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Phylogeny and evolutionary trends in subtribe *Hieraciinae* (*Asteraceae*)

Fylogeneze a evoluční trendy v subtribu *Hieraciinae* (*Asteraceae*)

Ph.D. Thesis

Supervised by: Mgr. Jindřich Chrtek, CSc.

In Prague, 29. 5. 2017

Content

Hereby I declare that I made this thesis independently, using the mentioned references. I have not submitted or presented any part of this thesis for any other degree or diploma.

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Jaroslav Zahradníček (in Prague 29. 5. 2017)

Author contribution statement

I declare that I made substantial part of the thesis papers (manuscripts). Contribution to each paper is specified below:

1. Jindřich Chrtek, Jaroslav Zahradníček, Karol Krak, Judith Fehrer: Genome size in *Hieracium* subgenus *Hieracium* (*Asteraceae*) is strongly correlated with major phylogenetic groups. Genome size measurement and analysis. Participation on material collection and manuscript preparation. Total contribution: 40%

2. Maria Zita Ferreira, Jaroslav Zahradníček, Jana Kadlecová, Miguel Menezes de Sequeira, Jindřich Chrtek, Judith Fehrer: Tracing the evolutionary history of the little-known Mediterranean-Macaronesian genus *Andryala* (*Asteraceae*) by multigene sequencing.

Participation on material collection, lab work, data analysis, interpretation and manuscript preparation. Total contribution: 40%

3. Jaroslav Zahradníček, Jindřich Chrtek: Cytotype distribution and phylogeography of *Hieracium intybaceum* (*Asteraceae*).

Material collection, lab work, data analysis, interpretation. Participation on manuscript preparation. Total contribution: 80%

4. Jaroslav Zahradníček, Maria Zita Ferreira, Anna Krahulcová, Judith Fehrer, Jindřich Chrtek: Genome size variation in the genus *Andryala* (*Hieraciinae*, *Asteraceae*).

Participation on material collection, cytometry, data analyses and interpretation, manuscript preparation. Total contribution: 60%

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Summary

Subtribe *Hieraciinae* includes taxonomically intricate polyploid and mostly apomictic genera *Pilosella* and *Hieracium* as well as diploid sexual genus *Andryala*. It offers a unique possibility to compare evolutionary trends and processes in closely related genera with contrasting frequency of polyploids, modes of reproduction, and geographical distrubution. The thesis is focused on *Hieracium* s.str. and *Andryala*; the genus *Pilosella* was studied by another authors.

Genome size of so called 'basic' *Hieracium* s. str. (*Hieracium* subgen. *Hieracium*) was estimated and correlated with results of phylogenetic analysis based on nuclear DNA marker ETS, ploidy level, breeding system and ecogeographical features. Inter- and intraspecific variability in genome size was also analyzed. Genome size variation corresponded with results of molecular phylogeny that separated three main clades reflecting geographical distribution in Europe ('western', 'eastern' and hybridogenous). The monoploid genome size in the 'western' species was significantly lower than in the 'eastern' ones. Intraspecific variability was generally low. Genome size downsizing was confirmed in monoploid C values comparison among diploid and polyploid cytotypes. Correlation of genome size with longitude was apparent for the whole data, correlations with latitude and altitude were not significant.

Evolutionary history and genome size pattern and evolution were explored in *Andryala*. A medium-size genus distributed mainly in the Mediterranean Basin and Macaronesia. To reconstruct the relationships within the genus we used three nuclear markers (ETS, ITS and single-copy gene *sqs*) and two chloroplast markers (*trn*T-*trn*L and *trn*V-*ndh*C). While cpDNA analysis confirmed a previously inferred chloroplast capture event with the sister genus *Pilosella*, nuclear markers supported the monophyletic origin of *Andryala*. None of phylogenetic analyses resulted in sufficient resolution, due to very low levels of nucleotide divergence of two nuclear and two chloroplast markers and a high degree of homoplasy and incomplete lineage sorting in the variable *sqs* marker. Only two well-supported basal lineages corresponding to relict species *A. laevitomentosa* and *A. agardhii* were separated. The rest of *Andryala* species collapsed to well-supported large group named here 'Major Radiation Group'. Relationships inside this group are largely unresolved. Regarding the genome size, highest C values were detected in basal relict species (*A. laevitomentosa, A. agardhii*) and in two populations of *A. ragusina*. Another two populations of *A. ragusina* have distinctly lower C values. Higher intraspecific variation of genome size in a few species might be explained by allopatric differentiation including island populations.

In addition, special attention was also payed to phylogeography and cytotype structure of *Hieracium intybaceum*, the sister member of all *Hieraciinae* genera forming transitional and still clearly unexplored lineage among the four *Hieraciinae* genera. 43 populations collected across the distribution range in the Alps and the Vosges Mts were explored using flow cytometry and AFLP molecular markers. We detected two ploidy levels, diploid and tetraploid with contrasting modes of reproduction (sexuality in diploids, apomixis in tetraploids). Diploids were found all across the Alps, while tetraploids only in the westernmost Alps and the Vosges Mts. Genetic variation was very low. Bayesian clustering identified four clusters/genetic groups, which are partly congruent with the ploidy pattern and geographical distribution. We suppose that diploids colonized the deglaciated areas from source populations most likely located in the southern part of the recent distribution range and in the western Alps.

General introduction

Taxonomy and evolutionary history of subtribe *Hieraciinae*

Evolution in closely related lineages of a monophyletic group is a timely topic with numerous studies published within the last decades. However, a detailed comparison of related lineages with strongly contrasting breeding systems with various proportions of apomixis and polyploidy is still lacking. Subtribe *Hieraciinae* (tribe *Cichorieae*) is a particularly well-suited model system due to the evolution of two different mechanisms of apomixis (diplospory, apospory) in two highly diverse and taxonomically intricate genera (*Hieracium* and *Pilosella*) and the presence of an entirely sexual sister genus (*Andryala*). This combination of features is unique, not only in the *Asteraceae* (Noyes 2007), but generally in plants.

Phylogenetic relationships, delimitation and taxonomy of the subtribe *Hieraciinae* was in the focus of botanists for a long time. Based on morphological cladistic analysis and chromosome numbers (Bremer 1994) it originally included *Hieracium* s.l. (incl. *Pilosella* Hill and *Chionoracium* Dumort.), *Andryala* L., *Hispidella* Barnad. ex Lam., *Hololeion* Kitam., *Tolpis* L. and *Arnoseris* L. Later on, Lack (2006) placed *Arnoseris* into the *Hypochaeridinae*, and Gemeinholzer et al. (in Kilian et al. 2009) came to a conclusion that it clusters with *Cichorium*. Further changes in delimitation of the subtribe refer to *Tolpis*, which clusters with *Cichorium* as part of the *Cichoriinae,* and to *Hololeion*, which falls into the *Heteracia-Soroseris* subclade of the *Crepidinae*. The exclusion of *Hololeion* is also supported by its basic chromosome number x = 8, different from the rest of *Hieraciinae* genera (x = 9) in the former circumscription. According to a recent molecular phylogeny based on two chloroplast and one nuclear DNA markers (Fehrer et al. 2007) the tribe *Hieraciinae* includes only *Hieracium* s.l. (incl. *Pilosella* and *Chionoracium*), *Andryala* and *Hispidella*, which is also in agreement with morphological characters (Krak & Mráz 2008). Three major lineages (Fehrer et al. 2007) generally match the generic circumscriptions: (i) *Pilosella* with *Hispidella* as a sister group, (ii) *Hieracium*/*Chionoracium*, and (iii) *Andryala* which is a sister genus of the whole group. All genera have the same basic chromosome number x = 9. Particular genera considerably differ in the frequency of polyploidy, frequency, mechanisms and manifestation of apomixis and consequently by their past and recent evolutionary potential. While the chloroplast DNA shows ancient intergeneric introgression from *Hieracium* into an 'Eastern European lineage' of *Pilosella*, and from this lineage into all *Andryala* species, nuclear DNA show monophyletic origin of this genera (Fehrer et al. 2007, Ferreira et al. 2015). Genetic exchanges among the lineages most likely occurred before the shifts from sexuality to apomixis, because they affected diploid taxa (Fehrer et al. 2009).

Various evolutionary processes contributed to present diversity in the subtribe *Hieraciinae*. In *Hieracium* and *Pilosella*, hybridization and polyploidization have been a pervasive evolutionary forces. In contrast, *Andryala* is a model example of allopatric and parapatric speciation and can serve as a suitable model group for studies aimed at Mediterranean and Macaronesian biogeography.

The thesis is focused on two genera with contrasting evolutionary histories – *Hieracium* s.str. with common polyploidy and gametophytic apomixis and *Andryala*, a purely diploid

genus.

Special attention was also payed to *Hieracium intybaceum*, which is a sister-group to all four genera of the subtribe Hieraciinae based on nrDNA, but clearly belong to *Hieracium* based on cpDNA (Fehrer et al. 2007).

Hieracium

Delimitation of the genus *Hieracium* has been a matter of debate. In the broadest sense, it includes subgenera *Hieracium, Pilosella* (Hill) S.F. Gray, *Chionoracium* Schultz-Bip. and *Mandonia* (Schultz-Bip.) Zahn (Zahn 1921–1923). The subgenus *Mandonia* was later merged with subgen. *Chionoracium* by the majority of authors. Most controversies referred to taxonomic status of subgen. *Pilosella*. Schultz & Schultz (1862) are among the first to accept it as a genus of its own, as partly did also 19th century *Hieracium* specialists Fries, Arvet-Touvet and Norrlin. During the last decades generic recognition of *Pilosella* has gained increased but not universal support. Recently, the nuclear ribosomal (ITS) data yielded a neat classification well compatible with morphological traits (Fehrer et al. 2007). Apart from *Hieracium intybaceum* Jacq., situated in a basal position, it shows a subdivision into three main clades (see also above): *Hieracium* (incl. subgen. *Chionoracium*), *Pilosella* with its sister *Hispidella*, and *Andryala*. A narrower generic concept reflecting these clades seems to be the best taxonomic solution, as the clades of *Hieracium* and *Pilosella* also considerably differ from each other by morphology, breeding system, frequency of polyploidy, geographic distribution and DNA content. With respect to morphology, *Hieracium* and *Pilosella* differ in achene traits, and by several other characters, not expressed in all species of the respective genera, such as stolons (widely occurred in *Pilosella*, extremely rare in *Hieracium*, red veins on the abaxial ligule surface in many *Pilosella* species, teeths on the leaf margin in many *Hieracium* species vs. entire leaves in *Pilosella* etc.). Both genera also significantly differ in the DNA content, which is about twice as high in *Hieracium* as in *Pilosella* (Bräutigam & Bräutigam 1996, Vladimirov & Greilhuber 2003). Differences in the frequency of polyploids, reproduction modes and geographic distribution are discussed below.

The genus *Hieracium* in the here accepted circumscription thus includes two subgenera, subgen. *Hieracium* and subgen. *Chionoracium*. Similarly to *Pilosella*, several authors proposed to evaluate subgen. *Chinoracium* as a separate genus (with a correct name *Stenotheca* Monn.; e.g. Sennikov & Illarionova 2002). This concept is supported by morphological data, mode of reproduction (most likely only diploid sexuals in subgen. *Chinoracium* in contrast to prevailing apomictic polyploids in subgen. *Hieracium*) and also geographical distribution (*Chionoracium* is a New World group, in contrast to mostly Euroasian *Hieracium*). Molecular data favor this separation. In *Hieracium* subgen. *Hieracium*, two major phylogenetic clades were recognized, composed of species with either western or eastern European origin. The ETS tree revealed that *Chionoracium* is derived from the 'Eastern' clade of *Hieracium* subgen. *Hieracium* species, which also fits well to the relatively high genome size of *Chionoracium* taxa. The 'Eastern' clade has significantly higher genome size than 'Western' clade. These two main lineages were separated in phylogenetic analysis and mostly correspond with geographical distribution of analysed species. Results of a combined analyses of two cpDNA regions suggest that the subgenus *Chionoracium* is monophyletic and nests near the base of several *Hieracium* subgen. *Hie-*

racium lineages, i.e. none of the present-day *Hieracium* subgen. *Hieracium* taxa show particular affinities to the American lineage (Fehrer et al. 2007).

Hieracium subgen. *Hieracium* comprises perennial plants which are distributed in temperate regions of Europe, Asia, rarely Mediterranean Africa, North America and have been also introduced into several other regions (e.g. New Zealand). The subgenus has a broad ecological amplitude, and its species occupy different habitats as forests, forest margins, various grasslands and rocks from the lowlands to the alpine belt.

Hieracium subgen. *Hieracium* is a huge diploid-polyploid complex with prevailing triploids and tetraploids (Merxmüller 1975, Schuhwerk 1996, Schuhwerk & Lippert 1998, Chrtek et al. 2007), and very rare pentaploids (Chrtek et al. 2004). Compared to polyploids, diploid species are very rare (cca 20 species) and mostly confined to refugial areas in southern Europe and the Eastern Carpathians, not affected by the Pleistocene glaciation (e.g. Merxmüller 1975, Chrtek 1996, Mráz 2003, Castro et al. 2007). *Hieracium umbellatum* L. seems to be the only one widely distributed (Euroasian) diploid species.

Traditionally, the polyploid *Hieracium* species have been considered near-obligate apomicts. The pathway taken to form chromosomally unreduced embryo sacs is diplospory of the Antennaria type (Bergman 1941, Gustafsson 1947, Skawińska 1963, Nogler 1984), embryo and endosperm are formed independently without fertilization. Recently, Hand et al. (2015) revealed meiosis and megaspore tetrad formation in 1–7% of ovules in 16 *Hieracium* species, confirming residual sexuality in this genus as suggested by Bergman (1941) and Skawińska (1963). The diploid species or cytotypes are sexual with regular micro- and megasporogenesis (Gustafsson 1947) and are self-incompatible (Rosenberg 1927, Bergman 1941). However, the presence of heterospecific pollen on stigma can induce the breakdown of self-incompatibility (mentor effect; Mráz 2003). Pollen production in sexual species is regular, resulting in viable pollen grains. In contrast, pollen amount, size heterogeneity and viability strongly vary among polyploid apomicts. Some tetraploids are pollen fertile, while others have more or less abnormal pollen development, resulting in non-viable pollen grains and male sterility. In triploids, abnormal pollen development and male sterility are more common compared to tetraploids (Mráz et al. 2005, Slade & Rich 2007). As expected, modes of reproduction also determine the capability for hybridization. It can recently proceed between diploid parents, both in field and experimental conditions, but hybrids are nearly or completely seed sterile (Mráz et al. 2005, Chrtek et al. 2006, Mráz & Paule 2006). Besides of them, pollen bearing tetraploids can serve as pollen parents in crosses with diploid sexual maternal plants (Mogie 1992). In contrast to the genus *Pilosella*, successful crosses with polyploids as maternal plants have not been reported yet.

There are two basic taxonomic concepts applied for intrageneric classification of *Hieracium*. Scandinavian, British and Russian botanists follow a narrow species concept (nearly all morphologically recognizable forms are treated at species rank (‘microspecies’)). A second concept, based on monographic studies by Nägeli & Peter (1885) and Zahn (1921-1923) and mostly used by Central European botanists accepts a broad species definition (species are further divided into subspecies, varieties, etc.). In the papers presented here, we follow the Central European concept, because, in our opinion, it better reflects the situation across the whole distribution range.

According to Central European concept, it is recognized ca 500 species of *Hieracium* subgenus *Hieracium* (Zahn 1921–1923), divided into so-called 'basic' and 'intermediate' species. Basic species were believed to be non-hybridogenous, either diploid, tentatively considered as the main units of the species evolution, or polyploid. Intermediate taxa share morphological characters of two or more basic species and are supposed to be of hybridogenous origin (hybrids stabilized by polyploidization and apomixis). Despite a high number of hybridogenous species, recent hybridization in *Hieracium* subgen. *Hieracium* is highly restricted, only a very few recent hybrids are found in nature, and only two cases have been published so far, namely *Hieracium krasanii* Woł. (Mráz et al. 2005, 2011) and *H. grofae* Woł. (Chrtek et al. 2006). Moreover, these hybrids are often sterile and produce only small amount of viable pollen.

Andryala

According to a recent taxonomic concept, the genus *Andryala* (including *Paua* Caball., *Rothia* Schreb., and *Pietrosia* Nyár. ex Sennikov) comprises ca. 17 perennial, less often annual or biennial species (Greuter 2006, Blanca 2011, Ferreira et al. 2014) distributed mainly in the Mediterranean Basin and Macaronesia with centers of diversity in North-West Africa, the Iberian Peninsula and Macaronesia. Only diploid (2n = 18) plants (Goldblatt & Johnson 1979-) with strictly sexual reproduction were found so far.

Taxonomic complexity and geographical distribution differ remarkably between most of the species. The genus includes both relict species confined to a few localities (*A. laevitometosa* (Nyár. ex Sennikov) Greuter with one locality (a few microlocalities) in the Romanian Carpathians (Kukula et al. 2003) and *A. agardhii* with a few localities in mountains of Andalusia and Morocco (Blanca et al. 1998) as well as widely distributed (often at localities strongly influenced by human activities) species as *A. integrifolia* s.l. with complex pattern of morphological variation.

Hieracium intybaceum

Hieracium intybaceum is a perennial herb scattered to locally common on siliceous bedrock in the subalpine and alpine belts of the Alps, and spatially isolated in the Vosges Mts and the Schwarzwald Mts. (the latter not confirmed recently). Three ploidy levels have been reported so far, namely diploids (2n = 18), triploids (2n = 27) and tetraploids (2n = 36; Favarger 1997, Chrtek et al. 2007). The diploid cytotype prevail across the distribution range, tetraploids are restricted to a small area in the southwestern Alps and the Vosges Mts.

Hieracium intybaceum forms a sister-group to all four genera of the subtribe *Hieraciinae* and is strongly divergent from other *Hieracium* species based on nrDNA analysis (Fehrer et al. 2007, 2009). In contrast, according to cpDNA (Fehrer et al. 2007), this species clearly belongs to *Hieracium*, a pattern that was attributed to a chloroplast capture event (Fehrer et al. 2007). A distinct morphology of *H. intybaceum* well matches phylogenetic data and was a principal reason for its separation into the genus *Schlagintweitia* Griseb., proposed and accepted by several authors in the past. Despite its strange position, there are many hybridogenous intermediate species with assumed parentage of *H. intybaceum* (Zahn 1921–1923). Moreover,

hybridization between *H. intybaceum* and another *Hieracium* species (*H. prenanthoides*) was also confirmed by experimental crosses (Zahradníček & Chrtek, unpubl. data).

Polyploidy

Polyploidization (whole genome duplication) is one of the most crucial mechanisms in the plant evolution, and very common also in several genera of the subtribe *Hieraciinae*. Ancient polyploidization was detected for almost all plant groups (Soltis et al. 2009). Two types of polyploidy can be distinguished based on origin of the duplicated genome: autopolyploids and allopolyploids (Clausen et al. 1945, Ramsey & Schemske 1998). Autopolyploids arise by genome duplication within individuals or by crossing of individuals (with participation of at least one unreduced gamete) of the same species. This type of polyploidy seems to be less frequent. However, the detection of autopolyploids and their recognition from allopolyploids is often difficult (for more details see Soltis et al. 2007, Parisod et al. 2010).

Probably more frequent is allopolyploidy. Allopolyploids have more than two chromosome sets which are dissimilar and derived from different species. This process is a result of interspecific hybridization of diploid taxa and subsequent polyploidization of hybrids (e.g. Mallet 2007) as well as simple genome duplication via unreduced gametes. Higher production of unreduced gametes in hybrid plants was detected and thus polyploidy is clearly related with hybridization (Ramsey & Schemske 1998). Polyploidy also plays a key role in establishment and stabilization of hybrids. In diploid hybrids, homologous chromosomes do not pair properly due to structural differences between chromosome sets and the hybrids often suffer from sterility (Grant 1981, Ramsey & Schemske 1998). In the allopolyploid genome, the parental chromosome sets are duplicated and pairing of homologous chromosomes is possible and thus, for many hybrids, polyploidy represents a way to escape from sterility (Ramsey & Schemske 1998).

The second most common mechanism of allopolyploids origin is probably through simple genome duplication of unreduced gametes (Ramsey & Schemske 1998, Thompson & Lumaret 1992, Kreiner et al. 2017). Polyploids may arise directly by fusion of two unreduced gametes or by fusion of one unreduced and one reduced gametes. The second path gives rise to triploids (triploid bridge) which consequently participate in forming of higher polyploids by backcrossing with diploids or crossing between triploids. The way is complicated by triploid block (triploids are often sterile; e.g. Levin 1975, Ramsey & Schemske 1998, Burton & Husband 2000, Husband 2004, Köhler et al. 2010). Polyploid cytotypes do not arise only once but probably frequently in various diploid populations (in case of autopolyploidy e.g. Segraves et al. 1999, Dobeš et al. 2004, Halverson at al. 2008).

Production of unreduced gametes in diploid populations is infrequent, but most likely widespread. However, nearly all estimates refer to male gametes, mostly due to methodical limitations as the estimation of the frequncy of unreduced female gametes needs more sophisticated approaches. Generally, production of 2n gametes averages from 0.1–2.0% (Ramsey 2007, Kreiner at al. 2017) in non-hybrid species and partly depends on reproduductive strategies as documented in the family *Brassicaceae* (Kreiner et al. 2017). Besides genetical control of meiosis, it also rely on environmental factors, such as temperature oscillations, herbivory, pathogens (e.g., Okuno 1952, Maceira et al. 1992).

Neopolyploids are expected to suffer for frequency-dependent mating disadvantage (minority cytotype exclusion; Levin 1975). It can be compensated by higher production of neopolyploids, assortative mating, shifts in reproduction modes (self-compatibility), phenological or ecological differentiation.

Apomixis

Apomixis is an asexual mode of seed formation that produces clonal progeny with a maternal genotype (Asker & Jerling 1992). It has been documented in more than 125 angiosperm genera (Carman 1997). Distribution of apomixis in the flowering plants (angiosperm) is not random. It clearly prevails in some families (*Poaceae, Rosaceae, Asteraceae*; Richards 2003) and almost all apomictic plants are polyploids.

The apomictic development of seeds includes three elementary steps: avoidance or strong modification of meiosis during megaspore formation, fertilisation-independent embryo formation and autonomous or fertilization-dependent (fusion of sperm cell with central cell of the embryo sac) generation of endosperm (Koltunow 1993, Richards 2003, Bicknell & Koltunow 2004). Regarding the embryo sac formation, two basic mechanisms of apomixis can be distinguished: (i) In diplospory, embryo sac originates from the megaspore mother cell, like in sexual reproduction, but meiosis is avoided in the first cell division and unreduced megaspores are formed by mitosis. (ii) In apospory, the embryo sac is formed from a somatic cell (e.g. Bergman 1941, Gustafsson 1947, Skawińska 1963, Nogler 1984, Richards 1997, Koltunow at al. 2000).

Despite parthenogenetic development of the egg cell, fertilization of central cell of the embryo sac is sometimes required for endosperm formation (pseudogamy; Asker & Jerling 1992, Koltunow 1993, Richards 1997). Obligatory apomixis was documented only in some diplosporous plant groups (Richards 1997). Also production of fertile pollen was detected in obligate apomictic plants (Mogie 1992), pollen of these plants could be involved in crosses with their sexual relatives.

Combination of apomictic reproduction with sexual reproduction (facultative apomixis) is more common and more or less corresponds with apospory type of apomixis. From the taxonomic point of view, classification of apomictic complexes is very difficult and often a matter of controversies, especially the concepts of large agamospecies vs. narrow microspecies (e.g., Dickinson 1998, Hörandl 1998, Stace 1998).

Apomixis in angiosperms is heritable and has evolved independently multiple times (Carman 1997, Van Dijk & Vijverberg 2005). However, the regulatory mechanisms are complex and still largely unknown. The components of apomixis – apomeiosis, parthenogenesis and fertilization-independent endosperm development, are at least in some species under different genetic control and can be uncoupled (Albertiny et al. 2001, Ozias-Akins & Van Dijk 2007). It is also supposed that polyploidy can cause temporal or spatial de-regulation of genes controlling the sexual pathway consequently leading to apomeiosis (e.g., Koltunow & Grossniklaus 2003, Curtis & Grossniklaus 2007). Recently, the role of epigenetic actions that are exerted on the sexual pathway by a set of genes which are inherited as a unit is stressed (Grimanelli 2012).

Sexual and asexual plant species seem to at least partly differ in the distribution pattern and ecological demands. Compared to sexuals, asexuals are wider distributed and rather tend to occur in higher latitudes and altitudes or otherwise devastated areas (Bierzychudek 1985, Van Dijk 2003, Hörandl 2006, 2009, Cosendai et al. 2013). Several hypotheses have been formulated, but none of them alone seems to be unequivocal (reviewed in Hörandl 2006).

Firstly, Baker´s law (Baker 1965, 1967) supposes that asexual plants are better colonizers, while their sexual relatives often depend on mating partners. Advantage of polyploidy and / or hybrid origin hypothesis assumes strong association between polyploidy and apomixis (asexual seed reproduction; Asker and Jerling 1992), Bierzychudek (1985) explains the distributional success of asexual plants by whole genome duplication providing the asexuals with higher level of genetic variation and heterozygosity, which might increase the ecological tolerance and buffered the negative effect of deleterious mutations. Moreover, polyploidy might change phenotypic traits (Linder & Barker 2014). Further hypotheses concern more efficient exploitation of ecological niches by asexuals due to either superior specialization (Frozen niche variation hypothesis; Vrijenhoek 1984, Vrijenhoek & Parker 2009) or superior generalization (General-purpose genotype hypothesis, lack of inbreeding depression during colonization/recolonization events in asexuals (Metapopulation hypothesis; Bierzychudek 1985, Haag & Ebert 2004) and evolutionary advantage of sexuals in environments rich in biological interactions (competitors, parasites and pests) (Asker & Jerling 1992, Red Queen hypothesis – Van Valen 1973).

Apomixis is widespread in *Hieracium* and *Pilosella* and strongly influences the pattern of morphological variation and evolutionary potential of particular groups. Detailed understanding of mechanisms and consequences of apomixis in *Hieraciinae* is thus crucial.

