glaucophyllum*.

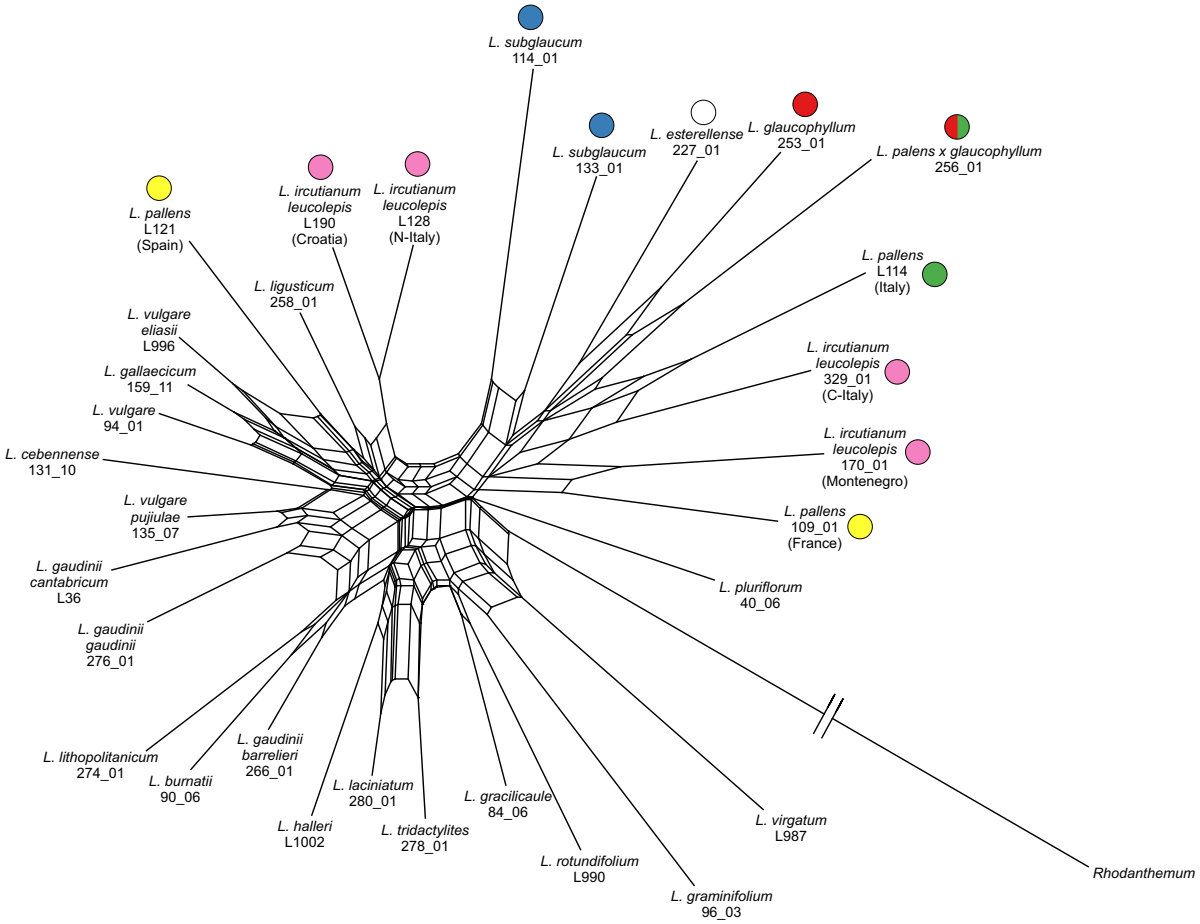

Figure 16 - Supernetwork as obtained from low copy nuclear genes and cpDNA. The coloured circle above species name indicates species membership.

4.5. Discussion

Biogeographical aspects

Considering the separation of *L. pallens* into the eastern and western group which is clearly marked in the chloroplast haplotype network it is assumed that it originated by differentiation into the eastern and western subpopulations rather than multiple formation. This separation is also observable in AFLP where these groups are visible likewise but are mixed along the E-W gradient forming a gradual change from one cluster to another (**Figure 16**). This may be explained by gene flow which occurs rather by pollen than seeds and is reported in literature as "pollen swamping" (Petit & Vendramin, 2007). Such cases occur in other Mediterranean plants as *Quercus ilex* where separation into lineages corresponding to western and eastern haplogroup was also reported (Lumaret et al. 2002). Moreover Lumaret et al. (2002) pointed to Rhône valley as a melting pot where lineages separated previously into refugia in Iberian and Apennine Peninsulas met. Similar patterns in the same area occurred as well in other species as *Quercus coccifera* (López de Heredia et al. 2007b) or *Pinus pinaster* (Burban & Petit 2003). Study done on *Pinus pinaster* by Hu et al. (2009) reveals even more analogous pattern where mitochondrial haplotypes (maternally inherited by seeds in *Pinus*) show east-west disjunction whereas chloroplast haplotypes (paternally inherited by pollen in *Pinus*) supports the same blurred pattern through the E-W gradient. Although all mentioned papers are dealing with wind-pollinated plants in contrary to *Leucanthemum* which is insect-pollinated, it appears that the pattern is very similar and not affected by the pollination type. Thus reported studies along with the present one suggest potential climatic oscillations in this part of the Mediterranean (Rhône valley, southern France) which caused the extinction of some taxa during glaciations to become then the meeting point of the previously separated lineages, during the interglacials.

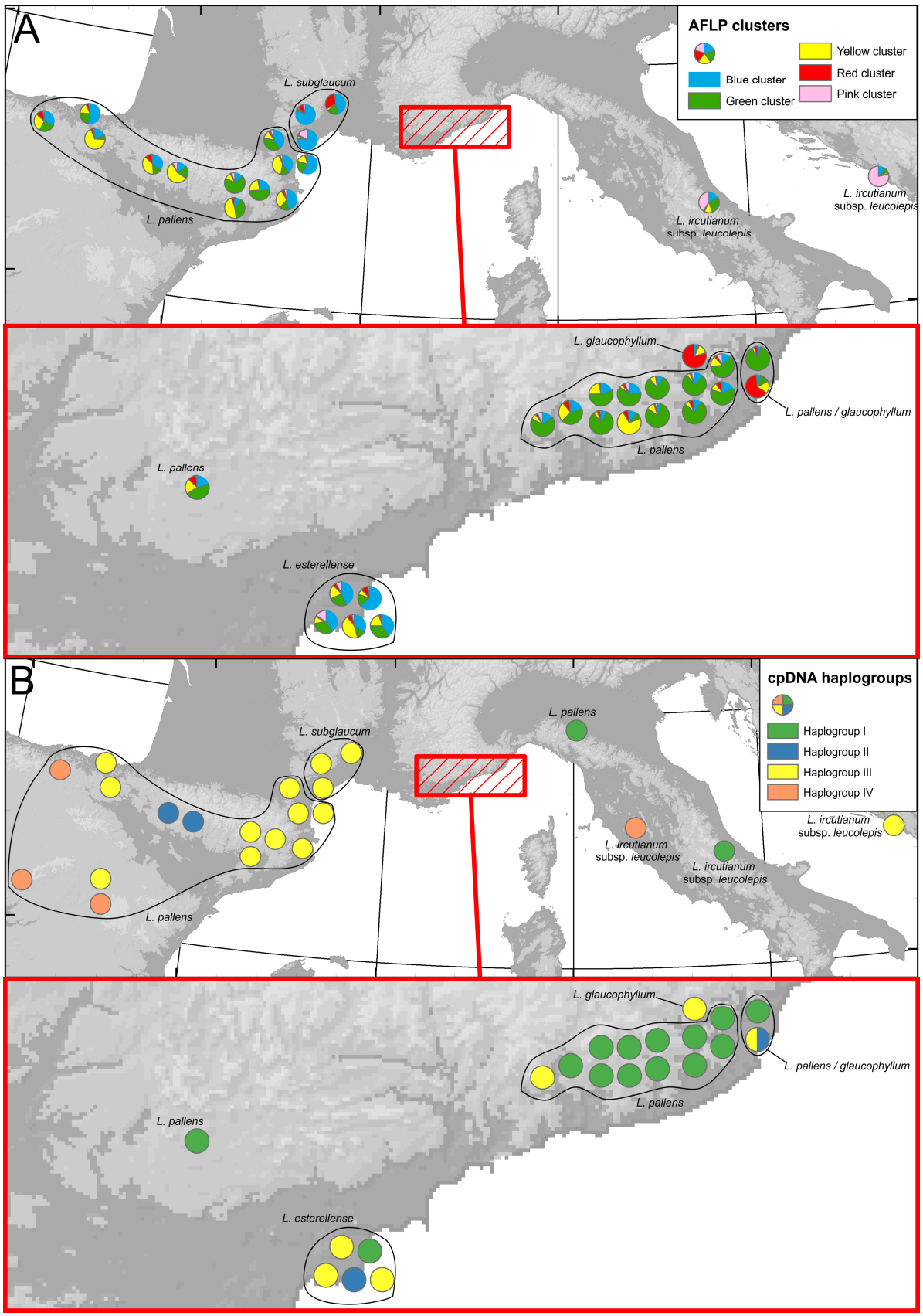

Figure 17 - Results of AFLP clustering averaged per population with program clumpp 1.1.2 (A) and nested clade analysis on chloroplast haplotype network (B). Meaning of the colors is explained in the insets. Members of the same taxon are enclosed in a black circle. For clarity and to display results on species level population numbers were not shown and circle placement may not exactly correspond to the geographic coordinates. Accessions of diploid species are not shown.

Another striking observation is the fact that many plants within haplogroups I and III are divided by more mutations than many diploid species from each other. This especially concerns *L. pallens* and at one hand points to old origin of that species which accumulated a lot of variability and on the other it may suggest that within both western and eastern distribution there was more than one refugial area and populations were separated from each other for significant amount of time which caused emergence of many sub-haplogroups. This pattern of "refugia within refugia" was reported for the Iberian Peninsula for e.g. *Quercus suber, Q, ilex, Q. coccifera, Pinus sylvestris, P. pinaster* and other plants as well as animals (Gómez & Lunt 2007, López de Heredia et al. 2007a). However it should not be restricted only to the Iberian Peninsula but may be applied also to Apennine Peninsula and other Mediterranean refugial areas (e.g *Triturus carnifex* in Apennine Peninsula – Canestrelli et al. 2012, *Rana temporaria* in Apennine Peninsula – Stefani et al. 2012.)) which comprise a vast area differentiated into many habitats with locally deviating environments creating diversity and allowing survival in the multiple refugia.

Evolutionary aspects

Cytometry indicated that species are rather homogenous in their ploidy level and hybrids of intermediate ploidy levels do not occur in the sampled individuals, except one mixed stand. Some variation exist among studied populations but it is rather attributable to the usage of silica dried material as those type of material is often affected by sample quality, time and conditions of storage (Suda & Trávníček 2006, Suda et al. 2007). Another factor affecting the results is that this method is not directly measuring ploidy but genome size that may vary within a species (Pellicer et al. 2009) and among taxa (Garcia et al. 2004). In the study of Greiner & Oberprieler (2012) which examined different polyploids from NW Iberian Peninsula results were similar to those and no intermediate ploidy levels were found. It suggests that interploidy mating is rare or that such hybrids may be least competitive with their progenitors.

Some of the *L. pallens* haplotypes are not connected to main groups of this species and are attached to diploids (accessions 70_01, M13_09, L121). Interestingly they all occur in the peripheral populations close to SW range limit (**Figure 16C**). Many studies reported "cytoplasmic capture" which is a cytoplasmic (chloroplast or mitochondrial) exchange that occurs without significant nuclear gene flow (Rieseberg & Soltis 1991, Petit & Vendramin 2007). In present study this is caused either by polyploidization process where ancestral plants harbored more than one chloroplast type during multiple origins or this species is able to form hybrids with other *Leucanthemum* taxa – not necessarily

diploids but also different polyploids not covered by this study. The second scenario is more probable because *L. pallens* plants with anomalous chromosome numbers (indicating possible hybridization) have been noted by Favarger (1975) (53 to 63 chromosomes) and Marchi et al. (1983) 63 to 65 chromosomes. Hybridization may be also present in other *Leucanthemum* taxa taking into consideration intermediate ploidy levels (e.g. 5*x* and 7*x* Papeš 1971, 5*x* Papeš 1972a, 7*x* Papeš 1972b, 5*x* Przywara 1974). Together with results obtained by Villard (1971) and Greiner & Oberprieler (2012) who were successful in performing artificial crossings among many polyploid species it suggests weak barriers among different cytotypes of *Leucanthemum*, especially those on higher ploidy levels. As concluded by Greiner & Oberprieler (2012) the species barriers in *Leucanthemum* are rather pre-zygotic (different flowering times, geographic isolation) than caused by genetic incompatibility. Observable fact of hybridization and range shifts during climatic oscillations in the last 3 millions years (Oberprieler et al. 2014) contributed significantly to the reticulate evolutionary history of the genus. Therefore three odd accessions separated from the main *L. pallens* groups are possibly indicating hybridization with other taxa which was accompanied by a chloroplast capture.

On the basis that hybrids between hexa- and deca-ploid species were found this study leads to the conclusion that different ploidy level is not necessarily a strong barrier for the gene flow. However, clear hybrids were found between *L. glaucophyllum* and *L. pallens* in flow cytometry analysis, in the AFLP cluster membership of the alleged hybrids was variable. Still within mixed populations there is a remarkably increased influence of green cluster as well as yellow (both attributed to *L. pallens*) compared to pure stand (population 253). Bayesian clustering method implemented in the program Structure can detect hybrids with very good efficiency (Vähä & Primmer 2006) but its performance is reduced as introgression increases (Sanz et al. 2009). This may be the case here since the hybrids in this mixed stand were recorded already in the 80's (Marchi et al. 1983) and if it is assumed that they are fertile they are likely far beyond the second generation. The introgression is especially visible in *L. glaucophyllum*-like plants since their cluster assignment is not as homogenous as in the *L. pallens* plants. This may indicate introgressive hybridization of *L. pallens* into *L. glaucophyllum*. Similar observation was made in *Capsella* (Slotte et al. 2008), *Epidendrum* (Pinheiro et al. 2010), and *Senecio* (Chapman & Abbott 2010) which show high potential for interspecific gene flow despite different ploidy levels.

Another taxon with signs of hybridization, but on the same ploidy level. is *L. subglaucum* which receives a high posterior probability of membership to one of the AFLP

clusters (blue) together with some of the *L. pallens* populations, especially those from the eastern Pyrenees. This would suggest a gene flow between *L. pallens* and *L. subglaucum*. *L. subglacum* situation appears to be more complicated when considering a haplotype network where it is not monophyletic and closely linked to *L. pallens*. In addition morphological characters attributed to *L. subglaucum* are not very stable and are rather variable (personal observation). This was noted as well by Faverger (1975), which observed that sometimes this taxon is similar to *L. pallens* (pale involuclar bracts). This author went even step further and suggested to synonymize *L. subglaucum* with *L. adustum*. Possibility of hybridization between this taxon and other *Leucanthemum* species may be indicated by population 133. It does not belong to the blue cluster entirely and all individuals received partial membership to the red cluster, therefore suggesting a hybrid origin when compared to the two other populations which are more similar to each other. However, it would be hard to specify which species may hybridize in that case, since *L. glaucophyllum* which received a high posterior probability of membership within the red cluster is separated by long geographical distance from that location. Instead it may be explained by participation of the third, not examined species related to *L. glaucophyllum* (by ancestry or hybridization) that crossed with population 133. Again this scenario does not exclude multiple origins of this species which is common in polyploid plants (Tayalé & Parisod 2012 and references therein).

The tetraploid plants on Apennine Peninsula are likely not pure *L. ircutianum* subsp. *leucolepis* nor pure *L. pallens*. On one hand AFLP suggests their close relationship with *L. ircutianum* subsp. *leucolepis* occurring on the SW Balkan Peninsula and NE Apennine Peninsula because they partially belong to the cluster solely formed by that species. Similar morphology and same ploidy level could support this connection furthermore explained by common history of Apennine and Balkan Peninsulas (land bridge during last glacial maximum, Lambeck et al. 2004) resulting in classifying these two regions into one biogeographical province (Rivas-Martínez et al. 2004). On the other hand, AFLP clustering indicates also relatively high probability of membership to cluster consisting of eastern *L. pallens* in those plants. Incongruence is even more visible on chloroplast haplotype network where these tetraploid population is sharing haplotype with eastern clade of *L. pallens* constituted solely by this species. Similarities between those two taxa led Marchi (1982) to classify both under *L. pallens* with indication that it posses two ploidy levels (4*x*, and 6*x*). But following the reasoning of Soltis et al. (2007) which signalized the problem of underrepresenting distinct evolutionary lineages (i.e. putative autopolyploids) as different species and cytology-based taxonomical concept adapted for

Leucanthemum (Vogt 1991) it appears more appropriate to treat tetra- and haxaploid plants as different species. Lastly two other populations treated as *L. ircutianum* subsp. *leucolepis* are placed within different haplogroups. Therefore it is hard to answer the question of origin of tetraploid plants classified here as *L. ircutianum* subsp. *leucolepis*. However, based on the results it appears that the plants from Apennine Peninsula may be hybrids or at least products of gene flow between *L. pallens* and *L. ircutianum* subsp. *leucolepis s. s.* which are still overlapping nowadays in the northern Apennine Peninsula. Furthermore, incongruence visible within examined *L. ircutianum* subsp. *leucolepis* accessions indicates that this species needs more detailed study to examine its identity. Another similar taxon which should be covered by such study is *L. ircutianum* subsp. *asperulum* growing in southern Apennine Peninsula.

Taxonomical aspects

Octoploid plants from Esterel Massif show some good characteristics that allow acknowledging them as an independent taxonomic unit. Distinctiveness of this new species was observed based only on morphological characters (Briquet & Cavillier, 1906) and it is further supported by its ploidy which is unique in this area. It has a well-defined range (Esterel Massif) and it is different morphologically from *L. glaucophyllum* under it was assigned previously (Briquet & Cavillier, 1906). In particular it possesses different ligule (oblong-obovate vs. oblong-linear), different leaf shape (oblong-obtuse vs. lanceolate-acuminate), and less glaucous leaves. Contrary to *L. glaucophyllum* where glaucousness is stable and always observable, in "*L. esterellense*" the intensity of this trait is variable and may diminish with the age of the individual (Briquet & Cavillier, 1906). Also some morphological similarity to *L. pallens* has been noted because of such characters as pale-margined involucral bracts (Briquet & Cavillier, 1906). Molecular analyses are suggesting a recent origin and reticulate evolution of this species. AFLP clustering never recognizes it as a separate group showing the highest mixture of different clusters in this species. Similarly, in the chloroplast haplotype network this species belongs to haplogroups I, II, and III. Based on this information the origin of this species appears to be quite complex. As indicated by morphological comparison it posses some similarity to *L. pallens* and *L. glaucophyllum*, and because the crossing between those two taxa will produce octoploid they are both immediate candidates for the parental species. Although it should be noted that "*L. esterellense*" is not similar to hybrids between those two species found on Monte Bignone and presently *L. glaucophyllum* endemic range is not reaching area of Esterel Massif. Based on the present results (AFLP clustering), *L. subglaucum* is also plausible candidate for the parenthood, and this species is also mentioned to occur close to this area

(French Regions: Alpes-de-Haute-Provence and Vaucluse, Rouy 1903). Hypothesis that it is a cross between *L. subglaucum* and *L. glaucophyllum* was also supported by the super network analysis where "*L. esterellense*" was located between those two taxa.

Also octoploid hybrids from the Monte Bignone (near Alassio, Italy) seem to be worth acknowledging as a separate unit at a rank of nothospecies. As discussed previously, it is apparent that they exist in this location at least since 80' which either suggest their repeated formation and/or ability to cross with each other.

5. GENERAL CONCLUSIONS

This thesis concentrated on investigating phylogenetic relationships among *Leucanthemum* species on the basis of species tree and network reconstruction methods with usage of low-copy nuclear genes and chloroplast markers. Whereas the phylogenetic reconstructions were in the central focus, particular chapters also provided additional threads. In the first Chapter presence of homoploid hybrid speciation was accentuated as results have shown that it may had a huge impact on the evolution on the diploid level. Afterwards in the second Chapter phylogenetic reconstruction including near complete sampling from the whole genus revealed complex network-like relationships among all taxa. The third Chapter examined with denser sampling a smaller study group comprising several polyploid species and revealed their basic phylogenetic relationships with the discovery that the gene flow among *Leucanthemum* polyploids is likely occurring.

Interbreeding within and across ploidy levels provide a major explanation for the phylogenetic complexity of *Leucanthemum*. It may be seen as a series of events in the past which through gene flow and homoploid hybrid speciation influenced the majority of the diploid species. Moreover, this process could also facilitate the formation of many polyploid species, and it is still contributing presently to the hybridization between some of them (e.g. *L. glaucophyllum* × *L. pallens*). Because it influences history of many species, it also has a certain impact on the phylogeny of the genus which seem to be hard to resolve. Additionally it complicates the taxonomy and has led to paraphyletic origin of some taxa (Appendix B).

Sometimes *Leucanthemum* taxonomic classification as currently used may be questionable e.g. as it is problematic whether all subspecies of *L. vulgare* or *L. gaudinii* should be treated as belonging to one species. In this study they are analyzed as separate taxa by specifying a separate leaf in species tree for each of them and this approach adds weight to not treating different subspecies as sister taxa and ascribing them to one species since they often fail to be monophyletic. Another problem illustrated by AFLP data highlights the situation that even different specimens of the same taxon do not form monophyletic groups (as in the case of some species in the ‘Group 2’, cf. Chapter 1). These may be the effect of hybridization between different species, effect of recent rapid radiation (ILS signal) or eventually incorrect taxonomical classification. Presently, *Leucanthemum* species delimitation is based on the ploidy level and geographical range (Vogt 1991) and it is likely the most satisfactory way to separate different taxa. But species defined in that way in some cases may not exactly correspond to the evolutionary units

because of changes occurring within species, and for example what is a clear species in the centre of the distribution may become blurred when approaching margins of its range. In these circumstances, "species" term here should be treated rather as an aid for classification based on morphological characters – however, there will be cases when it is matching evolutionary unit as well. The methods for coalescent-based species delimitation (Fujita et al. 2012) would be a way to further address those doubts but presently available data are not suited for that type of analysis.

The present work benefited a lot from technological advances and results obtained by 454 sequencing were far beyond traditional Sanger sequencing considering the number of obtained reads, amount of work and costs. Still, the whole procedure in the wet lab and further analyses could be optimized to decrease the amount of manual work and necessary time. Single-copy genes for the Compositae family (Chapman et al. 2007) are a valuable source of information although as it turned out even large screening could not find markers with divergence level sufficient to distinguish all species with significant posterior probability. This case may be more general and characteristic of the recently evolved group. As compared to other studies reporting universal single-copy genes (Strand et al. 1997, Steele et al. 2008), they were superior by obtained quality (e.g. variability, length) and the number of markers available for screening.

It seems that the range dynamics i.e. contraction-expansion phases were crucial for the *Leucanthemum* evolution and were the main cause for the formation of various hybrids and polyploids. *Leucanthemum* itself seems to be influenced firstly by Pre-Pleistocene events (separation of *Leucanthemum* and sister genera ca. 7.9 – 4.0 Mya) then followed by Pleistocene events especially linked with range expansions and contractions during glaciations (divergence within *Leucanthemum* ca. 3.1 – 1.4 Mya) (Hößl, 2006). This relatively recent origin and diversification may also explain some distributional patterns seen in the *Leucanthemum* related to the filling of eco-climatological niches (Appendix A). Filling of the niches is negatively correlated with the ascending ploidy, thus it may be assumed that it is dependent on the time of origin – because diploids exist for relatively longer time as compared to polyploids, they fill their niches more completely. On the contrary, the higher is the ploidy level, the lower is the filling of the potential niche. The time of origin seems to be the most important factor since there was no difference between potential ranges of diploids and polyploids which imply that no significant ecological advantages connected to polyploidy may be assumed.

Within all phylogenies, split between *Leucanthemum* and other *Leucanthemineae* genera (*Plagius*, *Coleostephus*, *Glossopappus*, *Chrysanthoglossum*, *Mauranthemum*) can

be noticed and suggest strong diversification of these two clades which are occupying northern and southern part of the Mediterranean, respectively. Previous analysis of joint ITS and cpDNA suggested *Coleostephus myconis* as sister genus to *Leucanthemum* placing their last common ancestor (LCA) sister to a clade formed by *Plagius* and *Mauranthemum* (Oberprieler & Vogt 2000). Another earlier analysis suggested close relationship between *Rhodanthemum* and *Leucanthemum* whereas *Mauranthemum* would be a sister to their LCA based on ITS (Oberprieler 2005). Based on this result, that study also suggested a colonization of Iberian Peninsula by *Leucanthemum* from N Africa, which could be somehow congruent with *L. gracilicaule* as one of the basal species in the genus reconstructed on a species tree. However, this hypothesis is not fully supported here since *Rhodanthemum* is not a sister to *Leucanthemum* in the present reconstruction. In Oberprieler et al. (2009) *Leucanthemum* was reconstructed as sister to a clade composed by *Chrysanthoglossum* and *Chlamydophora* whereas *Plagius* was basal to those three genera. Perhaps results of these previous studies were influenced by use of different techniques based on different analytical tools and markers, which could be again influenced not only by ILS and hybridization but also by different sampling. Although it has to be noted that in many respects they recover similar patterns as in present publication that closest genus to *Leucanthemum* needs to seek within the clade reconstructed as sister to it in this study (*Plagius*, *Coleostephus*, *Glossopappus*, *Chrysanthoglossum*, *Mauranthemum*), but instead being a presently occurring genus it is rather a last common ancestor of these group which in turn was sister to pre-*Leucanthemum*. Results of this study strongly favor monophyly of *Leucanthemum* which is followed similarly by its morphological circumscription.