Genome size

Genome size is a powerful biosystematic marker for many types of studies. As a rule, it relates to flow cytometry which si used by botanists for more than three decades (e.g. Galbraith et al. 1983, Laurie & Bennett 1985, Doležel et al. 1994, Bennett & Leitch 1995, Bennett & Leitch 2005, Suda et al. 2007) but first report of plant genome size is much older (Swift 1950).

In flowering plants C-values vary more than 2300-fold among smallest known values in *Gentiana tuberosa* and *G. aurea* (1C = 0.061pg; 1C= 0.064 pg; Fleischmann et al. 2014, Greilhuber et al. 2006) and highest known value in *Paris japonica* (1C=152.23 pg; Pellicer et al. 2010). The high genome size variation is explained by several mechanisms. Firstly, it was considered that the genome size can only increase (‘one-way path to genome obesity’) due to polyploidization and retrotransposon proliferation (Flavell et al. 1977, Barakat et al. 1997, Bennetzen & Kellogg 1997). More recent studies conclude that genome size increases and also decreases have played a key role in the evolution of many plant groups (Wendel et al. 2002, Hawkins et al. 2008). The mechanisms responsible for genome size changes include mainly genome duplication, transposable element activity, recombination mechanisms, double stranded break repair, insertion-deletion rate and even losses of whole chromosomes (e.g. Kirik et al. 2000, Morgan 2001, Petrov 2002, Wendel et al. 2002, Gregory 2004, Leitch & Bennett 2004, Bennetzen et al. 2005, Hawkins et al. 2009, Ågren & Wright 2011).

Interpretation of genome size variability is very difficult (sometimes impossible)

without a phylogenetic context (Albach & Greilhuber 2004, Price et al. 2005, Weiss-Schneeweiss et al. 2006, Chrtek at al. 2009, Mandák et al. 2016). In polyploid plants genome size reduction was proved (downsizing) in comparison with diploid cytotypes of the same species (Keelogg & Benetzen 2004, Leitch & Bennett 2004). However, a reverse path was detected in tetraploid cytotypes of *Orobanche transcaucasica* (Weiss-Schneeweiss et al. 2006), in *Chenopodium* (Mandák et al. 2016) and in the genus *Nicotiana* both processes have been recognized (Leitch et al. 2008).

Although the full consequence of high variation of genome size is still not completely known, it has been many times suggested that genome size can constrain some phenotypic traits and that it significantly affects plant development, phenology and ecology (Leitch & Bennett 2007, Greilhuber & Leitch 2013).

Classical studies showing a positive relationship between genome size and cell size and duration of cell division (reviewed by Leitch & Bennett 2007) were followed by studies that revealed correlations between genome size and seed mass, leaf mass per unit area, growth rate and/or photosynthetic rate (Knight & Beaulieu 2008). Further studies point out relationships between genome size and life form (higher DNA content in perennials compared to annuals) and genome size and selfing rate (lower genome size in selfers in comparison to outcrossers). However, confounding effect of life form and mode of reproduction must be considered, as annuals often display higher rates of selfing than perennials and the correlation might be caused by a reduction in transposable elements in selfers. Relations between genome size and ecogegraphical factors are still matter of controversies.

In a few species, correlation with altitude (Bottini et al. 2000, Albach & Greilhuber 2004, Bancheva & Greilhuber 2006) or continentality (Bureš et al. 2004, Garnatje et al. 2007) was found. The former can be explained by higher capacity for growth at low temperatures and frost resistance in larger genomes (MacGillivray & Grime 1995) and by the more phosphate-rich soils at higher altitudes (Körner 1989) as phosphate is often a limiting nutrient for DNA biosynthesis and plant growth (Raven et al. 1986). On the other hand, other studies revealed either negative or no correlation and the picture seems to be more complex, depending on the group studied and lacking any general trend (Knight et al. 2005, Loureiro et al. 2010). Furthermore, several studies revealed advantage of smaller genomes in stressful conditions caused by the presence of heavy metals, impairment of nutrient uptake, water stress and temperature stress (Knight et al. 2005, Šmarda et al. 2008, Temsch et al. 2010, Pustahija et al. 2013). Correlation between DNA amount and insularity has been repeatedly documented and seems to be a general rule (Suda et al. 2005, Kapralov & Filatov 2011), island species possessing lower genomes in comparison with their continental counterparts.

Often large differences between species together with a fairly constant amount of nuclear DNA within species (or evolutionary entity) make the genome size a useful supportive character for taxonomic decision-making, i.e. taxa delimitation (e.g. Buitendijk et al. 1997, Thalmann et al. 2000, Zonneveld 2001, Šiško et al. 2003, Bureš et al. 2004, Baack et al. 2005, Záveský et al. 2005, Suda et al. 2007, Chumová et al. 2015). It can also help to infer evolutionary relationships in homoploid plant groups (Loureiro et al. 2010). Last but not least, it can be a valuable tool for detection o hybrids between species with different DNA contents, and (with some limitations) for detection of aneuploids. Genome size can also have a predictive value in evolutionary studies, as plant groups with high genome size are supposed to have lower evoluti-

onary potential compared to group with lower genome size. In plant ecology, genome size can predict e.g. invasibility of a given species, as species with lower genome are usually better invaders (see more details Suda et al. 2014).

Genome size was proved to be very useful species-specific marker for taxa delimitation in closely related genus *Pilosella* (Suda et al. 2007). Therefore the aim of the thesis is to reveal if its importance is also applicable to rest of the subtribe *Hieraciinae*.

Aims of thesis

I. To explain the genome size variability in *Hieracium* subgen. *Hieracium* in phylogenetic context and in relations to ecogeographical factors and polyploidy (Paper 1)

II. To reconstruct the evolutionary history of a little-known Mediterranean-Macaronesian genus *Andryala* (*Asteraceae*) by multigene sequencing (Paper 2)

III. To clarify the cytotype distribution and geographical pattern of genetic variation of *Hieracium intybaceum* (Paper 3)

IV. To analyze the genome size variability in the genus *Andryala* within phylogenetic context and with respect to selected life history and geographical traits (Paper 4)

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Genome size in *Hieracium* subgenus *Hieracium* (Asteraceae) is strongly correlated with major phylogenetic groups

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• *Background and Aims Hieracium* subgenus *Hieracium* is one of the taxonomically most intricate groups of vascular plants, due to polyploidy and a diversity of breeeding systems (sexuality vs. apomixis). The aim of the present study was to analyse nuclear genome size in a phylogenetic framework and to assess relationships between genome size and ploidy, breeding system and selected ecogeographic features.
• *Methods* Holoploid and monoploid genome sizes (C- and Cx-values) of 215 cultivated plants from 89 field populations of 42 so-called 'basic' *Hieracium* species were determined using propidium iodide flow cytometry. Chromosome counts were available for all analysed plants, and all plants were tested experimentally for their mode of reproduction (sexuality vs. apomixis). For constructing molecular phylogenetic trees, the external transcribed spacer region of nuclear ribosomal DNA was used.
• *Key Results* The mean 2C values differed up to 2·37-fold among different species (from 7·03 pg in diploid to 16·67 in tetraploid accessions). The 1Cx values varied 1·22-fold (between 3·51 and 4·34 pg). Variation in 1Cx values between conspecific (species in a broad sense) accessions ranged from 0·24% to 7·2%. Little variation (not exceeding the approximate measurement inaccurracy threshold of 3·5%) was found in 33 species, whereas variation higher than 3·5% was detected in seven species. Most of the latter may have a polytopic origin. Mean 1Cx values of the three cytotypes (2*n*, 3*n* and 4*n*) differed significantly (average of 3·93 pg in diploids, 3·82 pg in triploids and 3·78 pg in tetraploids) indicating downsizing of genomes in polyploids. The pattern of genome size variation correlated well with two major phylogenetic clades which were composed of species with western or eastern European origin. The monoploid genome size in the 'western' species was significantly lower than in the 'eastern' ones. Correlation of genome size with latitude, altitude and selected ecological characters (light and temperature) was not significant. A longitudinal component was only apparent for the whole data set, but absent within the major lineages.
• *Conclusions* Phylogeny was the most important factor explaining the pattern of genome size variation in *Hieracium sensu stricto*, species of western European origin having significantly lower genome size in comparison with those of eastern European origin. Any correlation with ecogeographic variables, including longitude, was outweighed by the divergence of the genus into two major phylogenetic lineages.

Key words: Apomixis, chromosome numbers, Compositae, genome size, hawkweeds, *Hieracium* subgenus *Hieracium*, mode of reproduction, nuclear DNA content, phylogeny, polyploidy.

INTRODUCTION

Genome size has become a widely studied phenomenon, beginning in the 1950s when large differences in the nuclear content of different organisms were detected (e.g. Swift, 1950; Laurie and Bennett, 1985; Bennett and Leitch, 1995). Large differences in DNA content can be caused by several mechanisms. It has been found that nuclear DNA content is primarily influenced by the proportion of non-genic repetitive DNA, much of which is generated by transposable elements (Flavell *et al.*, 1977; Barakat *et al.*, 1997). In particular, it has been found that retrotransposon copy number can vary among genomes (Arumuganathan and Earle, 1991; Vicient *et al.*, 1999; Kalendar *et al.*, 2000; Piegu *et al.*, 2006; Wicker and Keller, 2007; Hawkins *et al.*, 2008). Decrease in genome size can result from a higher overall rate of deletions than insertions, selection against transposable elements, unequal crossing over and illegitimate recombination (Morgan, 2001; Petrov, 2002; Wendel *et al.*, 2002; Ma *et al.*, 2004; Bennetzen *et al.*, 2005).

Correlations between genome size and specific life traits, most importantly life history and breeding systems, have been documented. Selfers were found to have smaller Cx-values than related outcrossers (Labani and Elkington, 1987; Govindaraju and Cullis, 1991; Albach and Greilhuber, 2004). Annuals, especially weedy species, tend to have lower genome size in comparison with related perennials (Bennett, 1972; Rejmanek and Richardson, 1996; Bennett *et al.*, 1998; Garnatje *et al.*, 2004; Grotkopp *et al.*, 2004), probably due to an association of annual life history with selfing (e.g. Albach and Greilhuber, 2004). Relationships between genome size and ecological factors are less clear (see, for example, Knight *et al.*, 2005). Correlations between genome size and frost resistance in the British flora (MacGillivray and Grime, 1995), elevation in some groups of *Centaurea* (Bancheva and Greilhuber, 2006), *Veronica* (Albach and Greilhuber, 2004), *Dactylis* (Reeves *et al.*, 1998) and

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Berberis (Bottini *et al.*, 2000) and continentality and habitat conditions (moisture) in *Cirsium* (Bureš *et al.*, 2004) have already been documented. There are also more or less close associations between genome size and cell size and leaf anatomical traits (Bennett, 1972; Edwards and Endrizzi, 1975; Knight *et al.*, 2005; Sugiyama, 2005; Beaulieu *et al.*, 2008), cell cycle duration (e.g. Rees *et al.*, 1966; Bennett *et al.*, 1983; Lawrence, 1985), seed mass (e.g. Knight *et al.*, 2005; Beaulieu *et al.*, 2007) and photosynthetic rate (e.g. Knight *et al.*, 2005).

Polyploids often have smaller Cx-values than their diploid relatives (e.g. Leitch and Bennett, 2004; Weiss-Schneeweiss *et al.*, 2006). These decreases correlate with a mutational bias towards deletion over insertions (Petrov, 2002), and illegitimate recombination has been shown to eliminate retrotransposon sequences (Bennetzen, 2002; Devos *et al.*, 2002; Ma *et al.*, 2004). However, exceptions of this downsizing pattern have been found, e.g. in the genus *Orobanche* (tetraploid *O. transcaucasica*). It was hypothesized that such polyploids are relatively young and that there was not enough time for a substantial reduction in nuclear DNA content (Weiss-Schneeweiss *et al.*, 2006).

Genome size alone is of little value as a phylogenetic indicator at higher taxonomic levels, but can be helpful in infrageneric classification assessments, species delimitation or hybrid identification (Keller *et al.*, 1996; Buitendijk *et al.*, 1997; Morgan *et al.*, 1998; Thalmann *et al.*, 2000; Zonneveld, 2001; Šiško *et al.*, 2003; Bureš *et al.*, 2004; Baack *et al.*, 2005; Záveský *et al.*, 2005; Suda *et al.*, 2007). An important issue that is still largely neglected in the literature, mostly due to a lack of comparative analyses between DNA sequence and genome size data sets, is the understanding of how genome size variation is linked with species evolution (but see Wendel *et al.*, 2002; Albach and Greilhuber, 2004; Grotkopp *et al.*, 2004; Jakob *et al.*, 2004; Weiss-Schneeweiss *et al.*, 2006). Species relationships were therefore assessed based on the external transcribed spacer (ETS) of nuclear ribosomal DNA in order to relate genome size variation to their evolutionary history.

Hieracium subgenus *Hieracium* is distributed in temperate regions of Europe, Asia, Mediterranean Africa and North America and has been introduced to several other regions, e.g. New Zealand. The genus is suitable for the study of genome size variation due its remarkable diversity in ploidy (coupled with breeding systems), habitat preferences and distribution of particular species. Polyploid (triploid, tetraploid and rarely pentaploid, $x = 9$) taxa with asexual reproduction through parthenogenetic development of the unreduced egg cell (*Antennaria*-type diplospory) prevail in this group, i.e. they are (near-)obligate apomicts (e.g. Nogler, 1984). Sexual reproduction is rather rare and restricted to diploid species (Schuhwerk, 1996; Chrtek *et al.*, 2004). The species occupy forests, forest margins, various grasslands and rocks from the lowlands to the alpine belt.

Species concepts in *Hieracium* have long been a matter of discussion (e.g. Schuhwerk, 2003). The Central European school of 'hieraciology' (founded by Nägeli and Peter in the 19th century) accepts a broad species definition (species are then divided into subspecies, varieties, etc.), whereas Scandinavian, British and Russian botanists follow a narrow species concept, i.e. nearly all morphologically recognizable forms are treated at species rank ('microspecies'). We follow the Central European concept because, in our opinion, it better reflects the situation across the whole distribution area, especially in central and southern Europe where most diploids occur, from which the apomictic polyploids are thought to be derived. According to this concept, approx. 500 species (in the broad sense) are accepted (Zahn, 1921–1923; and species described since that time), being either so-called 'basic' or 'intermediate' taxa. The latter share morphological characters of two or more basic species and are supposed to be of hybridogenous origin (hybrids stabilized by agamospermy). Basic species (about 45, including diploids and polyploids) are tentatively considered as main units of species evolution in *Hieracium*.

Here, a nuclear DNA content analysis of 42 basic species of *Hieracium* subgenus *Hieracium* (*sensu* Zahn, 1921–1923, with a few exceptions, see Materials and methods) is reported. The following questions were addressed: (*a*) how does the level of intraspecific variation in holoploid and monoploid genome sizes relate to the circumscription of species *sensu* Zahn? (*b*) how does monoploid genome size (Cx) relate to ploidy (diploids, triploids and tetraploids), i.e. is there evidence for downsizing of genomes in polyploids? (*c*) is there any congruence between the phylogenetic structure and the pattern of genome size variation? and (*d*) how does nuclear genome size relate to selected ecogeographic features (latitude, longitude, altitude, temperature and light)?

MATERIALS AND METHODS

Plant material

Two hundred and fifteen samples from 89 populations of 42 *Hieracium* species were collected in the field (or grown from seeds in a few cases) throughout Europe and transferred to the experimental garden of the Institute of Botany in Průhonice (Table 1; for details of sample localities see Supplementary Data, available online). Taxon sampling was restricted to so-called 'basic', supposedly non-hybridogenous species, generally following Zahn (1921–1923) with a few exceptions. The species concept of section *Cerinthoidea* followed Mateo (2005), *H. plumulosum* (*H. waldsteinii sensu lato*) was treated as a separate species, and two newly described diploid Balkan species (*H. kittanae* and *H. petrovae*; Vladimirov, 2003; Vladimirov and Szeląg, 2006) were included. Complete analysis covering all recognized and mostly (allo)polyploid hybridogenous species (approx. 500 'broad' species) was not feasible, and interpretation of estimated genome sizes would be extremely complicated due to the often unknown origin of polyploids and the reticulate patterns of variation.

For diploid, sexually reproducing species and for agamospermous polyploids with a rather small distribution area, one or two populations were chosen. For sexual diploids with large geographic areas and for more widely distributed agamospermous polyploids, two to six populations were selected. The number of plants analysed per population varied from two in agamospermous species with likely clonal population structure (e.g. Shi *et al.*, 1996; Mráz *et al.*, 2001; Štorchová *et al.*,

TABLE 1. *Accession origin and genome size*

Species	Locality (no. of plants) (S): plant cultivated from seed	2*n*	2C (pg) mean ± s.e.	2C (pg) range	1Cx (pg)	1Cx (pg) species mean ± s.e.	1Cx (pg) species range	1Cx species variation (%)	ETS clade[†]
H. alpinum L.	Ukraine: Chornohora (4)	18	7·90 ± 0·01	7·88–7·92	3·95	3·95 ± 0·01	3·94–3·97	0·76	E
	France: Hautes Alpes (1)	27	11·87	–	3·96				n.d.
H. amplexicaule L.	Austria: Hohe Tauern, Fragant (2)	27*	10·70 ± 0·01	10·7–10·71	3·57	3·60 ± 0·02	3·54–3·68	3·95	X(W)
	Austria: Hohe Tauern, Mallnitz (1)	27*	10·74	–	3·58				n.d.
	Spain: prov. Gerona (1)	27*	10·8	–	3·6				n.d.
	France: Hautes Alpes (1)	27	10·62	–	3·54				n.d.
	Italy: Rhaetian Alps (2)	36*	14·66 ± 0·07	14·59–14·72	3·67				n.d.
H. bifidum Kit.	Slovakia: Roháče (1)	27	10·67	–	3·56	3·53 ± 0·01	3·51–3·56	1·42	W
	Czech Rep.: distr. Beroun	27	10·52 ± 0·01	10·52–10·53	3·51				n.d.
	Czech Rep.: Krkonoše Mts.	27	10·62 ± 0·01	10·61–10·63	3·54				n.d.
H. bracteolatum Sibth. & Sm.	Greece: Pilion (2) (S)	27	12·39 ± 0·08	12·31–12·47	4·13	4·13 ± 0·03	4·10–4·16	1·46	X
H. bupleuroides C.C.Gmel. I.	Slovakia: Biele Karpaty (2)	27*	11·95 ± 0·05	11·91–12·01	3·99				E(H)
H. bupleuroides C.C.Gmel. II.	Slovakia: Chočské vrchy (2)	27*	12·00 ± 0·02	11·96–12·00	3·99				n.d.
	Slovakia: Roháče (2)	27	11·73	–	3·91				E
	Austria: Dachstein massif (1)	27*	11·61 ± 0·05	11·56–11·66	3·87				n.d.
	Slovakia: Slovenský raj (1)	27*	12·03	–	4·01				n.d.
	Austria: Allgäuer Alpen (1)	27	11·63	–	3·88				n.d.
	H. bupleuroides s.l. mean					3·95 ± 0·06	3·85–4·01	4·20	
H. caesium (Fr.) Fr.	Sweden: prov. Gotland (3)	36*	14·66 ± 0·12	14·52–14·89	3·67	3·68 ± 0·01	3·64–3·72	2·20	X(W)
	Sweden: prov. Gästrikland (3)	36*	14·75 ± 0·05	14·66–14·83	3·69				n.d.
H. cerinthoides L.	Spain: Pyrenees (2)	27*	10·68 ± 0·03	10·66–10·71	3·56	3·56 ± 0·01	3·55–3·57	0·56	W(H)
H. cordifolium Lapeyr.	Andorra: Pyrenees (6)	18*	7·18 ± 0·02	7·11–7·22	3·59	3·59 ± 0·01	3·56–3·61	1·40	W(H)
H. eriophorum St.-Amans	France: Landes, Labenne (6) (S)	18*	8·51 ± 0·03	8·45–8·61	4·25	4·27 ± 0·01	4·22–4·31	2·13	E
	France: Landes, Vieux-Boucau-les-Bains (6) (S)	18*	8·55 ± 0·03	8·44–8·61	4·27				n.d.
H. glaucum All.	Slovenia: Julijske Alpe, Podklanec (2)	27*	11·31 ± 0·03	11·29–11·34	3·77	3·79 ± 0·01	3·76–3·81	1·33	n.d.
	Slovenia: Julijske Alpe, Zadnjica (2)	27*	11·42 ± 0·00	11·42–11·42	3·81				n.d.
	Slovenia: Julijske Alpe, izvir Soče (2)	27	11·39 ± 0·05	11·34–11·44	3·8				X
H. gouani Arv.-Touv.	Spain: prov. Gerona (8)	18*	7·10 ± 0·01	7·07–7·12	3·55	3·55 ± 0·00	3·54–3·56	0·56	X(W)
H. gymnocephalum Griseb. ex Pant.	Albania: Jezerce (2)	18	8·44 ± 0·01	8·43–8·45	4·22	4·23 ± 0·01	4·22–4·23	0·24	X
H. gymnocerinthe Arv.-Touv. & G.Gaut.	Spain: Serra del Cadí (2)	27*	10·6 ± 0·02	10·58–10·61	3·53	3·54 ± 0·01	3·53–3·54	0·28	W(H)
H. heterogynum (Froel.) Guterm.	Montenegro: Lovčen (2) (S)	27	12·52 ± 0·02	12·52–12·55	4·17	4·18 ± 0·01	4·17–4·18	0·24	X
H. humile Jacq.	Austria: Dachstein massif (2)	36*	14·25 ± 0·01	14·24–14·26	3·56	3·55 ± 0·01	3·54–3·57	0·85	W
	France: Corbières (2)	27	10·64 ± 0·01	10·61–10·66	3·55				W
H. kittanae Vladimirov	Bulgaria: Rodopi (3)	18*	8·41 ± 0·04	8·38–8·46	4·21	4·21 ± 0·01	4·19–4·23	0·95	E
H. lachenalii Suter	Czech Rep.: Křivoklátsko (2)	27*	11·25 ± 0·01	11·27–11·29	3·76	3·75 ± 0·01	3·73–3·76	0·80	n.d.
	Czech Rep.: distr. Znojmo (2)	27*	11·22 ± 0·03	11·19–11·24	3·74				X(W)
	Czech Rep.: distr. Praha-east (2)	27	11·24 ± 0·06	11·20–11·32	3·75				n.d.
H. laevigatum Willd.	Czech Rep.: Brdy (2)	27*	12·05 ± 0·02	12·01–12·08	4·02	4·05 ± 0·02	3·97–4·14	4·28	X
	Czech Rep.: Hradec Králové (2)	27*	12·00 ± 0·08	11·94–12·10	4				n.d.
	Germany: Kamenz (2) (S)	27	12·41 ± 0·01	12·40–12·42	4·14				n.d.
H. lawsonii Vill.	France: Corbières (2)	27	10·76 ± 0·02	10·74–10·78	3·59	3·62 ± 0·03	3·58–3·68	2·79	W
	France: Briançon (1)	36	14·71	–	3·68				n.d.
H. murorum L.	Czech Rep.: Plzeň (2)	27*	10·62 ± 0·06	10·55–10·68	3·54	3·56 ± 0·01	3·52–3·59	1·99	W
	Czech Rep.: Doupovské hory (2)	27*	10·67 ± 0·05	10·63–10·72	3·56				n.d.
	Czech Rep.: Český kras (2)	27*	10·74 ± 0·02	10·72–10·76	3·58				n.d.
H. naegelianum Pančić	Montenegro: Durmitor Mts (2)	27*	10·89 ± 0·08	10·81–10·97	3·63	3·63 ± 0·03	3·60–3·66	1·67	E
H. olympicum Boiss.	Bulgaria: Kaloferska Planina (2)	27*	12·27 ± 0·05	12·22–12·33	4·09	4·05 ± 0·01	4·04–4·06	0·50	X

Continued

TABLE 1. *Continued*

Species	Locality (no. of plants) (S): plant cultivated from seed	2*n*	2C (pg) mean ± s.e.	2C (pg) range	1Cx (pg)	1Cx (pg) species mean ± s.e.	1Cx (pg) species range	1Cx species variation (%)	ETS clade†
H. pannosum Boiss. I	Bulgaria: Trojanska Planina (2)	27*	11·71 ± 0·05	11·66–11·77	3·9				E
H. pannosum Boiss. II	Greece: Peloponnesos (2) (S)	36	16·67 ± 0·01	16·66–16·67	4·17				n.d.
	H. pannosum s.l. mean					4·04 ± 0·08	3·89–4·17	7·20	
H. petrovae Vladimirov & Szeląg	Bulgaria: Rodopi (1)	18*	7·9	–	3·95				E
H. pictum Pers.	France: Montegenèvre (2)	27	10·78 ± 0·27	10·5–11·05	3·59	3·59 ± 0·04	3·50–3·68	5·14	n.d.
	France: Briançon (2)	27	10·75 ± 0·13	10·62–10·88	3·58				W
H. piliferum Hoppe	Austria: Gurktaler Alpen (2)	36	15·57 ± 0·04	15·54–15·60	3·89	3·91 ± 0·02	3·86–4·01	3·89	E-int.
	Austria: Reisseck-Gruppe (1)	27	11·58	–	3·86				
	Italy: Alps, Spluga (1)	27	11·58	–	3·86				
	France: Hautes Alpes (2)	27	11·95 ± 0·07	11·89–12·02	3·99				
H. pilosum Schleich. ex Froel. I.	Slovenia: Julijske Alpe (1)	27	11·57		3·86				E
H. pilosum Schleich. ex Froel. II.	Slovenia: Julijske Alpe (1)	27	11·8		3·93				X
	H. pilosum s.l. mean					3·90 ± 0·04	3·86–3·93	1·81	
H. plumulosum A.Kern.	Montenegro: Mrtvica canyon (1)	18*	8·59	–	4·29				X(E)
H. porrifolium L.	Austria: Karawanken (6)	18*	7·76 ± 0·01	7·7–7·79	3·88	3·89 ± 0·01	3·85–3·93	2·08	E
	Austria: Karawanken (1)	18	7·7	–	3·85				n.d.
	Slovenia: Julijske Alpe (6)	18*	7·82 ± 0·02	7·74–7·85	3·91				n.d.
H. prenanthoides Vill. I.	Poland: Karkonosze (2)	27	10·82 ± 0·03	10·78–10·85	3·61				X(W)
	France: Hautes Alpes, La Grave (1)	18	7·11	–	3·55				X(W)
	France: Hautes Alpes, Briançon (2)	18	7·29 ± 0·01	7·24–7·24	3·64				n.d.
H. prenanthoides Vill. II.	Andorra: Canillo (2)	27*	11·41 ± 0·02	11·40–11·43	3·8				X(W)
	H. prenanthoides s.l. mean					3·67 ± 0·04	3·56–3·81	7·02	
H. racemosum Waldst. & Kit. ex Willd.	Czech Rep.: distr. Znojmo (2)	27	12·41 ± 0·02	12·39–12·44	4·14	4·11 ± 0·06	4·08–4·15	1·72	X
	Czech Rep.: Ústí nad Orlicí (2)	27	12·26 ± 0·01	12·26–12·26	4·09				n.d.
	Slovakia: Gemer (1) (S)	27	12·24	-	4·08				n.d.
H. ramondii Griseb.	Andorra: Encamp (2)	27*	10·63 ± 0·07	10·56–10·7	3·54	3·54 ± 0·03	3·51–3·57	1·71	W
H. recoderi De Retz	Spain: prov. Barcelona (8)	18*	7·09 ± 0·01	7·00–7·09	3·53	3·53 ± 0·01	3·50–3·68	1·43	W
H. sabaudum L.	Czech Rep.: Praha (2)	27	12·51 ± 0·05	12·47–12·56	4·17	4·17 ± 0·02	4·12–4·23	2·67	n.d.
	Germany: Oberlausitz (2)	27	12·65 ± 0·03	12·62–12·68	4·22				X
	Czech Rep.: České středohoří (2)	27	12·36 ± 0·01	12·35–12·37	4·12				n.d.
H. schmidtii Tausch	Czech Rep.: České středohoří (2)	27*	10·64 ± 0·05	10·59–10·69	3·55	3·54 ± 0·01	3·52–3·56	1·14	n.d.
	Czech Rep.: České středohoří (2)	27*	10·58 ± 0·00	10·61–10·61	3·54				W
	Czech Rep.: Křivoklátsko (2)	27	10·60 ± 0·01	10·60–10·61	3·53				n.d.
H. sparsum Friv.	Bulgaria: Vitoša (1)	18	8·15	-	4·08	4·03 ± 0·03	3·99–4·08	2·56	E(H)
	Bulgaria: Pirin (2)	18	8·01 ± 0·03	7·98–8·04	4·01				
H. stelligerum Froel.	France: Ardèche (3)	18*	7·03 ± 0·06	6·91–7·14	3·51	3·51 ± 0·03	3·47–3·57	2·89	W
H. tomentosum L.	France: Alpes Maritimes (8)	18*	7·48 ± 0·02	7·41–7·58	3·74	3·75 ± 0·01	3·71–3·79	2·16	W
	France: Briançon (1)	27	11·27	–	3·76				n.d.
	France: Hautes Alpes (2)	27	11·25 ± 0·08	11·17–11·33	3·78				n.d.
H. transylvanicum Heuff.	Ukraine: Marmaros'ki Al'py (8)	18*	8·56 ± 0·01	8·52–8·59	4·28	4·28 ± 0·00	4·26–4·30	0·94	W
H. umbellatum L.	Poland: Baltic coast, Jantar (1)	18	8·34	–	4·17	4·26 ± 0·01	4·17–4·30	3·12	E
	Czech Rep.: Praha (8)	18*	8·54 ± 0·01	8·48–8·59	4·27				n.d.
	Germany: Nordfriesland (2) (S)	18	8·48 ± 0·03	8·45–8·52	4·24				n.d.
H. villosum Jacq. I.	Slovakia: Strážovské vrchy (2)	36	15·71 ± 0·04	15·66–15·75	3·93				E
H. villosum Jacq. II.	France: Hautes Alpes (1)	27	11·6	–	3·87				X(E)
	H. villosum s.l. mean					3·91 ± 0·02	3·87–3·94	1·81	
H. virosum Pall.	Russia: Rostov-na-Donu (2) (S)	27	13·06 ± 0·03	13·02–13·09	4·35	4·34 ± 0·00	4·33–4·36	0·69	E
	Russia: Altajskij kraj (3) (S)	27	13·00 ± 0·01	12·98–13·03	4·34				E
'Hieracium' intybaceum All.	Italy: Rhaetian Alps (1)	18	7·5	–	3·75	3·76 ± 0·01	3·74–3·79	1·34	

	Italy: Alpi Oróbie (2)	18	7·53 ± 0·07	7·48–7·58	3·77
	Austria: Ötztaler Alpen (2)	18	7·53 ± 0·07	7·52–7·54	3·77
Pilosella lactucella (Wallr.) P.D.Sell & C.West	Germany: Erzgebirge (1)	18	4·07[‡]		2·04
Andryala integrifolia L.	Spain: Andalusia (1)	n.d.	n.d.		
Andryala levitomentosa (Nyár.) P.D.Sell	Romania: Pietrosul Bogolini (1)	18	5·31[§]		2·66
Hispidella hispanica Barnades ex Lam.	Spain: Sierra de Guadarrama (1)	18[#]	∼4·00		∼2·00

* From Chrtek *et al.* (2007).

† E, 'eastern' clade; W: 'western' clade; X, interclade hybrid; X(E) and X(W), interclade hybrids with predominant 'eastern' or 'western' sequence variant; E(H) and W(H), hybrids within 'eastern' or 'western' clade; hybrid origin of *H. sparsum* and *H. lachenalii* inferred from plastid DNA (J. Fehrer *et al.*, unpubl. res.). E-int. indicates ETS character additivity between the 'eastern' clade and '*Hieracium*' *intybaceum* indicative of hybrid origin.

‡ See also Suda *et al.* (2007).

§ With *Pilosella lactucella* standard.

Luque (1981); Elena-Rosselló *et al.* (1985).

2002) to eight in supposedly genetically variable populations of sexual diploids. Two species, *H. petrovae* and *H. plumulosum*, were represented by a single plant due to their rarity in the field or because of cultivation problems. When two or more ploidies have been reported for a species, this diversity was covered as far as possible. Voucher specimens of all samples are deposited in the herbarium PRA.

Chromosome numbers and breeding system

At least two plants per population were checked for their chromosome number using the method described in Chrtek *et al.* (2007); counts for selected accessions have been published (Chrtek *et al.*, 2007). The mode of reproduction was also tested, generally following Gadella (1987) and Krahulcová and Krahulec (1999). In diploids (in which sexual reproduction was expected), randomly selected capitula were bagged at the bud stage and tested for late-acting autogamy (in the absence of active pollination); results were compared with control capitula from the same plant in open pollination treatments. In polyploids (in which agamospermy was expected), the upper part of the capitulum was cut off at the bud stage (emasculation) and the number of 'full' achenes was counted as a measure of seed set and compared with the number of achenes from untreated capitula of the same plant. Percentages of 'full' achenes after emasculation in particular plants/species are available upon request.

Genome size estimation

Genome size was determined by flow cytometry using a Partec CyFlow cytometer equipped with a green (532 nm) solid-state laser. *Zea mays* 'CE−777' (2C = 5·48 pg; Lysák and Doležel, 1998) and *Pisum sativum* 'Ctirad' (2C = 8·85 pg; Doležel *et al.*, 1994; Suda *et al.*, 2007) were used as internal standards for diploid and polyploid species. The modified two step-procedure described by Otto (1990) was employed for sample preparation. Intact leaf tissue (approx. 1 cm^2) of the analysed species and an appropriate quantity of the internal standard were co-chopped with a sharp razor blade in a plastic Petri dish with 1 mL of ice-cold Otto I buffer (0·1 M citric acid, 0·5% Tween 20) as the nuclear isolating solution. The suspension was filtered through a 42-μm nylon filter and centrifuged at 15 *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 100 μL fresh Otto I buffer. Samples were incubated for at least 10 min at room temperature and mixed with 1 mL Otto II buffer (0·4 M Na_2HPO_4) supplemented with propidium iodide as the fluorochrome, RNase IIA (both at a concentration of 50 μg mL^{-1}) and β-mercaptoethanol (2 μL mL^{-1}). Samples were stained for 5 min at room temperature before measurement. Usually, 5000 nuclei were analysed for each sample. Nuclear genome size was calculated as a linear relationship between the ratio of 2C peaks of sample and standard. Each plant was measured at least three times on different days by the same operator to eliminate potential artefacts. If the difference between the three measurements exceeded 2%, the value was discarded, and the sample was re-analysed. The coefficients of variation (CVs) of G_0/G_1 peaks did not exceed 5% (with two exceptions in *Hieracium* samples).

Molecular methods

A representative subset of 49 *Hieracium* accessions was selected for phylogenetic analysis. As the outgroup, species of the most closely related genera *Andryala*, *Hispidella* and *Pilosella* (sometimes treated as a subgenus of *Hieracium*) and '*Hieracium*' *intybaceum* were chosen according to previous results (Fehrer *et al.*, 2007). Although the latter species was traditionally placed in *Hieracium* subgenus *Hieracium*, molecular data (ITS sequences) suggested it belongs to an older isolated lineage clearly separated from a cluster formed by *Hieracium s.l.* and its closely related genera *Andryala* and *Hispidella* (Fehrer *et al.*, 2007), which is also supported by the present data based on the ETS region.

Total genomic DNA was extracted from fresh or CTAB-preserved material using a sorbitol extraction method (Štorchová *et al.*, 2000). The ETS region of the nuclear ribosomal DNA was amplified using the primers Ast-8 and 18 S (Noyes, 2006). PCR amplifications were done in 25-μL reactions containing 2·5 mM $MgCl_2$, 0·2 mM of each dNTP, 0·5 μM of each primer, 0·5 unit of *Taq* DNA polymerase (Fermentas, Ontario, Canada), 1 × *Taq* buffer with KCl (Fermentas) and a few nanograms of genomic DNA. An initial denaturation step at 94°C for 3 min was followed by 35 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 40 s) steps, and a final extension at 72°C for 10 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced (GATC Biotech, Konstanz, Germany). Both strands were sequenced using the PCR primers. For one accession, *Pilosella lactucella*, direct sequencing was not successful and, therefore, the amplified fragment was subcloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, but downscaled to half reactions. Three clones of this sample were sequenced (GATC Biotech), using the primer Ast-8.

Molecular data analyses

Sequence chromatograms were inspected by eye. In many accessions intra-individual polymorphism, i.e. more than one allele of the ETS region, was detected. Polymorphic sites were represented by the NC-IUB ambiguity symbols (e.g. R for A or G).

Initial sequence alignment was done with Clustal X (Thompson *et al.*, 1997) and further edited manually in Bioedit (Hall, 1999). It was unambiguous due to low overall variation. Visual inspection of the alignment revealed the existence of two major groups within *Hieracium s.s.*, and a proportion of accessions were identified as hybrids among these groups according to the additive pattern of polymorphic sites (these are referred to as 'interclade hybrids'). Furthermore, the *Hieracium piliferum* accession analysed was identified as a hybrid between one of the major groups and '*Hieracium*' *intybaceum*. Sequences were submitted to GenBank (EU821362–EU821419).

Bayesian (MrBayes V3·1·2; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and maximum likelihood (RAxML V7·0·3; Stamatakis, 2006) analyses were performed (*a*) on the complete data set and (*b*) on a modified data set excluding interclade hybrids and *H. piliferum*. Bayesian analyses were run with two nucleotide substitution rates and gamma distribution. This corresponds to a HKY + G model found in hierarchical Likelihood Ratio Tests as the model of molecular evolution best fitting to the data as implemented in Modeltest V3·5 (Posada and Crandall, 1998). Two parallel runs with four chains each were used for both analyses, sampling every 1000th tree. The analysis of the complete data set was computed for 2 million generations until convergence. The first 500 trees per run were discarded as burn-in and the remaining 1501 trees per run were summarized. For the modified data set, 1 million generations were sufficient for reaching convergence, the first 250 trees per run were discarded as burn-in and the remaining 751 trees per run were summarized. Maximum likelihood analyses were done using the rapid BS algorithm in combination with a maximum likelihood search, using the GTR model of nucleotide substitutions with a gamma model of rate heterogeneity and 1000 bootstrap replicates.

Statistical analyses of genome size

Intraspecific variation in morphologically defined species *sensu* Zahn was assessed and species with variation exceeding the approximate inaccuracy threshold of 3·5% [following Suda *et al.* (2007) in *Hieracium* subgenus *Pilosella*] were marked (*H. amplexicaule*, *H. bupleuroides*, *H. laevigatum*, *H. pannosum*, *H. pictum*, *H. piliferum* and *H. prenanthoides*). In *H. bupleuroides* and *H. prenanthoides*, differences in ETS sequences among accessions corresponding with the differences in genome size were found (J. Fehrer *et al.*, unpubl. res.) which were caused by hybridization with other species. These heterogeneous species were therefore split into more natural units (accessions or groups of accessions) according to genome size and treated separately in the following analyses. The same was done for *H. pannosum*, in which the accessions differed distinctly in genome size (and in ploidy), and for *H. pilosum* and *H. villosum*, in which differences in ETS sequences due to introgression were found between accessions (although genome size variation was rather low in these cases). On the other hand, morphologically homogeneous species with high intrapopulation variation in genome size (*H. pictum*) and species with unclear patterns of genome size variation (*H. amplexicaule*, *H. laevigatum* and *H. piliferum*) were not split. The units after splitting held the name of the broad species and were numbered (I, II) (Table 1). For convenience, they are referred to as species in the following paragraphs. Forty six taxa were recognized after the split (Table 1). To test the correlation between monoploid DNA amount (1Cx) and chromosome number, the Spearman rank order correlation coefficient and the one-way ANOVA were used with a matrix of all samples ($2n = 18$, 27 and 36).

Evolution of genome size was investigated on a sample of trees using the generalized least-squares method implemented in BayesTraits (Pagel and Meade, 2007). For this purpose, the last 751 trees from each run of the Bayesian analysis of the modified data set (without hybrids) were sampled and merged into one file. All 1502 trees were rooted manually with the outgroup (*Andryala integrifolia*, the taxon used as

outgroup in the Bayesian analysis), using the program Dendroscope (Huson *et al.*, 2007). Analyses were conducted on three different data sets: (1) on a complete set of these species; (2) separately for species of the western clade; and (3) separately for species of the eastern clade. All genome size data were $\log_{10}$ transformed prior to analyses. Two models of trait evolution were compared, using likelihood ratio statistics (Huelsenbeck and Rannala, 1997) or BayesFactor. Model A (drift model) corresponds to the standard constant-variance random-walk model, and model B is a directional random-walk model (Pagel, 1999, 2004). The scaling parameters lambda (λ), kappa (κ) and delta (δ) were optimized for 1Cx-values. λ assesses the contribution of the phylogeny to a character, κ scales branch lengths and can be used to test punctual vs. gradual modes of trait evolution, and δ scales the overall path length in the phylogeny. Values of 1·0 correspond to the null model (tree topology and branch lengths accurately describe models A and B). To test whether a model with estimated scaling parameters is a better fit than the null model where all scaling parameters are set to 1 (i.e. a strict Brownian motion model) the likelihood ratio test or BayesFactor was used. Two different methods of analysis were used, namely maximum likelihood and Monte Carlo Markov chain (MCMC).

How the variation in genome size matched the two major lineages of *Hieracium* (named 'western' and 'eastern') suggested by molecular phylogenetic analyses of the ETS region was tested further. In addition, genome sizes of interclade hybrid accessions were analysed. Five informal groups were recognized and used for these analyses, restricting the genome size data set to the accessions for which sequence data were available (Table 1): (*a*) 'western', corresponding to the phylogenetically distinguished 'western' group and containing 'pure' accessions as well as hybrid/hybridogenous accessions within the 'western' group [W and W(H)]; (*b*) 'eastern', corresponding to the phylogenetically distinguished 'eastern' group and containing 'pure' accessions and hybrid/hybridogenous accessions within the 'eastern' group [E and E(H)]; (*c*) hybrid/hybridogenous accessions between 'western' and 'eastern' clade species with about equal contribution of ETS variants from each parent (X); (*d*) interclade hybrid accessions with strongly dominating 'western' ETS sequence type [X(W)]; and (*e*) interclade hybrid accessions with strongly dominating 'eastern' group ETS [X(E)] (Table 1). For the purpose of this analysis, no distinction was made between 'pure' and intraclade hybrids (*a*, *b*) because of the low sequence variation within each clade and because their genome sizes did not differ. Two comparisons were performed: (1) the 'western' and 'eastern' groups with a group comprising all E–W hybrids; and (2) the 'western' and 'eastern' groups with the three different groups of hybrids (*c*–*e*) specified above. More details about ETS sequence features and the identification of particular hybrids will be presented elsewhere (J. Fehrer *et al.*, unpubl. res.). Both comparisons were conducted separately with and without *H. transylvanicum* (which fell into the phylogenetically defined western lineage, but has a genome size and geographic range congruent with the 'eastern' group; see Discussion). The correlation between genome size and phylogenetic pattern (five groups, see above) was tested.

The Spearman non-parametric rank order correlation coefficient was used in testing whether DNA amounts correlated with selected Ellenberg's indicator values, namely for light and temperature (Ellenberg *et al.*, 1992). Mean 1Cx values for species *sensu* Zahn were used for this analysis; only a subset of central European species (for which these values are available) was chosen. Genome size variation was also tested against altitudinal and geographical position (longitude and latitude) for (*a*) the complete set of accessions (mean accessions' 1Cx values were used), and (*b*) excluding accessions of widely distributed species (*H. bifidum*, *H. lachenalii*, *H. laevigatum*, *H. murorum*, *H. sabaudum* and *H. umbellatum*) for which the results are strongly affected by the collection site of the samples.

The only significant correlations of genome size variation with other parameters concerned phylogeny and longitude. In order to distinguish between these two factors, three correlation tests concerning longitude were performed for each pairwise comparison, constrained to accessions for which sequence data were available: (1) across all accessions from the 'western' and 'eastern' clades; (2) within the 'western' clade only; and (3) within the 'eastern' clade only. If the correlation is significant across all species, but not significant within either of the two clades analysed separately, this would argue for a connection between phylogenetic relationships and genome size variation. If, however, significant correlations are found for each of these tests, a relationship of genome size to longitude independent of species relationships would be supported. Data were analysed using the statistical package 'Statistica for Windows 6·0' (StatSoft, 1984–2002).

RESULTS

Chromosome counts and mode of reproduction

Chromosome numbers for plants of 43 populations analysed in the present paper belonging to 28 species were published elsewhere (Chrtek *et al.*, 2007), and counts for the remaining 46 populations are presented here (Table 1). A new ploidy is reported for *H. gymnocephalum* ($2n = 18$). Other counts confirmed previously published chromosome numbers. All diploids studied were found to be sexual and allogamous, and all polyploids ($3x$, $4x$) were agamospermous (data not provided).

Flow cytometry

Flow cytometric analyses yielded high-resolution histograms with CVs of G_0/G_1 peaks for *Hieracium* samples ranging from 0·83 to 5·76% (mean 2·28%), the values for internal reference standards were 0·97 to 5·0% (mean 2·19%). Generally, CVs of *Pisum sativum* were lower than those of *Zea mays*.

Nuclear DNA content: within-species variation

Intraspecific variation was assessed in 40 of 42 species *sensu* Zahn. Variation within accessions (populations) was generally low (Table 1). Mean values with standard errors (ranges for 2C and means for 1Cx genome sizes) for each population and

mean values with standard errors and ranges for 1Cx for each species are summarized in Table 1. Variation in 1Cx values between conspecific accessions (in both homo- and multiploid species) ranged from 0·24% in *H. gymnocephalum* and *H. heterogynum* to 7·2% in *H. pannosum*. Variation exceeding the approximate measurement inaccuracy threshold of 3·5% was detected in seven species, namely *H. amplexicaule*, *H. bupleuroides*, *H. laevigatum*, *H. pannosum*, *H. pictum*, *H. piliferum* and *H. prenanthoides*.

However, variation in the more naturally delimited (without heterogeneity in ETS sequences and inter-population genome size; see Materials and methods) *H. bupleuroides* was below the threshold of 3·5% variation. Variation within the separately treated populations of morphologically heterogeneous *H. pannosum* was also below 3·5% (Table 1). In further paragraphs, these 'narrower' taxa (a total of 46 taxa) are used.

C-values in the total set of 'basic' species

The mean 2C values differed up to 2·37-fold among different species (from 7·03 pg in diploid *H. stelligerum* to 16·67 in a tetraploid accession of *H. pannosum*). The 1Cx values varied 1·22-fold between 3·51 pg in *H. stelligerum* and 4·34 pg in *H. virosum* (mean 1Cx value of 3·87, s.d. 0·27; Fig. 1). The 1Cx values of diploids (including diploid accessions of multiploid species, means for species/cytodemes) varied 1·22-fold between 3·51 pg in *H. stelligerum* and 4·29 pg in *H. plumulosum* (mean 1Cx value 3·92 pg, s.d. 0·30), in triploids [including triploid accessions of multiploid species (means for species/cytodemes)] 1·23-fold between 3·53 pg in *H. bifidum* and 4·35 pg in *H. virosum* (mean 1Cx value of 3·81 pg, s.d. 0·25), and in tetraploids 1·17-fold between 3·56 pg in *H. humile* and 4·17 pg in *H. pannosum* II (mean 1Cx value of 3·79, s.d. 0·19).

Correlation between genome size, ploidy and breeding system

Diploids differed significantly in their 1Cx values from both triploids ($t = 2{\cdot}71$, d.f. $= 196$, $P = 0{\cdot}007$) and tetraploids ($t = 2{\cdot}01$, d.f. $= 109$, $P = 0{\cdot}047$), but triploids did not differ significantly from tetraploids ($t = 0{\cdot}72$, d.f. $= 119$, $P = 0{\cdot}476$) (Fig. 2). The value of the Spearman non-parametric rank order correlation coefficient was $r = -0{\cdot}179$, $P = 0{\cdot}009$. The mean 1Cx value was 3·93 pg in diploids, 3·82 pg in triploids and 3·78 pg in tetraploids, suggesting a trend towards smaller genome size with increasing ploidy.

In multiploid species, there was no general trend to either genome downsizing or upsizing. In *H. prenanthoides* 2*x*/3*x* there was 2·47% upsizing, in *H. villosum* 3*x*/4*x* 0·06% downsizing, in *H. tomentosum* 2*x*/3*x* 0·36% upsizing, in *H. humile* 3*x*/4*x* 0·36% upsizing and in *H. alpinum* 2*x*/3*x* 0·17% upsizing.

Comparison between 1Cx values of sexually reproducing plants (i.e. all diploids; polyploids were exclusively apomictic) and apomicts (triploids and tetraploids) revealed significant differences at $\alpha = 0{\cdot}01$ (*t*-test, $t = 3{\cdot}04$, d.f. $= 213$, $P = 0{\cdot}003$); the mean 1Cx value in sexuals was 3·93 pg, whereas in apomicts it was 3·82 pg, corresponding to the value for triploids due to the low number of tetraploid accessions.

Molecular phylogenetics of Hieracium

Analysis of ETS data including all sequenced accessions resulted in the same tree with both methods. It indicates monophyly of *Hieracium* subgenus *Hieracium*, but species relationships remained completely unresolved as reflected by a large polytomy with only two small subclusters that received low support (Fig. 3). However, as two major species groups could be identified by visual inspection of the alignment and many sequences showed additive patterns indicative of hybridization involving both groups, these accessions were deleted from subsequent analysis, because reticulation is known to collapse branches (Feliner *et al.*, 2001; Soltis *et al.*, 2008). With the reduced data set, a clear separation into two major clades with strong statistical support was found with both methods (Fig. 4). These lineages were designated 'eastern' and 'western' clade because they contained species of predominantly eastern or western European origin. A large number of accessions (18) showed ETS variants of both clades in either equal proportion or with the 'eastern' or 'western' sequence type dominating as indicated in Fig. 4. Details of these analyses will be given in a parallel paper (J. Fehrer *et al.*, unpubl. res.).

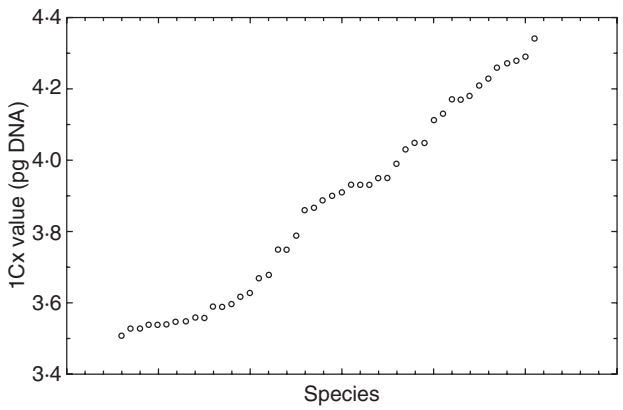

FIG. 1. 1Cx-value variation in 46 taxa of *Hieracium* subgenus *Hieracium*.

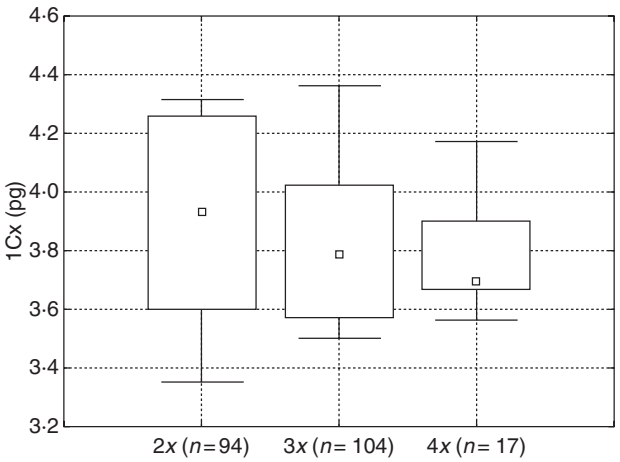

FIG. 2. Variation of genome size among diploids, triploids and tetraploids (all samples). 1Cx values of all accessions are shown. Differences between diploids and triploids, and between diploids and tetraploids are significant. The box indicates the interquartile (25–75%) range, the small square within the box is the median. The whiskers indicate minimum and maximum values.