Perhaps one of the most interesting questions to answer would be, how this polyploid complex was formed? At first instance, several biological reasons may be proposed. From the available literature on *Leucanthemum* it seems that the most common explanation involves unreduced gametes. This is the prevailing mode of polyploid formation in the plant kingdom (de Wet 1979, De Storme & Geelen 2013) and was also found in the genus under study. In particular, Dowrick (1952) observed that the pollen from tetraploids (*L. ircutianum*) sometimes consisted of 36 chromosomes which is the same as the somatic number. He made the same observation in *L. atratum* where the double number of chromosomes in pollen occurred after irregularities in meiosis (Dowrick 1953). Probably inspired by these two findings, Pearson (1967) pinpoints the unidirectional gene flow from diploids to tetraploids via unreduced gametes as the most probable explanation and argues that interploidy crosses (i.e. triploid hybrids) may have little contribution to the gene flow. Because polyploidy is often invoked as an escape from the

hybrid sterility (e.g. Levin 1983) and if most of the presumed natural hybrids (Chapter 1) and artificial hybrids (Villard 1971) are fertile, one could expect that the polyploids should be less numerous in *Leucanthemum*. However, the other feature is that a common hybridization may actually provide a higher error rate in meiosis and induce a higher production of unreduced gametes. For example in *Brassica* hybrid plants produced unreduced gametes up to two orders of magnitude higher than parental plants and in some plants it constituted up to 33.5% of all gametes (Mason et al. 2011). Moreover, pollen producing unreduced gametes were more vigorous than normal pollen in hybrids (Mason et al. 2011). In this way hybridization could indirectly facilitate polyploid formation. As *Leucanthemum* seems to be a group of relatively recent origin, the likelihood of successful hybrid formation is increased, since the fertility of a hybrid is negatively correlated with the phylogenetic distance (Buggs et al. 2008). This does not imply for a polyploid formation and basically whether species are closely or distantly related does not play a role in the success of forming successful polyploids (Buggs et al. 2008). Already Grant (1981) proposed that some genera which are rich in polyploids may be influenced by genetic background such as alterations in certain genes controlling meiosis. Such candidate genes were reported recently in such model plants as *Arabidopsis* or *Lycopersicon* (De Storme et al. 2013, De Storme & Geelen 2013). Whether such genes may be responsible for a frequent polyploid formation in *Leucanthemum* is unknown but even if not, it is logical to assume that after the first polyploid incidences the next ones are just a consequence of the first ones and in a favorable circumstances may form a "chain reaction". And when the higher ploidies come into existence, the possibility of interploidy crosses and uneven hybrids (3*x*, 5*x*, 7*x*,...) increases and their contribution to the formation of other polyploids may also become significant. As studies on pentaploid (Papeš 1972a) and heptaploid (Papeš 1972b) populations have shown, plants with intermediate ploidy levels which are a result of hybridization between higher polyploids produce seedlings with variable number of chromosomes (5*x* – 35 to 71) as well as pollen with variable chromosome number (7*x* – 59 to 64 with up to 10 B chromosomes). This scenario proposes the hypothetical factors behind the origin and the formation of the *Leucanthemum* polyploid complex. It should also highlight the complexity of the polyploid formation which is not a process occurring within one step but may consist of several smaller and bigger steps which all in the end contribute to the complex and network-like history of the genus.

One point that should be mentioned concerning the origins of *Leucanthemum* polyploid complex together with the biological background is the influence of ecological factors. Conditions that could be associated with the formation of polyploids may be linked

to the Quaternary climatic oscillations. Obvious factor of the glacial age is the oscillation of the temperature and it is possible to link the cold phases with the increased number of unreduced gametes. For example studies on *Brassica* (Mason et al. 2011) and *Arabidopsis* (De Storme et al. 2012) provided indication that cold-stressed plants produced higher frequencies of diploid and polyploid pollen grains. This demonstrates that male gametogenesis in plants is sensitive to environmental stress and as De Storme et al. (2012) hypothesize: "the prevalence of polyploids in adverse climates may be linked to the abiotic stress conditions which can induce or stimulate diploid gamete production". This highlights the possible way of origin which together with previously mentioned hybridization may be treated as the first step while the second step would lie in the establishment of newly arisen polyploids in the landscape. There must be some mechanisms for the promotion of polyploid plants in the environment and this resembles commonly stated principle that polyploids are more frequent in extreme climates (Hagerup 1932) because of such advantages as e.g. increased heterozygosity (Brochmann et al. 2004). Deglaciated areas or even those that were under the influence of changing climate are places of shifts between vegetation types and provide opportunity for the establishment as the competence is much lower than in undisturbed habitats (Van de Peer & Fawcett 2010). Therefore it is possible to link all these processes and assume their active role in the formation of polyploids in *Leucanthemum*. In particular, study done on *L. pluriflorum*-clan shows that species on Iberian Peninsula experienced significant range shifts since the last glacial maximum (LGM) (Appendix B). As discussed there, in some cases species enlarged their potential ranges since LGM, but in some cases potential ranges contracted. Moreover, as shown in the *L. pluriflorum*-clan example, those range shifts may explain the present day haplotype diversity found within the group which contain two major haplotypes L1 and L2 (cf. also Greinter et al. 2013). It is hypothesized that one of the two diploid *L. pluriflorum* lineages went extinct since LGM (southern-lineage) but its haplotype is still found within polyploids occupying the same area (Appendix B). Quaternary climatic oscillations are often invoked when the history of polyploid plants is discussed (e.g. Säll et al. 2003, Rebernig et al. 2010, Casazza et al. 2012). In accordance to the observations on *Leucanthemum*, studies on *Draba* (Jordon-Thaden & Koch 2008) have shown that polyploid species are more frequent in areas with high species richness.

6. SUMMARY

The genus *Leucanthemum* consists of ca. 56 taxa occurring mainly in Southern and Central Europe. While there are 19 diploid taxa ($2n = 2x = 18$) the rest of the genus is formed by an unbroken polyploid chain ranging from tetraploid ($2n = 4x = 36$) to dokosaploid ($2n = 22x = 198$). Because of richness in polyploids this genus provides an ideal study system to examine various aspects of polyploidy. This thesis is the starting point to unravel phylogenetic relationships within the whole genus.

In this study sampling of all recognized taxa in the genus *Leucanthemum* is provided with the main aim to reconstruct phylogenetic relationships. The thesis is divided into three chapters with focus on: 1) phylogeny of diploids and homoploid hybrid speciation incidence, 2) phylogeny of all taxa including polyploids, 3) phylogeny and evolutionary processes within *L. glaucophyllum* group and closely related species.

Phylogenies are reconstructed with usage of low-copy nuclear genes sequenced by 454 sequencing accompanied by chloroplast markers. Species tree is constructed in the first Chapter together with species network and hybrid index for all diploid taxa. It reveals that *Leucanthemum* is monophyletic and that fifteen out of nineteen diploid taxa experienced homoploid hybrid speciation or severe gene flow among species. As a consequence the particular branches in the phylogeny gain rather low support but with robust division of diploids into two groups present in all analyses. In the second Chapter the phylogeny of all taxa is reconstructed as species network which recovers high level of reticulation among them with main finding that taxa from the same geographic area show affinity irrespective of their ploidy level. The third Chapter focuses in more detail on a smaller group of high polyploids related to a decaploid *L. glaucophyllum*. With reconstruction of species network, nested clade analysis on chloroplast network, and AFLP clustering it brings insights into relationships in this group. It also sheds light on the phylogeography of *L. pallens* and suggest that gene flow among the same and different ploidy levels is likely occurring. Furthermore new octoploid race is found in Esterell Massif and its relation to other taxa is discussed.

The results pinpoint hybridization as one of the major processes in the evolution of the genus *Leucanthemum*. It influences speciation on the diploid level, enables formation of polyploids and contributes to the reticulate evolution among them in general, and specifically in taxa related to *L. glaucophyllum*.

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8. APPENDIX A

8.1. Gene trees for diploids

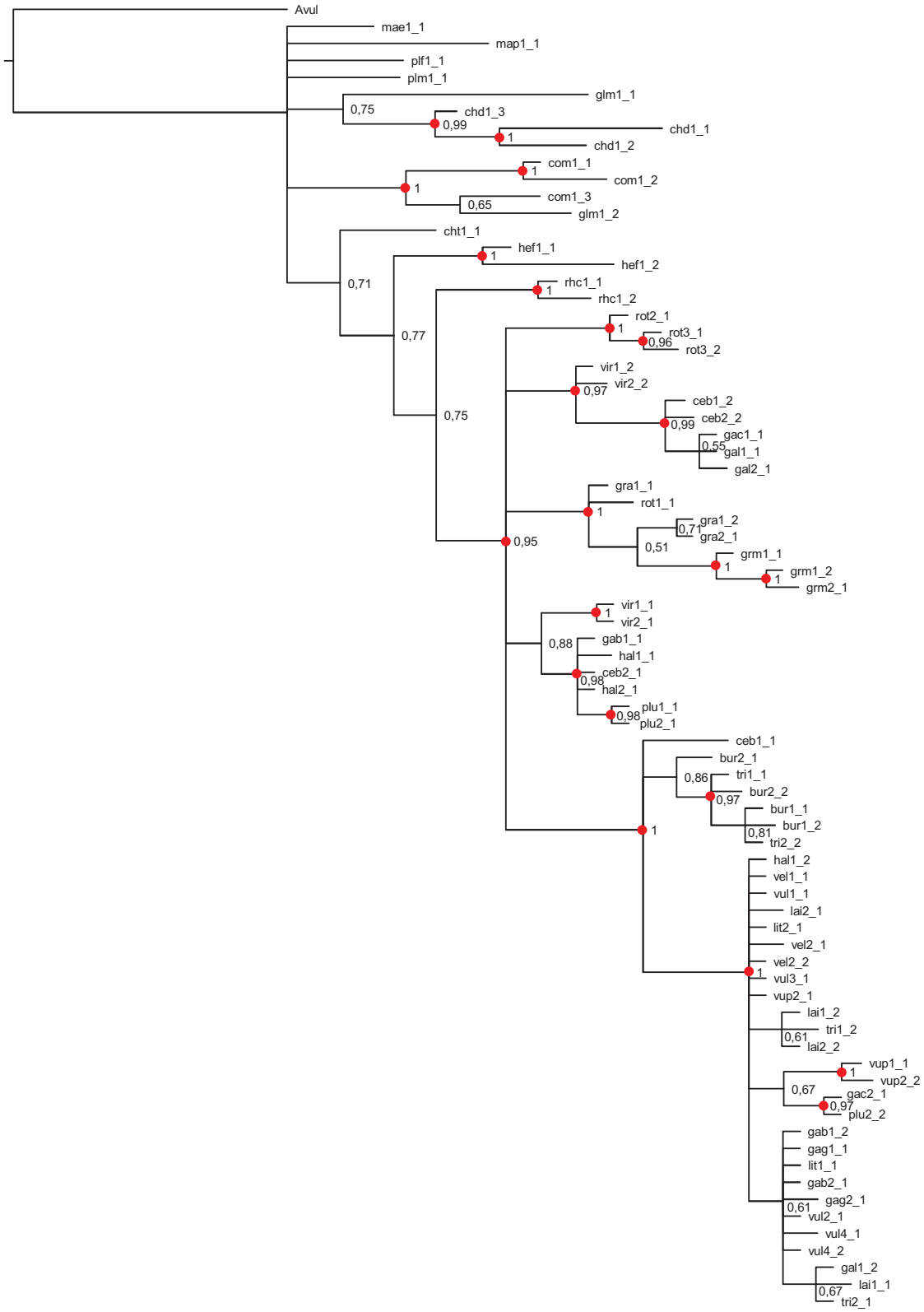

Figure 18 - Gene tree for marker A39 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

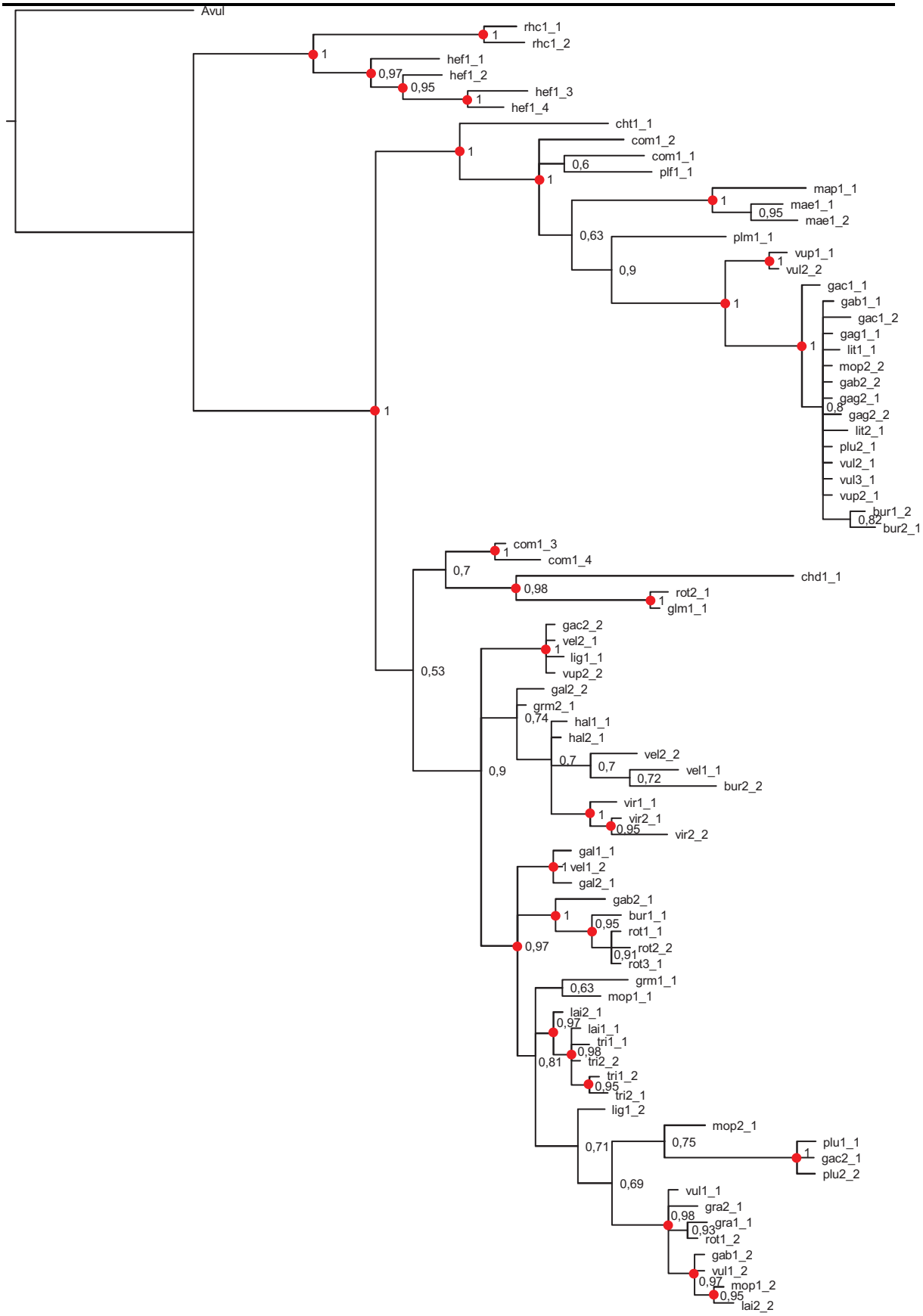

Figure 19 - Gene tree for marker B12 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

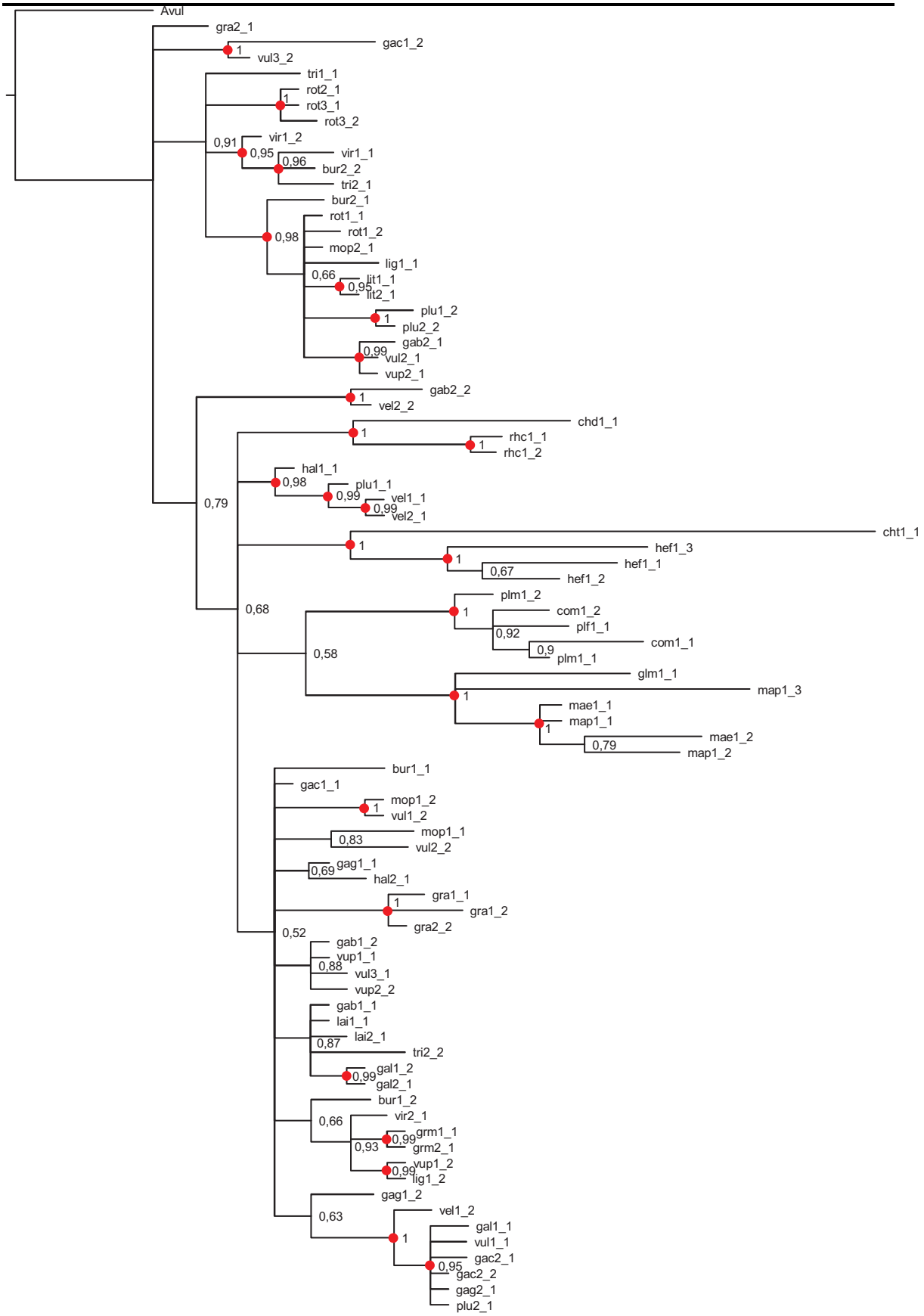

Figure 20 - Gene tree for marker B20 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

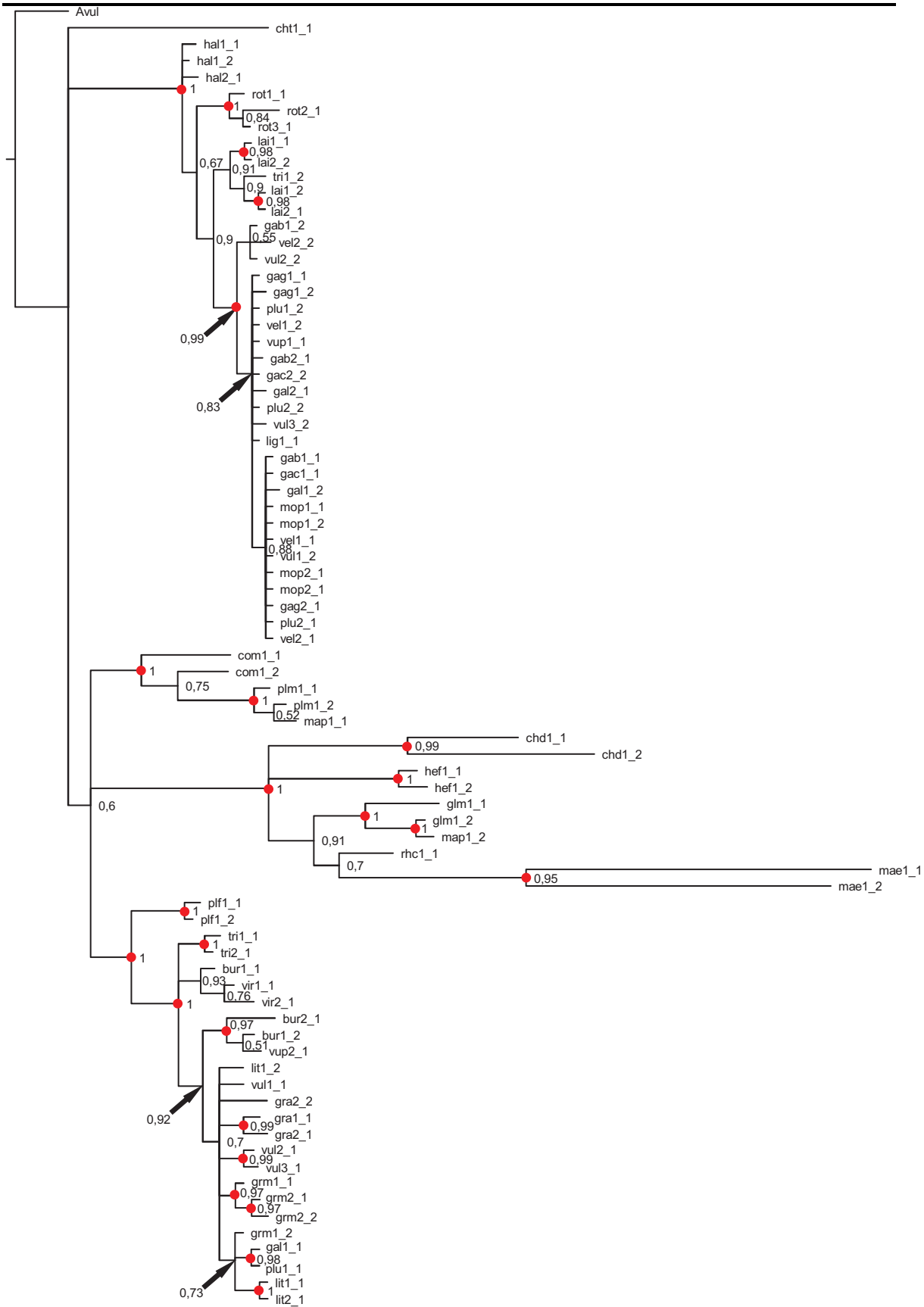

Figure 21 - Gene tree for marker C12 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

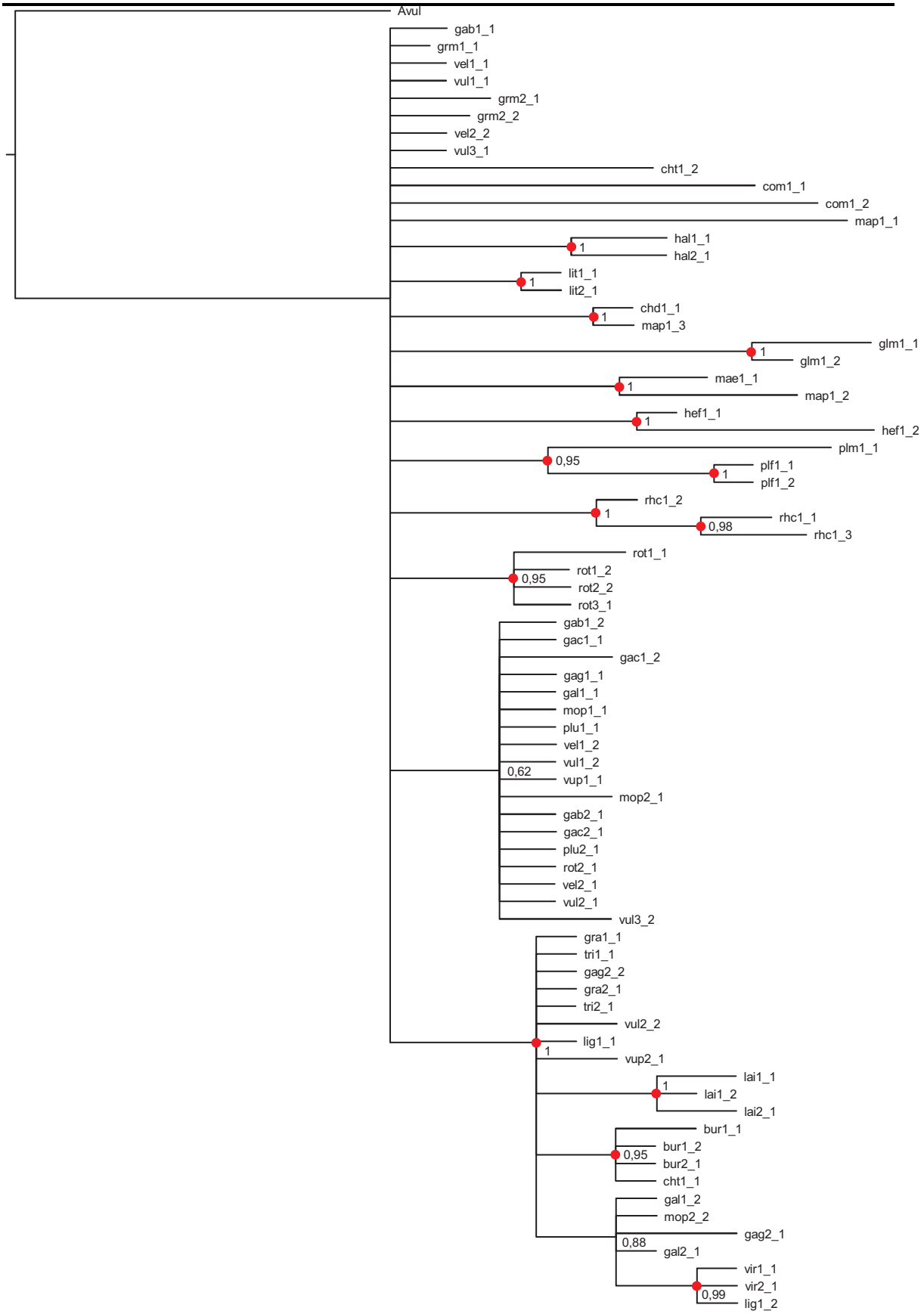

Figure 22 - Gene tree for marker C20 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

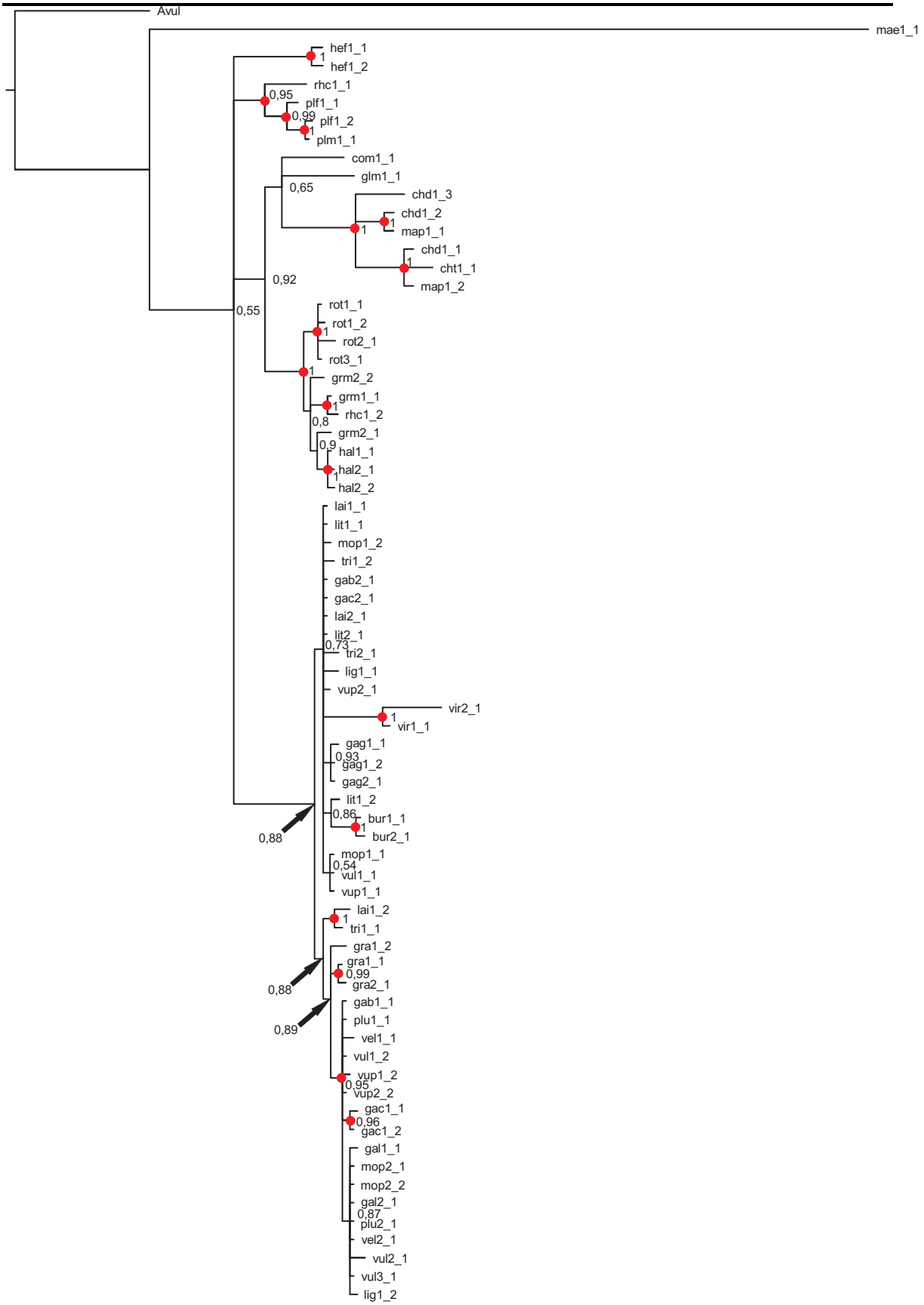

Figure 23 - Gene tree for marker C33 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

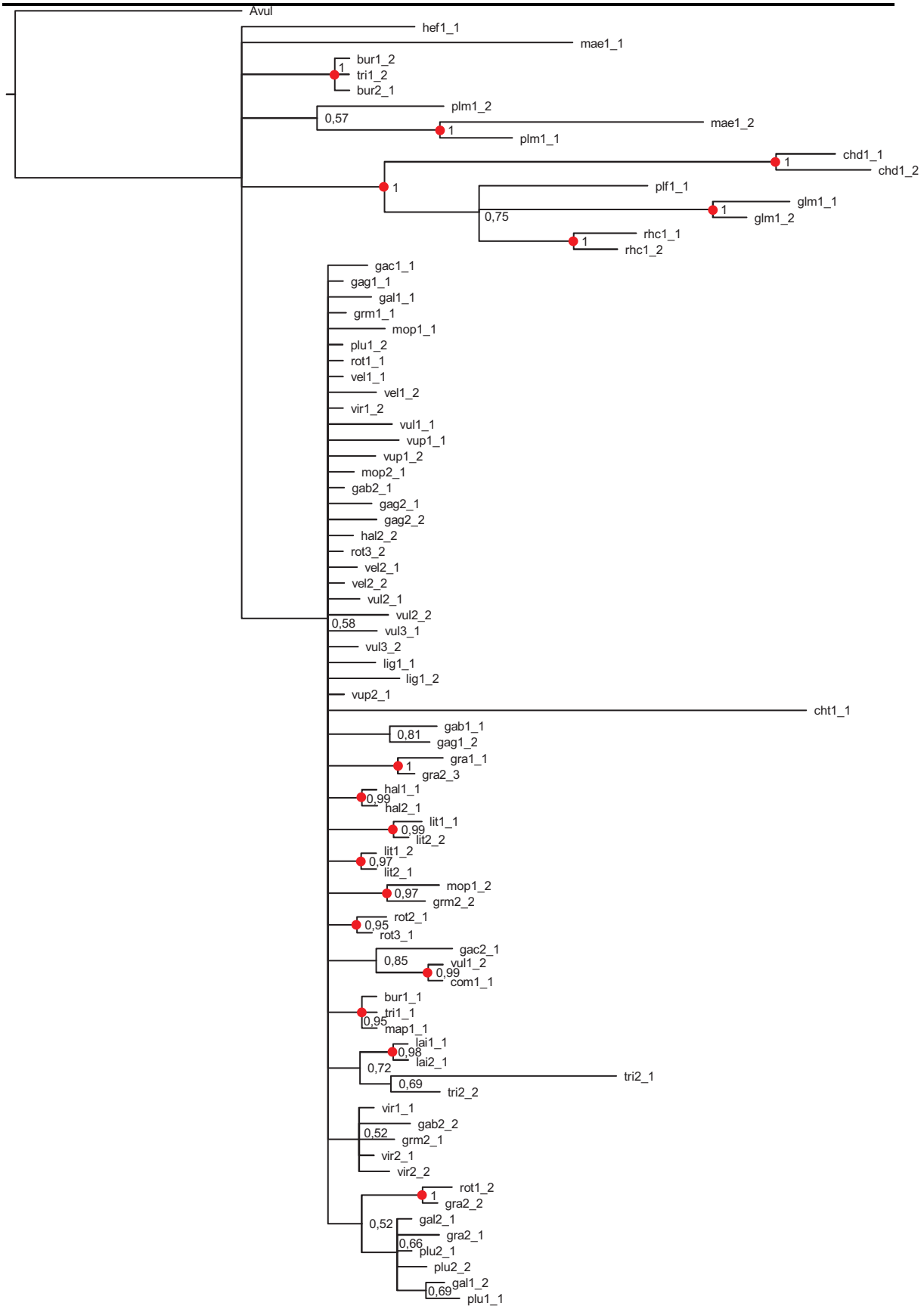

Figure 24 - Gene tree for marker D18 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

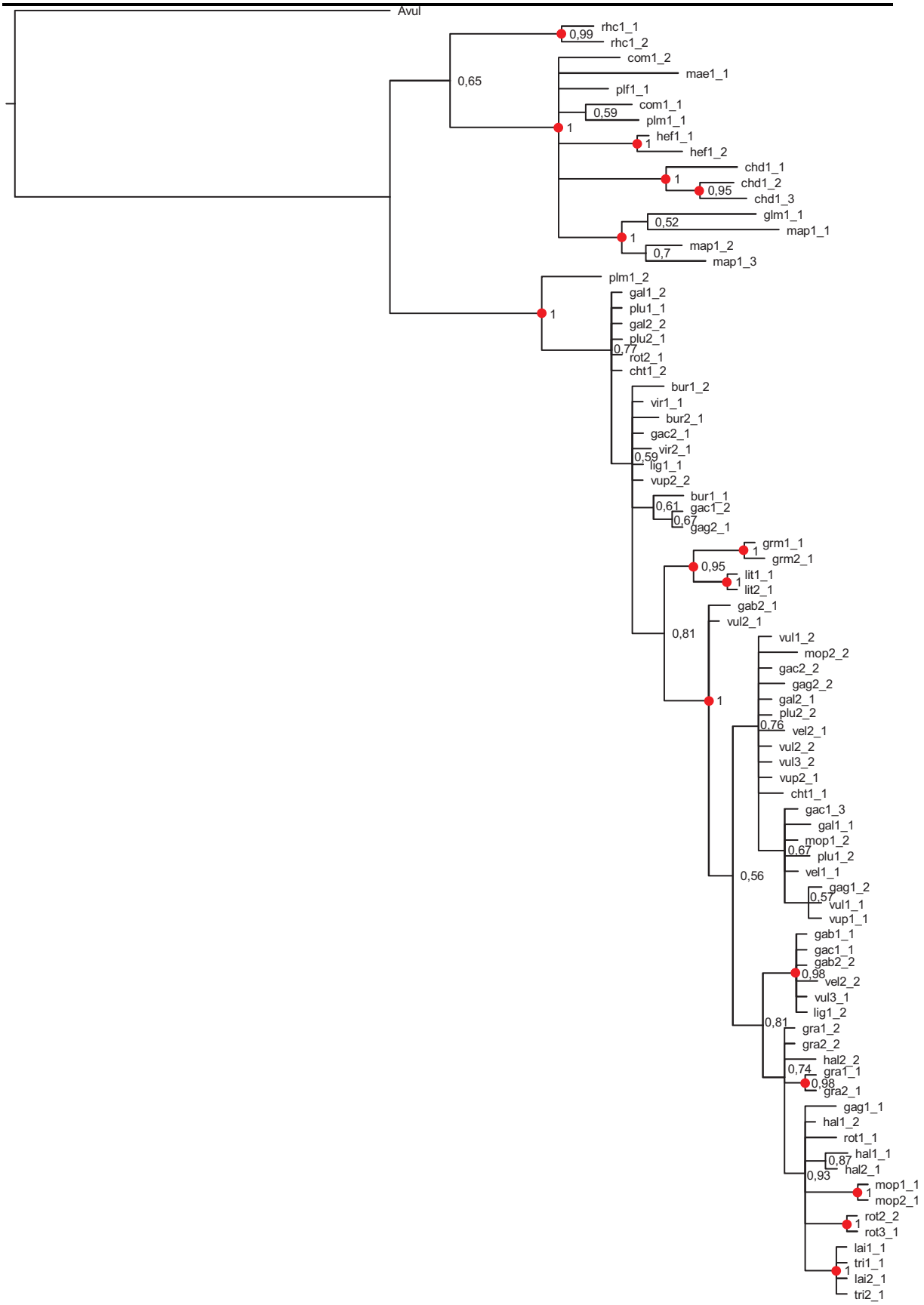

Figure 25 - Gene tree for marker D18 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

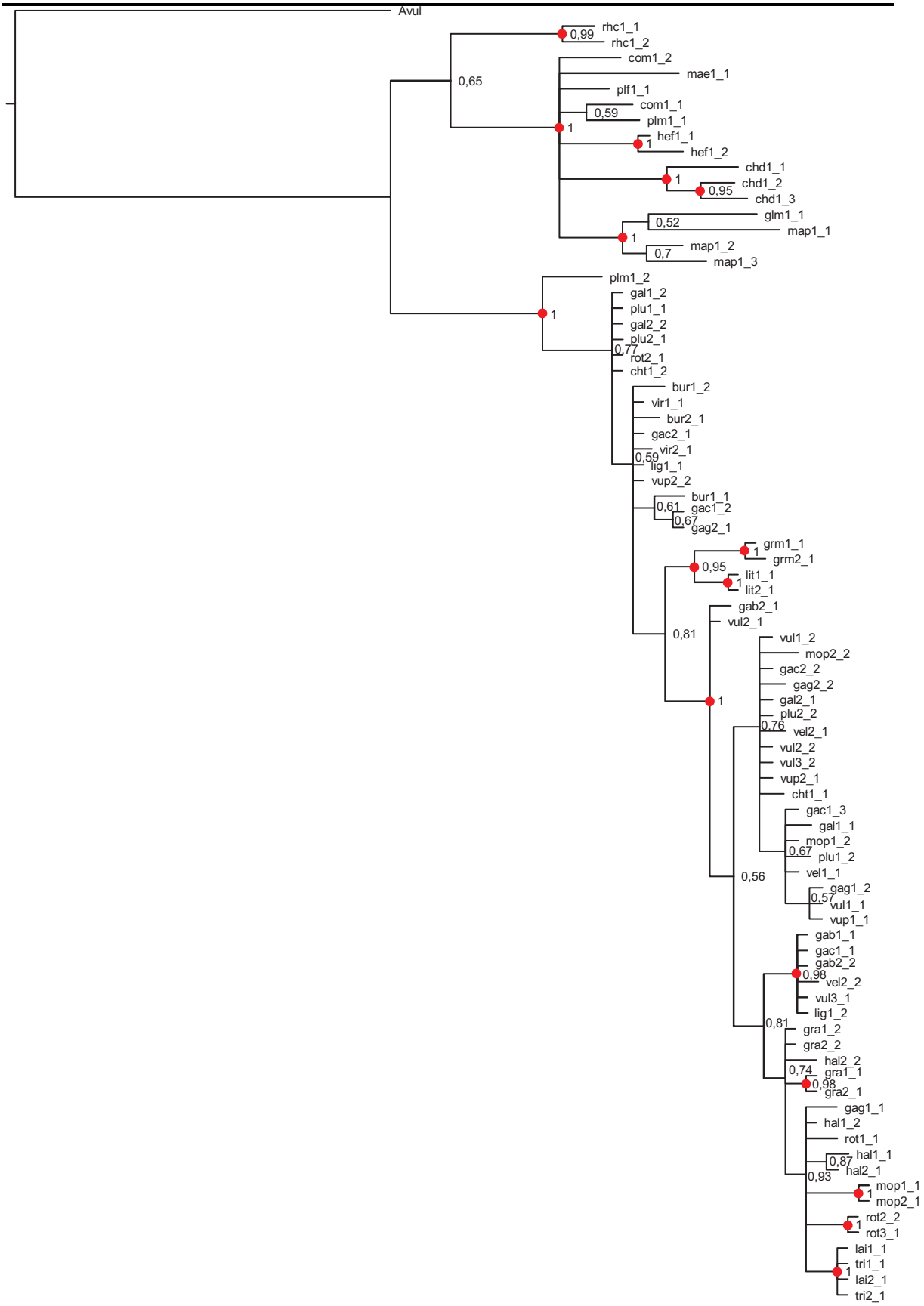

Figure 26 - Gene tree for marker D23 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

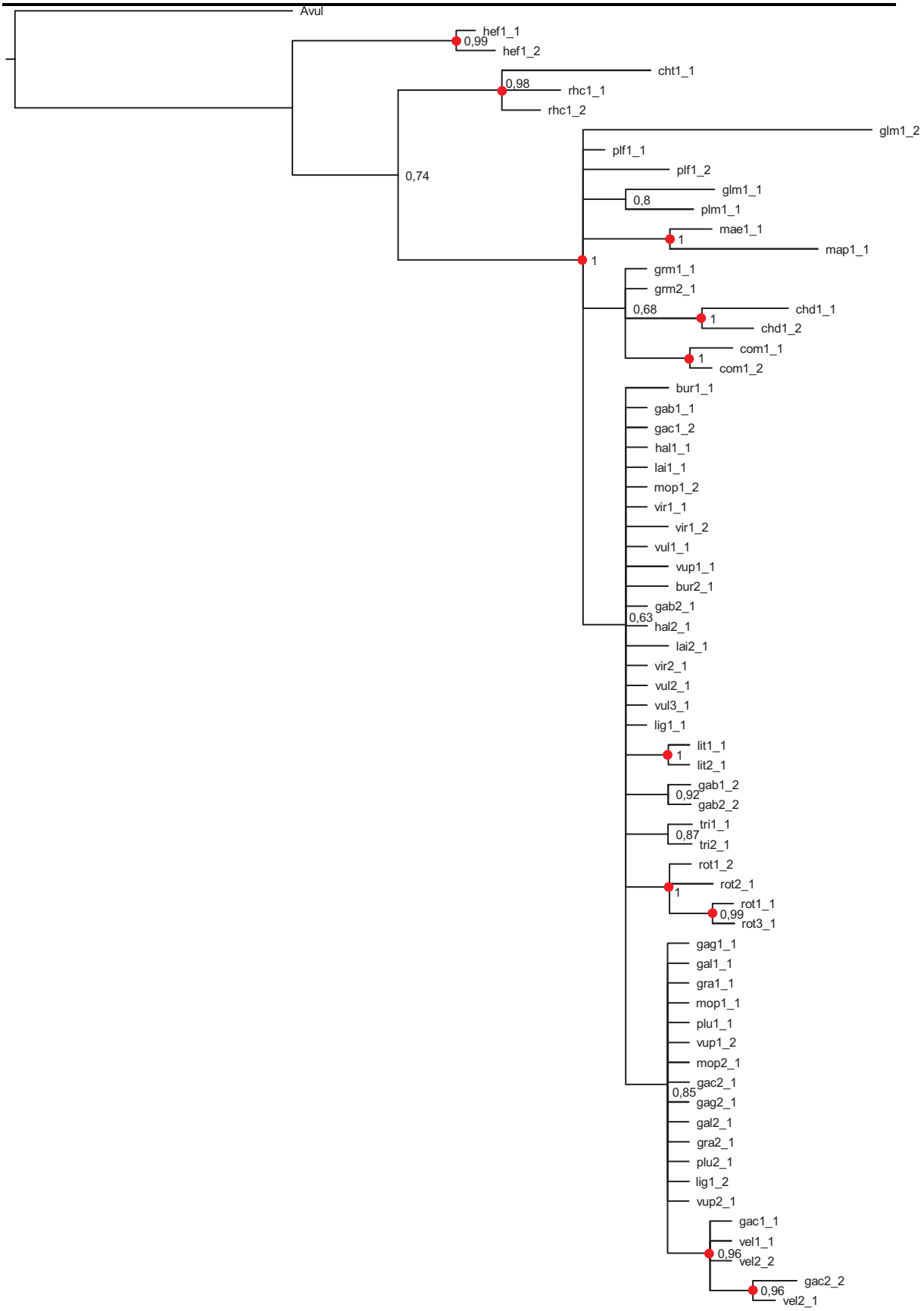

Figure 27 - Gene tree for marker D27 containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

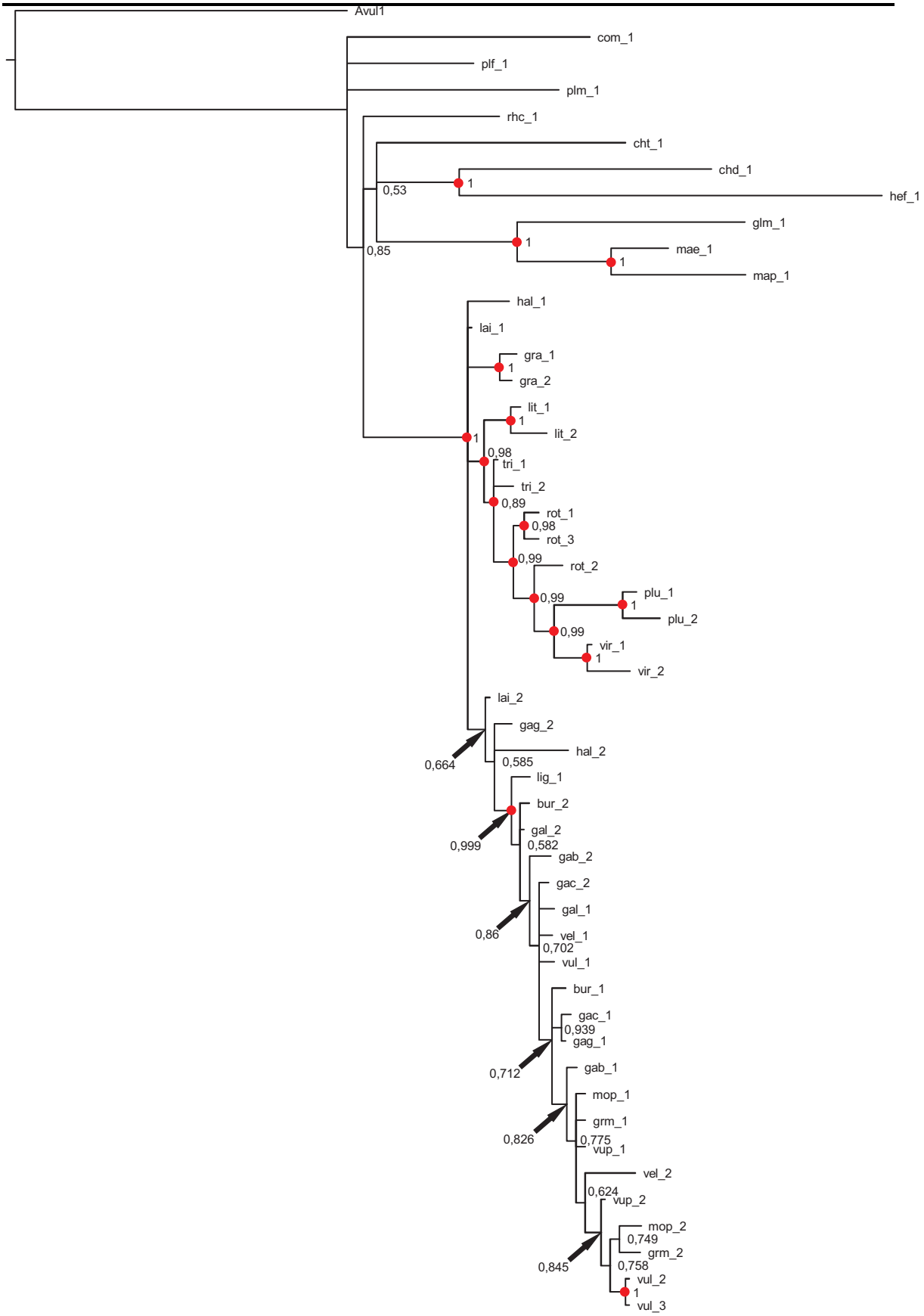

Figure 28 - Gene tree for five cpDNA markers containing diploid *Leucanthemum* taxa and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