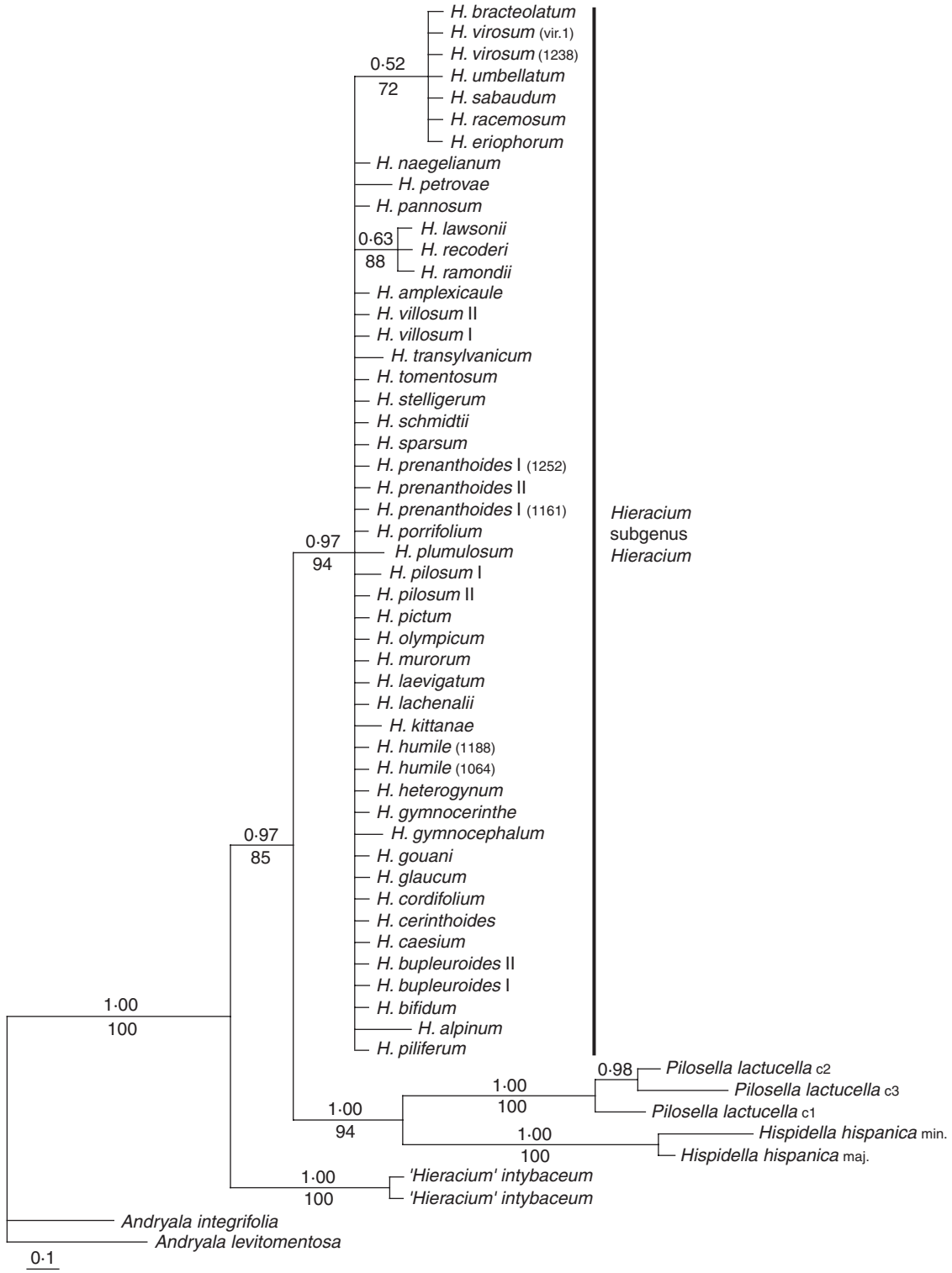

FIG. 3. Phylogenetic analysis of ETS sequences based on all accessions. A Bayesian consensus tree of 3002 saved trees is shown with posterior probabilities above branches. The maximum likelihood tree has the same topology; bootstrap values are indicated below branches. *Hieracium* subgenus *Hieracium* (=*Hieracium sensu stricto*) is monophyletic, but species relationships are completely unresolved when hybrids are included in the analysis. Support for the two subclusters is low.

Correlation of genome size with phylogenetic signal

The 'western' clade included 15 accessions: 2C values ranged from 7·03 pg in diploid *H. stelligerum* to 14·25 pg in a tetraploid accession of *H. humile*; 1Cx values ranged from 3·51 pg in *H. stelligerum* to 4·28 pg in *H. transylvanicum* (mean ± s.d.: 3·61 ± 0·19 pg; with *H. transylvanicum*

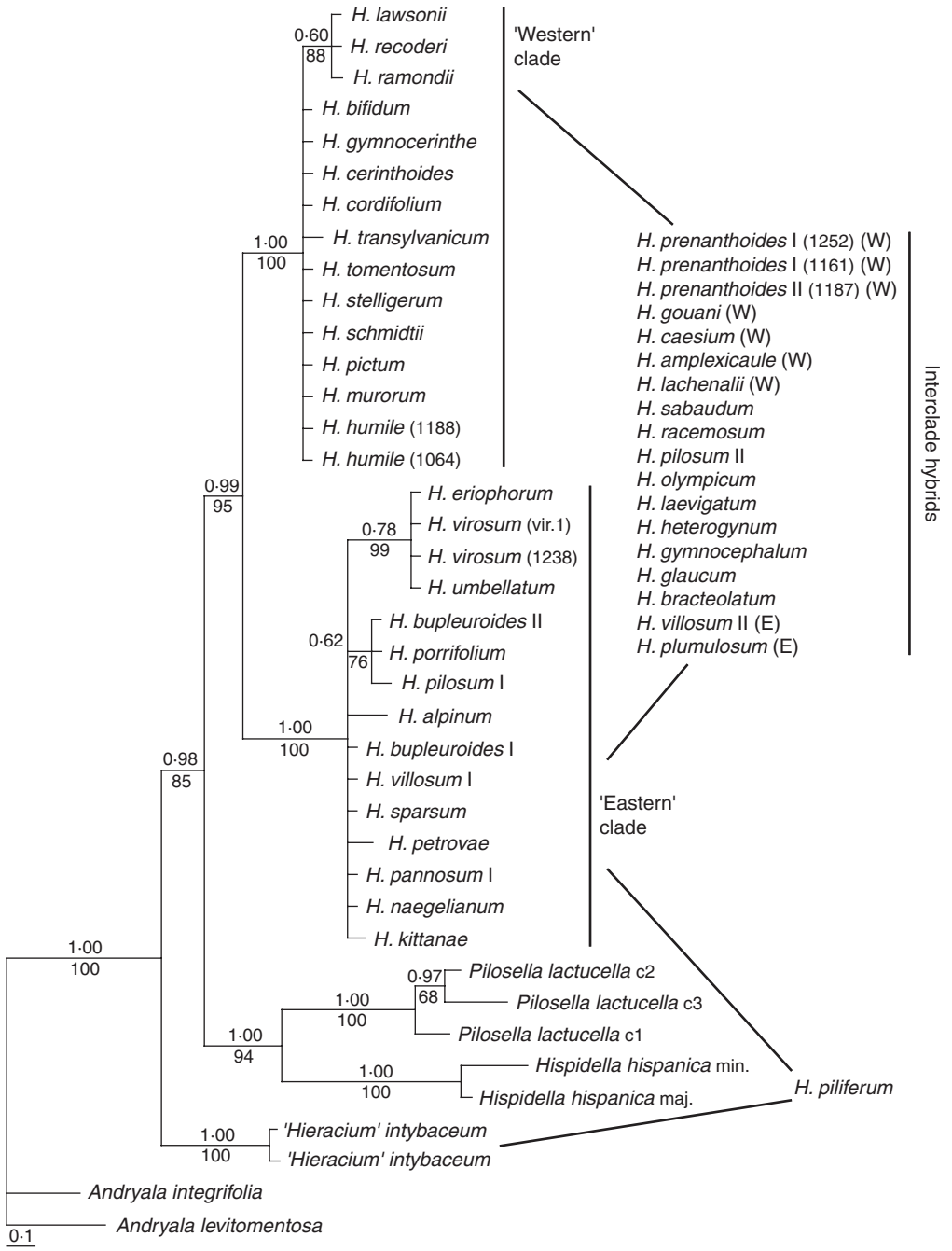

FIG. 4. Phylogenetic analysis of ETS sequences excluding interclade hybrid accessions. A Bayesian consensus tree of 1502 saved trees is shown with posterior probabilities above branches. The maximum likelihood tree has the same topology; bootstrap values are indicated below branches. After exclusion of hybrids based on character additivity, two major groups are resolved (referred to as 'eastern' and 'western' clades). Hybrid accessions composed of parents from both clades (interclade hybrids) are listed to the right. (W), Interclade hybrids with predominant 'western' ETS type; (E), interclade hybrids with predominant 'eastern' ETS type. *Hieracium piliferum* is a hybrid between '*Hieracium*' *intybaceum* and an 'eastern' clade taxon. For details about accessions, see Table 1 and Supplementary Data, available online.

excluded: up to 3·74 pg in *H. tomentosum*, 3·57 $\pm$ 0·06 pg). The 'eastern' clade also comprised 15 accessions: 2C values ranged from 7·78 pg in diploid *H. porrifolium* to 15·71 pg in a tetraploid accession of *H. villosum*; 1Cx values ranged from 3·63 pg in *H. naegelianum* to 4·35 pg in *H. virosum* (4·02 $\pm$ 0·20 pg). Significant differences in 1Cx values were found between the clades at $\alpha = 0{\cdot}001$ (Student's *t*-test), with ($t = -5{\cdot}71$, d.f. = 28, $P < 0{\cdot}001$) and without ($t = -8{\cdot}23$, d.f. = 27, $P < 0{\cdot}001$) *H. transylvanicum*.

Differences in 1Cx values between accessions of the 'western' (W) and 'eastern' (E) clades and of interclade hybrid accessions (X) are significant, independent of the inclusion of *H. transylvanicum* ($F = 13{\cdot}79$, d.f. = 45, $P < 0{\cdot}001$ with *H. transylvanicum*, $F = 20{\cdot}87$, d.f. = 44,

$P < 0{\cdot}001$ without *H. transylvanicum*; Fig. 5A). However, *post hoc* comparison (Scheffé test) revealed only two groups at $\alpha = 0{\cdot}05$, the first comprising all 'western' accessions, and the second embracing 'eastern' and 'hybrid' accessions. Thus, 'eastern' and 'hybrid' accessions do not differ significantly from each other. Significant differences were also found between five groups, i.e. after splitting the bulk of hybrids into three groups, namely hybrids with intermediate position (X) and hybrids with strongly dominating 'western' [X(W)] or 'eastern' [X(E)] ETS sequences ($F = 17{\cdot}07$, d.f. $= 43$, $P < 0{\cdot}001$ with *H. transylvanicum*, $F = 28{\cdot}86$, d.f. $= 42$, $P < 0{\cdot}001$ without *H. transylvanicum*). The Scheffé test revealed only two groups at $\alpha = 0{\cdot}05$, the first including W and X(W) accessions, the second X, X(E) and E accessions (Fig. 5B). A significant correlation (Spearman rank coefficient $r = 0{\cdot}705$, $P < 0{\cdot}001$) between phylogenetic signal and hybrid origin [all five groups – W, E, X, X(W) and X(E)] and the pattern of genome size variation was found. *Hieracium piliferum* (1Cx = 3·9) occupies an isolated position, and it was identified as a hybrid between an 'eastern' clade taxon and '*Hieracium*' *intybaceum* (1Cx = 3·76).

Evolution of genome size

Maximum likelihood method. For the complete data set (all species), a directional model of evolution (model B) did not result in significantly higher likelihood scores than the drift model of evolution (model A; 74·856 vs. 75·746), indicating that there is no general trend to either genome size increase or decrease. Scaling parameters leading to the highest likelihood for 1Cx values were $\lambda = 0{\cdot}908$, $\delta = 0{\cdot}819$ and $\kappa = 1{\cdot}035$ in model A and $\lambda = 0{\cdot}701$, $\delta = 0{\cdot}637$ and $\kappa = 1{\cdot}201$ in model B, respectively. For both models, the values of scaling parameters did not differ significantly from 1 (the null expectation, LR test), indicating that the phylogenetic tree correctly predicts the pattern of covariance among species on the trait (1Cx) and that there is no evidence of accelerated evolution.

For the western clade, likelihood scores of models A and B did not differ significantly (44·061 vs. 44·790). The maximum likelihood values for λ (<1; 0·550 in model A, 0·523 in model B) show a role of adaptive response to some external factors. Values of δ and κ are >1 in both models (not shown) indicating that longer paths contribute more to 1Cx evolution (accelerated evolution as time progresses) and that longer branches contribute more to the trait evolution. Likelihoods of the null model (with scaling paramaters set to 1·0) are significantly lower in both models, indicating that scaling parameters improve the fit of the data to the models.

For the eastern clade, likelihood scores of models A and B also did not differ significantly (40·915 vs. 42·141). Scaling parameters are not significantly different from 1 (the null expectation, Brownian motion, data not shown) indicating that the phylogenetic tree correctly predicts the pattern of covariance among species and that there is no evidence of accelerated evolution.

MCMC method. For the complete data set, comparison of harmonic means of log maximum likelihoods of models A and B showed a somewhat higher value in the latter (82·544 vs. 84·670). The model with estimated scaling parameters is a better fit than the null model (with scaling parameters set to 1) for both models A and B, showing that the scaling parameters improve the fit of the data to the model. The values of λ did not differ significantly from 1, and relatively high values of κ (3·379 and 3·606, respectively) indicate that longer branches contribute more to genome size evolution.

For the western clade only, the harmonic mean of model B is also higher than that of model A (49·888 vs. 45·494) and the harmonic mean of the null model is significantly lower than that for the model with estimated scaling parameters. Scaling parameters are similar to those found with the maximum likelihood method, i.e. λ and $\delta < 1$, $\kappa > 1$ (for interpretation see above).

For the eastern clade, harmonic means of models A and B do not differ significantly. Values of λ and δ do not differ from 1, higher values of κ (1·988 in model A and 2·212 in model B) again indicate accelerated rates of evolution within long branches.

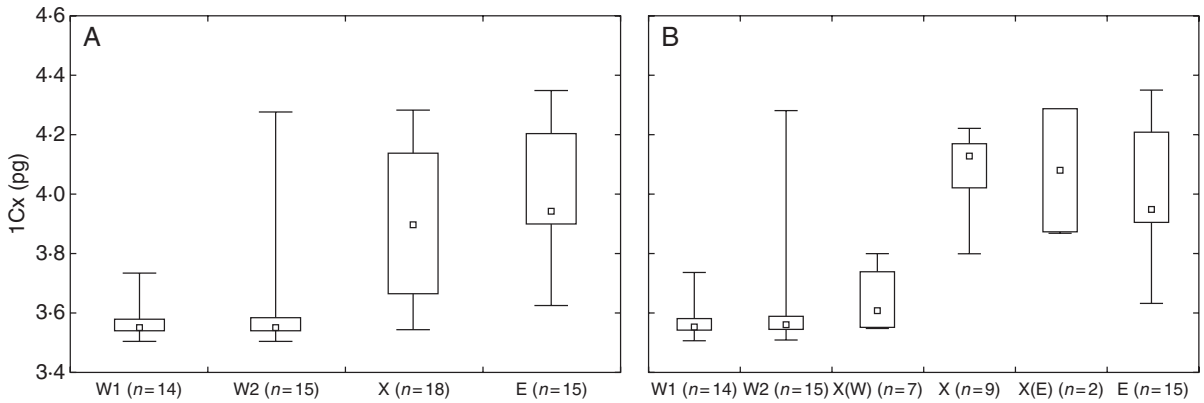

FIG. 5. Correlation of 1Cx values with phylogeny. Only accessions for which sequence data were available are included. (A) W1, 'western' clade accessions without *H. transylvanicum*; W2, 'western' clade accessions including *H. transylvanicum*; X, interclade hybrid accessions; E, accessions of the 'eastern' clade. (B) W1, W2 and E as before, hybrids divided into those with predominant 'western' [X(W)], equal (X) and predominant 'eastern' [X(E)] ETS sequence composition (see also Table 1). The box indicates the interquartile (25–75%) range, the small square within the box is the median. The whiskers indicate minimum and maximum values.

Correlation between genome size and ecogeographic features

The genome size of particular accessions was significantly correlated with their geographic position (longitude) in a west–east direction, both in the complete set of accessions (Spearman rank coefficient $r = 0{\cdot}562$, $P < 0{\cdot}001$; Fig. 6A) and after exclusion of widely distributed species ($r = 0{\cdot}617$, $P < 0{\cdot}001$; i.e. without *H. bifidum*, *H. lachenalii*, *H. laevigatum*, *H. murorum*, *H. sabaudum* and *H. umbellatum*; Fig. 6B). The correlation was stronger in the second case due to the strong dependence on the part of the geographic area from which the target plants of widespread species were sampled.

No correlation between genome size and latitude ($r = 0{\cdot}049$, $P = 0{\cdot}646$) or genome size and altitude ($r = -0{\cdot}224$, $P = 0{\cdot}034$) was found (complete set of accessions, results not shown). Also, no significant correlation was found between genome size and selected ecological parameters (Ellenberg's indicator values), namely temperature ($r = 0{\cdot}194$, $P = 0{\cdot}427$) and light ($r = -0{\cdot}236$, $P = 0{\cdot}331$) in a subset of species occurring in central Europe (results not shown).

Distinction between longitudinal and phylogenetic correlation

In order to determine whether the increase in genome size towards the east/'eastern' clade is based on geographic distribution or on species relationships, the longitudinal correlation was re-analysed for those accessions for which molecular data were available. Even stronger correlation was found between longitude and genome size in a set of accessions with known ETS sequences independent of including ($r = 0{\cdot}656$, $P < 0{\cdot}001$) or excluding ($r = 0{\cdot}688$, $P < 0{\cdot}001$) accessions of widely distributed species (Fig. 7A, B). In contrast, no significant correlation was found either among species of the 'western' ($r = 0{\cdot}161$, $P = 0{\cdot}567$) or among species of the 'eastern' ($r = 0{\cdot}394$, $P = 0{\cdot}245$) clade when tested separately (Fig. 7C and D). These results reveal that the evolutionary history due to eastern or western origin of the species is the dominant parameter affecting genome size in *Hieracium* rather than longitude.

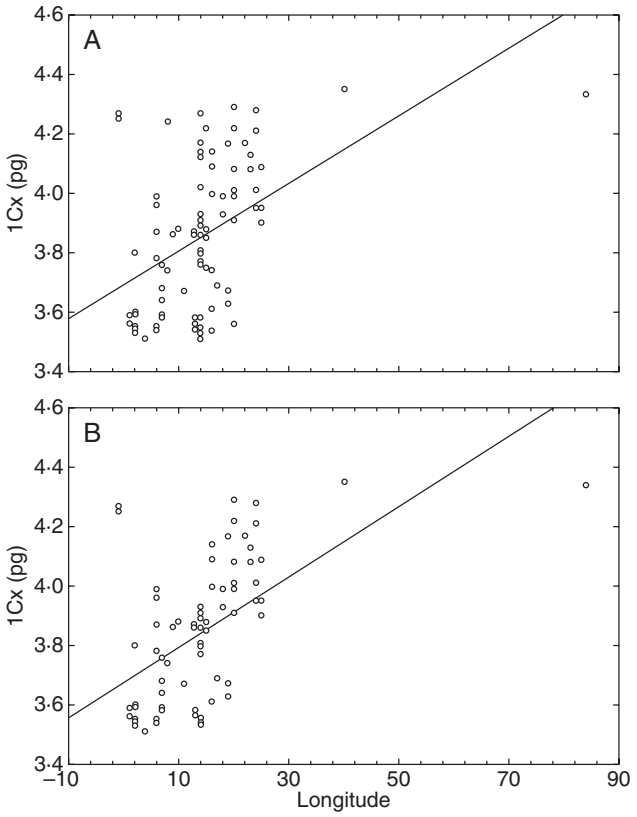

FIG. 6. Distribution of 1Cx values versus longitudinal position of collection sites: (A) based on the complete set of accessions/populations (Spearman rank coefficient $r = 0{\cdot}562$, $P < 0{\cdot}001$); (B) based on a subset after excluding accessions of widely distributed species (*H. bifidum*, *H. lachenalii*, *H. laevigatum*, *H. murorum*, *H. sabaudum* and *H. umbellatum*; $r = 0{\cdot}617$, $P < 0{\cdot}001$).

DISCUSSION

Chromosome numbers and mode of reproduction

Chromosome numbers for plants from 46 populations belonging to 26 species are published here for the first time, and counts for the remaining accessions have been published elsewhere (Chrtek *et al.*, 2007). Among the new data, a new ploidy (diploid) is reported for *H. gymnocephalum* (*s.l.*); previously reported counts (Niketić *et al.*, 2006) refer only to triploids ($2n = 3x = 27$). After *H. petrovae*, this is the second diploid count within section *Pannosa*. Also worth mentioning is the diploid ($2n = 2x = 18$) count for *H. prenanthoides* from the western Alps. Although this number had been reported from the same area in the 1960s (Favarger, 1969; Löve, 1969), it has not been confirmed until now. The remaining chromosome numbers correspond to previously published counts for the target species [cf. Schuhwerk (1996) and other standard reference manuals]. Analysis of the mode of reproduction confirmed the pattern already published for selected species and suggested it to be generally valid throughout the genus: diploid species reproduce sexually and are allogamous whereas polyploids are agamospermous.

Intraspecific genome size variation

Variation beyond arbitrary fluctuation (3·5%) was found in seven species *sensu* Zahn, namely *H. piliferum* (3·89%), *H. amplexicaule* (3·95%), *H. bupleuroides* (4·2%), *H. laevigatum* (4·28%), *H. pictum* (5·14%), *H. prenanthoides* (7·02%) and *H. pannosum* (7·2%). All are agamospermous polyploids, two of them including two cytodemes (*H. amplexicaule*, 3*x* /4*x*; *H. prenanthoides*, 2*x* /3*x*). As only a subset of populations was used for phylogenetic analysis, these results need to be interpreted with caution. In several cases in which sequence data were obtained for more than one accession, multiple origins of a given taxon, namely in *H. bupleuroides*, *H. pilosum*, *H. prenanthoides* and *H. villosum*, were found, which allows a plausible explanation of genome size variation at least for *H. prenanthoide*s. In this species, sequences of three accessions, one being diploid and two triploid, were analysed. All three showed identical signatures of an ancient interclade hybridization already affecting the diploid (J. Fehrer *et al.*, unpubl. res.). The triploids resulted from different subsequent hybridizations, one involving a 'western' and one an 'eastern' lineage. This fits well with

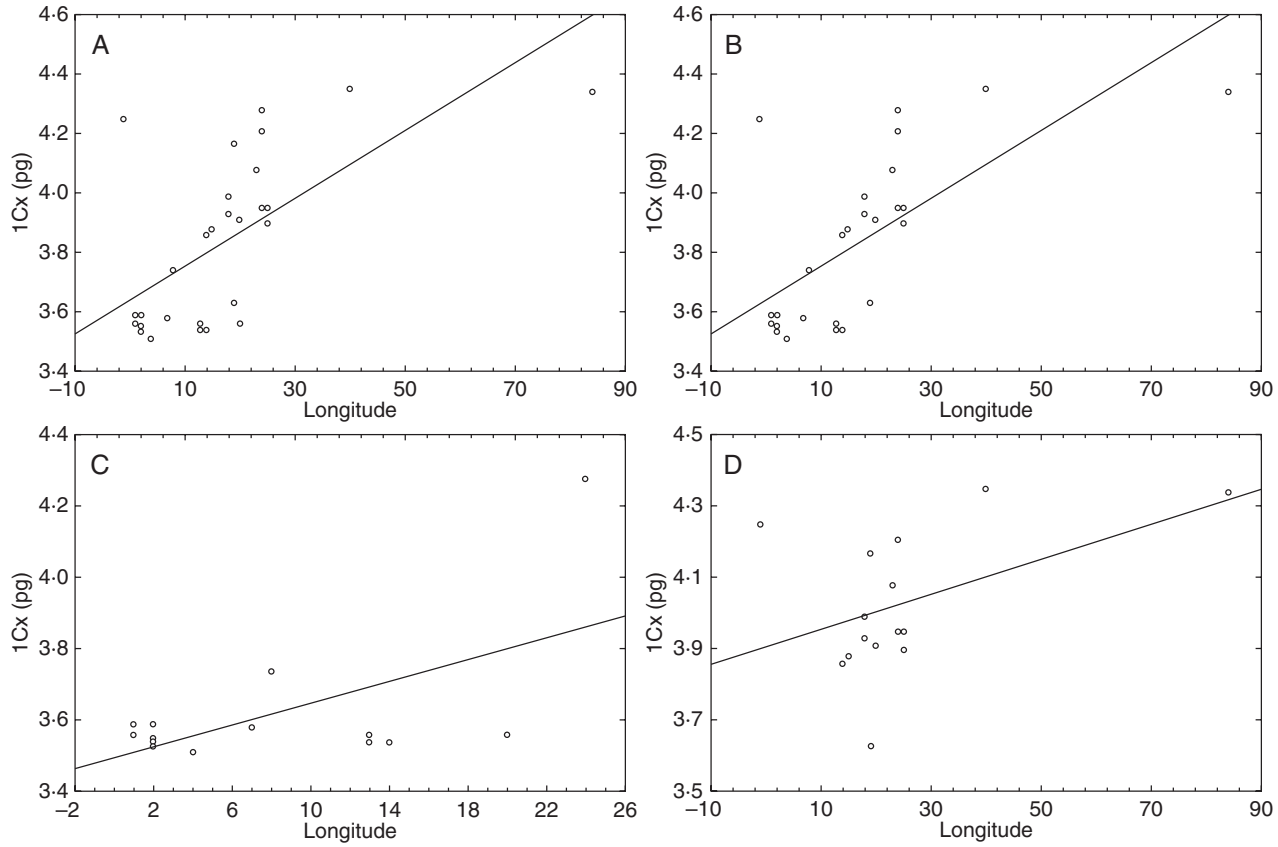

FIG. 7. Longitudinal component of genome size variation for accessions of known phylogenetic origin: (A) based on a complete set of 'western' and 'eastern' accessions/populations analysed by molecular methods (excluding interclade hybrid accessions; Spearman rank coefficient $r = 0{\cdot}656$, $P < 0{\cdot}001$); (B) based on a subset after excluding accessions of widely distributed species (*H. bifidum*, *H. murorum* and *H. umbellatum*; $r = 0{\cdot}688$, $P < 0{\cdot}001$); (C) based on a subset of accessions with 'western' ETS type ($r = 0{\cdot}161$, $P = 0{\cdot}567$); (D) based on subset of accessions with 'eastern' ETS type ($r = 0{\cdot}394$, $P = 0{\cdot}245$).

the 1Cx values: whereas values of the diploid and 'western' introgressed triploid populations ranged from 3·56 pg to 3·67 pg, the 1Cx value of the 'eastern' introgressed triploid was higher (3·81 pg). *Hieracium laevigatum*, *H. amplexicaule* and *H. piliferum* are, according to the ETS sequences, hybrids/hybridogenous types (at least the analysed accessions), and higher intraspecific variation might reflect recurrent polytopic origins. High inter-population variation in *H. pannosum* could also be related to multiple origins as suggested by the different ploidies of the analysed accessions. The high variation in *H. pictum* cannot be explained by the present data. Broader sampling for molecular analysis in these species might reveal hybrid accessions that have not yet been discovered. Thus, unequivocal evidence for intraspecific genome size variation was not found in 'good' species of subgenus *Hieracium*.