9. APPENDIX B

9.1. Gene trees for polyploids

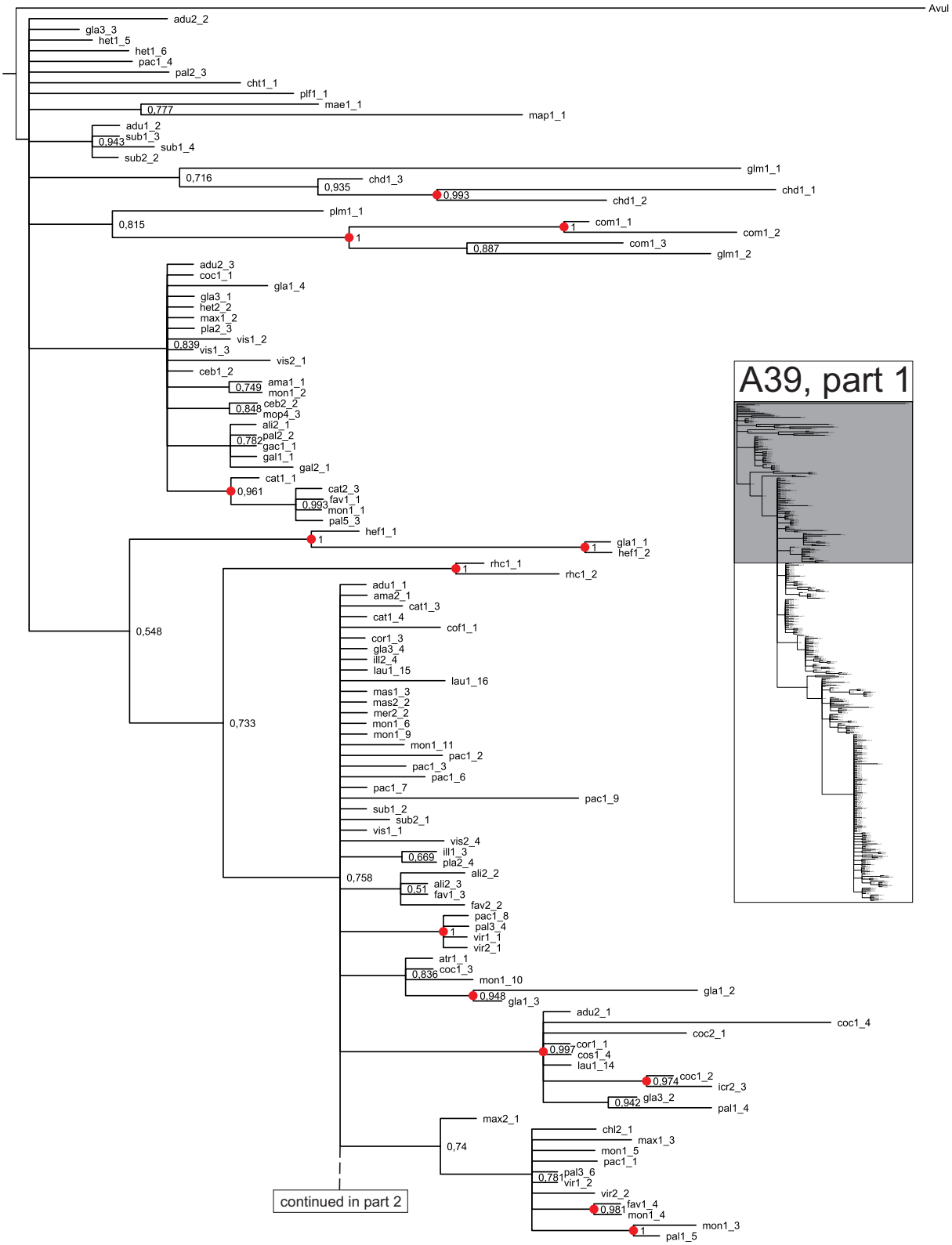

Figure 29 – Gene tree for marker A39 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

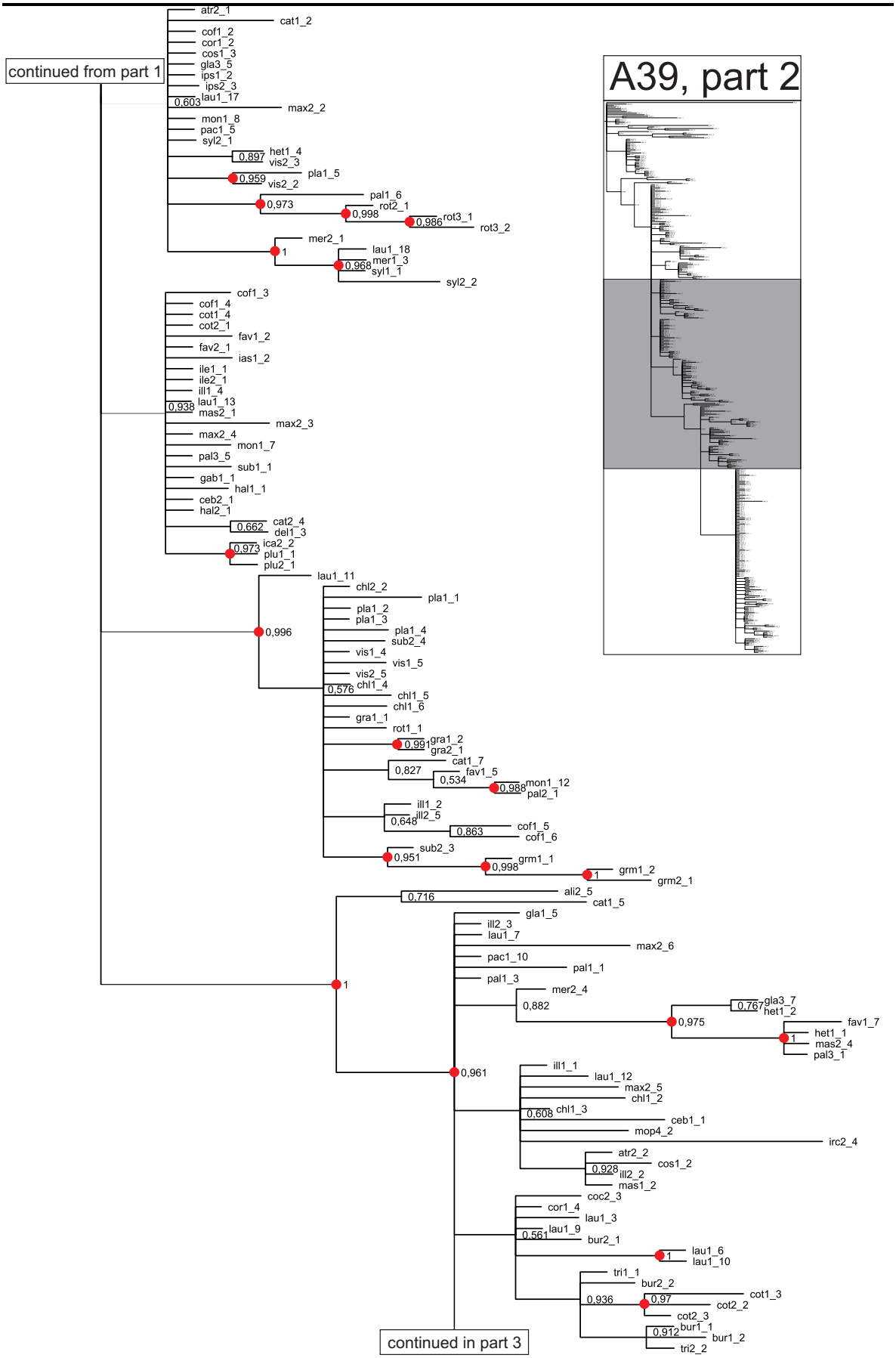

Figure 30 - Gene tree for marker A39 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

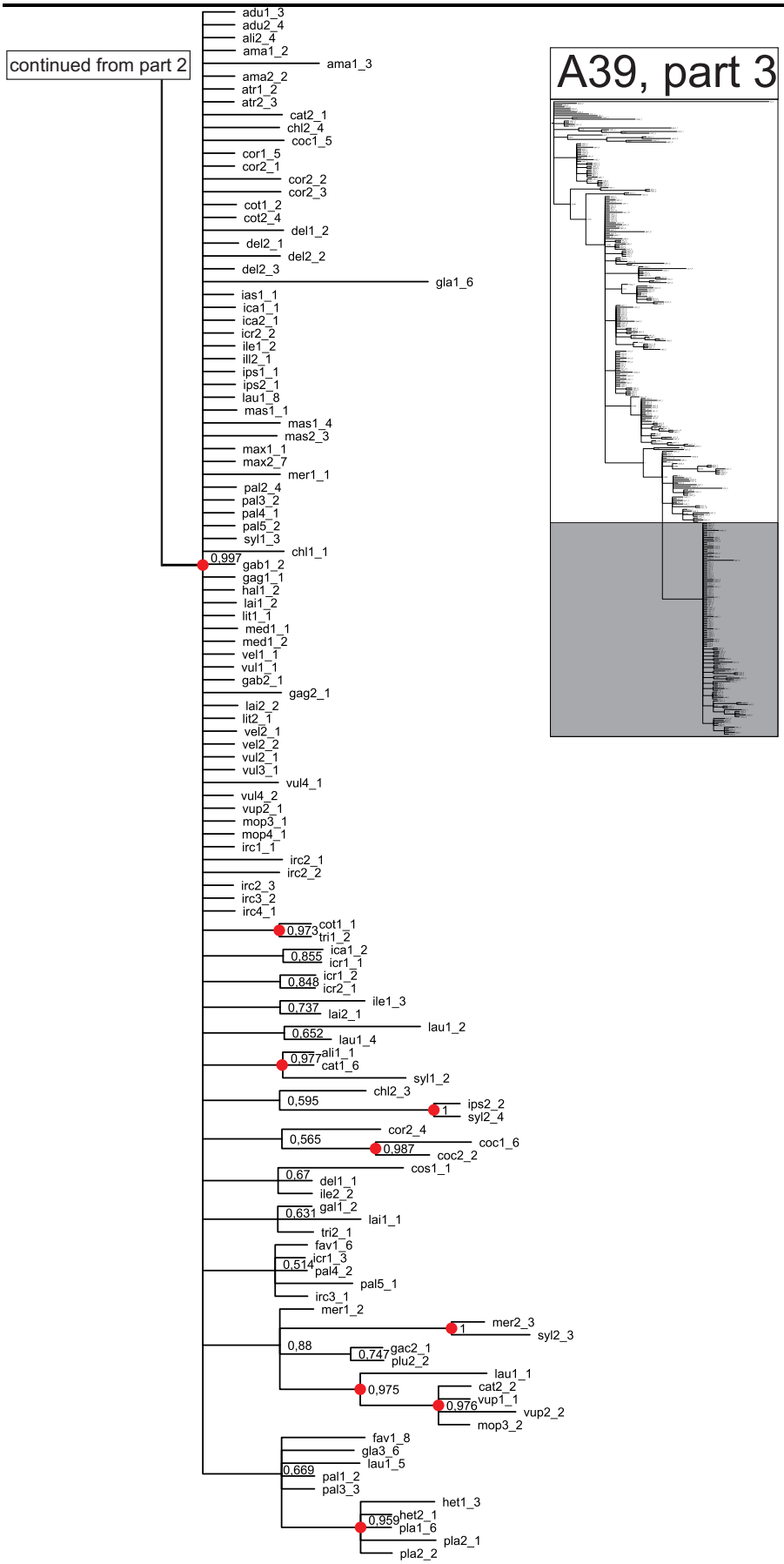

Figure 31 - Gene tree for marker A39 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

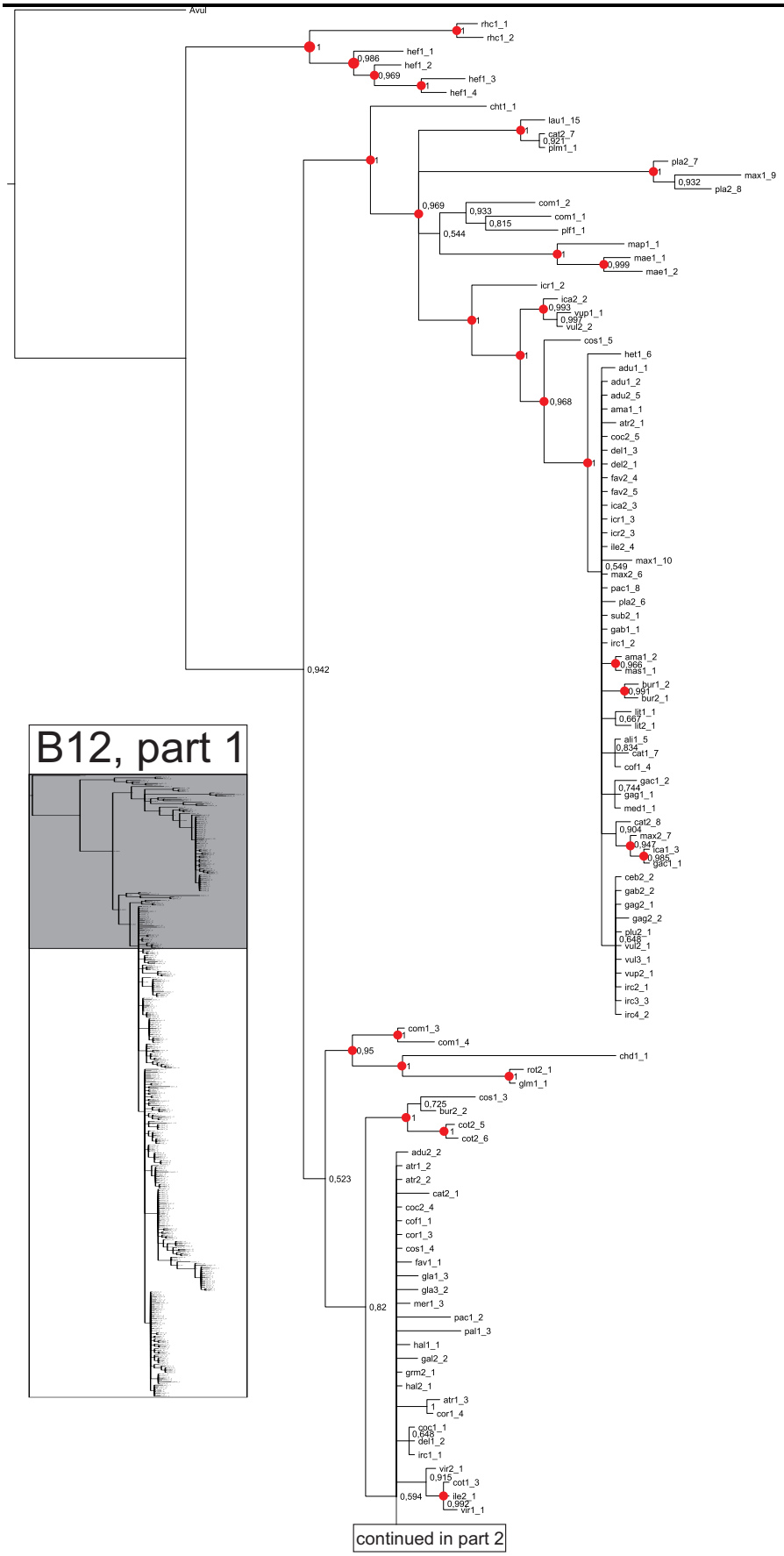

Figure 32 - Gene tree for marker B12 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

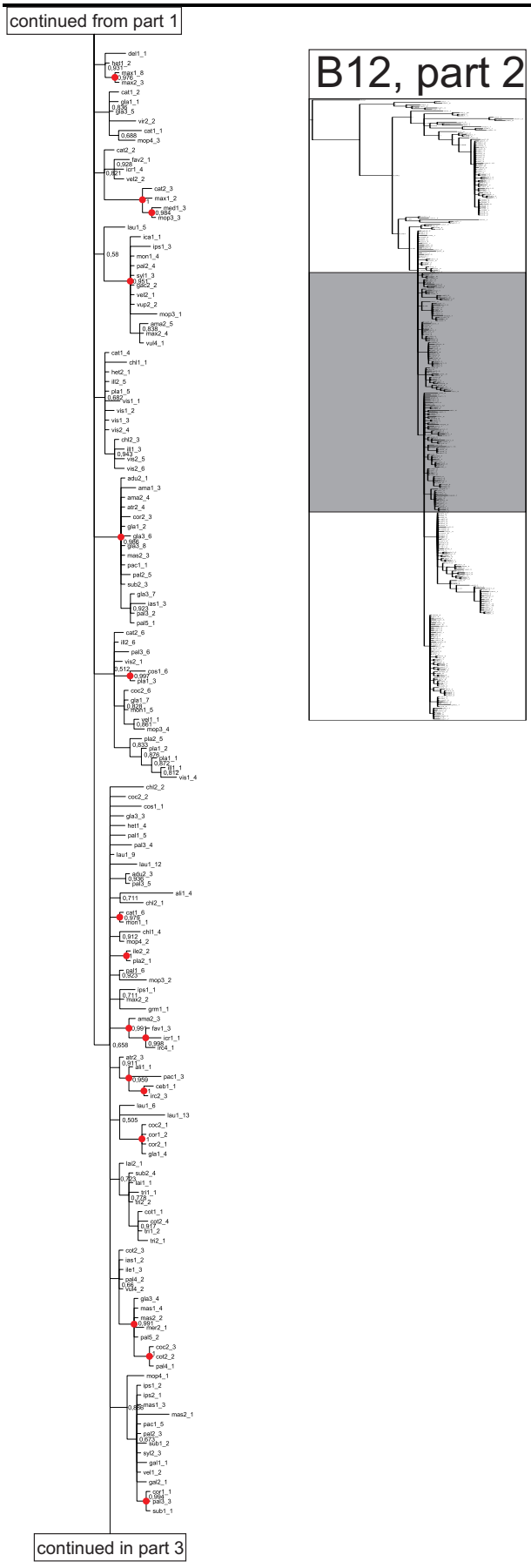

Figure 33 - Gene tree for marker B12 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

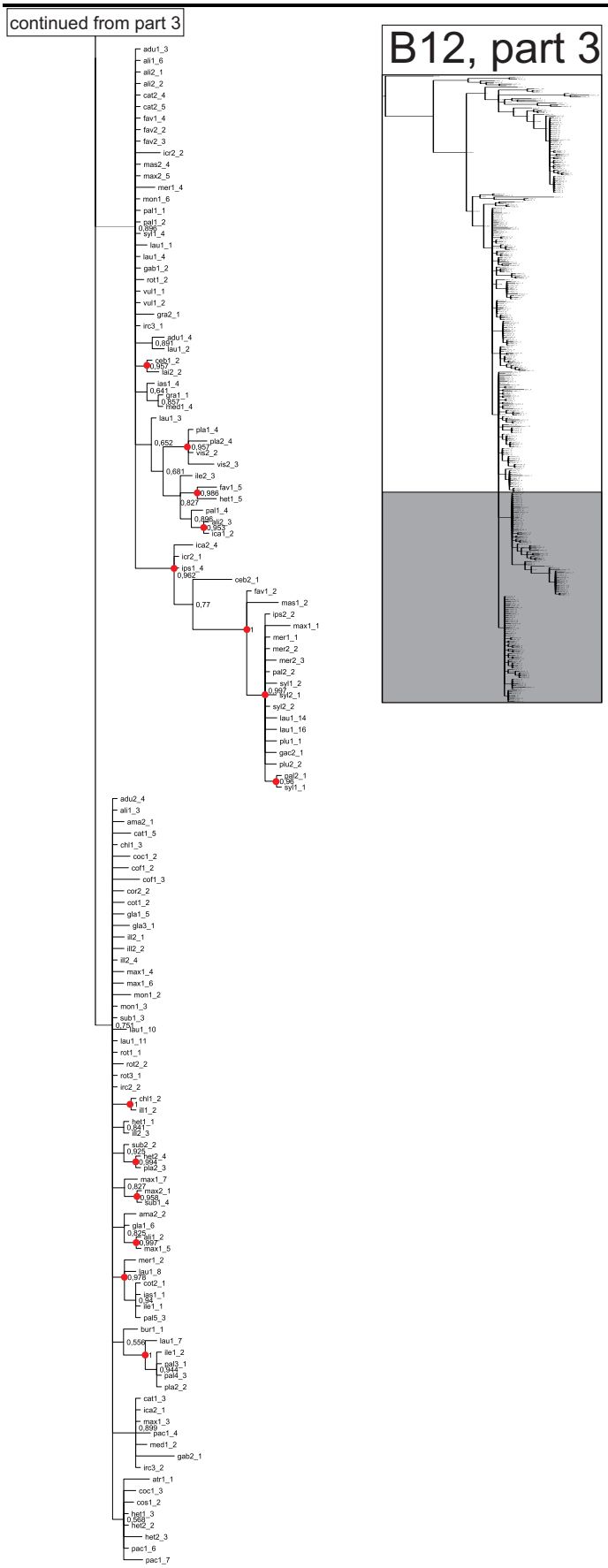

Figure 34 - Gene tree for marker B12 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

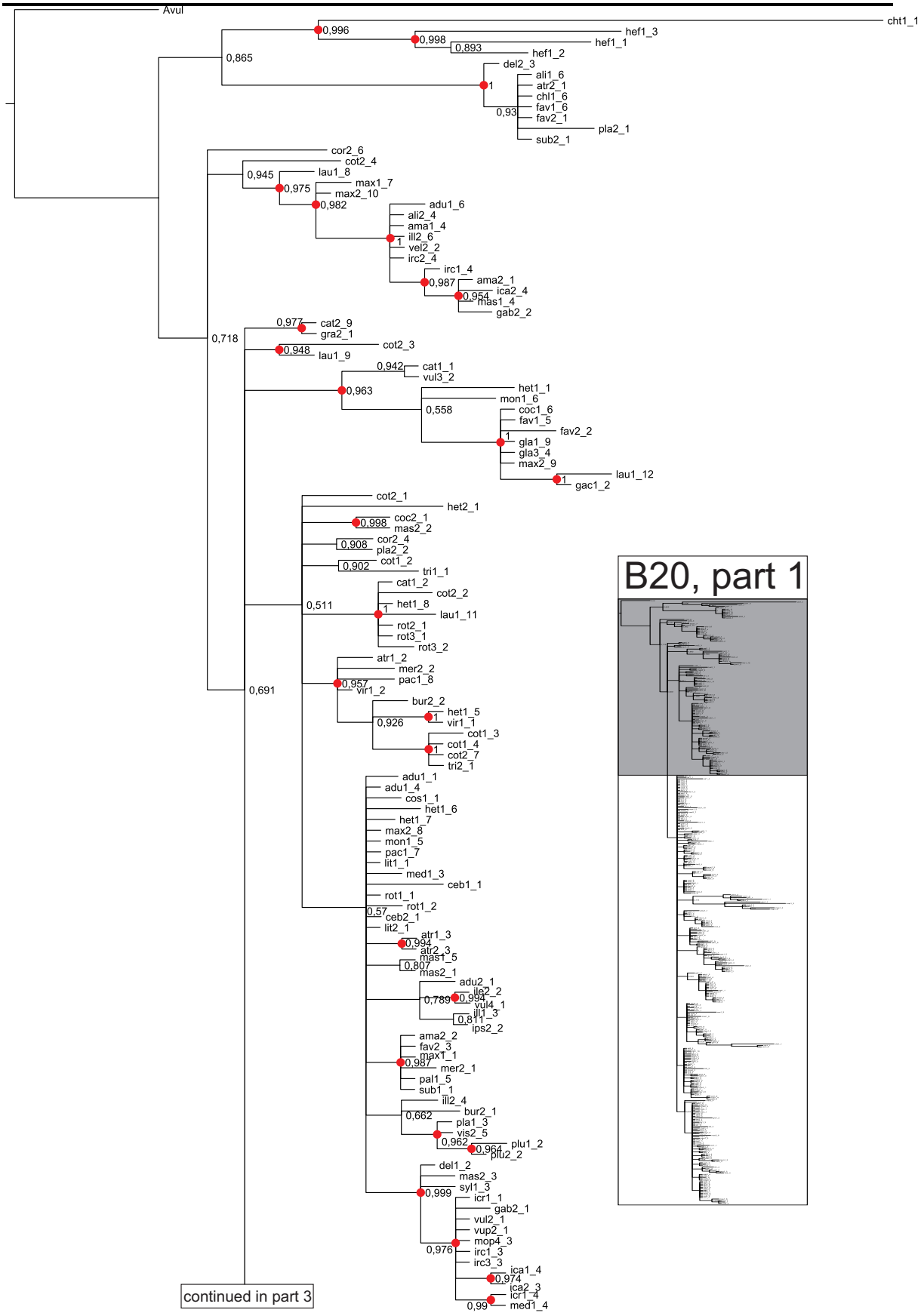

Figure 35 - Gene tree for marker B20 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

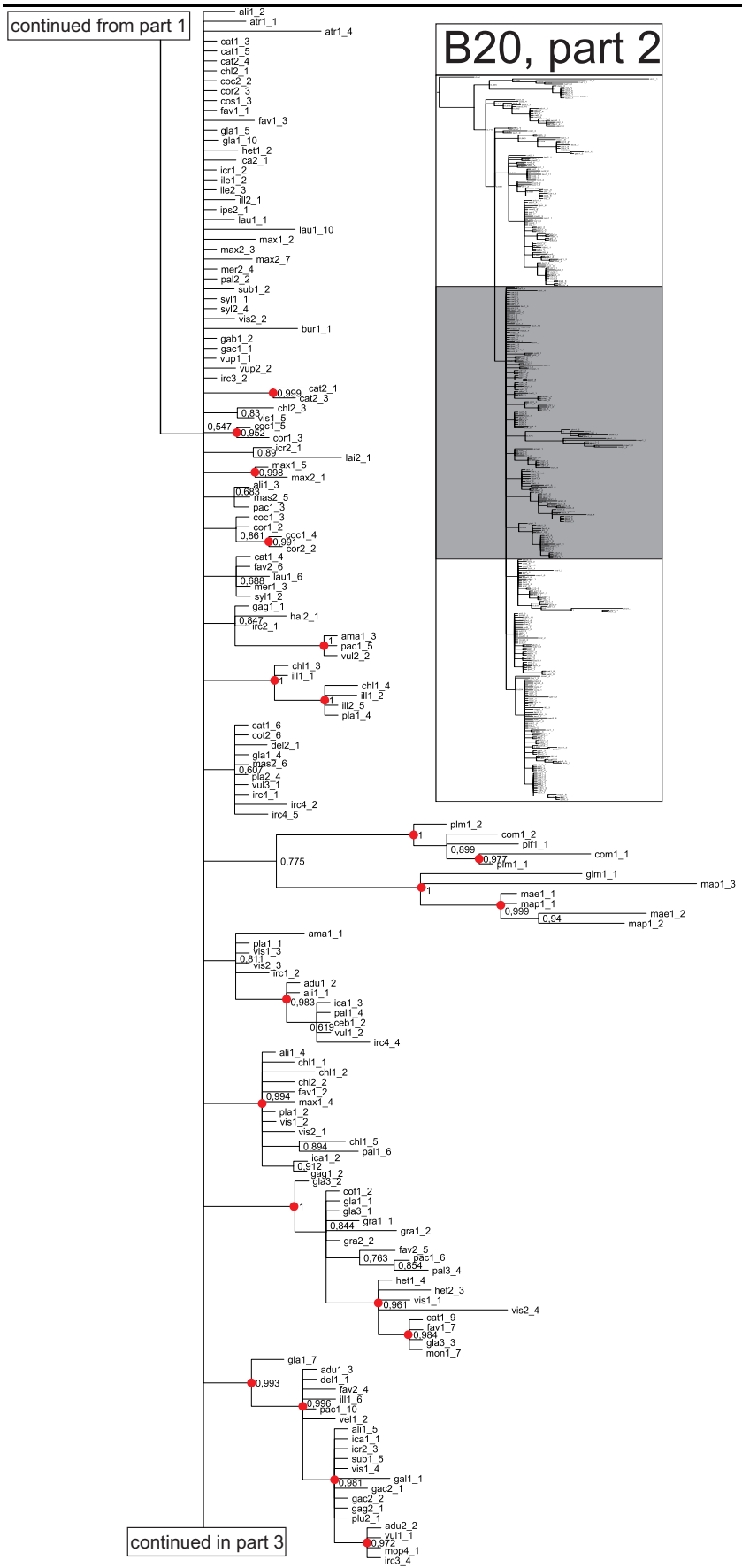

Figure 36 - Gene tree for marker B20 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

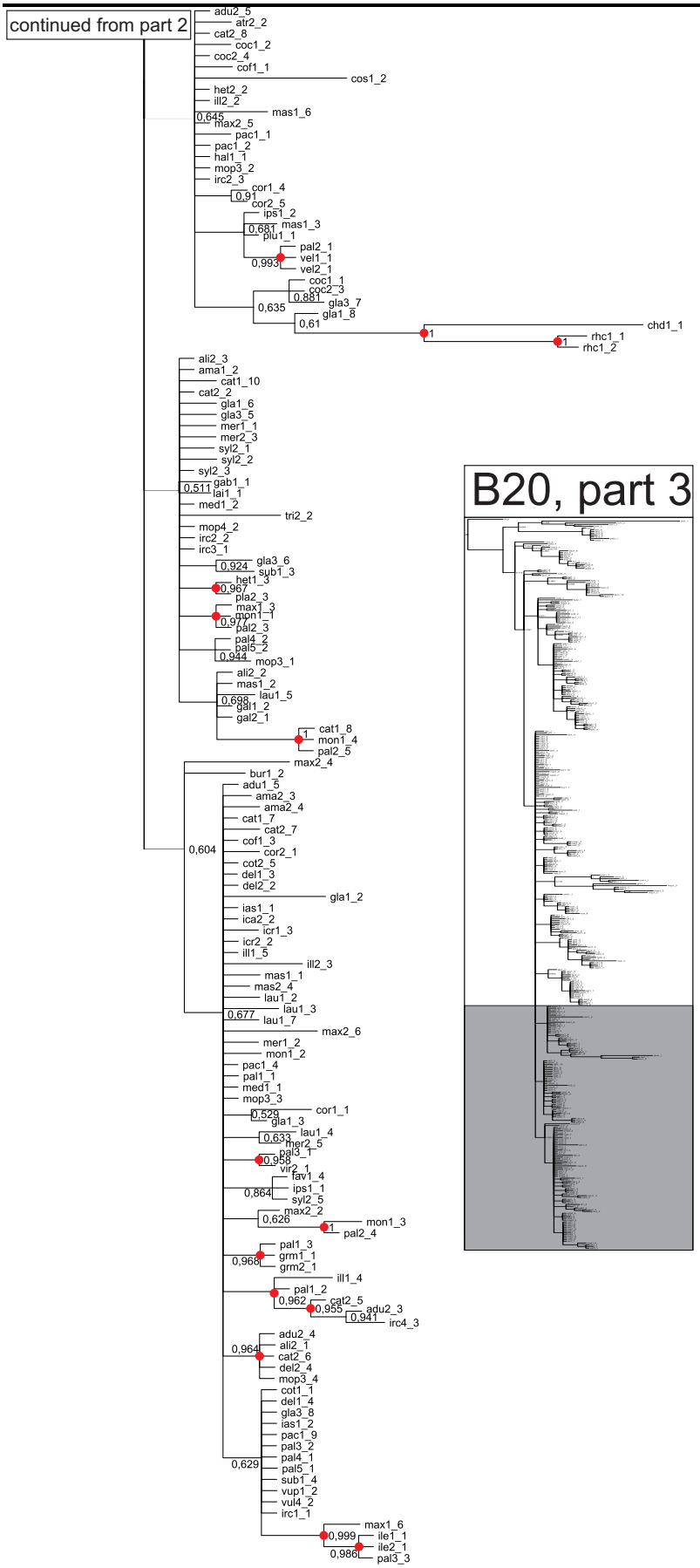

Figure 37 - Gene tree for marker B20 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

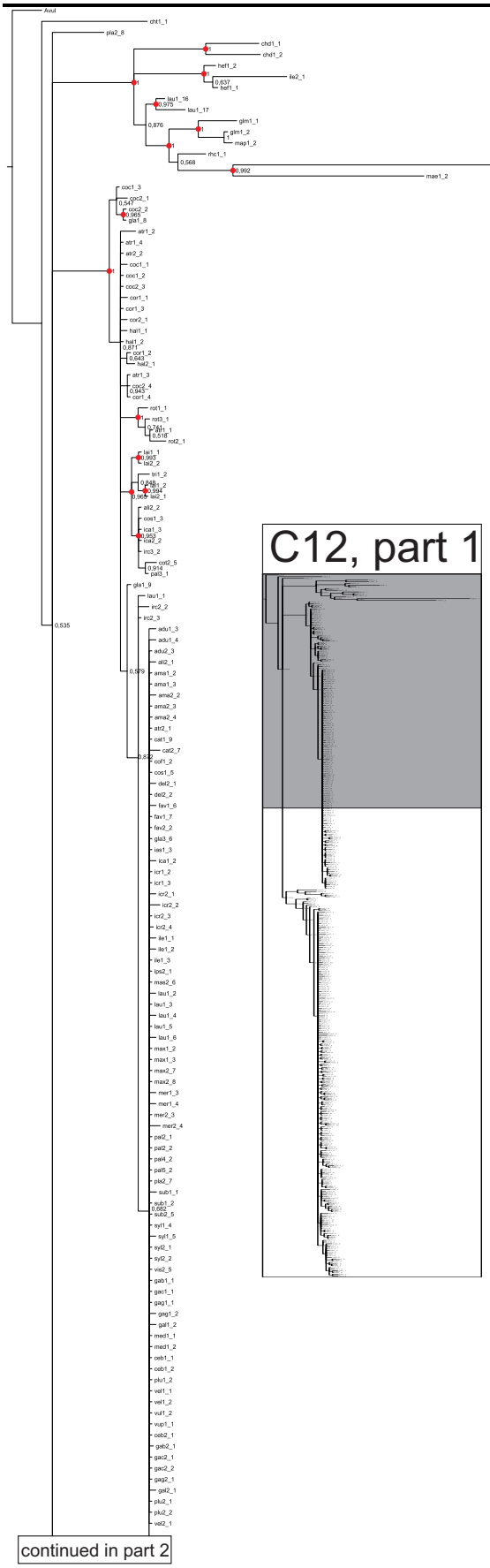

Figure 38 - Gene tree for marker C12 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

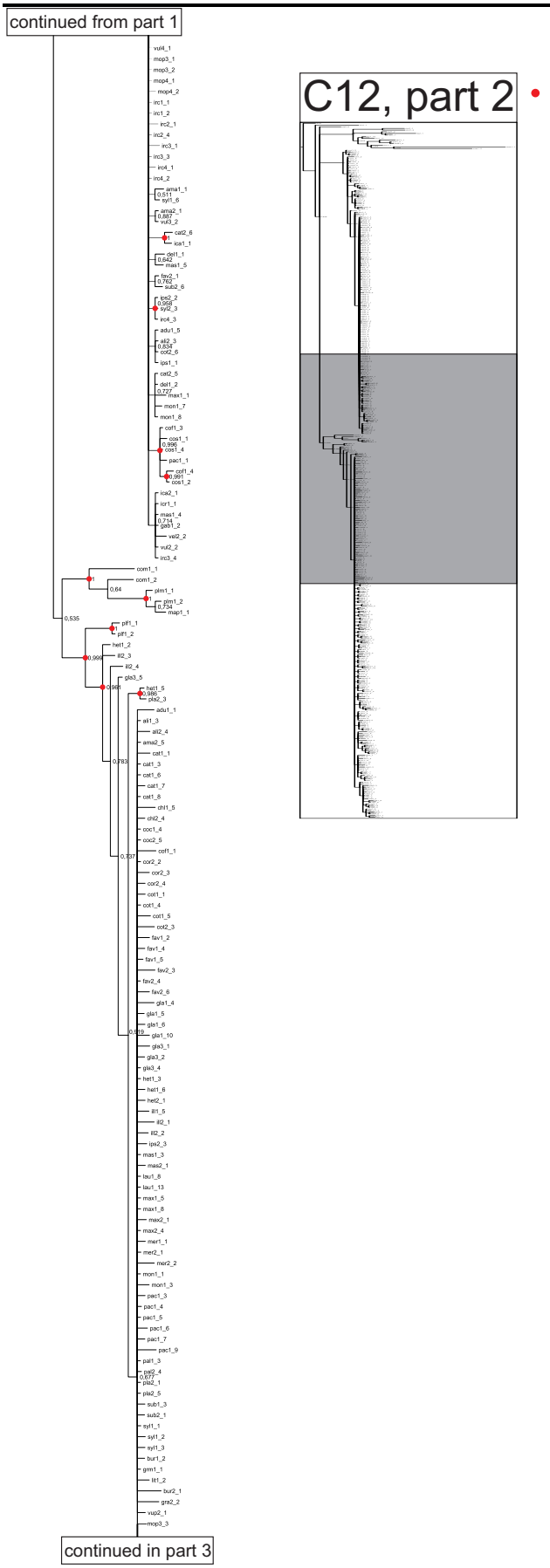

Figure 39 - Gene tree for marker C12 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

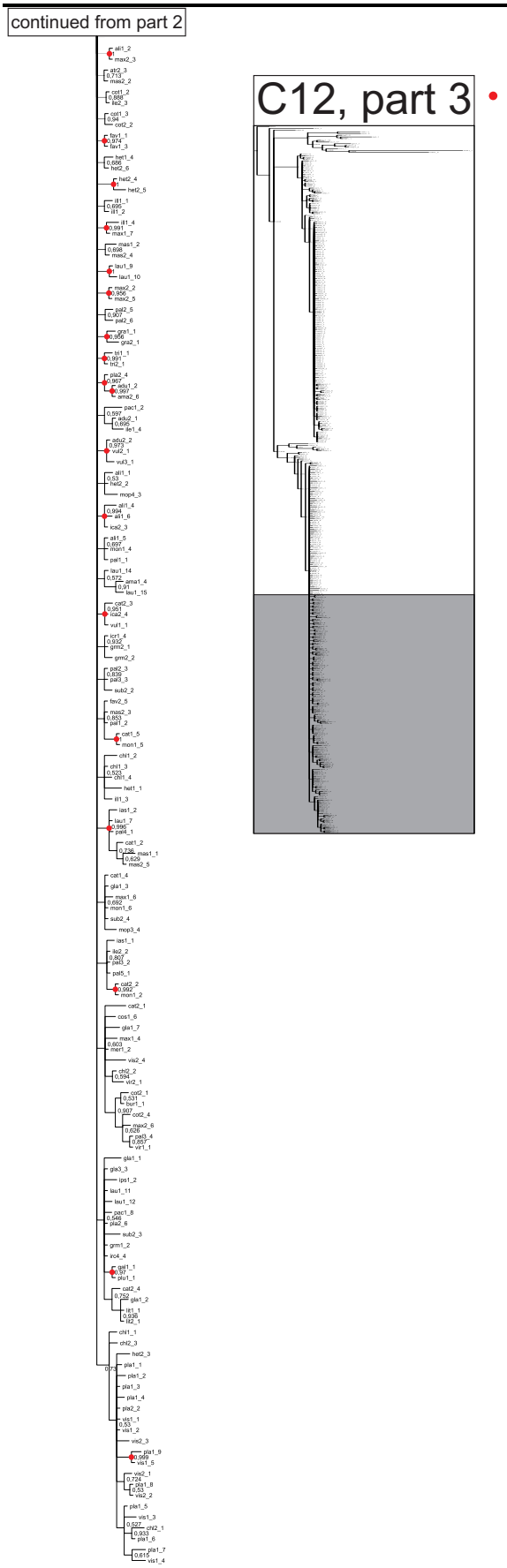

Figure 40 - Gene tree for marker C12 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

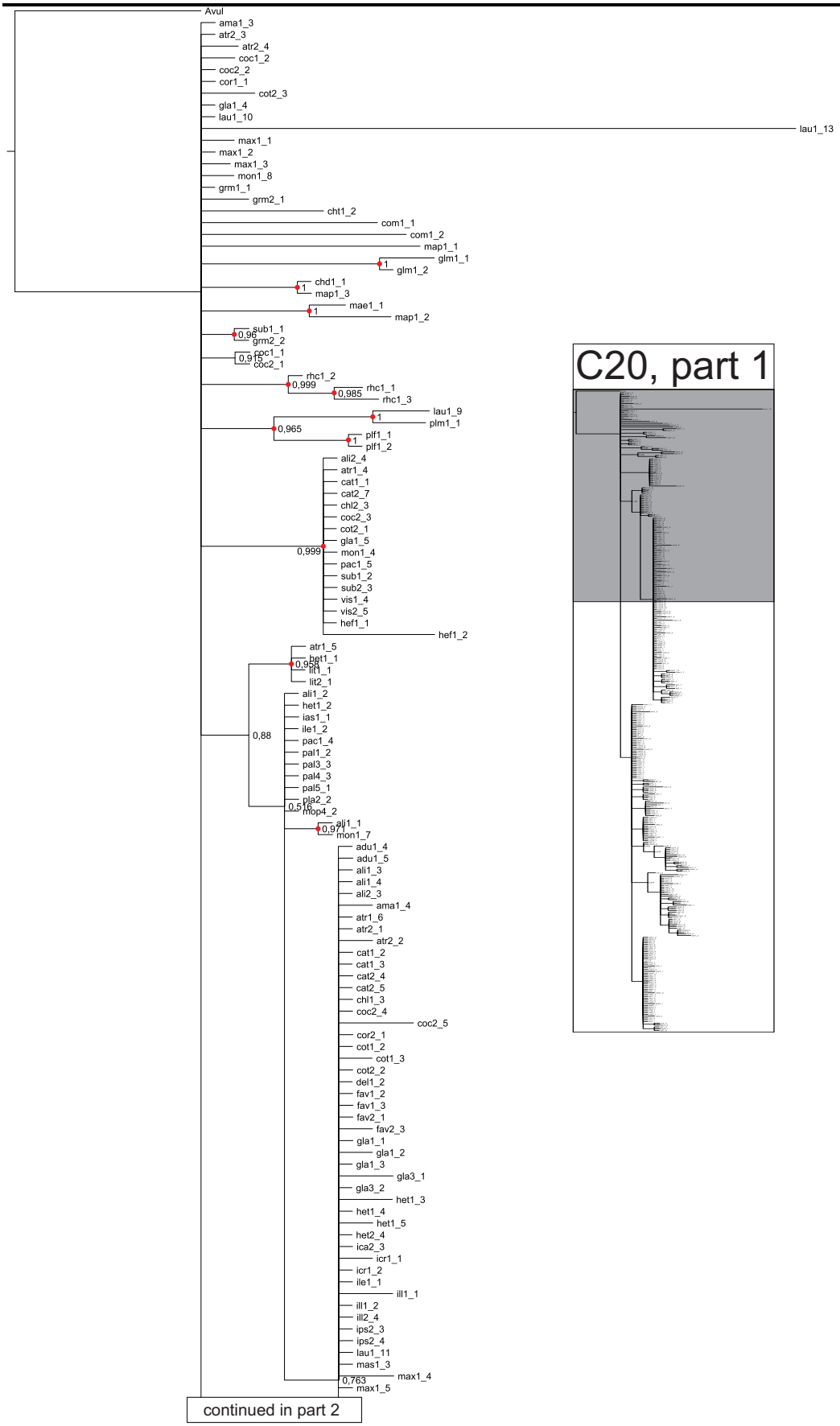

Figure 41 - Gene tree for marker C20 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

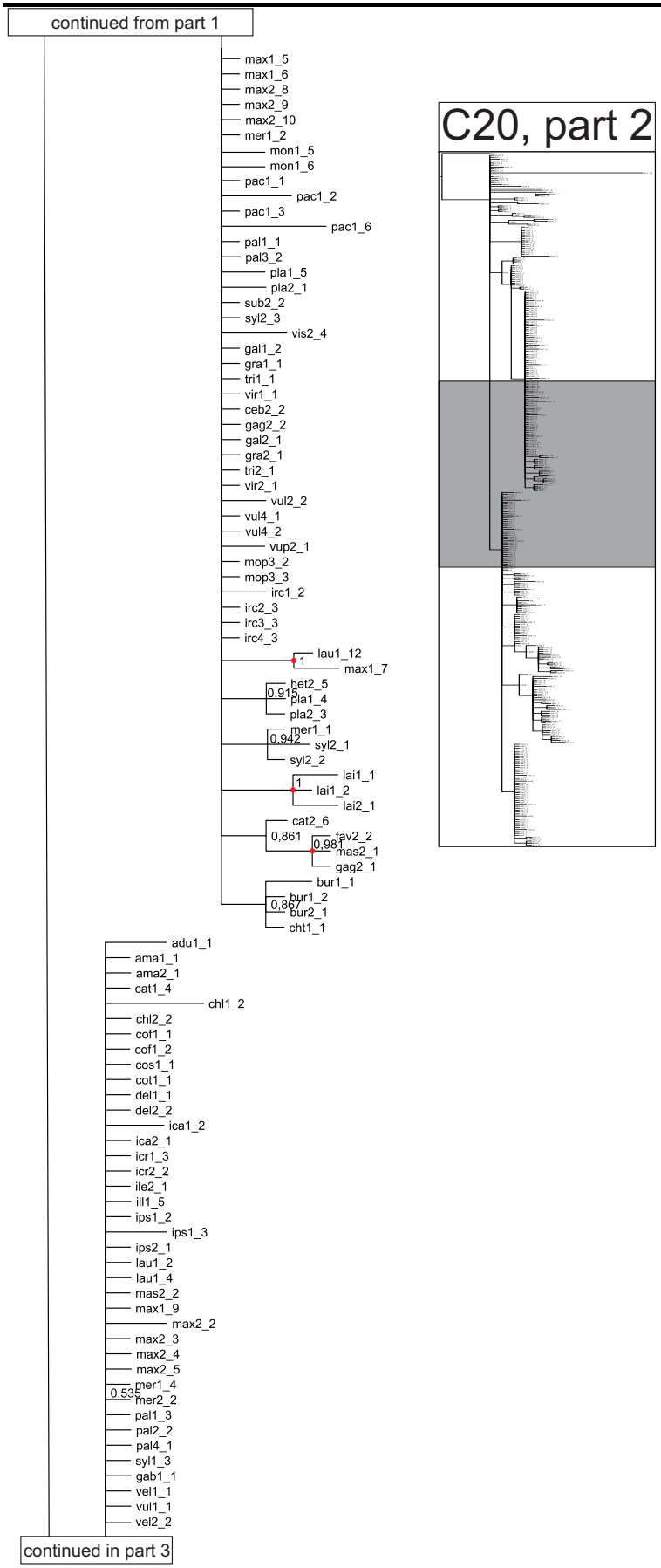

Figure 42 - Gene tree for marker C20 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

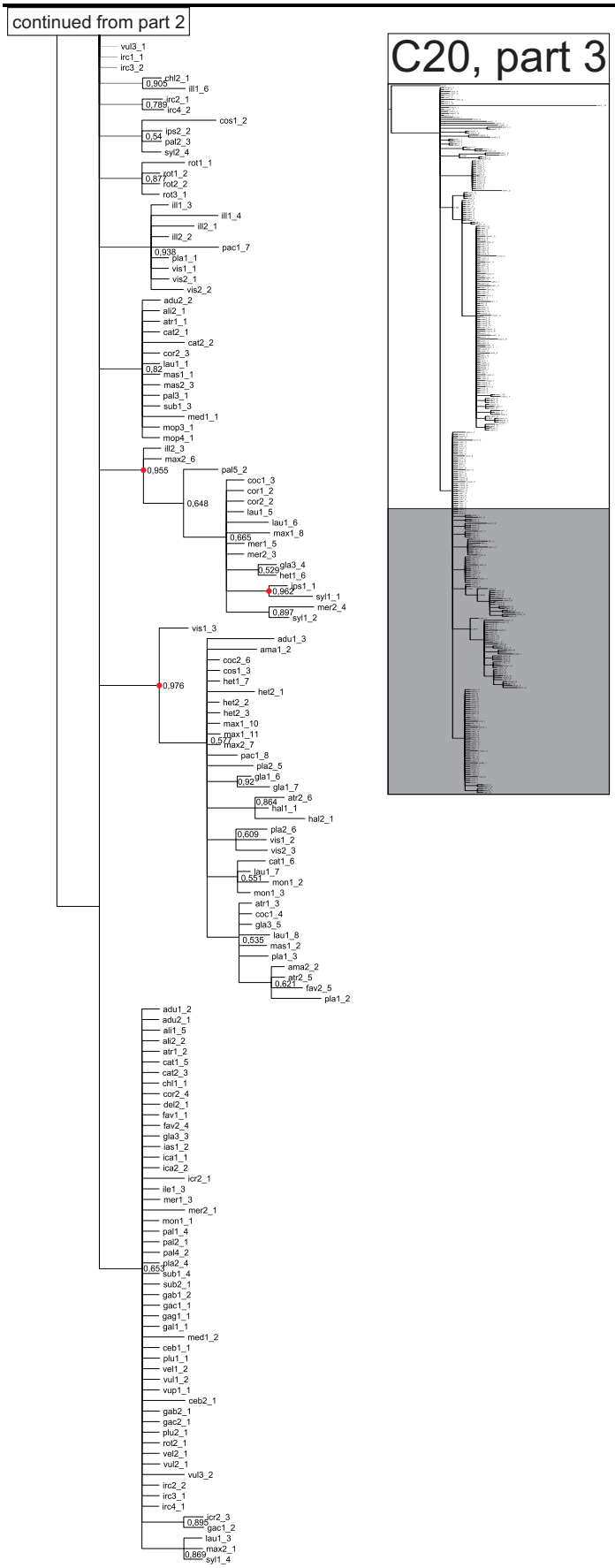

Figure 43 - Gene tree for marker C20 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

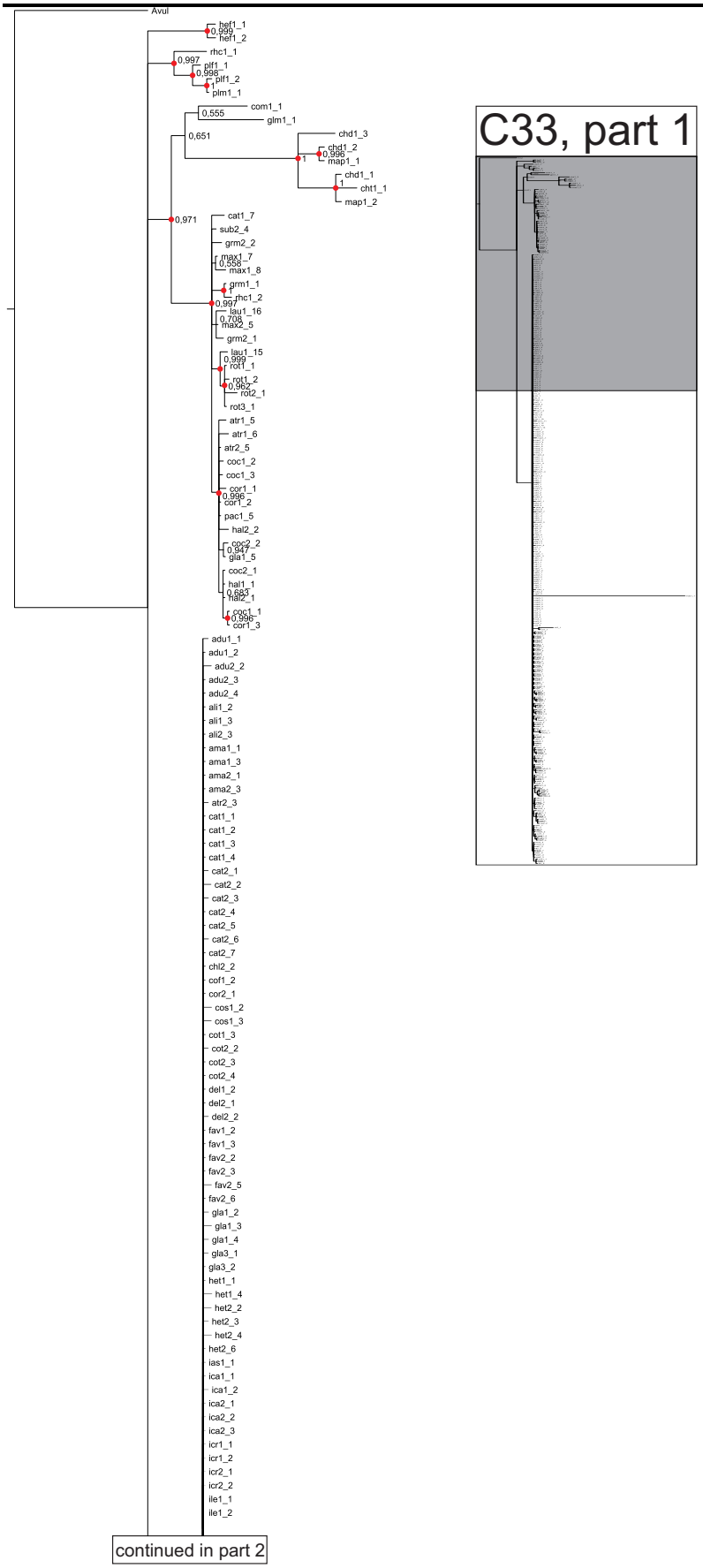

Figure 44 - Gene tree for marker C33 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