Similar results were obtained for *Hieracium* subgenus *Pilosella* in which the majority of wild species/cytotypes possess constant nuclear DNA amounts (variation in fluorescence intensity lower than 3·5%). Nevertheless, higher divergence was observed in six cytotypes belonging to three 'intermediate' species (the same terms as in subgenus *Hieracium*, i.e. hybrids/hybridogenous species) and among genetically variable F_1 offspring of experimental crosses between hexaploid *H. rubrum* and tetraploid *H. pilosella* (Suda *et al.*, 2007).

Intraspecific genome size variation in 'non-hybridogenous' species recently became a matter of debate (Murray, 2005). Whereas many of the examples of variation have been shown to be artefacts of measurement methods (e.g. Teoh and Rees, 1976; Greilhuber, 1998, 2005), there are some reports documenting C-value variation where appropriate controls and standards have been used (Reeves *et al.*, 1998; Hall *et al.*, 2000; Moscone *et al.*, 2003; Pecinka *et al.*, 2006).

Interspecific genome size variation

The present estimates of nuclear DNA content in *Hieracium* subgenus *Hieracium* are the first to be published for this group; all other data on *Hieracium* available so far refer to species of (subgenus) *Pilosella* (Bennett and Leitch, 2005), genomes of which are considerably smaller than those of subgenus *Hieracium* (see below). Holoploid (2C) genome sizes in *Hieracium* species included in the present study ranged 2·37-fold from 7·03 pg to 16·67 pg (mean 2C value 10·16 pg, median 10·61 pg). As almost all so-called 'basic' species were investigated, the present results should cover the genome size variation within the subgenus. A few rare pentaploid hybridogenous (i.e. not 'basic') taxa exist which were not analysed, and thus the upper limit could be higher. Variation in Cx values among species is relatively high (up to about 20%), but more or less continuous.

In *Hieracium* subgenus *Pilosella* with the same basic chromosome number ($n = 9$), holoploid (2C) genome size differs 4·33-fold and ranges from 3·53 pg to 15·30 pg (Suda *et al.*, 2007). However, *Pilosella* has more extensive variation in ploidy, ranging from diploid to octoploid. Monoploid genome sizes (1Cx values) in subgenus *Hieracium* ranged 1·22-fold from 3·51 pg to 4·29 pg (mean 2C value 3·86 pg, median 3·85 pg), whereas genome size in *Pilosella* is distinctly lower (it varies 1·23-fold from 1·72 pg to 2·16 pg). Thus, *Pilosella* has consistently about half the DNA content compared with *Hieracium*. The reasons for these large differences among closely related groups (Fehrer *et al.*, 2007) are unclear at the moment. Chromosomes of subgenus *Hieracium* are distinctly larger than those of *Pilosella* (no quantitative assessments available). Accumulation of repetitive sequence elements as in other plant groups might be one of the causes, but insights into *Hieracium* genomes are still lacking.

Genome size and ploidy

Diploid hawkweeds differ significantly in their 1Cx values from both triploids and tetraploids, but the latter do not differ from each other. This might indicate general downsizing of genomes in polyploid hawkweeds. However, there is no general trend to either downsizing or upsizing within multiploid species. Their origin remains to be elucidated in many cases and could involve autopolyploid origin as well as participation of another taxon (introgression which cannot always be detected by morphology). In Asteraceae, a similar situation was documented in the genus *Centaurea s.l.* in four multiploid (consisting of diploid and tetraploid cytotypes) species: downsizing was found in two species, upsizing in one species and equal monoploid genome size in one species (Bancheva and Greilhuber, 2006). However, downsizing of the genome after polyploidization is widely supposed to be a general trend in angiosperms (Kellogg and Bennetzen, 2004; Leitch and Bennett, 2004; Weiss-Schneeweiss *et al.*, 2006), as seems to be the case for *Hieracium*. The present data also suggest that species of autopolyploid origin might have more uniform genome size (and morphology) than allopolyploids of multiple (hybrid) origin.

Genome size and phylogeny

Genome size distribution basically matches two phylogenetically defined major lineages, i.e. a 'western' and an 'eastern' group (Figs 4 and 5). It thus reveals a strong correlation of nuclear DNA content with the basal evolutionary divergence of *Hieracium* subgenus *Hieracium*. Both clades include sexual diploids, agamospermous triploids and rare tetraploid apomicts. A similar pattern has also been observed for the geographic ranges. Both groups comprise local endemics (e.g. *H. stelligerum* and species of section *Cerinthoidea* in the 'western' group and *H. kittanae*, *H. petrovae* and *H. eriophorum* in the 'eastern' group) and widely distributed species (e.g. *H. murorum* and *H. bifidum* in the 'western' and *H. umbellatum* in the 'eastern' group). Differences of genome size between the two clades were also significant when only diploid or triploid accessions were compared (despite indication for some genome downsizing in polyploids), and thus all cytodemes were analysed together.

As mentioned above, *H. transylvanicum* falls into the western lineage but has a genome size and geographic range congruent with the 'eastern' group. Two alternative scenarios for its origin can be proposed: (1) the species has an eastern origin as suggested by its current distribution and DNA content and shows some ancient introgression from western species, some of which are widespread. Its ETS sequence then became completely homogenized towards the western type by concerted evolution (Arnheim, 1983); and (2) the species originated in western Europe, spread towards the east, the original populations became extinct probably during the Ice Ages and only the eastern populations survived in an eastern glacial refuge like the Carpathian basin. In this case, the high DNA content may be due to other reasons than phylogenetic relationships. Another accession of a 'western' clade species, *H. lachenalii*, has plastid DNA matching some 'eastern' clade species which suggests ancient introgression (J. Fehrer *et al.*, unpubl. res.). Correspondingly, its DNA content is also slightly higher than that of most other species of the 'western' clade. *Hieracium eriophorum*, a local endemic of the Atlantic coast near Arcachon in western France, is another species with an incongruent geographic range and position in the phylogenetic tree. Despite its western European distribution, it is most likely derived from widespread *H. umbellatum*, a species belonging to the same 'eastern' clade (Fig. 4). The morphology of *H. eriophorum* could therefore be interpreted as a local adaptation to sand dunes along the sea coast. The lowest genome size within the 'eastern' clade ($2C = 10{\cdot}89$ pg, $1Cx = 3{\cdot}63$ pg) was detected in triploid *H. naegelianum*. The distribution of this species fits well with other 'eastern' species as it occurs in the Balkan Peninsula and in the Abruzzi Mountains in central Italy, mostly in refugial areas. With respect to morphology, it is the only species of *Hieracium* subgenus *Hieracium* with long underground stolons, which enable the plant to spread vegetatively. Although no evidence of introgression from a 'western' species is apparent from the present molecular data, its occurrence in Italian glacial refuges could be indicative of past contacts and introgression from which only an unusually small genome size is left. Its plastid DNA is unique, and its relationships with other 'eastern' clade species are unresolved.

Hybrid (hybridogenous) 'basic' species

Fourteen 'basic' species were found to be of hybrid origin between 'eastern' and 'western' clade species (Fig. 4): *H. amplexicaule*, *H. bracteolatum*, *H. caesium*, *H. glaucum*, *H. gouanii*, *H. gymnocephalum*, *H. heterogynum*, *H. lachenalii*, *H. laevigatum*, *H. olympicum*, *H. plumulosum*, *H. prenanthoides*, *H. racemosum* and *H. sabaudum*. In addition, individual accessions of *H. pilosum* and *H. villosum* also had this type of hybrid origin. Based on the known mean 1Cx values for the 'western' and 'eastern' groups (3·61 and 4·02 pg, respectively), intermediate genome sizes of the previously mentioned species might be expected. However, hybrid accessions were more similar to the 'eastern' species group and significantly different from the

'western' group. The median 1Cx values of intermediate hybrid taxa and those with dominating 'eastern' ETS sequence were even higher than those for both clades (Fig. 5B). Potential interpretations could be to assume extinct parents with higher genome sizes or, more likely, an increase in DNA content in species of hybrid origin in comparison to their parents, as has been documented in *Helianthus* by Baack *et al.* (2005). The present results also show that hybrids/hybridogenous types with strongly dominating 'western' type ETS (e.g. *H. amplexicaule*, *H. caesium*, *H. gouani*, *H. lachenalii* and *H. prenanthoides*) have similar DNA content in comparison with 'western' species, which is significantly different from hybrids with equal contribution of 'eastern' and 'western' parents. This might suggest repeated backcrossing towards 'western' species at the diploid level before genomes became fixed by apomixis. Intermediate hybrids with dominant 'eastern' ETS, as expected, did not differ significantly from 'intermediate' hybrids or 'eastern' species.

One accession of *H. piliferum*, a basic species distributed in European mountains was found to be a hybrid between an unidentified 'eastern' taxon and '*H.*' *intybaceum* by character additivity in ETS sequences. Comparing 1Cx values of '*H.*' *intybaceum* (3·76 pg, Table 1), *H. piliferum* (3·91 pg) and members of the two ('western' and 'eastern') major clades, past hybridization between '*H.*' *intybaceum* and an 'eastern' species might be expected, which is also congruent with the results of the molecular analyses. Intraspecific genome size variation beyond the arbitrary fluctuation in *H. piliferum* could be indicative of multiple origins.

Evolution of genome size

Two different approaches implemented in BayesTraits for continuous data were applied, namely the maximum likelihood method and the MCMC approach, and two models of evolution (A, drift model; B, directional model) were compared. In most comparisons, our data fit (higher log-likelihoods and harmonic means) model B better, indicating that there is some but no strong trend to genome size increase.

For the complete data set and the maximum likelihood method, the values of scaling parameters did not depart significantly from the default values (1·0) suggesting that tree topology and branch lengths accurately describe the constant variance random-walk model A or B. Thus, genome size is evolving, as expected, given the tree topology, fitting well with the basal split into the two clades. Using the MCMC method, more changes on longer branches were indicated (longer branches contribute more to genome size evolution).

The tempo and mode of evolution differ between the western and eastern clade. In the western clade, values of scaling parameters depart from the default value (1·0) indicating that genome size evolution has not followed the topology or the branch lengths. Phylogenetic history has lower impact ($\lambda < 1$, presumed adaptive response to some external pressures) in this case which may also be reflected by the almost complete lack of resolution of species within the western clade (Fig. 4). Nevertheless, longer paths and branches contribute more to 1Cx evolution (accelerated evolution as time progresses). In contrast, for the eastern clade, the maximum likelihood method revealed that topology and branch lengths accurately describe the constant variance random-walk model A or B. However, using the MCMC method, accelerated rate of evolution in long branches is indicated.

Studies using a phylogenetic approach to evaluate the directionality of genome size evolution revealed both DNA decrease and increase and often different tendencies in genome size diversification in different phylogenetic lineages (e.g. Wendel *et al.*, 2002; Jakob *et al.*, 2004; Caetano-Annolés, 2005; Price *et al.*, 2005). Ancient genome size enlargement followed by more or less drastic parallel reduction in the main phylogenetic lineages was found in *Festuca* (Šmarda *et al.*, 2008).

Genome size and ecogeographic features

In order to identify further components of genome size variation for *Hieracium*, correlations were tested with a number of other factors. A significant, positive correlation was found between 1Cx value and longitude of sampling sites, both in the complete set of accessions and in a restricted set without species with large distribution areas for which the results depend strongly on sampling (Fig. 6). Restriction of these analyses to accessions analysed by molecular data showed that these correlations were even stronger when accessions of ambiguous origin were excluded (Fig. 7A, B). As no significant correlation was found within either of the two clades ('western' and 'eastern'; Fig. 7C, D), it can be concluded that the basal divergence into two phylogenetic lineages is most likely the determining factor of genome size variation in *Hieracium* (or vice versa) rather than longitudinal distribution.

In *Hieracium* subgenus *Pilosella* (Suda *et al.*, 2007), a longitudinal component of genome size distribution was also found: the highest 1Cx values were detected in *H. echioides*, a species distributed mainly in steppic habitats in Asia and eastern Europe (and well differentiated from the remaining species by the absence of a basal leaf rosette at flowering time). However, no comparison with species relationships is available. An opposite relationship between genome size distribution and geographic ranges has been observed in the genus *Cirsium* (Bureš *et al.*, 2004). At the intraspecific level, a geographically correlated variation in DNA content with an increase towards the east has been documented, e.g. in several taxa of *Koeleria* (Pecinka *et al.*, 2006), but no correlation was found in *Sesleria albicans* (Lysák *et al.*, 2000). Thus, there does not seem to be a general trend in genome size variation in relation to longitude. The same holds for a relationship between genome size and latitude, where positive, negative, or non-significant correlations with genome size have been found (reviewed in Knight *et al.*, 2005).

Altitude was also studied. The correlation between this parameter and genome size has been a matter of debate in the past years, and divergent results have been obtained. In subgenus *Hieracium*, genome size variation does not depend on altitude. However, the present data had to be based on altitudes of the sampling sites and are therefore biased by this selection, especially in species occurring across a large range of different altitudes (e.g. *H. bifidum*). Similarly, no correlation between genome size and altitude in Asteraceae was found in *Cirsium*

(Bureš *et al.*, 2004) or *Artemisia* and *Tripleurospermum* (García *et al.*, 2004, 2005). In other families, no or even a negative correlation between genome size and altitude was found by Creber *et al.* (1994), Reeves *et al.* (1998) and Vilhar *et al.* (2002) (all on intraspecific variation in *Dactylis glomerata*). On the other hand, an increase in genome size with higher altitute was found in *Centaurea s.s.* (Bancheva and Greilhuber, 2006) and in some groups of grasses (Bennett, 1976; Laurie and Bennett, 1985; Rayburn and Auger, 1990). Thus, altitudinal genome size variation also seems to be dependent on the particular plant group analysed (Knight *et al.*, 2005). In *Hieracium*, there are specifically montane or alpine taxa, but they are distributed in the Pyrenees, the Alps or the Balkan mountains, i.e. in western, central and eastern European regions, and therefore, the geographic origin of the species strongly dominates any altitudinal genome size variation that might be found.

Also no correlation was found between Cx values and two selected approximate ecological parameters published for German plant species by Ellenberg *et al.* (1992), namely light and temperature. However, the use of Ellenberg's indicator values for *Hieracium* is ambiguous. Many *Hieracium* species have large ecological amplitudes and therefore these approximate values could indeed be useful indicators, but these values are only available for central European species, and therefore species confined to either western or eastern Europe had to be excluded.

Many published correlations between ecogeographical factors (and others such as life form, etc.) and genome size must be interpreted with caution, as phylogenetic information is lacking. Albach and Greilhuber (2004) showed quite different correlations between selected factors (habitat, life history and breeding system) and genome size and DNA C-values in the genus *Veronica* if using a simple statistical test without phylogenetic information or more sophisticated methods incorporating phylogeny (independent contrast, GLSM). Furthermore, the use of linear regression analysis could obscure patterns in relationships if they are not linear. Knight and Ackerly (2002), Knight *et al.* (2005) and Beaulieu *et al.* (2007) used quantile regression analysis and showed that, although the relationship between genome size and a particular parameter was poor for species with small genomes, as genome size increased, the relationship became increasingly significant.

Conclusions

Genome size variation in *Hieracium* subgenus *Hieracium* is congruent with the phylogenetic pattern, with species of putative western European origin having significantly lower genome size than those of eastern European origin. Consequently, a significant longitudinal correlation can also be inferred. Separate analyses of closely related species (i.e. within each phylogenetic clade) clearly show that despite considerably overlapping scales, no significant geographic component is apparent. Thus, in *Hieracium*, any correlation of genome size with longitude, and with other ecogeographic variables such as latitude, altitude, light and temperature, is outweighed by the basal phylogenetic divergence into species of eastern or western European origin.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org/ and provide detailed information about the sample localities.

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Tracing the evolutionary history of the little-known Mediterranean-Macaronesian genus *Andryala* (Asteraceae) by multigene sequencing

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Abstract *Andryala* (Asteraceae: Cichorieae) is a little-known Mediterranean-Macaronesian genus whose taxonomy is much in need of revision. In order to elucidate species relationships in the genus, we performed phylogenetic analyses of nucleotide sequences of the internal transcribed spacers (ITS) and the external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA), two chloroplast (cpDNA) markers (*trnT-trnL* and *trnV-ndhC* intergenic spacers), and one single-copy nuclear gene (*sqs*) using Bayesian and maximum parsimony methods of inference. While cpDNA analysis confirmed a previously inferred chloroplast capture event with the sister genus *Pilosella*, all nuclear markers supported the monophyletic origin of *Andryala*. However, determining accurate phylogenetic relationships within the genus was quite challenging due to very low levels of nucleotide divergence of all nrDNA and cpDNA markers and a high degree of homoplasy and incomplete lineage sorting in the variable *sqs* marker. Although none of the phylogenies were well resolved, all markers identified two well-supported basal lineages corresponding to the relict species *A. agardhii* (Spain, Morocco) and *A. laevitomentosa* (Romania). The remaining *Andryala* taxa under study, whose relationships were largely unresolved, formed a well-supported clade ("Major Radiation Group"). The capacity of the markers to resolve taxonomic entities within this group varied. While congruent genetic evidence was found for some taxa, several morphologically unambiguous species could not be distinguished at all with most or even all markers. The extremely low level of genetic divergence among most of the species, in spite of high morphological diversity, along with a basal polytomy found with all markers, suggests their relatively recent and rapid speciation. Phylogenetic analyses of the single-copy marker advocate for a single colonization event of the common ancestor of two endemic species (*A. glandulosa*, *A. crithmifolia*) from the Mediterranean region to Madeira and that of two other endemics (*A. perezii*, *A. pinnatifida*) to the Canary Islands. The frequently observed evolutionary pattern of continental dispersion followed by insular speciation also holds for *Andryala*.

Keywords *Andryala*; colonization; Macaronesia; Mediterranean Basin; molecular phylogeny; speciation

Supplementary Material Electronic Supplement (Table S1; Figs. S1–S5) and alignment are available in the Supplementary Data section of the online version of this article at http://www.ingentaconnect.com/content/iapt/tax

■ INTRODUCTION

The Mediterranean Basin is one of the world's major biodiversity hotspots (Médail & Myers, 2004). This region comprises two major centers of biodiversity: one in the west that includes the Iberian Peninsula and Morocco, and one in the east comprising Turkey and Greece (Médail & Quézel, 1997). Considerable plant biodiversity, as well as endemism, in the Mediterranean region, are the result of the interaction of complex historical geological and environmental factors (Médail & Quézel, 1997; Thompson, 2005).

Macaronesia is an Atlantic region widely considered to comprise five volcanic archipelagos (Azores, Madeira, Selvagens, Canaries, Cape Verde), located at distances varying from 96 to 1500 km off the Iberian Peninsula and North Africa (Fernández-Palacios & al., 2011). This region also contains high plant diversity, where levels of endemic taxa reach 16.2% in Madeira and 67.8% in the Canary Islands (Jardim & Sequeira, 2008). The Macaronesian islands exhibit a wide range of geological ages, varying from 0.8 to 21 million years (Carracedo & al., 2002). Besides the presently emerged archipelagos, Macaronesia includes several seamount archipelagos that constitute Palaeo-Macaronesia. These seamount archipelagos, serving as "stepping stones" during glacial periods, might have facilitated dispersal and colonization from the European or African mainland to Macaronesia and occasionally in the opposite direction as well as inter-archipelago dispersal (García-Talavera, 1999; Carine & al., 2004; Fernández-Palacios & al., 2011).

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The Macaronesian islands have lately become the subject of several phylogenetic studies to clarify origin and diversification of various vascular plant groups. While very few of these studies have identified species endemic to Macaronesia as relicts of Tertiary origin, for example, *Lavatera phoenicea* Vent. (Fuertes-Aguilar & al., 2002), the majority of molecular studies suggested a general pattern of dispersal from the continent followed by insular speciation. In effect, molecular data have revealed single or multiple colonizations of Macaronesia from the Mediterranean region in several plant groups, such as the *Olea europaea* L. complex (Hess & al., 2000), *Lavatera* L. (Fuertes-Aguilar & al., 2002), *Hedera* L. (Valcárcel & al., 2003), *Cheirolophus* Cass. (Garnatje & al., 2007), *Festuca* L. (Díaz-Pérez & al., 2008), and *Echium* L. (Mansion & al., 2009). The Macaronesian islands show considerable habitat diversity, which most likely promoted adaptive radiation. One of the most spectacular cases of rapid speciation of Macaronesian endemics is *Argyranthemum* Webb ex Sch.Bip. (Asteraceae), a morphologically highly variable genus. Molecular studies supported the Mediterranean origin of this genus and revealed low levels of nucleotide divergence among species (Francisco-Ortega & al., 1997). The lack of sequence divergence in cpDNA and nrDNA has been an obstacle to achieve phylogenetic resolution in various groups of island plants. The pace of diversification in some insular groups has apparently been too rapid for fixation of sufficiently many shared mutations to allow robust phylogenetic reconstruction using a limited number of cpDNA and nrDNA characters (Baldwin & al., 1998).

Both the Mediterranean and Macaronesian regions host a little-known genus, *Andryala* L. This plant group is a member of tribe Cichorieae (Asteraceae), included in subtribe Hieraciinae, along with *Hieracium* L., *Hispidella* Barnad. ex Lam., *Pilosella* Vaill. (Fehrer & al., 2007a; Krak & Mráz, 2008) and *Schlagintweitia* Griseb., a segregate of *Hieracium*, including *S. intybacea* (All.) Griseb. (also known as *Hieracium intybaceum* All.), and two of its hybridogeneous derivates (Bräutigam & Greuter, 2007; Kilian & al., 2009). According to a divergence time estimate of tribe Cichorieae (Tremetsberger & al., 2013), the split of *Andryala* from other genera of the Hieraciinae occurred in the late Tertiary (Pliocene). *Andryala* (in its present circumscription, including *Paua* Caball., *Rothia* Schreb., and *Pietrosia* Nyár. ex Sennikov) comprises ca. 17 (Greuter, 2006–; Blanca, 2011; Ferreira & al., 2014a, b) perennial, less often annual or biennial, diploid ($2n$ = 18) species distributed mainly in the Mediterranean Basin and Macaronesia with centers of diversity in NW Africa, the Iberian Peninsula and Macaronesia. Thus, *Andryala* is an excellent model system for Mediterranean and Macaronesian biogeography, as its diversity centers are located in these regions. Far apart from the present-day distribution center of this genus, few populations of the endemic relict species *Andryala laevitomentosa* (Nyár. ex Sennikov) Greuter can be found (Kukuła & al., 2003; Negrea & Pricop, 2009) in the Romanian Carpathians, that are known as a glacial refuge (Zhang & al., 2001; Petit & al., 2003). Since it survived outside the present distribution center of the genus, probably the distribution area of *Andryala* was wider in the past (Fehrer & al., 2007b). A morphologically similar species, *Andryala agardhii* DC., occurs in high-altitude regions of southeastern Spain and Morocco and has been considered a Tertiary relict and paleoendemic species (Blanca & al., 1998). According to these authors, *A. agardhii* survived the last glacial persisting in the Iberian Peninsula, a Pleistocene refugium (Taberlet & al., 1998).

No taxonomic revision of the genus *Andryala* as a whole has ever been performed, which accounts for its poorly known taxonomy. The numerous taxonomic studies in *Andryala* include both splitting and lumping approaches (the first recognizing small but consistent variation at species level and the latter emphasizing the close relationship among variants) as it comprises many morphologically highly variable and unclearly delimited taxa. For instance, according to some authors, *Andryala integrifolia* L., an extremely variable species, should probably be divided into many closely related taxa (e.g., Sell, 1976; Greuter, 2006–). Many new names were proposed (Greuter, 2003; Greuter & Raab-Straube, 2007) based on erroneous taxonomy or misidentified specimens (e.g., Ball, 1878; Jahandiez & Maire, 1934; for further details see Ferreira & al., 2014b). Taxonomic treatments of *Andryala* suffer from a lack of molecular data showing relationships among intrageneric taxa. So far, only one or few *Andryala* species were included as outgroup for phylogenetic studies of other genera of tribe Cichorieae (Fehrer & al., 2007a, 2009; Tremetsberger & al., 2013). Fehrer & al. (2007a) suggested that an ancient chloroplast capture event occurred between *Pilosella* and *Andryala*. In the same publication, nuclear (ITS) sequence data clearly revealed the monophyly of *Andryala* and showed three main lineages, i.e., two relict species *A. agardhii* and *A. laevitomentosa* as separate lineages and a well-supported clade including the Macaronesian and Mediterranean taxa.

In the present study, a taxonomically and geographically fairly comprehensive set of *Andryala* samples was analysed (see Electr. Suppl.: Table S1), using the ITS and ETS regions of nrDNA as multi-copy nuclear markers, the intergenic spacers *trnT-trnL* and *trnV-ndhC* as chloroplast markers, and part of squalene synthase (*sqs*) as a highly variable low-copy nuclear marker (Krak & al., 2012). The last has recently been used in a phylogenetic analysis of *Hieracium* (Krak & al., 2013), one of the sister genera of *Andryala*. Phylogenetic analyses were performed to (1) elucidate species relationships in the genus, (2) investigate whether the current classification of *Andryala* is consistent with molecular data, and (3) examine colonization patterns in the Macaronesian region.