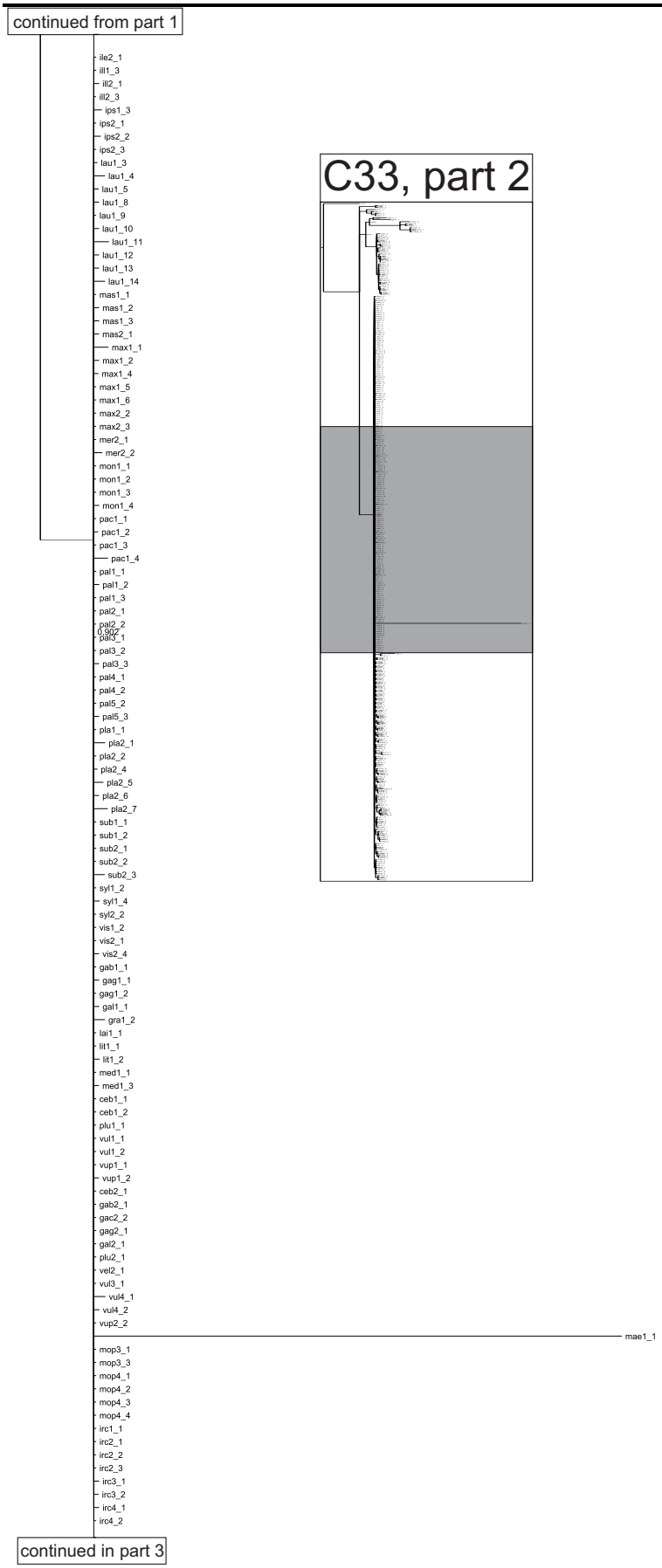

Figure 45 - Gene tree for marker C33 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

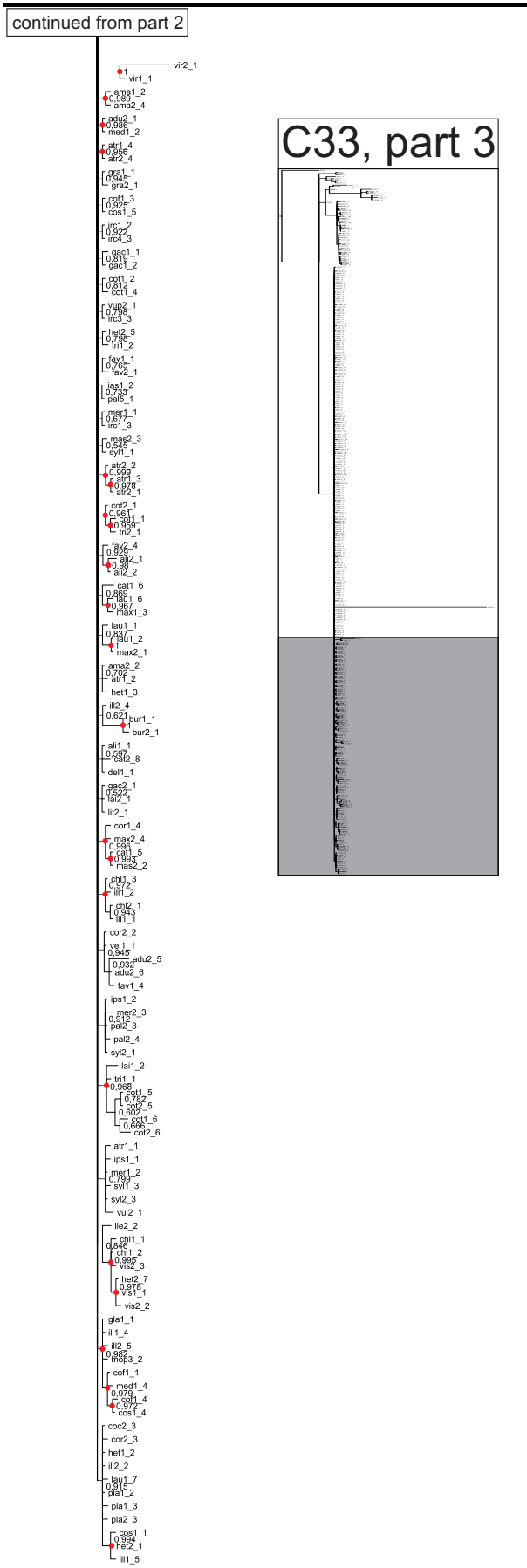

Figure 46 - Gene tree for marker C33 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

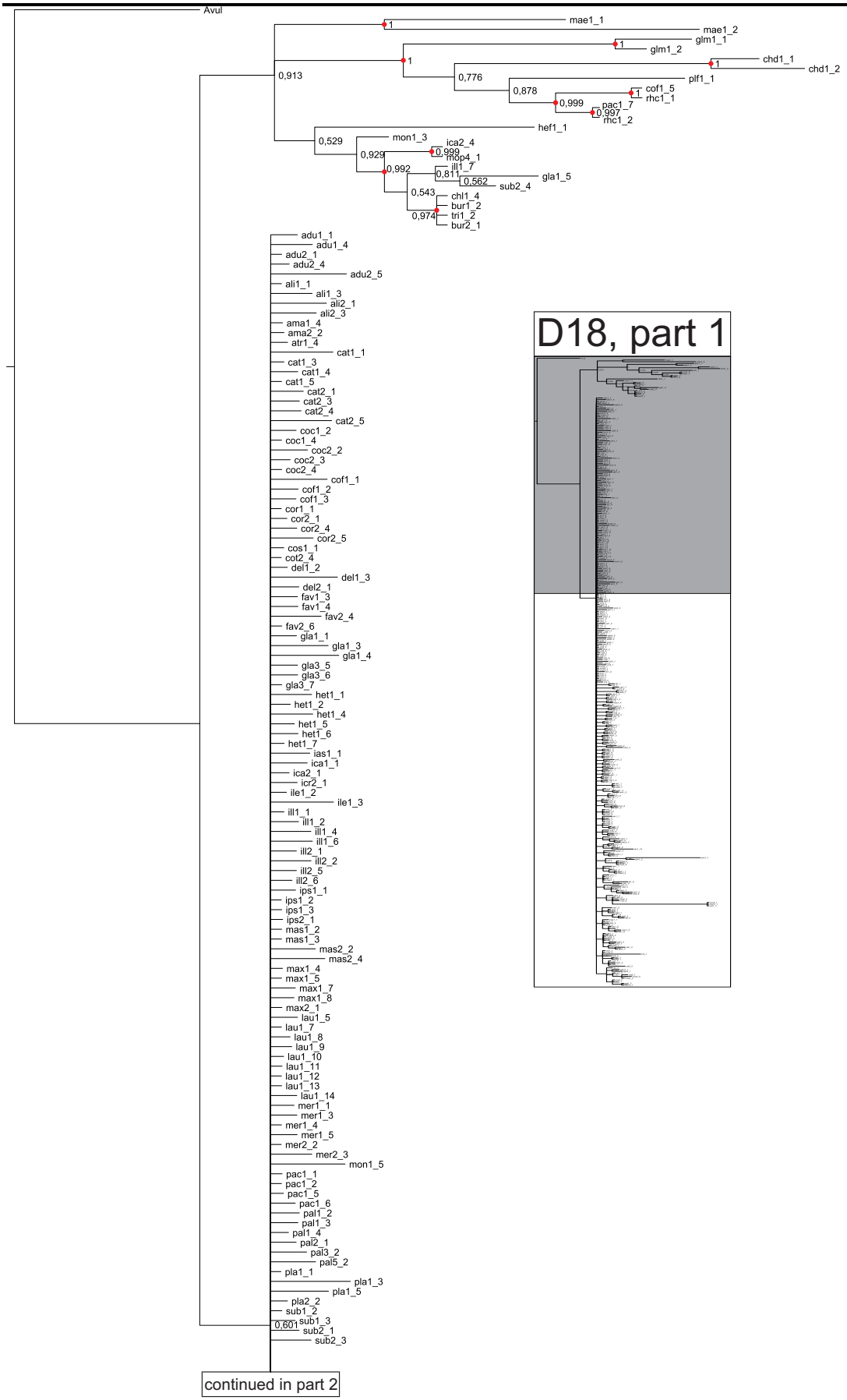

Figure 47 - Gene tree for marker D18 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

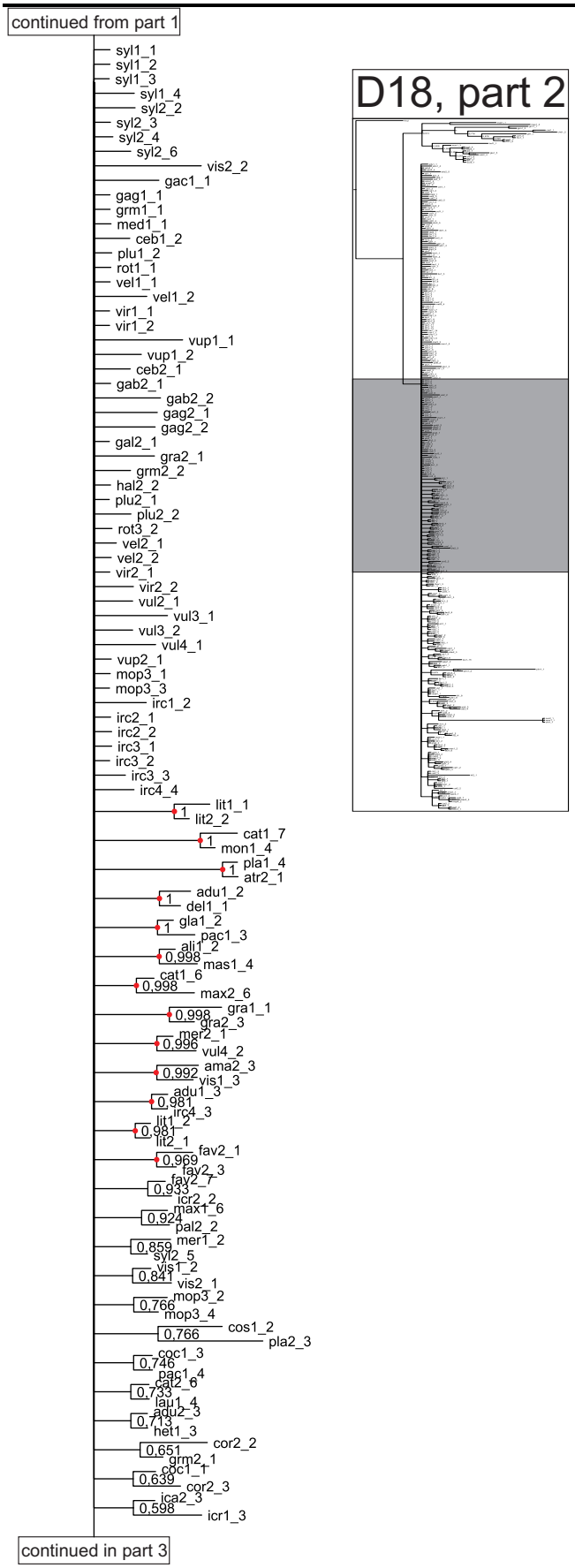

Figure 48 - Gene tree for marker D18 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

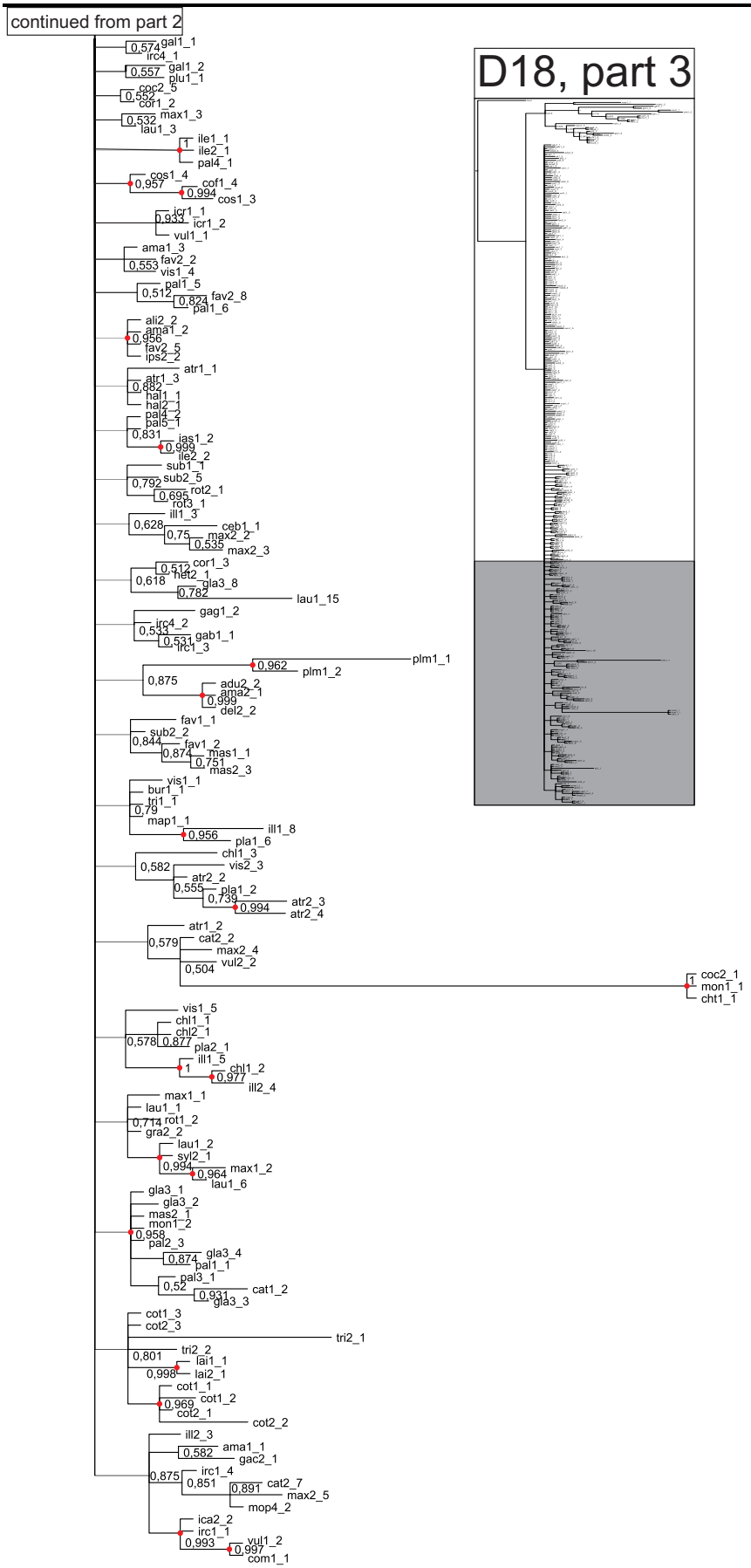

Figure 49 - Gene tree for marker D18 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

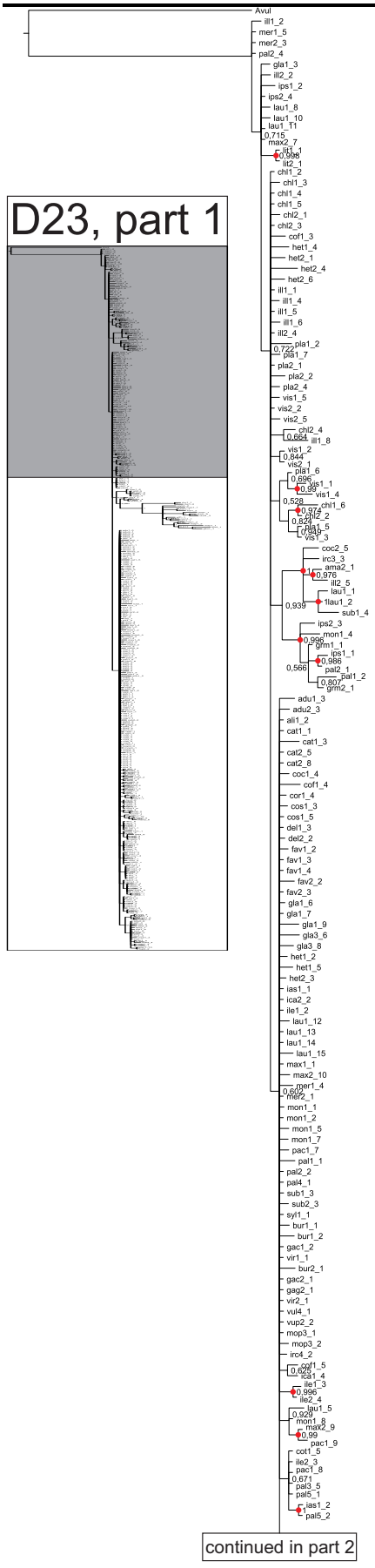

Figure 50 - Gene tree for marker D23 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

Figure 51 - Gene tree for marker D23 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

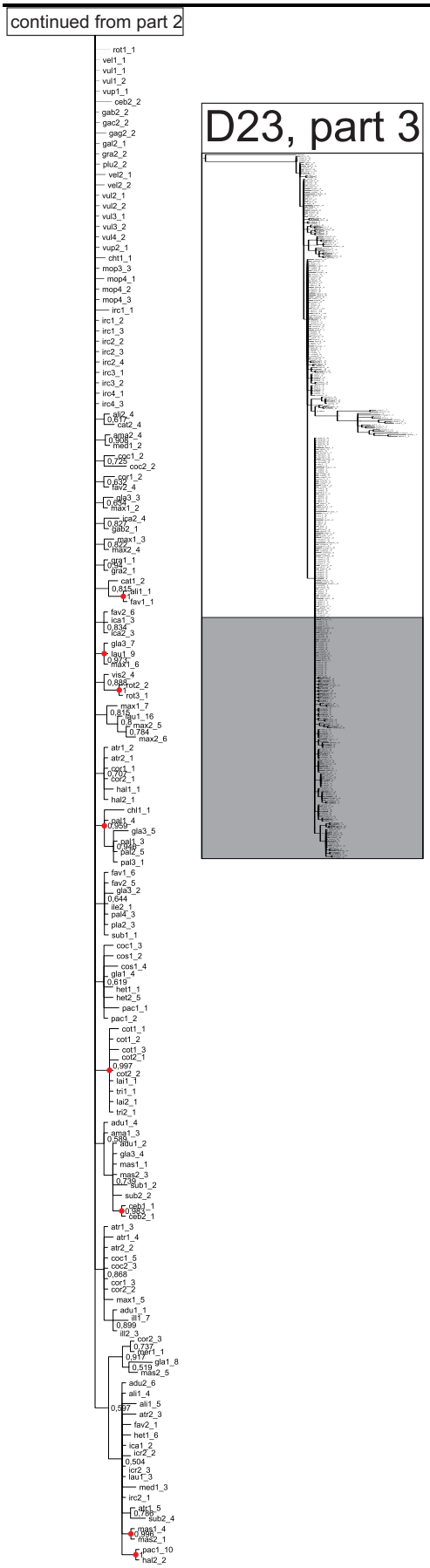

Figure 52 - Gene tree for marker D23 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

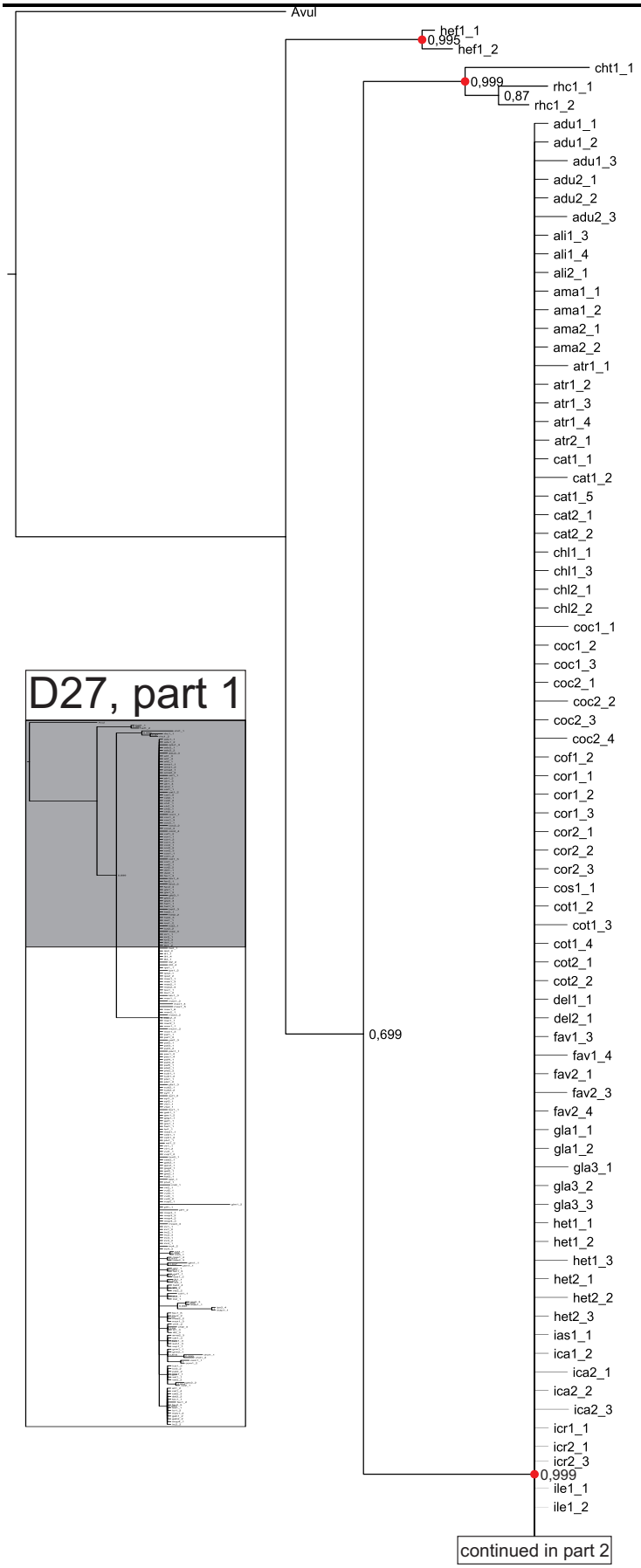

Figure 53 - Gene tree for marker D27 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 1)