■ MATERIALS AND METHODS

Plant material. — Living plants or seeds were collected during field trips between 2010 and 2012 or provided by international collaborators and were cultivated in the experimental garden of the Institute of Botany (Průhonice). Almost all European, North African, and Macaronesian *Andryala* species were included (ca. 90%). Due to difficulties in collecting new material in some countries/regions or inadequate documentation of localities on herbarium labels, three currently recognized North African species could not be included: *A. chevallieri*

Barratte ex L.Chevall., *A. nigricans* Poir., and *A. spartioides* (Pomel ex Batt. & Trab.) Barratte. Although recognized as distinct species (Greuter, 2006–), morphological studies support the inclusion of *A. floccosa* Pomel in *A. laxiflora* DC. as a mere variety (Battandier & Trabut, 1905; Ferreira & al., unpub. data), and in recent Floras *A. rothia* Pers. was synonymized with *A. laxiflora* (Blanca, 2009, 2011), the latter included in this study. For a comparison of the treatment of *Andryala* in the Euro+Med PlantBase (Greuter, 2006–) with the updated nomenclature and species concept we are applying in the present paper, see Electr. Suppl.: Table S1. For molecular analyses, we attempted to cover the range of morphological variation within a species as far as possible in order to assess its intraspecific genetic variation and correspondence to species boundaries. Therefore, morphologically variable taxa were represented by several accessions from different populations as far as possible. To avoid confusion with improperly identified material, plants of unclear taxonomic position or intermediate morphology and obvious hybrid individuals co-occurring with their parental species were excluded from molecular analyses. Altogether, a total of 49 accessions was analyzed. Based on previous studies (Fehrer & al., 2007a; Krak & Mráz, 2008), 12–16 samples of 10–11 species of the most closely related genera of Hieraciinae were chosen as outgroup for the phylogenetic analysis: these were species of *Pilosella* (Bräutigam & Greuter, 2007; formerly treated as a subgenus of *Hieracium*), *Hieracium*, *Hispidella* as well as *Schlagintweitia intybacea* (Fehrer & al., 2007a). Voucher specimens were deposited at PRA and MA. The taxa examined in this study are listed in Appendix 1, along with voucher data.

Molecular procedures. — Total genomic DNA was isolated from CTAB-preserved or silica-gel dried material, as well as from fresh or herbarium material, either by sorbitol extraction (Štorchová & al., 2000) or by use of the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Modifications to the sorbitol extraction were introduced: fresh samples were frozen in liquid nitrogen and crushed in a porcelain mortar, and poly(vinylpolypyrrolidone) (P 6755, Sigma-Aldrich, Prague, Czech Republic) as well as 1 µl of EDTA (ethylenediamine tetraacetic acid disodium salt dihydrate, 0.5 M, pH 8.19) were added between two additions of 650 µl of extraction buffer. DNA quality was checked through electrophoresis and its quantity measured using a spectrophotometer. PCR amplifications of the *trnT-trnL* intergenic spacer were performed as in Fehrer & al. (2007a). For ITS amplification, primers ITS-A and ITS-B (Blattner, 1999) were used with the same PCR conditions. The ETS region was PCR-amplified using primers Ast-8 and 18 S (Baldwin & Markos, 1998) as described in Fehrer & al. (2009). The chloroplast *trnV-ndhC* intergenic spacer and the part of the low-copy nuclear *sqs* gene spanning exon 4 through intron 8 were amplified following Krak & al. (2013). PCR products were purified and sequenced as described in Fehrer & al. (2009). All DNA regions under study were directly sequenced in both directions using the PCR primers; for *trnV-ndhC* and *sqs*, additional internal sequencing primers were utilized as described in Krak & al. (2013). Samples that were heterozygous for *sqs* were cloned as described in Fehrer & al. (2009). Cloned *sqs* sequences were also often sequenced with several primers due to the length of the amplification product and difficulties with polynucleotide runs. Correction of polymerase errors, elimination of recombinant clones and allelic interpretation were done as described in Krak & al. (2013). Four samples showed a single polymorphism in direct sequencing and were represented by two sequences with the alternative character states, designated as alleles A or B. No more than two alleles per plant were found, no extraordinary branch lengths occurred (i.e., individual lineages with strongly accelerated rates of molecular evolution which may be indicative of paralogs or pseudogenes), variation in exon regions was very low, and despite a number of outliers (see below), alleles of the same individual or species most often grouped together. Taken together, this indicates that the low-copy nuclear marker *sqs* is a functional single-copy gene in *Andryala* as it is in the closely related *Hieracium* (Krak & al., 2013). However, the proportion of homozygous plants was much higher in *Andryala*. GenBank accession numbers for each sequence are listed in Appendix 1.

Sequence and phylogenetic analysis. — Chromatograms were edited manually using Chromas v.1.45 or Chromas Lite v.2.01 (http://technelysium.com.au/) and aligned using the Bioedit Sequence Alignment Editor v.7.0.9.0 (Hall, 1999). For the multicopy nuclear markers ITS and ETS, both directions of sequence reads were checked for polymorphisms. Ribotypes of two accessions showed additive polymorphisms of other species indicative of introgression. The parental ribotypes (i.e., their specific character states) were present in unequal amounts (respective peak heights in electropherograms) and were sorted into major and minor ribotypes, respectively. Polymorphisms in other samples were treated as described in Fehrer & al. (2009). ITS and ETS polymorphic sequences grouped together with major ribotypes in preliminary analyses and thus, the last were used for final nrDNA tree reconstruction.

Phylogenetic analyses were conducted using maximum parsimony and Bayesian inference applying the same parameters for each marker (ITS, ETS, *trnT-trnL*, *trnV-ndhC*, *sqs*) unless stated otherwise. The datasets at first were analyzed separately. The topologies of the individual trees were in many respects conflicting between the nuclear ribosomal and plastid data (see Electr. Suppl.: Figs. S1–S4). Therefore, only the two nrDNA and the two cpDNA datasets, respectively, were combined, concatenating ITS+ETS and *trnT-trnL+trnV-ndhC* sequences. Many samples were heterozygous for *sqs*, sometimes with strongly divergent alleles, and the *sqs* tree topology was in conflict with the other trees as well. Therefore, this dataset was also analyzed separately. Parsimony analyses were performed with PAUP* v.4.0b10 (Swofford, 2002). Heuristic searches were done with 1000 random sequence addition replicates, saving no more than 1000 trees of length greater than or equal to 1 per replicate and TBR branch swapping. Gaps were coded using simple gap coding (Simmons & Ochoterena, 2000) as implemented in SeqState v.1.4.1 (Müller, 2005). Support for internal nodes was assessed using bootstrap analyses (Felsenstein, 1978) with 1000 replicates and the same settings as above. Bayesian analyses were carried out using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). For these analyses, at first, the model best fitting the presumed molecular evolution of

the respective datasets was determined using MrModeltest v.2.3 (Nylander, 2004). The best models found under the Akaike information criterion were used: GTR+G for ITS, ETS and the combined nrDNA dataset; GTR+I for *trnV-ndhC*; GTR for *trnT-trnL*; GTR+G for the combined cpDNA dataset; and GTR+I+G for the *sqs* dataset. Two replicate analyses with four chains each were performed with the default parameters and computed for 1.5 million generations, sampling every 1000th tree. All statistical parameters indicated that convergence was reached. The first 25% of the trees per run were discarded as burn-in, and the remaining trees were summarized. Multiple sequence alignments on which Figs. 1–3 are based are provided as supplementary data.

To assess the degree of interspecific variation for all markers, mean and maximum sequence divergences were calculated with MEGA v.5 (Tamura & al., 2011) using *P*-distances. To visualize character conflict observed in the alignment of *sqs* sequences, this dataset was also subjected to Neighbor Net analysis as implemented in SplitsTree v.4.11.3 (Huson & Bryant, 2006) using the default settings.

To assess the potential of combining the largely unresolved and incongruent ITS, ETS, *trnV-ndhC*, *trnT-trnL* and *sqs* datasets for species tree inference under coalescence, we used *BEAST v.1.8.1 (Drummond & al., 2012). This analysis was reduced to the ingroup due to the difficulty in aligning outgroup sequences for the low-copy marker. Individuals for which introgression was inferred (*A. laxiflora* JC 19/2 and *A. ragusina* L. JC 2011/2) were excluded. As the analysis requires at least two individuals per species, taxa represented by only one individual were combined: *A. atlanticola* H.Lindb.

Table 1. Species diagnosability by each molecular marker used in this study.

	Molecular marker				
	ETS	ITS	*trnV-ndhC*	*trnT-trnL*	*sqs*
A. laevitomentosa (Nyár. ex Sennikov) Greuter	+	+	+	+	+
A. agardhii DC.	+	+	+	+	+
A. maroccana (Caball.) Maire	+	+	+	+	+
A. ragusina L.	+ (JC 2011/2/1 introgressed)	+ (JC 2011/2/1 introgressed)	+	+ (weak support)	+ (except introgressed JC 2011/2/1)
A. dentata Sm.	+ (weak support)	+	+ (weak support)	–	+
A. perezii M.Z.Ferreira & al.	–	–	+	+ (weak support)	+ (paraphyletic)
A. pinnatifida Aiton	+ (weak support)	–	–	–	+
A. laxiflora DC.	–	+ (JC 19/2 introgressed)	–	–	–
A. arenaria (DC.) Boiss. & Reut.	+	–	–	–	–
A. mogadorensis Coss. ex Hook.f.	– (only shared polymorphisms)	– (only shared polymorphisms)	+ (except subspecies *jahandiezii*)	–	+ (comprising one allele of *A. atlanticola* 10JZ 08/1)
A. crithmifolia Aiton	–	–	–	–	+ (comprising one allele of *A. glandulosa* ZF 233)
A. glandulosa Lam.	–	–	–	–	+ (lacking one allele of *A. glandulosa* ZF 233)
A. cossyrensis Guss.	– (only shared polymorphisms)	–	–	–	–
A. integrifolia L.	–	– (partly shared polymorphisms, comprising *A. atlanticola* 10JZ 08/1)	–	–	–
A. atlanticola H. Lindb.	–	–	–	–	–

Notes: Branches with significant support for Bayesian and maximum parsimony analysis in the individual phylogenetic analyses (Electr. Suppl.: Figs. S1–S4; Fig. 3) are represented by "+"; "weak support" refers to bootstrap values <70% and posterior probabilities <0.95; markers that fail to identify species are represented by "–"; shared polymorphisms in nrDNA: the same double peaks or length variations (shifts) occur in different samples.

was included in *A. integrifolia* of which it may be only a variant (see below), *A. mogadorensis* subsp. *jahandiezii* (Maire) M.Z.Ferreira & al. was included in *A. mogadorensis* Coss. ex Hook.f., and the two subspecies of *A. glandulosa* Lam. were not distinguished. Models of molecular evolution were determined as above for each dataset, and GTR+I was used for *trnV-ndhC* and ITS; GTR for *trnT-trnL*; HKY+G for ETS and GTR+I+G for *sqs*. We applied the following parameters to the *BEAST analysis of each dataset: Yule tree prior, uncorrelated relaxed clock using a randomly generated starting tree; 0/1s representing the coded gaps were transformed to A/Cs. Two independent MCMC analyses were run for 50 million generations, sampling every 1000th tree. To check the stabilization of the analysis, ESS values of parameters were evaluated in Tracer v.1.6 (Rambaut & al., 2014). The two independent runs were merged by LogCombiner v.1.8 and the Maximum clade credibility tree was created with a burn-in period of 25% and a posterior probability limit of 0.5 with Treeannotator (both programs are included in the *BEAST package).

■ RESULTS

Altogether, 49 individuals of 15 species of *Andryala* (Table 1) and 12–16 outgroup samples belonging to 10–11 species of closely related genera were used for each sequence dataset. Two samples, *A. laxiflora* JC 19/2 and *A. ragusina* JC 2011/2, whose ITS and/or ETS sequences showed signs of introgression, were deleted from the nrDNA combined dataset prior to final phylogenetic analyses. According to character additivity, both were introgressed by *A. integrifolia* (Electr. Suppl.: Figs. S1–S2), the most widespread and common species of *Andryala*. These putative hybrids were, however, included in the cpDNA analyses to determine their maternal origin, and also in the phylogenetic analyses based on the nuclear *sqs* marker for which they were homozygous.

Nuclear ribosomal markers showed that all *Andryala* taxa formed a well-supported monophyletic group (Fig. 1). Chloroplast DNA analyses expectedly showed haplotypes of outgroup species that belong to the *Pilosella* II lineage in basal positions

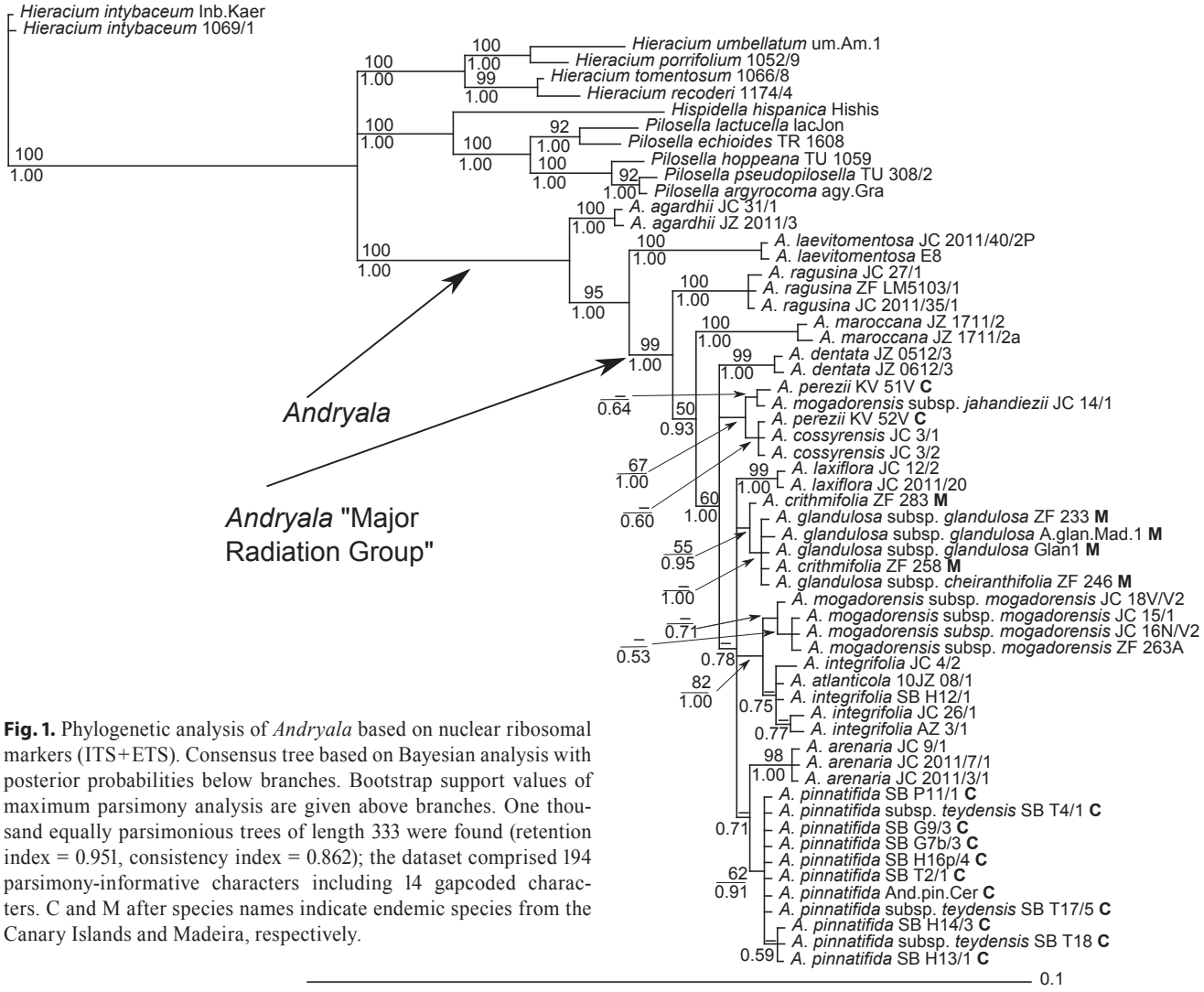

Fig. 1. Phylogenetic analysis of *Andryala* based on nuclear ribosomal markers (ITS+ETS). Consensus tree based on Bayesian analysis with posterior probabilities below branches. Bootstrap support values of maximum parsimony analysis are given above branches. One thousand equally parsimonious trees of length 333 were found (retention index = 0.951, consistency index = 0.862); the dataset comprised 194 parsimony-informative characters including 14 gapcoded characters. C and M after species names indicate endemic species from the Canary Islands and Madeira, respectively.

of the *Andryala* clade (Fig. 2), confirming a previously inferred ancestral chloroplast capture event (Fehrer & al., 2007a, b). The nrDNA and cpDNA markers revealed very low levels of sequence divergence within *Andryala* (mean/maximum *P*-distance including coded gaps: ETS, 0.9%/3.3%; ITS, 0.7%/2.2%; *trnV-ndhC*, 0.3%/2.0%; *trnT-trnL*, 0.5%/2.3%). The single-copy nuclear marker *sqs* had high genetic variation within *Andryala* (mean/maximum *P*-distance including coded gaps 3.9%/6.0%, mostly in intron regions), in contrast to the other four markers; the genus was also monophyletic according to *sqs* (Fig. 3).

All trees showed three main lineages in *Andryala*, namely the two relict species, *A. laevitomentosa* and *A. agardhii*, and a clade comprising all other taxa whose relationships were largely unresolved. Genetic variation within this clade was extremely low for the nrDNA and cpDNA markers (mean/maximum *P*-distance 0.1%–0.6%/1.0%–2.2%). We refer to these taxa as the "Major Radiation Group" (MRG) as they constitute the majority of the species and were monophyletic in all analyses. Although genetic variation in *sqs* for the MRG was relatively high (mean/maximum *P*-distance 3%/5.7%), relationships were mostly unresolved due to character conflict within the dataset (Electr. Suppl.: Fig. S5). PCR or cloning artifacts can be excluded, because most of the alleles that appeared in odd positions in the tree (Fig. 3) were from direct sequences of homozygous samples.

Within the MRG, the North African *A. maroccana* (Caball.) Maire was well distinguished from the other taxa and occurred in a basal position in all trees, along with *A. ragusina* in the nrDNA tree (Fig. 1). No other interspecific relationships within the MRG were supported (considering only bootstrap support [BS] of ≥70% and posterior probabilities [PP] of ≥0.95 as well-supported; Hillis & Bull, 1993; Larget & Simon, 1999) with the exception of a group consisting of *A. integrifolia*, *A. atlanticola* and *A. mogadorensis* subsp. *mogadorensis* (without *A. mogadorensis* subsp. *jahandiezii*) in the nrDNA tree (PP = 1.00, BS = 82%, Fig. 1). A group comprising *A. cossyrensis* Guss., *A. perezii* M.Z.Ferreira & al. and *A. mogadorensis* subsp. *jahandiezii* (North Africa and eastern Canary Islands)

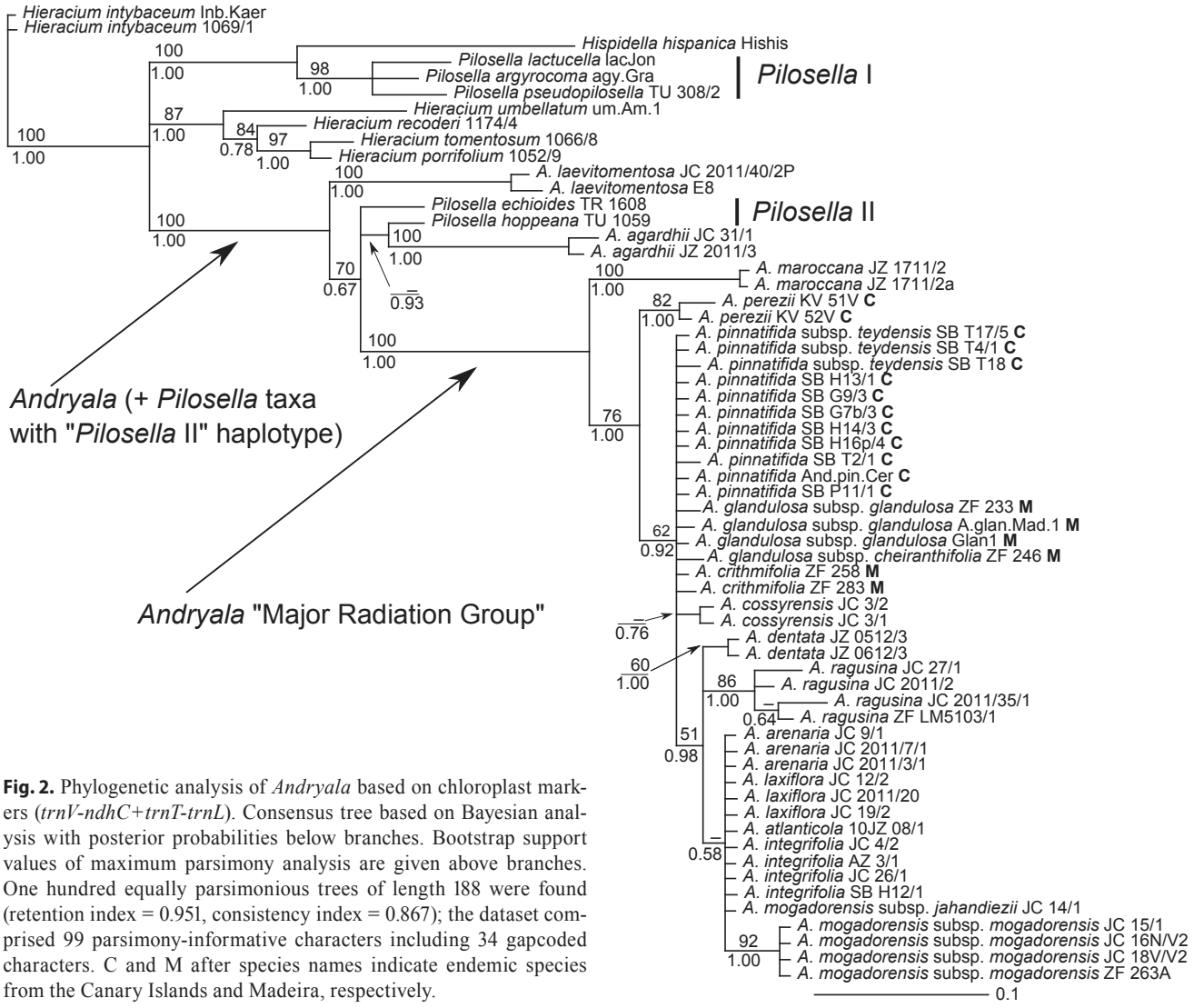

Fig. 2. Phylogenetic analysis of *Andryala* based on chloroplast markers (*trnV-ndhC+trnT-trnL*). Consensus tree based on Bayesian analysis with posterior probabilities below branches. Bootstrap support values of maximum parsimony analysis are given above branches. One hundred equally parsimonious trees of length 188 were found (retention index = 0.951, consistency index = 0.867); the dataset comprised 99 parsimony-informative characters including 34 gapcoded characters. C and M after species names indicate endemic species from the Canary Islands and Madeira, respectively.

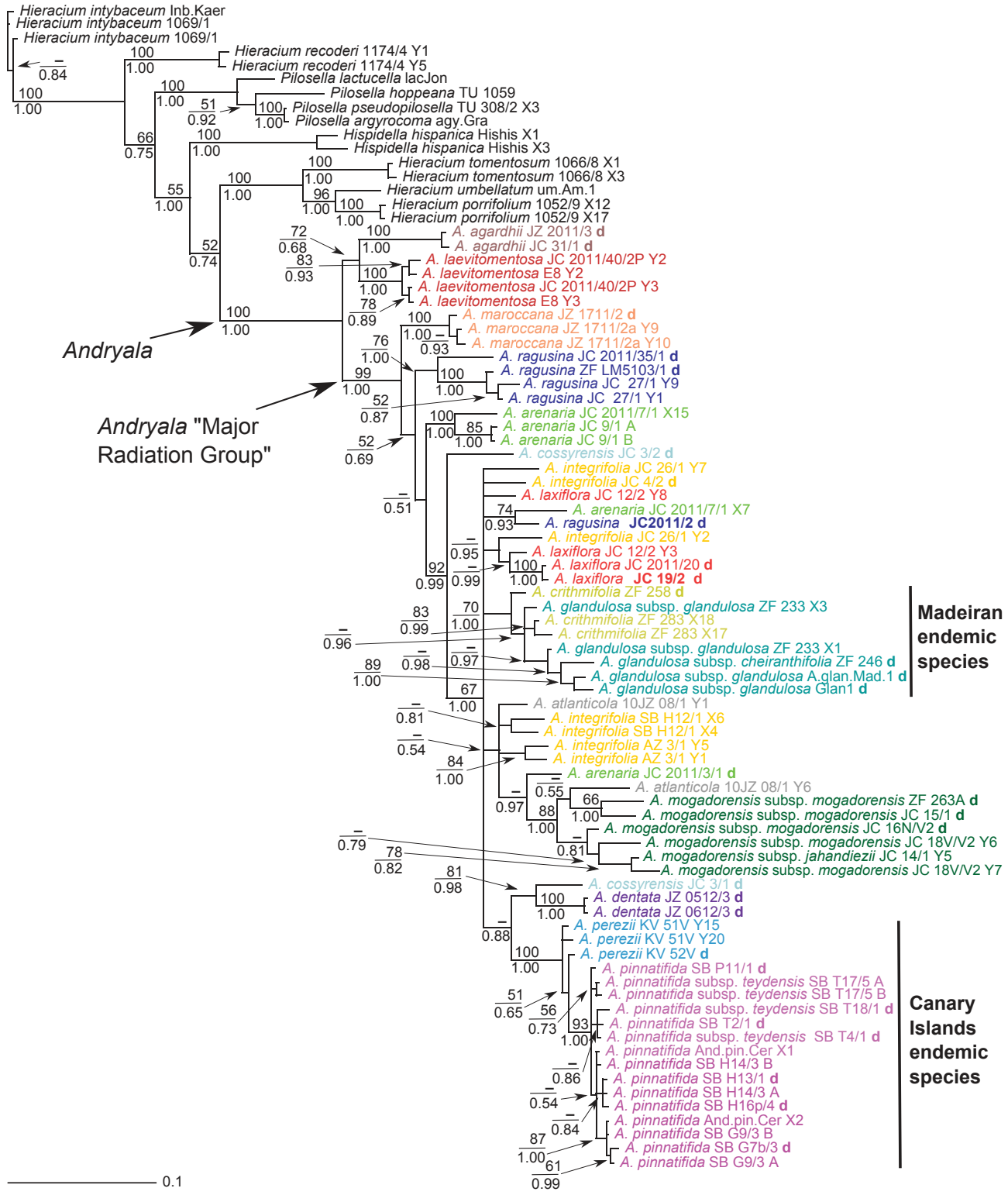

Fig. 3. Phylogenetic analysis of *Andryala* based on the single-copy nuclear marker *sqs*. Consensus tree based on Bayesian analysis with posterior probabilities below branches. Bootstrap support values of maximum parsimony analysis are given above branches. Four hundred equally parsimonious trees of length 1372 were found (retention index = 0.823, consistency index = 0.532); the dataset comprised 474 parsimony-informative characters including 169 gapcoded characters. Heterozygous individuals are represented by clones (X or Y plus number) or by alleles (A and B) if they differed only by a single polymorphism; direct sequences (i.e., homozygous individuals) are labeled with "d" after the taxon identifier. The direct sequences of the introgressed samples *A. ragusina* JC 2011/2 and *A. laxiflora* JC 19/2 are shown in bold. Colours of *Andryala* species are for easier visualization of the taxa.

(PP = 1.00, BS = 67%, Fig. 1) and a branch formed by *A. crithmifolia* Aiton and *A. glandulosa* from Madeira (PP = 0.95, BS = 55%, Fig. 1) were well-supported only in the Bayesian analysis of this dataset. The only well-supported group in the cpDNA data (also Bayesian analysis only) comprised the predominantly Iberian taxa *A. ragusina*, *A. laxiflora* and *A. arenaria* (DC.) Boiss. & Reut., the eastern Mediterranean *A. dentata* Sm., the widespread *A. integrifolia* and the North African taxa *A. atlanticola* and *A. mogadorensis* including both subspecies (PP = 0.98, BS = 51%, Fig. 2). According to the *sqs* tree (Fig. 3), many taxa within the MRG were not monophyletic (e.g., *A. arenaria*, *A. cossyrensis*). In addition, some of the samples that were heterozygous for this marker showed two strongly divergent alleles (e.g., *A. atlanticola*, *A. arenaria* JC 2011/7/1, *A. integrifolia* JC 26/1, *A. laxiflora* JC 12/2, *A. glandulosa* ZF 233). Consequently, species relationships within this group remain unclear with two notable exceptions: the Madeiran endemics *A. crithmifolia* and *A. glandulosa* (PP = 1.00, BS = 70%) and also the Canary Island endemics *A. perezii* and *A. pinnatifida* Aiton (PP = 1.00, BS = 100%) formed two well-supported groups. Of these, only the alleles of the accessions of *A. pinnatifida* were monophyletic.

Despite the failure to resolve species relationships in *Andryala*, individuals assigned morphologically to the same taxon often formed recognizable entities with one or several markers (Table 1). The potential of different markers to show species-specific features varied: the most divergent species *A. laevitomentosa*, *A. agardhii* and *A. maroccana* were unequivocally distinguishable with all markers; *A. ragusina* and *A. dentata* were distinguished by most markers, *A. perezii*, *A. pinnatifida*, *A. laxiflora* and *A. arenaria* by one or few markers. Some species, however, showed only shared polymorphisms or their accessions were not monophyletic (*A. mogadorensis*, *A. crithmifolia*, *A. glandulosa*, *A. cossyrensis*, *A. integrifolia*); one species did not show any diagnostic feature with any of the markers (*A. atlanticola*).

Species tree inference under coalescence based on all five datasets also resulted in mostly unresolved relationships. As before, *A. agardhii* and *A. laevitomentosa* formed separate lineages and *A. maroccana* occurred in an early branching position within the MRG (Fig. 4). The backbone of the rest of that group was again unsupported. The only species relationships within the MRG that were supported were between the Madeiran species *A. glandulosa* and *A. crithmifolia* as already found in the nuclear datasets (Figs. 1, 3).

■ DISCUSSION

Species relationships of the little-known Mediterranean-Macaronesian genus *Andryala* (Asteraceae), the exclusively sexually reproducing sister genus of the predominantly apomictic *Hieracium* and *Pilosella*, were investigated here for the first time with molecular markers, using a multigene approach based on two nuclear ribosomal regions, two chloroplast intergenic spacers and one single-copy nuclear gene marker. The most comprehensive and representative sampling possible was used to trace the evolutionary history of *Andryala*.

***Andryala* is a well-defined genus. —** Phylogenetic analyses of the nuclear markers showed that *Andryala* forms a well-supported monophyletic group (PP = 1.00, BS = 100%, Figs. 1, 3). However, cpDNA haplotypes of some species of

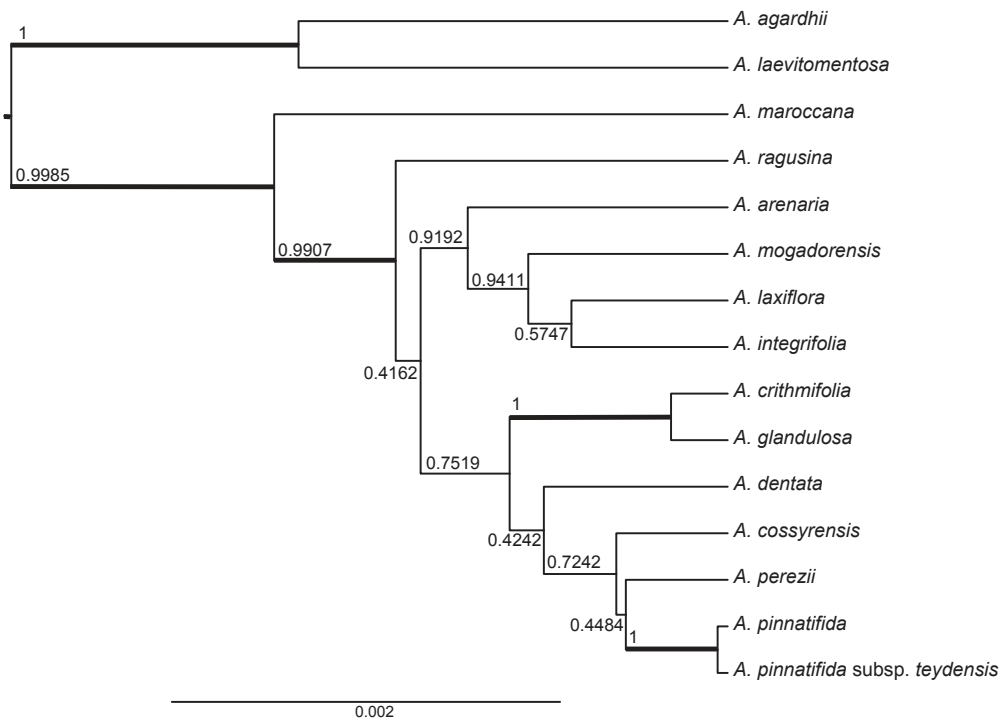

Fig. 4. Phylogenetic analysis of *Andryala* based on coalescence analysis of five datasets. Consensus tree based on Bayesian analysis as implemented in *BEAST with posterior probabilities at branches. Well-supported branches are shown in bold.

Pilosella were nested in *Andryala* (Fig. 2). This is the result of ancient wide hybridization as suggested by a previous molecular study that included only few species of *Andryala* (Fehrer & al., 2007a). The monophyly of *Andryala* (except in the cpDNA analysis) along with the non-monophyly of the two basal-most species, *A. agardhii* and *A. laevitomentosa*, contradicts the taxonomic placement of these two species in the genus *Pietrosia* as proposed by Sennikov (1999), although morphologically they are more similar to each other than to the remaining *Andryala* species (Sell, 1975, 1976). Thus, our data support the inclusion of these two species in *Andryala*, as recently suggested by Greuter (2003). Both *A. agardhii* and *A. laevitomentosa* can be regarded as relict species; they branched off earliest in the history of the genus, and sufficient time has elapsed for molecular divergence to occur. Likewise, our molecular data also strongly support the inclusion of the North African *A. maroccana* in *Andryala*, and not in a separate genus, *Paua*, as done by Caballero (1916). *Andryala maroccana* is morphologically more similar to *A. laevitomentosa* and *A. agardhii* than to the rest of *Andryala* in habit and cypsela morphology. Indeed, *A. maroccana* shares some features with *A. agardhii* and *A. laevitomentosa* (e.g., woody branched stock, covered with persistent bases of leaf petioles; several stems each usually bearing only one capitulum). Sell (1975) also highlighted the similarity of *A. agardhii* and *A. laevitomentosa* to North African species of *Andryala*, referring most likely to *A. maroccana*. This Moroccan-Algerian species branched off later than *A. agardhii* and *A. laevitomentosa*. It can probably be considered as a potential relict, as it seems to represent a relatively old lineage according to all molecular markers employed here (Fig. 4). Both *A. agardhii* and *A. laevitomentosa* are endangered species confined to mountain summits with similar ecological conditions. *Andryala agardhii* grows on calcareous rocky soils, screes and limestone-dolomite sands, and sometimes in rock crevices, at altitudes between 1600 and 3400 m (Jahandiez & Maire, 1934; Emberger & Maire, 1941; Blanca & al., 2001), while *A. laevitomentosa* occurs on metamorphic rocky alpine grasslands as well as in soil pockets on steep slopes or vertical cliffs, at 1600–1700 m (Lucas & Synge, 1978; Negrea & Pricop, 2009). Conversely, *A. maroccana* dwells on coastal sands as well as on steep quartzite sea cliffs (Caballero, 1916; Doumergue, 1921).

The major radiation of *Andryala*. — Despite high morphological and ecological diversity, all species of *Andryala*, except for *A. laevitomentosa*, *A. agardhii*, and, to a lower extent, also *A. maroccana*, showed very low nucleotide sequence divergence in all nuclear ribosomal and chloroplast datasets. Consequently, species relationships of the *Andryala* taxa designated as the "Major Radiation Group" remained almost completely unresolved (Figs. 1–2). Given that the nrDNA and cpDNA markers consisted mostly of non-coding sequences that are supposed to provide reasonably good resolution of interspecific relationships (Baldwin & Markos, 1998; Shaw & al., 2007), their extremely low overall genetic variation suggests that the majority of the *Andryala* taxa have undergone a very recent speciation. Despite higher interspecific sequence variation of *sqs*, the structure of the *sqs* tree (Fig. 3) revealed an almost complete lack of support of the basal nodes, which is consistent with a rapid divergence of the respective taxa (Fehrer, 1996; Stanley & al., 2011). The same lack of resolution was obtained using the five combined datasets in a coalescent approach (Fig. 4).

Similarly, the Mediterranean-Macaronesian *Cheirolophus* (Asteraceae: Cynareae) originated and radiated recently, an inference which was also based on low levels of nucleotide divergence (Garnatje & al., 2007). Unresolved phylogenetic groupings are suggestive of rapid diversification, as shown in the Mediterranean species of *Senecio* L. sect. *Senecio* (Asteraceae: Senecioneae; Comes & Abbott, 2001). These results are also consistent with molecular phylogenetic studies on Macaronesian genera of tribe Cichorieae (Asteraceae) such as the *Sonchus* L. alliance (Kim & al., 1996, 1999) and *Tolpis* Adans. (Gruenstaeudl & al., 2013). Likewise, the silversword alliance (Asteraceae: Madiinae) from Hawaii, as well as *Tetramolopium* Nees from the Hawaiian and Cook Islands, showed little genetic differentiation in spite of phenotypic and ecological diversity, supporting the hypothesis of a recent origin and rapid diversification on these islands (Baldwin & al., 1990; Okada & al., 1997). Thus, the example of *Andryala* fits the often observed evolutionary pattern of recent radiations that are characterized by a combination of low genetic diversity and large morphological differentiation.

Molecular evidence for introgressions. — Two samples, *A. laxiflora* JC 19/2 and *A. ragusina* JC 2011/2, showed additive patterns in nrDNA sequences that suggested a contribution of *A. integrifolia*. In the few *Andryala* hybrids described until now on the basis of morphology, *A. integrifolia* has repeatedly been reported as one of the parental species (e.g., Maire, 1937; García Adá, 1992). Indeed, re-inspection of cultivated plants or their herbarium vouchers revealed some morphological evidence of introgression that would have been overlooked without the molecular data. For *A. laxiflora* JC 19/2, only ITS indicated the influence of a second species (Table 1). The *A. integrifolia*–specific character states were consistently present only as smaller peaks at additive positions in sequence electropherograms, corresponding to the minor ribotype (PCRs were repeated in triplicate and pooled to ensure that this did not result from amplification bias), which may, along with its closer resemblance to *A. laxiflora*, indicate a later generation backcross. In contrast, the major ITS and ETS ribotypes of *A. ragusina* JC 2011/2 did not belong to this species, but to *A. integrifolia* (as indicated by ITS; ETS was equivocal due to a lack of species-specific characters). The nuclear *sqs* gene was homozygous in this sample and grouped near *A. integrifolia*, i.e., the sample had apparently lost its *A. ragusina*–specific allele. However, the chloroplast DNA belonged to *A. ragusina*. Thus, this sample may also represent a later generation backcross to *A. integrifolia*. The contrasting influence of hybridizations on various molecular markers observed in these two cases suggests an apparently random loss of at least some genetic evidence of these hybridization events. Differences in intraspecific genome size of *A. ragusina* of up to 50% (the usual level of intraspecific variation in other species of *Andryala* is below 5%; Zahradníček & al., unpublished data) may indicate

that even morphologically well-defined species of *Andryala* (see also below) could have a complex past whose genetic and morphological traces may be largely erased.

Character conflict within and among datasets. — The nrDNA (Fig. 1) and cpDNA (Fig. 2) data resulted in an almost complete lack of resolution within the MRG, indicating stochastic variation because of low levels of genetic divergence of these markers. This is most probably the reason why weakly supported relationships do not correspond well between nuclear ribosomal and chloroplast data (e.g., the positions of *A. dentata*, *A. perezii*, or *A. ragusina*). Theoretically, also hybridization could be responsible for incongruence between these datasets. However, apart from the two individuals of *Andryala* discussed above and the chloroplast capture event involving *Pilosella*, there is no further indication of additional cases of introgression (such as, for example, individuals of a particular species with the cpDNA of another species). The character conflict in the *sqs* data (visualized in Electr. Suppl.: Fig. S5) is not caused by too low variation, however, but by a high degree of homoplasy as observed in the multiple sequence alignment (see alignment file in the online supplementary data and explanations therein). Patterns in the MRG across all trees and a failure of individual or all markers to identify even a morphologically unambiguous species such as *A. integrifolia* (Table 1) suggest that taxa in the MRG are not yet well differentiated genetically. Thus, the taxa that seem to have evolved very recently may be situated between a process of differentiation at the population level (characterized by a reticulate pattern of relationships) and a completed speciation process with reproductive or at least geographic isolation (characterized by bifurcating patterns). Both, incomplete lineage sorting and hybridization are typical phenomena at this level (Comes & Abbott, 2001; Trewick & al., 2004; Richardson & al., 2012; Vitales & al., 2014). The lack of resolution at the backbone of the MRG in the species tree inferred under coalescence (Fig. 4) can also be interpreted in terms of incomplete lineage sorting (Degnan & Rosenberg, 2009; Wielstra & al., 2014). As hybrids age they become historical genome mosaics, therefore, it is almost impossible to distinguish (ancient) hybridization from ancestral polymorphism with deep coalescense. In addition, the ease with which alleles of a single-copy marker can get lost in cases of introgression that are still traceable with nuclear ribosomal markers, as discussed above, may suggest that at least partly unrecognized hybridization events that have occurred after speciation (i.e., much more recently) could be responsible for alleles that occur in odd positions within the MRG (Fig. 3). These findings also confirm the susceptibility of this kind of marker to population genetic processes (Sang, 2002). While this is a handicap for the establishment of species relationships, it does allow the inference of gene drift and population bottlenecks as will be shown in the next section.

Macaronesian colonization and insular speciation. — The nuclear *sqs* gene, specifically developed as a low-copy marker for phylogenetic studies at low taxonomic level in Asteraceae (Krak & al., 2012), showed the highest variation among the markers employed. It revealed sister relationships and monophyly for the two pairs of endemic species occurring on the Canary Islands and Madeira, respectively (Fig. 3). This can be interpreted as evidence for single colonization events by the respective ancestors, followed by insular speciation. Due to the lack of tree node resolution, the continental sister species could not be identified. In Madeira, geographic separation may have facilitated speciation of *A. glandulosa* and *A. crithmifolia*. Indeed, *A. glandulosa* subsp. *glandulosa* occurs mainly along the northern coast of Madeira Island, Porto Santo and Desertas Islands and *A. glandulosa* subsp. *cheiranthifolia* (L'Hér.) Greuter grows almost everywhere in Madeira Island, chiefly in inland sites, whereas *A. crithmifolia* is found in only a few sites along the southern coast of Madeira Island (Press, 1994; Ferreira & al., in press). The occurrence of one allele of the heterozygous sample *A. glandulosa* subsp. *glandulosa* ZF 233 among *A. crithmifolia* sequences (Fig. 3) can be either explained by introgression or by incomplete lineage sorting. Similarly, the Canary Island endemic *A. perezii*, a recently described species (Ferreira & al., 2014a), is confined to the easternmost islands Lanzarote and Fuerteventura that are closest to the African Atlantic coast (Fuerteventura is only 100 km from the African continent), whereas *A. pinnatifida* occurs mostly on the central and western islands (Gran Canaria, Tenerife, La Palma, La Gomera, El Hierro). The *sqs* alleles of *A. perezii* are paraphyletic (Fig. 3) with *A. pinnatifida* sequences appearing as a single well-supported lineage emerging basal to these. This is consistent with an ancestor that may have colonized the Canaries from the nearby continent, followed by diversification proceeding from East to West.

There are several well-documented examples of biogeographic connections between northwest Africa and Macaronesia. Molecular studies unequivocally identified northwestern Africa as the likely place of origin of Canarian *Lotus* L. (Fabaceae; Allan & al., 2004) and supported a recent colonization of the Macaronesian islands by the *Asteriscus* (L.) Less. alliance (Asteraceae) from northern Africa (Francisco-Ortega & al., 1999). Another interesting example is *Tolpis* (distributed in Macaronesia, Mediterranean Europe and North Africa), which originated from at least three independent dispersal events from the European and North African mainland to the islands (Gruenstaeudl & al., 2013), in which the north–east trade winds seem to have played an important role (Moore & al., 2002; Gruenstaeudl & al., 2013). It seems possible that in *Andryala* the seamount archipelagos between continental areas and Macaronesia emerged during glacial periods (i.e., Palaeo-Macaronesia, according to Fernández-Palacios & al., 2011) could have served as colonization stepping stones in the dispersal process, since the deciduous pappus does not seem suitable for long-distance wind dispersal.

The populations of Madeiran *A. glandulosa* and Canarian *A. pinnatifida* exhibit high morphological variation, which can be explained by the considerable habitat diversity on oceanic islands, created by topology and humidity gradients, which, combined with their isolation, results in lower competition and empty ecological niches. This provides a template for the evolution of species radiations (Juan & al., 2000).

The most widely distributed species, *A. integrifolia*, also occurs on Macaronesian islands. It is considered introduced

in Madeira and the Azores (Silva & al., 2005; Ferreira & al., 2011), but in the Canary Islands, it was thought to be probably native (Acebes Ginovés & al., 2010). Kunkel (1980) considered the species as originally Mediterranean, and probably introduced to the Canary Islands El Hierro and Gran Canaria, and the species has very recently been recorded for La Palma (Santos-Guerra & al., 2013). However, our results suggest a neophyte status of *A. integrifolia* also for the Canary Islands: a sample from El Hierro (SB H12/1) was most similar to plants of that species from the Iberian Peninsula and northern Africa and did not group with the Canary Island endemic species with any of the markers. As in Madeira, it can be found growing in roadside communities, as well as abandoned fields and pastures, and it has never been seen in more or less natural habitats (S. Bräutigam, pers. comm.). The occurrence of this species in habitats with strong anthropogenic influence corroborates the notion of its neophyte nature.

Species delimitation and taxonomy of *Andryala*. — Despite the low sequence divergence of the nrDNA and cpDNA markers and the extensive homoplasy of the *sqs* gene for taxa in the MRG, all five datasets showed some resolution at species level (summarized in Table 1). In all phylogenetic trees, both accessions of *A. maroccana* grouped together (PP = 1.00, BS = 100%, Fig. 1–3). Likewise, the three non-hybrid accessions of *A. ragusina* formed a strongly supported group (PP = 1.00, BS = 76%–100%, Figs. 1–3), which is in agreement with morphological data. Indeed, both are morphologically very distinct and generally accepted species.

The capacity of the markers to recognize further taxonomic entities within this group varied strongly. A good example for this is *A. integrifolia* and morphologically similar Mediterranean taxa with rather confusing taxonomy and unresolved nomenclature (Table 2; Electr. Suppl.: Table S1): *A. cossyrensis*, *A. arenaria*, and *A. dentata*. Accessions of *A. dentata* and *A. laxiflora* are well supported in the nrDNA tree (both PP = 1.00, BS = 99%, Fig. 1), in the latter case also supported by consistent diagnostic morphological characters. Indeed, *A. laxiflora* can easily be identified and is accepted at species level in the majority of recent Floras (e.g., Coutinho, 1939; Nègre, 1962; Pottier-Alapetite, 1981; Talavera, 1987; Blanca, 2009, 2011); it was even placed in a separate genus, *Rothia*, by earlier authors (e.g., Gaertner, 1791; Schreber, 1791; Roth, 1797). The same cannot be said regarding *A. dentata*, *A. cossyrensis*, and *A. arenaria*. The taxonomic delimitation of *A. dentata* has been very unclear; several authors suggested inclusion in *A. integrifolia* (Davis, 1975; Sell, 1976; Mouterde, 1983; Tohmé & Tohmé, 2007). Although *A. integrifolia* is a very common Mediterranean species, it is replaced by *A. dentata* in the East Mediterranean (Table 3). The contact zone of the two species is located in continental Italy and on Sicily and Pantelleria (Ferreira, unpub. results). Nonetheless, *A. dentata* is morphologically distinct from *A. integrifolia* (Table 2), and the present phylogenetic study supports this taxonomic point of view since *A. dentata* did not group with any *Andryala* species, including *A. integrifolia*, and was distinguished by most markers (Table 1). Similarly, accessions of *A. arenaria* are strongly supported in the nrDNA tree (PP = 1.00, BS = 98%, Fig. 1), but not by other markers (Table 1). *Andryala arenaria* has either been treated as a distinct species (Amo y Mora, 1872; Coutinho, 1939; Talavera, 1987; Blanca, 2009, 2011) or included in *A. integrifolia* (Ball, 1878; Barratte, 1896; Sell, 1976; Greuter, 2006–). In addition to the present molecular results (Fig. 1), its distinctive morphological features (Table 2) and a well-defined geographical range (distribution center in sandy habitats of the southwestern Iberian Peninsula and, to a lesser extent, in northwestern Africa) seem to justify

Table 2. Species distinction based on morphological features.

Species	Diagnostic characters
A. integrifolia L.	Upper leaves ovate-lanceolate or lanceolate, base rounded to cuneate Involucre usually stellate-tomentose All involucral bracts flat, not enfolding a floret Receptacle with usually long setae (1.7–)2–4.7 mm Ligules usually pale yellow, greatly exceeding involucral bracts
A. dentata Sm.	Upper leaves ovate-oblong to ovate-lanceolate, base rounded or subcordate Involucre slightly stellate-tomentose External involucral bracts involute, enfolding a floret, and purplish at the apex Receptacle with short setae 0.4–2 mm Ligules pale yellow, slightly exceeding involucral bracts
A. arenaria (DC.) Boiss. & Reut.	Upper leaves usually ovate-oblong, base cordate Involucre densely stellate-tomentose External involucral bracts involute, enfolding a floret Receptacle with usually short setae 0.3–2(–2.7) mm Ligules golden yellow, greatly exceeding involucral bracts
A. cossyrensis Guss.	Upper leaves narrowly ovate-lanceolate or ± linear-lanceolate, base rounded or rarely cordate Involucre slightly stellate-tomentose External involucral bracts involute, enfolding a floret, sometimes purplish at the apex Receptacle with usually short setae 0.8–2.5(–3) mm Ligules golden yellow, greatly exceeding involucral bracts

Note: Only species with traditionally controversial taxonomic delimitation are compared.

species status. On the other hand, *A. cossyrensis* showed almost no distinct molecular features (Table 1). Following our concept, it is a morphologically distinct species (Table 2), occurring predominantly in northwest Africa and on Sicily and Pantelleria, of which the latter island is situated only 60 km east of the Tunisian coast. In summary, our results show that *A. integrifolia*, *A. laxiflora*, *A. arenaria*, *A. cossyrensis*, and *A. dentata* do not form a monophyletic group and that the evaluation of the latter three at subspecific level proposed by some authors (e.g., Emberger & Maire, 1941; Pignatti, 1982) is not supported. Furthermore, their morphological distinctness (Table 2) and distinct distributions (Table 3) provide further support for their species status.

In all phylogenetic trees (Figs. 1–3) there was no resolution within *A. integrifolia*. The ancestor of the MRG may have survived the last glacial and then rapidly colonized the entire Mediterranean Basin, diversifying into several species, including *A. integrifolia*. This extremely polymorphic species seems to have successfully occupied different habitats, currently occurring in almost the entire distribution area of the genus (Table 3). In addition, there are several records of new species published in the past which actually correspond

Table 3. Geographical distribution of taxa used in this study.