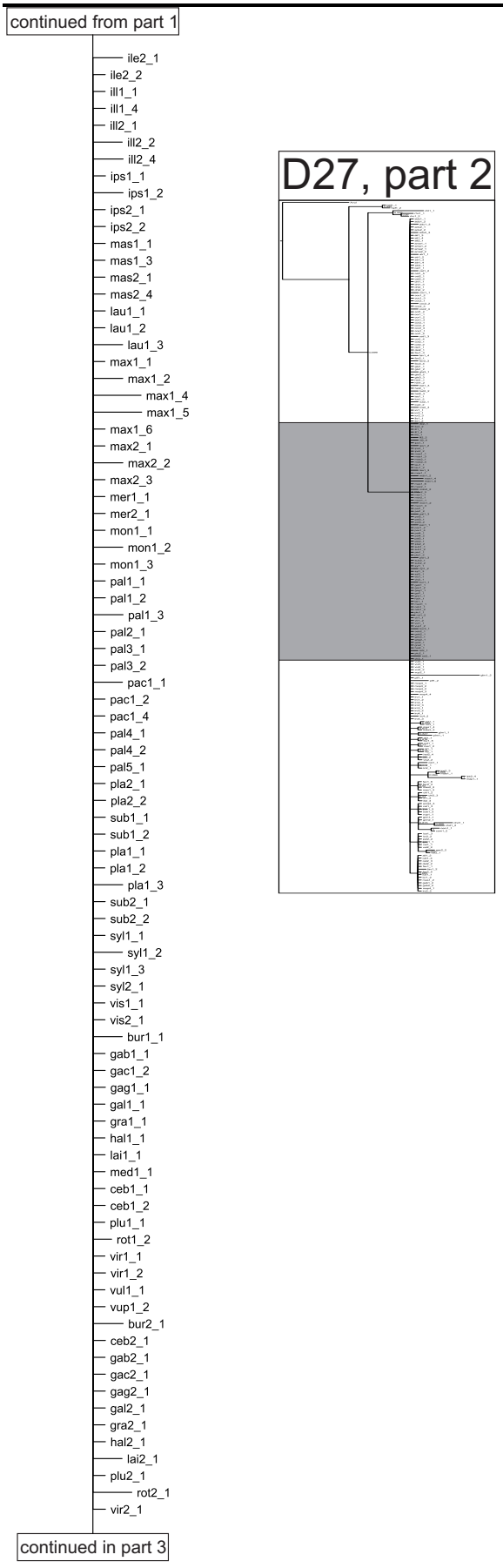

Figure 54 - Gene tree for marker D27 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 2)

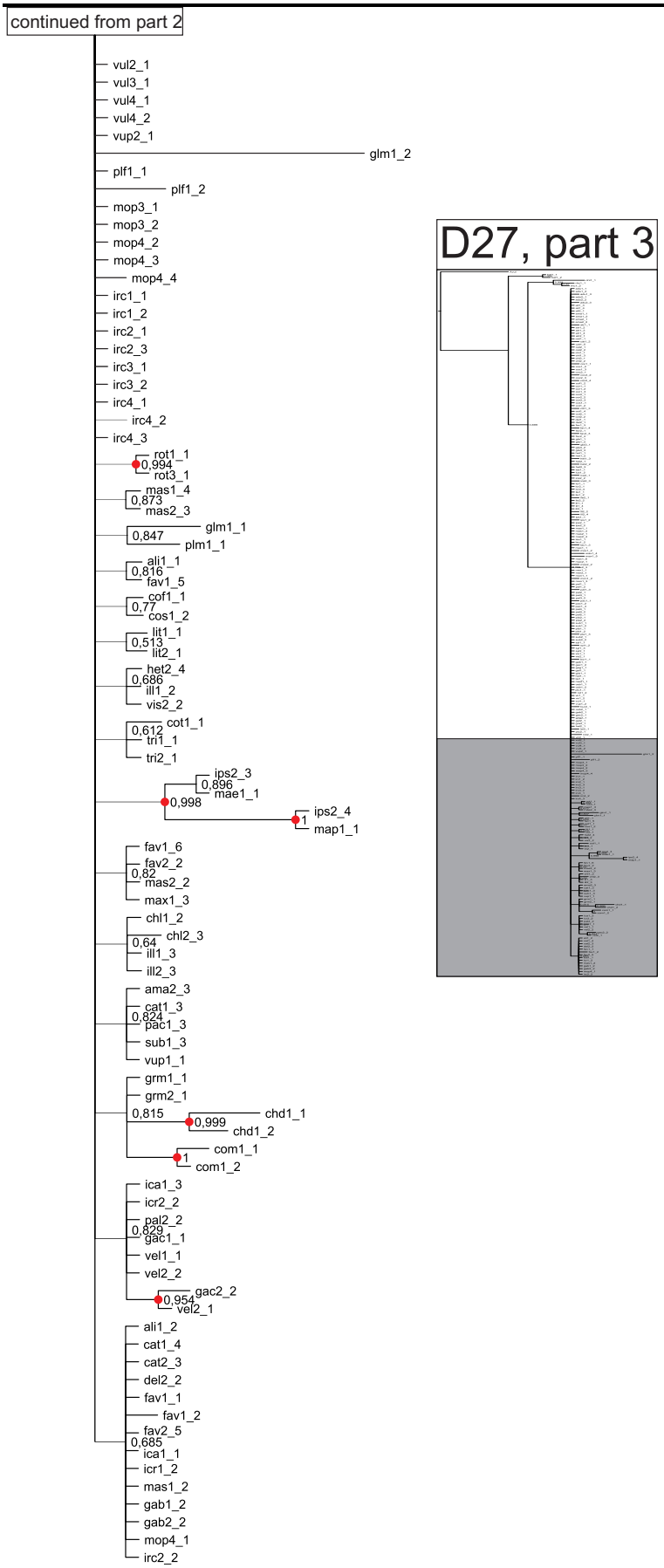

Figure 55 - Gene tree for marker D27 containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots. (part 3)

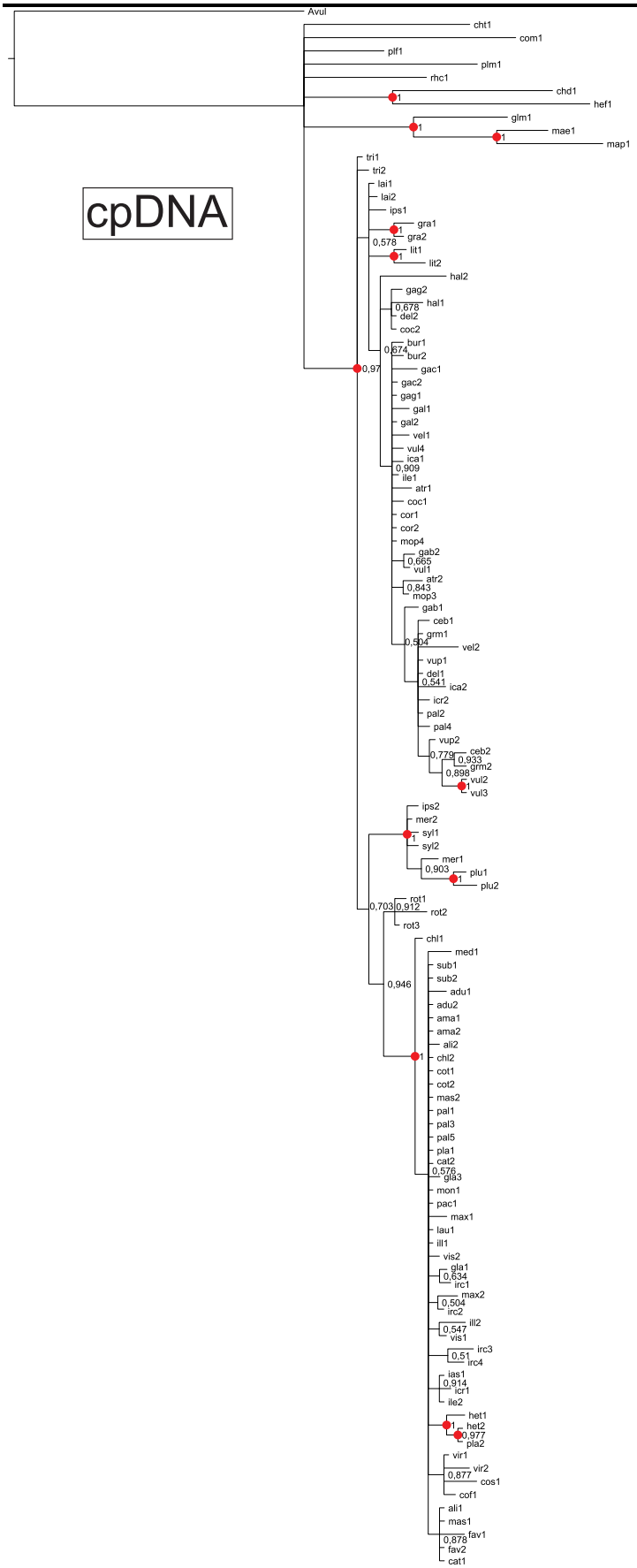

Figure 56 - Gene tree for three cpDNA markers containing diploid and polyploid *Leucanthemum* taxa, and the outgroups. Nodes with posterior probability support above 0.95 a markerd with red dots.

10. APPENDIX C

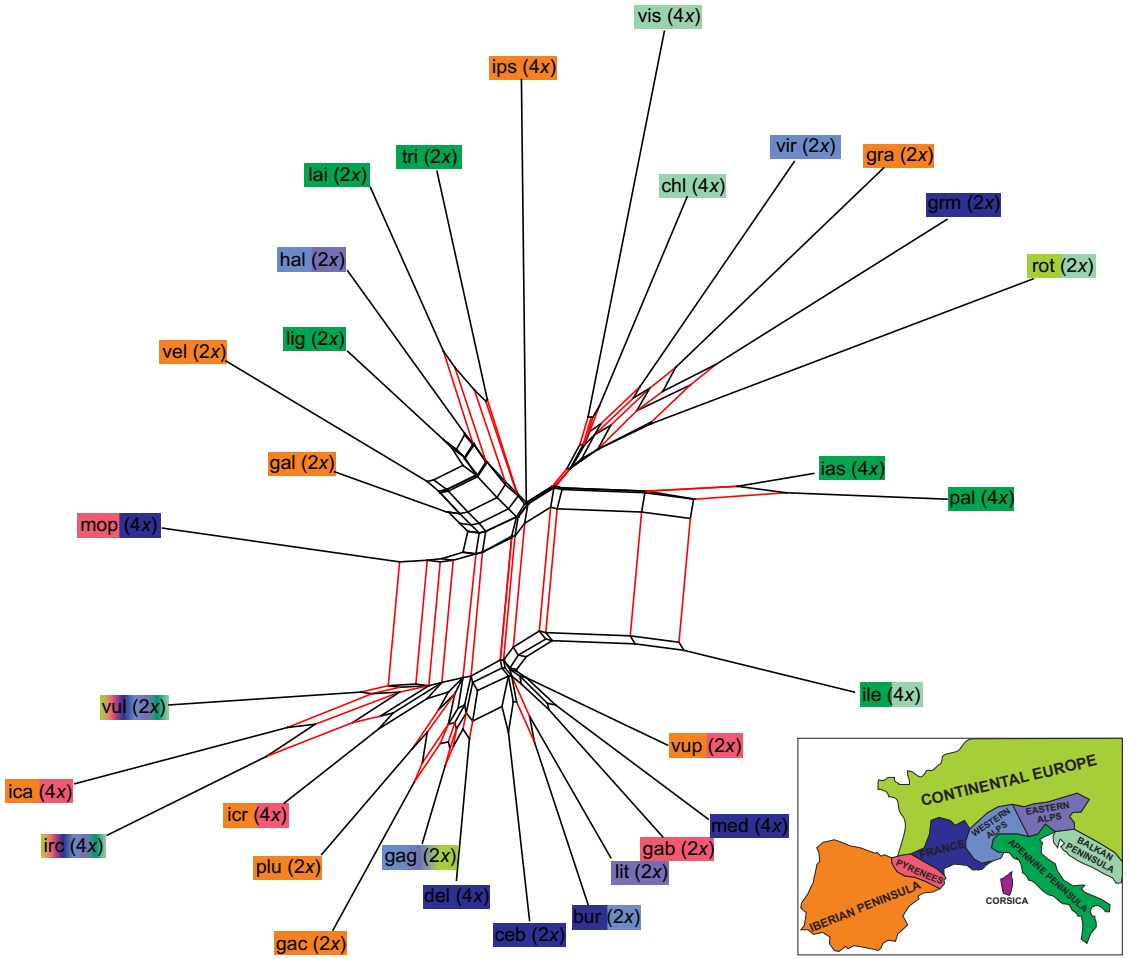

Figure 57 - Supernetwork as obtained from low copy nuclear genes and cpDNA including only *Leucanthemum* taxa on diploid (2*x*) and tetraploid (4*x*) levels. The red lines belong to the splits with bootstrap support greater than 50. Taxon shortcuts are explained in the corresponding chapter. Each name is followed by ploidy level in brackets. Labels are coloured according to their geographical origin (colours are explained in the inset).

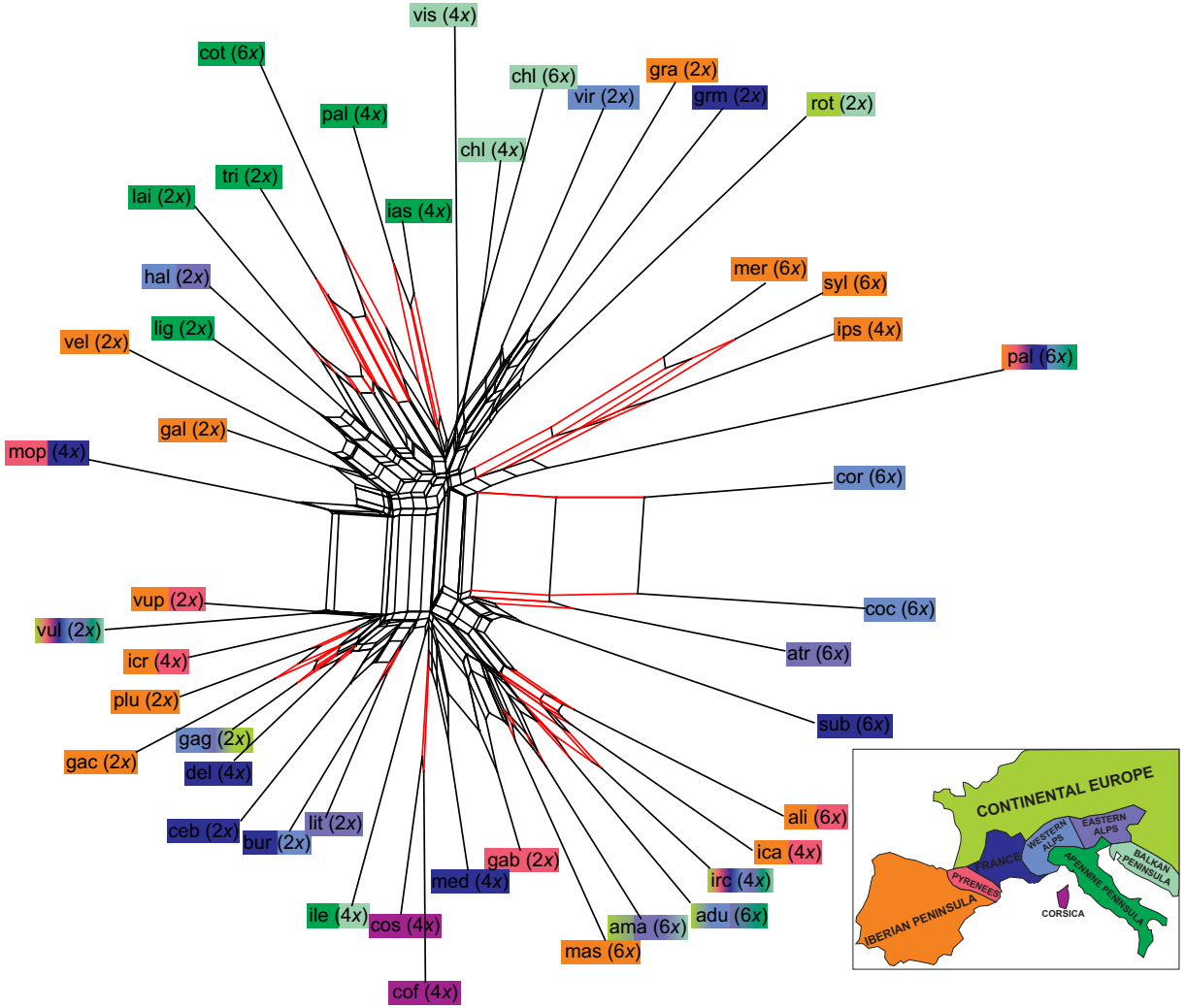

Figure 58 - Supernetwork as obtained from low copy nuclear genes and cpDNA including only *Leucanthemum* taxa on diploid (2*x*), tetraploid (4*x*), and hexaploid (6*x*) levels. The red lines belong to the splits with bootstrap support greater than 50. Taxon shortcuts are explained in the corresponding chapter (2). Each name is followed by ploidy level in brackets. Labels are coloured according to their geographical origin (colours are explained in the inset).

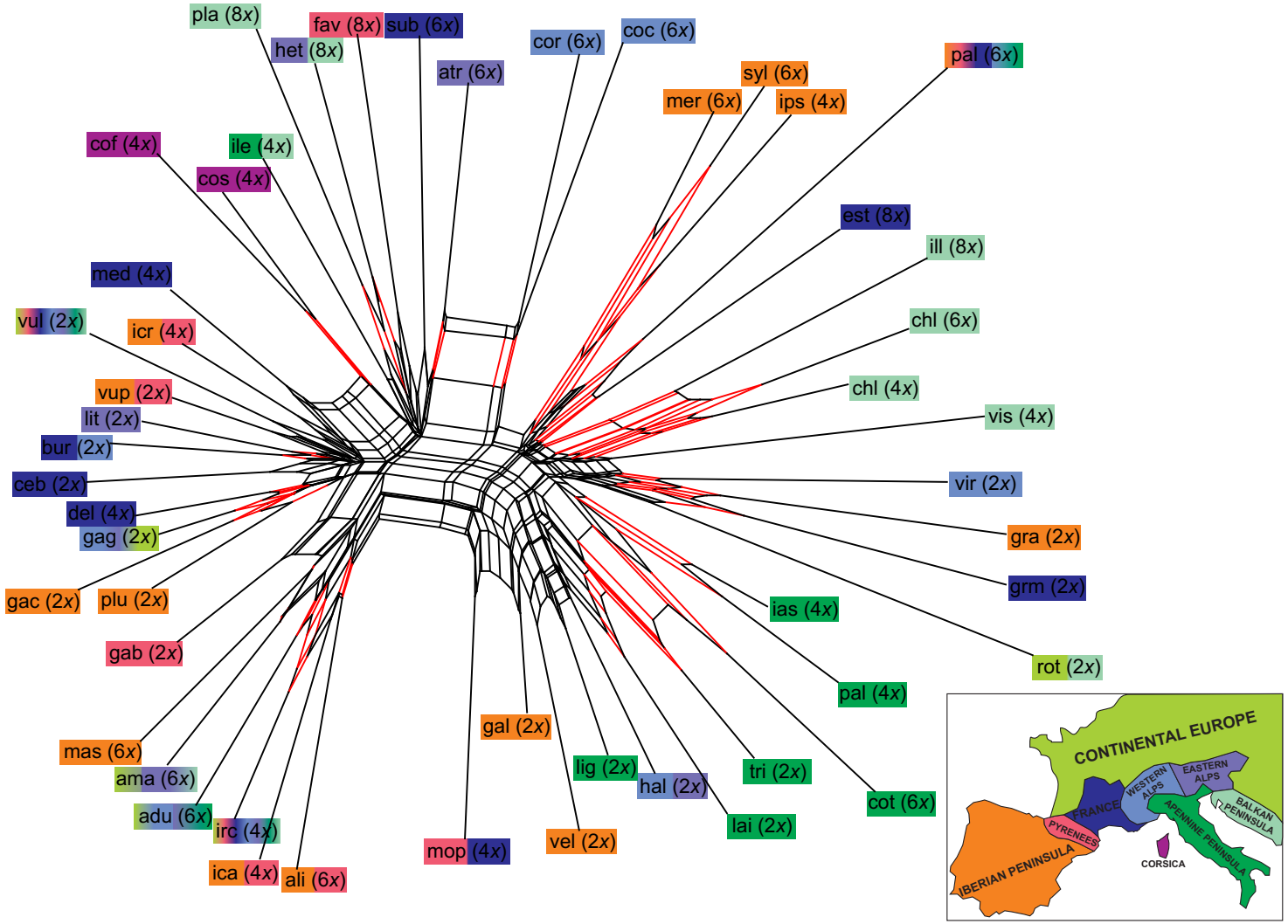

Figure 59 - Supernetwork as obtained from low copy nuclear genes and cpDNA including only *Leucanthemum* taxa on diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*), and octoploid (8*x*) levels. The red lines belong to the splits with bootstrap support greater than 50. Taxon shortcuts are explained in the corresponding chapter (2). Each name is followed by ploidy level in brackets. Labels are coloured according to their geographical origin (colours are explained in the inset).

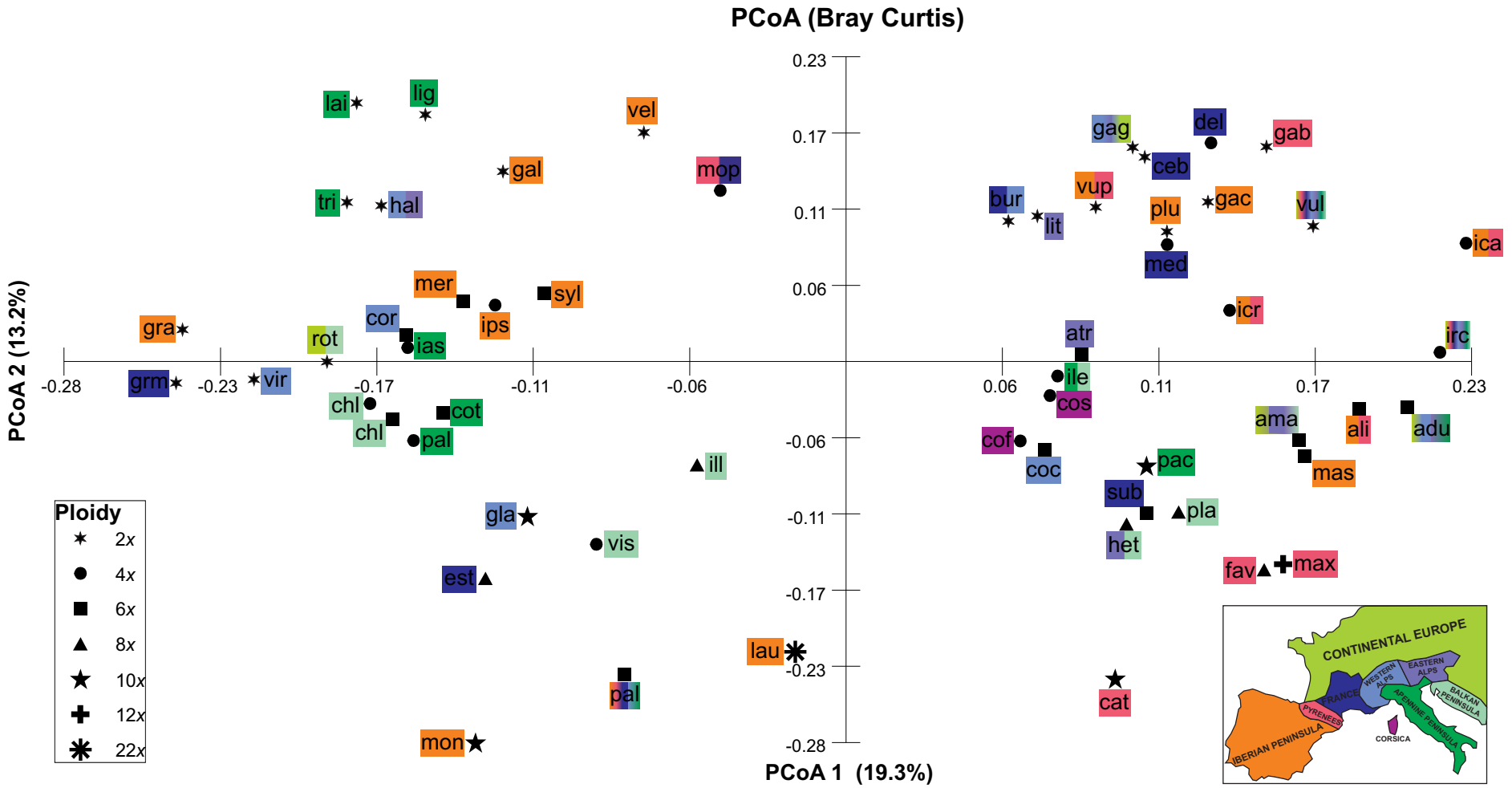

Figure 60 – Principal coordinates analysis (PCoA) graph using Bray-Curtis using dissimilarity on the reduced 0/1 matrix from Chapter 2. The first axis explain 19.3% of variation and the second axis explain 13.2% of variation observable within the dataset. Taxon shortcuts are explained in the corresponding chapter (2). Each taxon is represented with its ploidy symbol explained in the inset on the left side. Labels are coloured according to their geographical origin (colours are explained in the inset on the right side).

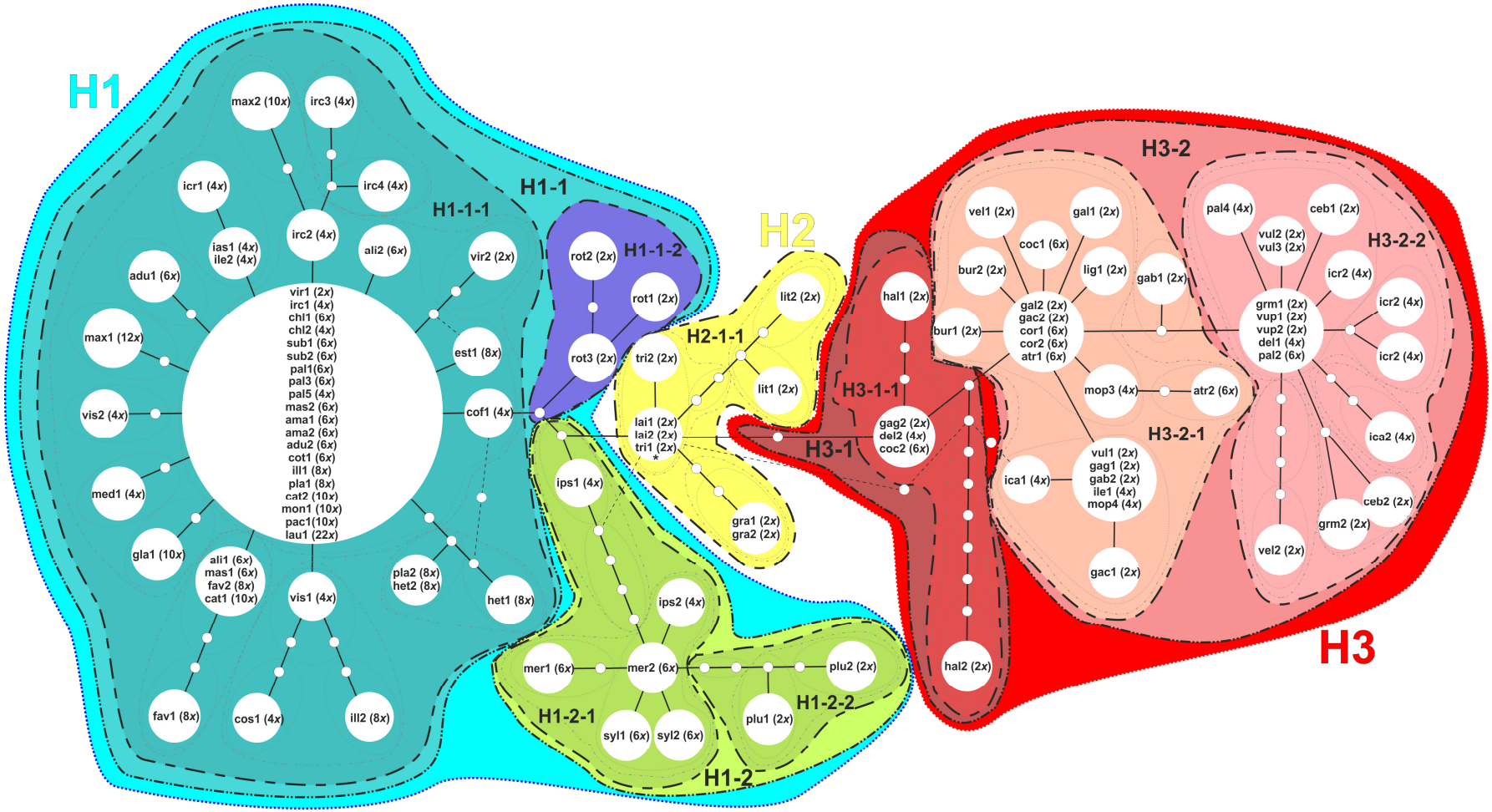

Figure 61 – Chloroplast haplotype network containing accessions sampled in the Chapter 2. Empty circles represent missing haplotypes, and in case when the connection between two haplotypes was equally parsimonious, two lines are shown where the dashed one is regarded as less likely. Each name is followed by ploidy in brackets and taxon shortcuts are explained in the corresponding chapter (2). Grouping was made manually according to the nesting rules of nested clade analysis (Templeton et al. 1987, Templeton & Sing 1993, cf. Chapter 3). The differentiation of outgroup haplotypes excessed 95% connection limit and their exact placement could not be estimated (~19 mutational steps), but based on the tree obtained with Bayesian interference (cf. Figure 56), the most probable connection with the outgroup would occur in the haplogroup H2-1-1 marked with "*".

11. APPENDIX D

11.1. Protocol of data analysis for 454 sequencing

Part I: Wet lab protocol.

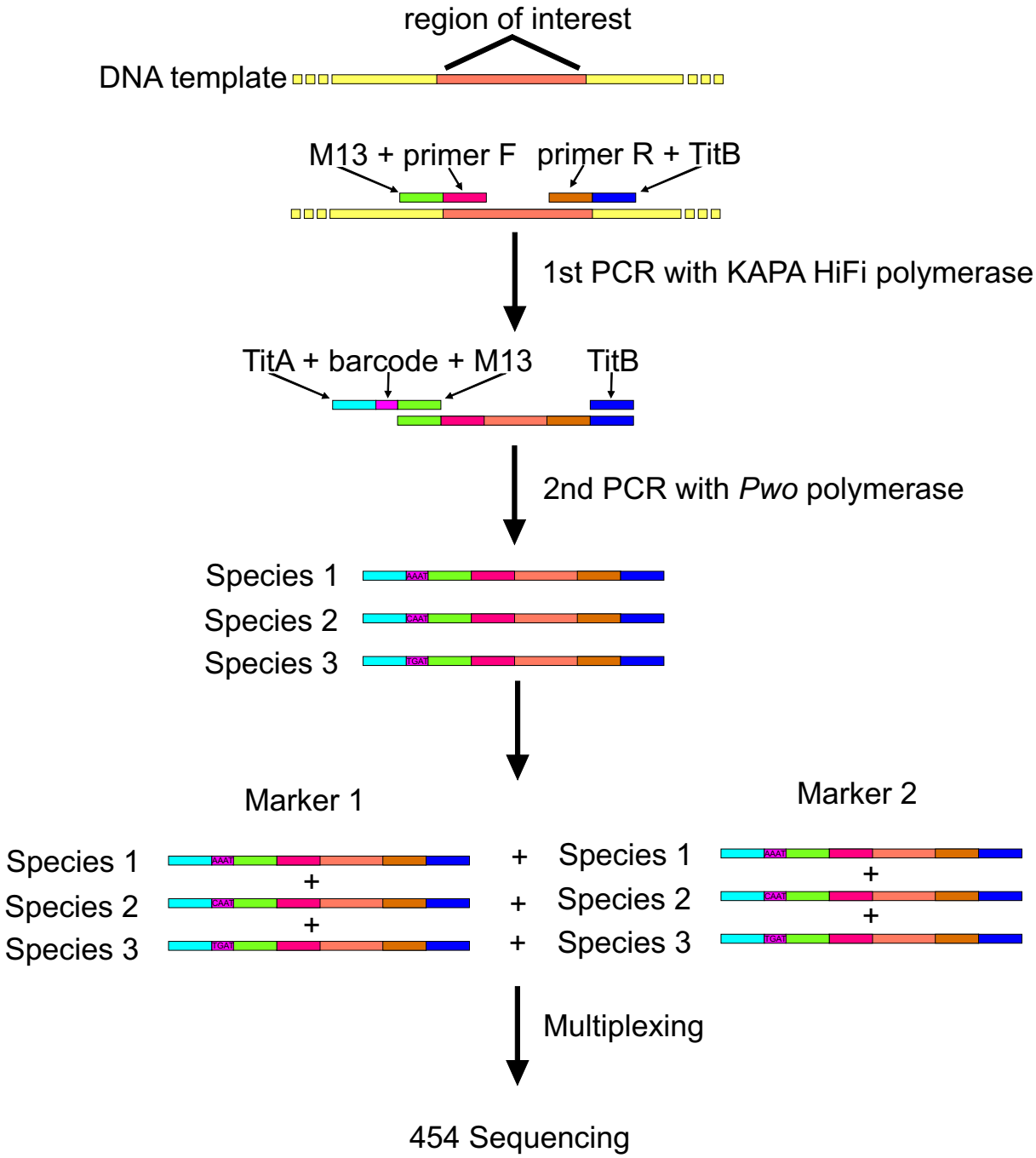

Figure 62 - Schema for the wet lab protocol..

Part II: Putting sequences and names together.

Recommended paper: *Griffin, P., Robin, C., Hoffmann, A., Mar. 2011. A next-generation sequencing method for overcoming the multiple gene copy problem in polyploid phylogenetics, applied to Poa grasses. BMC Biology 9 (1), 19+. URL http://dx.doi.org/10.1186/1741-7007-9-19*

Galaxy

1. From NGS company you should get three files: nnn.fna, nnn.qual, nnn.sff. The first one is FASTA file and the second one is quality file. You need to upload these two files to galaxy (http://main.g2.bx.psu.edu/). The third one is original 454 file which is not useful in our case.
2. Combine FASTA and quality file into FASTQ
   Tool: Combine FASTA and QUAL (Format: fastqsanger; Force Quality Score encoding: Use Source Encoding)
3. Convert FASTQ to Tabular (Tool: FASTQ to Tabular)
4. Add a column with number of the sequence to tabular file
   Tool: Add column (Format: tabular; Input Parameter: Value; Add this value: 1; Iterate?: YES)
5. Keep this file you will need it later. Now save it also on your local computer.

R & Excel (This section uses a method described by Griffin et al. 2011 check this paper and supplementary material 4 for details!)

6. Create a file where name of each species and sequences of each forward primer are matched. It should look like that:

Name	Seq
Bur_A39	(AAAGCACGACGTTGTAAAACGACAATGGTGTTTCAATTGGTTTTC)
Bur_B12	(AAAGCACGACGTTGTAAAACGACCAAGTGGCTGCAGCCATGGG)
Mop_A39	(AATGCACGACGTTGTAAAACGACAATGGTGTTTCAATTGGTTTTC)
Mop_B12	(AATGCACGACGTTGTAAAACGACCAAGTGGCTGCAGCCATGGG)

In the first column bur and mop indicate species accession, and A39 and B12 indicate marker. In the second one blue base pairs are species specific barcode, followed by M13 sequence, and finally by forward primer sequence. It is not necessary that they have the same length. In fact if your primer contained a wobble base pair it is not possible to include whole sequence here and the sequence should cut before it. This file should be saved in coma delimited .csv format and named IDs.csv

7. The other file you need is the one containing numbered sequences created in step 5. It should look like that:

No	Seq
1	ACTGCACGACGTTGTAAACGACAGTGGTATTAGTGGGCTTCTTGTTCGTGGTGG
2	AGGACGACGTTGTAAACGACGGAAGGCTATTGCAGGGAGCAATTGATTGTTGCAG

Export it to .csv format with the name Readnos.csv

8. Load both files to R.

```
readnos<-read.csv("Readnos.csv")
ids<-read.csv("IDs.csv")
```

9. And use the code:

```
sink(file="C://Matches.csv", append=TRUE)
for(i in c(1:171)){
posmatch<-c(grep(pattern=ids$Seq[i], x=readnos$Seq))
howlong<-ifelse(length(posmatch)>=1, length(posmatch), 1)
posmatch2<-rep(x=ifelse(length(posmatch)>=1,        "some",
"none"), each=howlong)
posmatch3<-ifelse(posmatch2>1, posmatch, posmatch2)
tempdata<-data.frame(posmatch3, ids$Name[i])
print(tempdata)
}
sink()
```

171 in this case is representing the number of species x marker combinations

10. File Matches.csv will be created. Open it in excel and delete first column containing number created by R. The two remaining columns will contain: sequence number and species & marker specific ID. Delete headers leaving two columns and save it in tab-delimited format. Load this file into galaxy.

Galaxy

11. Assign names to sequences using file from step 5 and 10.

Tool: Compare two Datasets (Format: tabular; Input Parameter: Value; Compare: numbered sequences; Using column: 1 (number); against: numbers matched to ID; and: column 1 (number); To find: Matching rows of 1st dataset)

* If you somehow worked separately with sequences and quality scores you should repeat all above steps for both of them and then unite quality and sequences into one file. It shouldn't be a problem until here since the order wasn't changed.

Excel

12. From previously created file extract species name and marker name into two separate columns.

13. Sort sequences alphabetically first by marker and then by species

14. Now this manual is considering working only at one marker at a time. You can simply select marker and all species in excel, copy it into new file and save as tab tab-delimited file (remember about keeping name, sequence and quality). Each of the following steps is then repeated for the each marker separately.

Galaxy

15. Import file with first marker. Here following schema is applied: first column is the name of the sequence, second column is the sequence and third column is quality score

16. Delete barcode, M13 and whole primer sequence (e.g. 4 bp barcode – 19 bp M13 – 23 bp primerF = 46 bp)

Tool: Trim (Trim this column only: 2; Trim from the beginning to this position: 46; Is input dataset in fastq format?: No)

17. Now the same step needs to be applied to the quality scores. The difference is that difference score consist of 2 numbers which are separated by 1 space. So to delete corresponding number of quality scores previous number have to be multiplied by 3 and from the final result 2 needs to be subtracted (e.g. (46 x 3)-2=136)

Tool: Trim (Trim this column only: 3; Trim from the beginning to this position: 136; Is input dataset in fastq format?: No)

18. Convert tabular to FASTQ

Tool: Tabular to FASTQ (Identifier column: 1; Sequence column: 2; Quality column: 3)

19. Apply FASTQ Groomer tool to make FASTQ file you created readable by the service
Tool: FASTQ Groomer (Input FASTQ quality scores type: Sanger)
* optionally here you can check quality visually with fastqc (Tool: Fastqc: Fastqc QC)

20. Remove reads with low quality. Here arbitrary we are removing all sequences which have quality below 20 in more than 80% of the read
Tool: Filter by quality (Quality cut-off value: 20; Percent of bases in sequence that must have quality equal to / higher than cut-off value: 80)

21. Convert FASTQ to FASTA (Tool: FASTQ to FASTA) and save in your local computer. Go back to step 15 and proceed with the next marker.

Mafft & BioEdit

* Since in this type of study length of the sequence may vary with the species and the marker is wasn't possible to remove reverse primer previously with the same tool as forward primer. In the end sequences are also sometimes difficult to analyze and have worst quality. Additionally we used wobble base pairs so it wasn't possible to exclude reverse primer with tool in galaxy designed for that purpose. Therefore the method applied here may seem to be a little bit coming around.

22. Align sequences with MAFFT. Default options are usually sufficient and fast.

23. Open file in Bioedit. Go to the end and locate reverse primer (in some sequences Titanium B adaptor may be present). Check whether primer is aligned correctly in all sequences (sometimes mafft is putting gaps or moving part of the primer in some). If it is correct select all columns and remove them.

24. Degap the alignment. (Sequence→Gaps→Degap)

25. Now the analysis of number of alleles per species and creating consensus sequences may start. Select all sequences belonging to one species, copy them to new file.

26. Align file with mafft.

27. Open in BioEdit. If the is a gap in the beginning of any sequence it should be removed. Check the alignment whether is properly done. Now you can do the analysis in BAPS, following steps will describe this procedure. If you don't want to use it proceed to step 37.

28. Gaps should be saved as "-" not "~" (lock the gaps). Export to tab delimited file.

29. Open file in excel. Insert column on the left side and number the sequences. The name of the column 1 should be ST and the other one e.g. gene1

ST	gene1
1	Ccccttcgcaaaggtcatggatgaggaattcggtaagtatatttttgtcc-tttgtc
2	Ccccttcgcaaaggtcatggatgaggaattcggtaagtatatttttgtcc-tttgtc

Save in excel format (.xls)

30. Open BAPS. Select: clustering with linked loci

31. Select MLST-format

32. Select concatenate allelic sequences (EXCEL) and open your file

33. Specify the linkage model (linear or codon)

34. Upper bound may be as high as the number of sequences, BAPS will lower the level very quickly.

35. You will see the window with sequences coloured according to the group they belong. You may save the data for the admixture analysis and use them later to check for the recombination.
*Check result window (log(ml), probability for number of clusters). Sometimes even if you have one allele BAPS will force bigger number of alleles if some variation is present in the dataset. This variation is mostly coming from the sequencing errors, such as missing base pair or additional base pair. Such results should be interpreted with caution. While BAPS analysis is applied here to make judgement more objective it is not always reliable.

36. Open your sequence in BioEdit and group them according to the BAPS results.

37. For reach allele create consensus sequence (Alignment→Create consensus sequence) here we adopt arbitrary rule that if sequence is not present in a group of sequences more than 20% it should be discarded, conversely if there a variation higher than 20% is counted as an allele or allele variant
*before analysis go to options→preferences→Consensus and set the threshold frequency to 80%

38. Each consensus sequence is exported into a new alignment. After scoring all variants for all species alignment after aligning (mafft + manual checking) is ready for the analysis.

12. APPENDIX E

12.1. Protocol for flow cytometry measurement

Recommended paper: *Estimation of nuclear DNA content in plants using flow cytometry; Dolezel J., Greilhuber J. & Suda J. in Nature protocols vol. 2 no. 9 pp. 2233-2244, 2007*

For a detailed measurement of DNA content in a population you need 5 specimens and each specimen should provide 2 samples alternatively 3 accessions per species each analyzed 2-3 times (publication purpose). Otherwise (e.g. ploidy determination) you can take less samples (even 1 or 2 per population).
For a total DNA content you can use ethydium bromide (EtBr) or propidium iodide (PI).
For a ploidy and dried samples analysis you may prefer a DAPI staining (measurement is less affected by operator, staining time etc. and it may have higher peaks in dry material).
Solutions:
Buffers (it is recommended to make 500 ml or 250 ml of a buffer, they shouldn't be stored too long). All buffers should be filtered through the 0.22-µm filter after preparation! The pH value should be around 7-7.5.

LB01 (modified)

	for 1 l	for 0.5 l	for 0.25 l	for 0.1 l
15 mM Tris	1.8171 g/l	0.9086 g/0.5l	0.4543 g/250ml	0.1817 g/0.1l
2 mM Na_2EDTA	0.7446 g/l	0.3723 g/0.5l	0.1862 g/250ml	0.0745 g/0.1l
0.5 mM Spermine-HCL	0.1741 g/l	0.0871 g/0.5l	0.0435 g/250ml	0.0174 g/0.1l
80 mM KCL	5.9680 g/l	2.9840 g/0.5l	1.4920 g/250ml	0.5968 g/0.1l
20 mM NaCl	1.1688 g/l	0.5844 g/0.5l	0.2922 g/250ml	0.1169 g/0.1l
6 mM Dithiothreitol (DTT)	0.9258 g/l	0.4629 g/0.5l	0.2315 g/250ml	0.0926 g/0.1l
0.1% (v/v) Triton X-100	1 ml/l	0.500 ml/0.5l	0.250 ml/250ml	0.1 ml g/0.1l
1% (w/v) PVP 40	1.2 g/l	0.6000 g/0.5l	0.3000 g/250ml	0.12 g/0.1l
fluorochrome optional:				
(4 mg/l DAPI)	*160 µl*[*]	*80 µl*[*]	*40 µl*[*]	*16 µl*[*]
(60 mg/l EtBr)	*6 ml*[**]	*3 ml*[**]	*1.5 ml*[**]	*0.6 µl*[*]

(store at -20°C or +4°C, in case when fluorochrome is added the solution should be stored in darkness in glass prohibiting the access of the light e.g. covered with aluminium foil)
[*] *values for DAPI are given for stock solution 25 mg/ml*
[**] *values for EtBr are given for stock solution 10 mg/ml*

Otto I

	for 1 l	for 0.5 l	for 0.25 l	for 0.1 l
0.1 M citric acid	19.213 g/l	9.6065 g/0.5l	4.8033 g/250ml	1.921 g/0.1l
0.5% v/v Tween 20	5 ml/l	2.5 ml/0.5l	1.250 ml/250ml	0.5 ml/0.1l

(store at +4°C)

Otto II

	for 1 l	for 0.5 l	for 0.25 l	for 0.1 l
0.4 M $Na_2HPO_4 \cdot 12H_2O$	143.256 g/l	71.628 g/0.5l	35.814 g/250ml	14.3256g
fluorochrome optional:				
(4 mg/l DAPI)	*160 µl*[*]	*80 µl*[*]	*40 µl*[*]	*16 µl*[*]
(60 mg/l EtBr)	*6 ml*[**]	*3 ml*[**]	*1.5 ml*[**]	*0.6 µl*[*]

(store at room temperature! In fridge the salt will precipitate. In case when fluorochrome is added the solution should be stored in darkness in glass prohibiting the access of the light e.g. covered with aluminium foil)

[*] *values for DAPI are given for stock solution 25 mg/ml*

[**] *values for EtBr are given for stock solution 10 mg/ml*

Two step protocol for fresh samples:

1. Prepare the Petri dishes and from each specimen take two samples and mark them as 1 and 1'.
2. Place a leaf fragment ca. 1-2,5 cm^2 of your sample and leaf fragment from your standard ca. 1 cm^2 on a plastic Petri dish (sample ca. 2 times bigger than a standard, you can also measure up to 50 mm^2)
3. Put 1 ml of Otto I buffer[!!!] on the plate with the leaf fragments. Chop them with razor blade until the buffer turns green and you won't be able to distinguish leaf fragments (but overchopping is dangerous too! Some experience is needed here).
   [!!!] In case of using PI or EtBr you should also add 10 µl of RNAse (100 µg/ml) on this step or after the filtration.
4. Take the buffer from the plate with 1 ml pipette and rinse cut samples again. Afterwards put it through 50 µm filter into new 1.5 ml tube or cytometer tube (don't throw out the filters or Petri dishes). The samples should be kept on ice.
5. Centrifuge your samples for 5 min in 150 G (+/- 1400 rpm) (in room temperature or preferentially in 4°C). After centrifugation put samples on ice again. Several samples may be prepared ahead since the nuclei are stable in Otto I buffer.
6. Prior to analysis remove the Otto I buffer leaving ca. 20-50 µl of it with remaining nuclei. Add Otto II buffer supported with stain (stain may be also added separately). Keep the samples in room temperature for appropriate staining (usually 5-10 min up to 30-60 min is sufficient).
7. First measure the DNA content in your standard. Afterwards proceed with your samples.
8. After the analysis wash the Petri dishes and filters (with water, possibly with help of ethanol).

Two step protocol for silica dried samples:

Note: silica dried samples are more difficult to analyze and sometimes the results could be very bad. General hint in this measurement is usage of samples which were dried within one year (usually after 3 years some plants are not possible to analyze anymore, however I sometimes analyzed successfully 6 year old samples, a lot depends on the quality of the material, the method of the preservation, and the species...). Sometimes using of more material is also helpful (but be careful not to put too much as this will increase the concentration of secondary metabolites in the suspension).

Sample preparation:

1. Put a tiny bit of the leaf of *Pisum* (Standard) together with about 2 times the amount of the unknown plant in a Petri dish
2. Add 1ml of Otto-I buffer (4°C)
3. Chop the plant material
4. Wash the chopped material with the rest of the buffer for about 2 times
5. Mark a 1,5 ml cup, put there a 50µm filter and place everything on ice and add your sample
6. Centrifuge at 1400 rpm at 4°C for 5 minutes
7. Keep the samples on ice or put them in the fridge until the measurement is done

It's possible to keep the samples for about 2 or 3 hours in the fridge after the centrifugation.

The following last preparation steps should be done about 5 minutes before the measurement:

8. Remove the buffer in the cups
9. Add 1ml of LB01 buffer and 20µl of DAPI (c=200 µg/ml)
10. Resuspend the pellet
11. Transfer the suspension to a cytometer tube
12. After few (about 5) minutes you can do the measurement

Experimentally this buffers were tried:

Ingredients (for 200 ml)		
Buffer 1 (works for *Leucanthemum*)	Buffer 2 **(works for *Leucanthemum*)**	Buffer 3
0,363g Tris	0,363g Tris	0,363g Tris
0,149g Na_2EDTA	0,149g Na_2EDTA	0,149g Na_2EDTA
0,0348g Spermine	0,0348g Spermine	0,0348g Spermine
1,193g KCl	1,193g KCl	1,193g KCl
0,234g NaCl	0,234g NaCl	0,234g NaCl
		0,1852g DTT
200µl Triton X-100	200µl Triton X-100	200µl Triton X-100
	209,3µl ß-mercaptoethanole	

ß-mercaptoethanole and PVP are optional here. There are used to compensate the effect of secondary metabolites. The buffer may be completely without them, with one of them, or with both. Experiments in *Leucanthemum* show good results without any of them but results with both are also good – it may depend on the freshness of the material and its quality e.g. it is more probable that older samples will work better with ß-mercaptoethanole and PVP added.

Preparation of DAPI

For preparing your sample you take 20µl of a DAPI-solution with the concentration 200µg/ml, the concentration of the stock solution is 25 mg/ml

→ 16µl DAPI stock solution and 1984µl water

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***Leucanthemum monspeliense* (L.) Coste (2*n* = 4*x* =36) growing among grass in the mountain meadow on the northern slope of Roc de Fraussa in the Catalan Pyrenees at the border between Spain and France (42°25´22.4´´N, 2°43´36.2´´E), 4 km westwards from the Salines sanctuary, Alt. 1416 m, 7.7.2010.**