Taxon	Distribution	Biogeographic region
Andryala agardhii DC.	southern Spain (few mountains of the Baetic System), Morocco (Atlas Mts., very rare)	CD
A. arenaria (DC.) Boiss. & Reut	mainly Iberian Peninsula, Morocco and Algeria	CD
A. cossyrensis Guss.	SW Italy (incl. Sicily and Pantelleria islands), Tunisia, Algeria and Morocco	DF[a]
A. crithmifolia Aiton	Madeira (southern coast, rare)	A
A. dentata Sm.	S and SW Italy (incl. Sicily and Pantelleria islands), S and E Greece, W Turkey and Lebanon (disjunct occurrences, rare)	F
A. glandulosa Lam. subsp. *glandulosa*	Madeira (mainly northern coast), Porto Santo and Desertas Islands	A
A. glandulosa subsp. *cheiranthifolia* (L'Hér.) Greuter	Madeira (mainly inland)	A
A. integrifolia L. (incl. *A. atlanticola* H.Lindb.)	Iberian Peninsula, France, Italy (incl. Sardinia and Sicily islands), Morocco, Algeria, Tunisia, Canary Islands, Madeira, Azores	CDEF[a,b]
A. laevitomentosa (Nyár. ex Sennikov) Greuter	Romanian Eastern Carpathians, very rare	G
A. laxiflora DC.	mainly Iberian Peninsula, Morocco, Algeria, Tunisia, Canary Islands (Tenerife)	CD[c]
A. maroccana (Caball.) Maire	Morocco and Algeria, very rare	D
A. mogadorensis Hook.f. subsp. *mogadorensis*	Morocco (between the regions of Grand Casablanca and Souss-Massa-Drâa)	E
A. mogadorensis subsp. *jahandiezii* (Maire) M.Z.Ferreira & al.	Morocco (Souss-Massa-Drâa Region)	E
A. perezii M.Z.Ferreira & al.	eastern Canary Islands (Fuerteventura, Lanzarote)	B
A. pinnatifida Aiton (excl. subsp. *teydensis*)	central and western Canary Islands (Gran Canaria, Tenerife, La Palma, La Gomera, El Hierro)	B
A. pinnatifida subsp. *teydensis* (Sch.Bip.) Rivas Mart. & al.	Canary Islands (Tenerife)	B
A. ragusina L.	Iberian Peninsula, southernmost France, Balearic Islands, (Algeria and Tunisia)[d]	C(D)[d]

Biogeographic regions: A, Madeira; B, Canary Islands; C, Mediterranean SW Europe (mainly Iberian Peninsula); D, Mediterranean N Africa; E, Atlantic NW Africa; F, Central and NE Mediterranean Basin; G, Romanian Eastern Carpathians.
[a] excluding the NE Mediterranean Basin; [b] neophytic occurrences on the Canary Islands, Madeira and the Azores; [c] neophytic occurrences in the Canary Islands; [d] the very rare *A. spartioides* (Pomel ex Batt. & Trab.) Barratte (not included here) occurring in Algeria and Tunisia might be a synonym of *A. ragusina* L.

to putative varieties of *A. integrifolia* (e.g., Coutinho, 1939; Dobignard, 2009). This seems to be also the case for *A. atlanticola* which morphologically resembles *A. integrifolia* when considering taxonomically important reproductive characters such as involucral bract convolution, receptacle indumentum, and ligule colour (Table 2).

Accessions of the northwest African *A. mogadorensis* form a well-supported clade in the cpDNA tree (PP = 1.00, BS = 92%, Fig. 2) and are clearly distinct from the Canarian *A. pinnatifida* according to all markers (Figs. 1–3). Although *A. mogadorensis* was traditionally recognized as a subspecies of *A. pinnatifida* (Jahandiez & Maire, 1934; Greuter, 2003), molecular data of the present study suggest to rank it at species level, which is in agreement with a recent morphological re-evaluation (Ferreira & al., 2014b).

Regarding the Macaronesian species, although the Canarian *A. perezii* was mistaken for *A. glandulosa* by earlier authors (e.g., Kunkel, 1980; Bramwell & Bramwell, 2001) and later recognized as a subspecies of *A. pinnatifida* (Greuter & Raab-Straube, 2009), morphological data support the distinction of these three taxa at species level (Ferreira & al., 2014a), which is also in accordance with the present molecular data. As for the Madeiran *A. glandulosa* and *A. crithmifolia*, although the molecular markers did not separate them completely (see above), these are morphologically distinct species with well-defined distribution areas (Menezes, 1914; Press, 1994; Ferreira & al., in press).

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Appendix 1. Accessions used for the molecular analyses and GenBank accession numbers.

Taxon name and authority, sample identifier: country: voucher collection records, altitude, *collector's name*, *collection number* (herbarium acronym), ITS accession number(s) / ETS accession number(s) / *trnT-trnL* accession number / *trnV-ndhC* accession number / *sqs* accession number(s) [sequences new for this study are marked by an asterisk].

Andryala agardhii DC., **JZ 2011/3:** Spain: Andalusia, province Jaén, Sierra Mágina, road to Pico Mágina, 1806 m, *Zahradníček & al.*, *1937/11* (PRA), KM372007*, KM372008* / KM371903*, KM371904* / KM371732* / KM386663* / KM371831*; **JC 2011/31/1:** Spain: Andalusia, province Granada, Sierra Baza, Calar del Desabezedo, 1195 m, *Chrtek & Dočkalová*, *1924/11* (PRA), KM372009*, KM372010* / KM371905*, KM371906* / KM371733* / KM371781* / KM371832*; ***A. arenaria*** (DC.) Boiss. & Reut., **JC 2011/9/1:** Spain: Andalusia, province Huelva, Almonte, 55 m, *Chrtek & Dočkalová*, *1931/11* (PRA), KM372011*, KM372012* / KM371907*, KM371908* / KM371734* / KM371782* / KM371833*, KM371898*; **JC 2011/3/1:** Spain: Andalusia, province Málaga, Artola near Marbella, 25 m, *Chrtek & Dočkalová*, *1929/11* (PRA), KM372013*, KM372014* / KM371909*, KM371910* / KM371735* / KM371783* / KM371834*; **JC 2011/7/1:** Spain: Andalusia, province Cádiz, Bornos, 237 m, *Chrtek & Dočkalová*, *1932/11* (PRA), KM372015*, KM372016* / KM371911*, KM371912* / KM371736* / KM371784* / KM371835*, KM371836*; ***A. atlanticola*** H.Lindb., **10JZ 08/1:** Morocco: region Marrakech-Tensift-El-Haouz, High Atlas, Imlil, 1960 m, *J. Zahradníček, 2012/10* (PRA), KM372017*, KM372018* / KM371913*, KM371914* / KM371737* / KM371785* / KM371837*, KM371838*; ***A. cossyrensis*** Guss., **JC 3/1:** Morocco: region Oriental, province Berkane, Berkane, Zegzal (Zegzel) valley, 260 m, *Chrtek & Dočkalová, 2016/10* (PRA), KM372019*, KM372020* / KM371915*, KM371916* / KM371738* / KM371786* / KM371839*; **JC 3/2:** Morocco: region Oriental, province Berkane, Berkane, Zegzal (Zegzel) valley, 260 m, *Chrtek & Dočkalová, 2017/10* (PRA), KM372021*, KM372022* / KM371917*, KM371918* / KM371739* / KM371787* / KM371840*; ***A. crithmifolia*** Aiton, **ZF 258:** Portugal: Madeira, Cabo Girão, base of the sea cliff, *Ferreira & Ferreira,* 258 (MA), KM372023*, KM372024* / KM371919*, KM371920* / KM371740* / KM371788* / KM371841*; **ZF 283:** Portugal: Madeira, São Gonçalo, Pináculo, 238 m, *Sequeira & Ferreira, 283* (MA), KM372025*, KM372026* / KM371921*, KM371922* / KM371741* / KM371789* / KM371842*, KM371843*; ***A. dentata*** Sm., **JZ 0512/3:** Italy: Isola di Pantelleria, Tracimo, 220 m, *Zahradníček & Chrtek, 689/12* (PRA), KM372027*, KM372028* / KM371923*, KM371924* / KM371742* / KM371790* / KM371844*; **JZ 0612/3:** Italy: Isola di Pantelleria, Siba-Roncone, 450 m, *Zahradníček & Chrtek, 691/12* (PRA), KM372029*, KM372030* / KM371925*, KM371926* / KM371743* / KM371791* / KM371845*; ***A. glandulosa*** Lam. subsp. ***glandulosa*****, A.glan.Mad.1:** Portugal: Madeira, Ponta do Pargo, ca. 312 m, *Bräutigam*, *148659* (GLM), KM372033*, KM372034* / KM371929*, KM371930* / AY573356 / KM371793* / KM371847*; **Glan1 (ZF 292):** Portugal: Madeira, Porto Moniz, 263 m, *Ferreira*, *292* (MA), KM372035*, KM372036* / KM371931*, KM371932* / KM371745* / KM371794* / KM371848*; **ZF 233:** Portugal: Madeira, Seixal, ca. 42 m, *Ferreira*, *233* (MA), KM372037*, KM372038* / KM371933*, KM371934* / KM371746* / KM371795* / KM371849*, KM371850*; ***A. glandulosa*** subsp. ***cheiranthifolia*** (L'Hér.) Greuter, **ZF 246:** Portugal: Madeira, Pico do Areeiro, 1542 m, *Ferreira & al., 246* (MA), KM372031*, KM372032* / KM371927*, KM371928* / KM371744* / KM371792* / KM371846*; ***A. integrifolia*** L., **JC 4/2:** Morocco: region Oriental, province Nador, Beni Chiker (Beni Sikar), 140 m, *Chrtek & Dočkalová*, *2020/10* (PRA), KM372039*, KM372040* / KM371935*, KM371936* / KM371747* / KM371796* / KM371851*; **SB H12/1:** Spain: Canary Islands, El Hierro, Valverde near Tinor, 980 m, *Bräutigam & Bräutigam*, *2046/10* (PRA), KM372041*, KM372042* / KM371937*, KM371938* / KM371748* / KM371797* / KM371852*, KM371853*; **JC 26/1:** Spain: Andalusia, province Granada, Guadix, 1075 m, *Chrtek & Dočkalová*, *1928/11* (PRA),

Appendix 1. Continued.

KM372043*, KM372044* / KM371939*, KM371940* / KM371749* / KM371798* / KM371854*, KM371855*; **AZ 3/1:** Algeria: Algiers, Kouba town district, 90 m, *Abida Zeddam*, *678/12* (PRA), KM372045*, KM372046* / KM371941*, KM371942* / KM371750* / KM371799* / KM371856*, KM371857*; ***A. laevitomentosa*** (Nyár. ex Sennikov) Greuter, **JC 2011/40/2P:** Romania: Judeţul Suceava, Munţii Bistriţei,Vatra Dornei, Pietrosul Bogolin, 1740 m, *Chrtek & al.*, *970/11* (PRA), KM372047*, KM372048* / KM371943*, KM371944* / KM371751* / KM371800* / KM371858*, KM371859*; **E8:** Romania: Judeţul Suceava, Munţii Bistriţei,Vatra Dornei, Pietrosul Bogolin, 1740 m, *Chrtek & al.*, *971/11* (PRA), KM372049*, KM372050* / KM371945*, KM371946* / KM371752* / KM371801* / KM371860*, KM371861*; ***A. laxiflora*** DC., **JC 2011/12/2:** Spain: Andalusia, province Huelva, Niebla, 40 m, *Chrtek & Dočkalová*, *1934/11* (PRA), KM372051*, KM372052* / KM371947*, KM371948* / KM371753* / KM371802* / KM371862*, KM371863*; **JC 2011/19/2:** Spain: Andalusia, province Córdoba, Hornachuelos, 280 m, *Chrtek & Dočkalová*, *1930/11* (PRA), KM372053*, KM372054* KM372055* / KM371949*, KM371950* / KM371754* / KM371803* / KM371864*; **JC 2011/20/1:** Spain: Andalusia, province Jaén, Linares, 405 m, *Chrtek & Dočkalová*, *1926/11* (PRA), KM372056*, KM372057* / KM371951*, KM371952* / KM371755* / KM371804* / KM371865*; ***A. maroccana*** (Caball.) Maire, **JZ 1711/2:** Morocco: region Oriental, province Nador, Ifrin-Dounacht, 74 m, *Zahradníček & Krak*, *2011/10* (PRA), KM372058*, KM372059* / KM371953*, KM371954* / KM371756* / KM371805* / KM371866*; **JZ 1711/2a:** Morocco: region Oriental, province Nador, Ifrin-Dounacht, 74 m, *Zahradníček & Krak*, *2010/10* (PRA), KM372060*, KM372061* / KM371955*, KM371956* / KM371757* / KM371806* / KM371867*, KM371868*; ***A. mogadorensis*** Coss. ex Hook.f. subsp. ***mogadorensis***, **ZF 263A:** Morocco: region Marrakech-Tensift-El-Haouz, province Essaouira, Essaouira (Mogador), 8 m, *Ferreira & Alvarez Fernández*, *263* (MA), KM372062*, KM372063* / KM371957*, KM371958* / KM371758* / KM371807* / KM371869*; **JC 16N/V2:** Morocco: region Marrakech-Tensift-El-Haouz, province Essaouira, Essaouira (Mogador), 8 m, *Chrtek & Dočkalová*, *2013/10* (PRA), KM372064*, KM372065* / KM371959*, KM371960* / KM371759* / KM371808* / KM371870*; **JC 15/1:** Morocco: region Souss-Massa-Drâa, prefecture Agadir-Ida ou Tanane, Tamri, 30 m, *Chrtek & Dočkalová*, *2019/10* (PRA), KM372066*, KM372067* / KM371961*, KM371962* / KM371760* / KM371809* / KM371871*; **JC 18V/V2:** Morocco: region Doukkala-Abda, province El Jadida, Moulay Abdallah, 12 m, *Chrtek & Dočkalová*, *2014/10* (PRA), KM372068*, KM372069* / KM371963*, KM371964* / KM371761* / KM371810* / KM371872*, KM371873*; ***A. mogadorensis*** subsp. ***jahandiezii*** (Maire) M.Z.Ferreira, **JC 14/1:** Morocco: region Souss-Massa-Drâa, prefecture Inezgane-Aït Melloul, Inezgane, 7 m, *Chrtek & Dočkalová*, *2018/10* (PRA), KM372070*, KM372071* / KM371965*, KM371966* / KM371762* / KM371811* / KM371874*; ***A. perezii*** M.Z.Ferreira & al., **KV 51V:** Spain: Canary Islands, Lanzarote, Mirador de Haría, 380 m, *Vazačová*, *838/10* (PRA), KM372072*, KM372073* / KM371967*, KM371968* / KM371763* / KM371812* / KM371875*, KM371876*; **KV 52V**: Spain: Canary Islands, Lanzarote, El Jurado, Guinate, 440 m, *Vazačová*, *839/10* (PRA), KM372074*, KM372075* / KM371969*, KM371970* / KM371764* / KM371813* / KM371877*; ***A. pinnatifida*** Aiton, **And.pin.Cer:** Spain: Canary Islands, La Gomera, El Cercado, 1000 m, *Bräutigam*, *158131* (GLM), KM372076*, KM372077* / KM371971*, KM371972* / AY573358 / KM371814* / KM371878*, KM371879*; **SB H16p/4:** Spain: Canary Islands, El Hierro, San Andrés, 1175 m, *Bräutigam & Bräutigam*, *2049/10* (PRA), KM372078*, KM372079* / KM371973*, KM371974* / KM371765* / KM371815* / KM371880*; **SB H13/1:** Spain: Canary Islands, El Hierro, San Andrés, 980 m, *Bräutigam & Bräutigam*, *2041/10* (PRA), KM372080*, KM372081* / KM371975*, KM371976* / KM371766* / KM371816* / KM371881*; **SB P11/1:** Spain: Canary Islands, Isla de la Palma, Roque del Faro, 1030 m, *Bräutigam & Bräutigam*, *2043/10* (PRA), KM372082*, KM372083* / KM371977*, KM371978* / KM371767* / KM371817* / KM371882*; **SB H14/3:** Spain: Canary Islands, El Hierro, Sabinosa, 720 m, *Bräutigam & Bräutigam*, *2051/10* (PRA), KM372084*, KM372085* / KM371979*, KM371980* / KM371768* / KM371818* / KM371883*, KM371884*; **SB T2/1:** Spain: Canary Islands, Tenerife, Puerto de la Cruz, 780 m, *Bräutigam & Bräutigam*, *2045/10* (PRA), KM372086*, KM372087* / KM371981*, KM371982* / KM371769* / KM371819* / KM371885*; **SB G7b/3:** Spain: Canary Islands, La Gomera, Arures, 940 m, *Bräutigam & Bräutigam*, *2050/10* (PRA), KM372088*, KM372089* / KM371983*, KM371984* / KM371770* / KM371820* / KM371886*; **SB G9/3/1:** Spain: Canary Islands, La Gomera, Las Rosas, 980 m, *Bräutigam & Bräutigam*, *2042/10* (PRA), KM372090*, KM372091* / KM371985*, KM371986* / KM371771* / KM371821* / KM371887*, KM371888*; **SB T17/5:** Spain: Canary Islands, Tenerife, Montañas de Anaga, Cruz de Carmen, 800 m, *Bräutigam & Bräutigam*, *2044/10* (PRA), KM372096*, KM372097* / KM371990*, KM371991* / KM371774* / KM371824* / KM371891*, KM371892*; ***A. pinnatifida*** subsp. ***teydensis*** (Sch.Bip.) Rivas Mart. & al., **SB T18/1:** Spain: Canary Islands, Tenerife, Cañadas, El Portilla, 1950 m, *Bräutigam & Bräutigam*, *2047/10* (PRA), KM372092*, KM372093* / KM371987*, KM371988* / KM371772* / KM371822* / KM371889*; **SB T4/1:** Spain: Canary Islands, Tenerife, Cañadas, La Escalona, 1190 m, *Bräutigam & Bräutigam*, *2103/10* (PRA), KM372094*, KM372095* / KM371989* / KM371773* / KM371823* / KM371890*; ***A. ragusina*** L., **JC 27/1:** Spain: Andalusia, province Granada, Guadix, 1075 m, *Chrtek & Dočkalová*, *1935/11* (PRA), KM372098*, KM372099* / KM371992*, KM371993* / KM371775* / KM371825* / KM371893*, KM371894*; **JC 2011/2/1:** Spain: Andalusia, province Málaga, Alhaurín el Grande, 230 m, *Chrtek & Dočkalová*, *1925/11* (PRA), KM372100*, KM372101*, KM372102* / KM371994*, KM371995*, KM371996* / KM371776* / KM371826* / KM371895*; **ZF LM5103/1:** Spain: Madrid, Guadalix de la Sierra, 850 m, *Sequeira & Medina*, *LM5103* (MA), KM372103*, KM372104* / KM371997*, KM371998* / KM371777* / KM371827* / KM371896*; **JC 2011/35/1:** Spain: Comunitat Valenciana, province Alicante, Sax, 610 m, *Chrtek & Dočkalová*, *1933/11* (PRA), KM372105*, KM372106* / KM371999*, KM372000* / KM371778* / KM371828* / KM371897*; ***Hispidella hispanica*** Barnades ex Lam., **His.his.2:** Spain: Sierra de Guadarrama, *Pizarro & Navarro*, *CN 2460* (M), KM372107* / EU821365 / AY573355 / JX129534 / JX129601, JX129602; ***Pilosella argyrocoma*** (Fr.) F.W.Schultz & Sch.Bip., **agy.Gra:** Spain: Province Granada, plant cultivated in Botanic Garden Munich, *Merxmüller & Gleisner, culture H11* (M), KM372108* / KM372001* / AY573320 / JX129536 / JX129605; ***P. lactucella*** (Wallr.) P.D.Sell & C.West, **lac.Jon.1:** Germany: Oberlausitz, Jonsdorf, *Bräutigam*, *140619* (GLM), KM372109* / KM372002* / AY192669 / JX129535 / JX129603; ***P. pseudopilosella*** (Ten.) Soják, **TU308/2:** Spain: Sierra Nevada, between Granada and Pradollano, *Urfus, 308/2* (PRA), KM372110* / KM372003* / JX129599 / JX129537 / KM371899*; ***P. hoppeana*** subsp. ***macrantha*** (Ten.) S.Bräut. & Greuter, **TU1059:** Romania: Banat, Gârnic, pastures on karst plateau SW of village, near the road to S. Helena, *Skála & Skálová*, *1059* (PRA), KM372111* / KM372004* / KM371779* / KM371829* / KM371900*; ***P. echioides*** (Lumn.) F.W.Schultz & Sch.Bip., **TR1608:** Czech Republic: Central Bohemia, Trubín, *Chrtek*, *258/2013* (PRA), KM372112* / KM372005* / KM371780* / KM371830* / -; ***Hieracium intybaceum*** All., **inb.Kaer:** Austria: Kärnten, *S. Jagalski*, *4* (M), KM372113* / EU821370 / AY573323 / JX129561 / JX129745; **1069/1:** Italy: Trentino-Alto Adige, Passo del Tonale, *Chrtek & Mráz*, *1069/2005* (PRA), HQ131821 / KM372006* / JX129600 / JX129560 / HQ131846, HQ131847; ***H. porrifolium*** L., **1052/9:** Austria: Carinthia, Karawanken Mts., Bad Eisenkappel, near the road to Bad Vellach, *Chrtek & Mráz*, *1052/2005* (PRA), HQ131823 / EU867631 / EU867730 / JX129578 / HQ131843, JX129701; ***H. recoderi*** De Retz, **1174/4:** Spain: Catalunya, province Barcelona: Berga, monastery of Queralt, *Chrtek*, *1174/2006* (PRA), KM372114* / EU867603 / EU867721 / JX129584 / KM371901*, KM371902*; ***H. tomentosum*** L., **1066/8:** France: dépt. Alpes Maritimes, valley of la Roya, Tende, *Chrtek & Mráz*, *1066/2005* (PRA), KM372115* / EU867596 / EU867731 / JX129590 / JX129726, JX129727; ***H. umbellatum*** L., **um.AM.1:** Germany: Upper Lusatia, SE Schönau-Berzdorf, *Bräutigam*, *46889* (GLM), KM372116* / EU867644 / AY573335 / JX129594 / JX129732

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Cytotype distribution and phylogeography of *Hieracium intybaceum* (Asteraceae)

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Using flow cytometry and amplified fragment length polymorphism (AFLP), we explored the cytogeography and phylogeography of *Hieracium intybaceum*, a silicicolous species distributed in the Alps and spatially isolated in the Vosges Mountains and the Schwarzwald Mountains. We detected two ploidies, diploid and tetraploid, but no triploid or mixed-ploidy populations. Whereas diploids are sexual and distributed all across the Alps, tetraploids are apomictic and seem to be confined to the western Alps and the Vosges. We detected a low level of genetic variation. Bayesian clustering identified four clusters/genetic groups, which are partly congruent with the ploidal pattern. The first two groups consisting exclusively of diploids dominate the whole distribution range in the Alps and show east–west geographical separation with a diffuse borderline running from eastern Switzerland to the eastern part of North Tyrol. The third genetic group lacks a defined geographical range and includes diploid and tetraploid plants. The last genetic group comprises tetraploid plants in the French Alps and the Vosges. We suppose that diploids colonized the deglaciated areas from source populations most likely located mainly in the southern part of the recent distribution range and occasionally also in the western Alps. Gene flow and further differentiation likely took place. Apomictic tetraploids most likely originated in the western Alps or in the refugium at the south-western foot of the Alps. Their rather limited geographical range (partly contrasting with the theory of geographical parthenogenesis) can be explained by their rather recent origin. *Botanical Journal of the Linnean Society*, 2015, ••, ••–••.

ADDITIONAL KEYWORDS: AFLP – Alps – geographical parthenogenesis – glacial refugia – polyploidy.

INTRODUCTION

Quaternary climatic changes have fundamentally influenced the present-day distribution and genetic variation of high-mountain species, which had to endure induced migrations and repeated range fragmentation, undergo diversification in isolated refugial areas and recolonize previously glaciated areas, or face local extinction (Hewitt, 1996; Bennett, 1997; Comes & Kadereit, 1998; Tribsch & Schönswetter, 2003).

The Alps and surrounding areas are a suitable model system for studies focused on evolutionary processes influenced by glacial cycles and on locating glacial refugia. Numerous phylogeographic studies carried out in the Alps over the last decades, coupled with previously acquired biogeographical, geological and palaeoenvironmental data, have allowed some more general trends and patterns to be postulated. They have shown that alleles and species sharing a common history of glaciation faced more or less the same distinctive alpine topography during their range retractions and expansions, resulting in two principal breaking zones. One zone is in the western Alps, in the area of the valleys of Aosta and Valais, and the other at the transition between the western and eastern part of the Alps between Lake Garda and Innsbruck (e.g. Thiel-Egenter *et al.*, 2011). Species have also undergone parallel processes in peripheral refugia on both siliceous (mainly in the eastern Alps and along the south and west border of the

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range) and calcareous bedrocks (mainly in the southern and eastern Alps; Brockmann-Jerosch & Brockmann-Jerosch, 1926; Merxmüller, 1952; Stehlik, 2000; Tribsch & Schönswetter, 2003; Schönswetter *et al*., 2005; Schönswetter & Tribsch, 2005; Burnier *et al*., 2009; Slovák *et al*., 2012) or in combined peripheral refugia and nunataks, albeit potentially at different time periods (Schneeweiss & Schönswetter, 2011; García *et al*., 2012). Pleistocene climatic changes also most likely promoted polyploid speciation (Stebbins, 1984, 1985; Brochmann *et al*., 2004; Dixon *et al*., 2009; Casazza *et al*., 2012).

Glacial advancement fragmented once continuous diploid populations that then differentiated in isolation. Partially differentiated populations may have come into contact again during glacial retreats, occasionally giving rise to hybrids that were then stabilized by polyploidization (secondary contact model, Stebbins, 1984, 1985). Alternatively, polyploids may have originated in diploid populations via unreduced gametes (primary contact model, triploid bridge). Formerly glaciated areas were then recolonized by polyploids, which today are dominant in central parts of the Alps (e.g. Stebbins, 1950; Parisod & Besnard, 2007; Schönswetter, 2008). Enhanced frequency of polyploids at higher elevations and latitudes can also be explained by the fact that cold temperatures can trigger the formation of unreduced gametes (Ramsey & Schemske, 1998).

Polyploidization and hybridization influenced by range fluctuations of species in the Pleistocene have also been thought to promote genetic and epigenetic changes that caused shifts to gametophytic apomixis (e.g. Carman, 1997; Grimanelli *et al*., 2001; Hörandl, 2009a, b). Apomictic taxa have generally larger geographical ranges that are often shifted to higher elevations and altitudes (often in formerly glaciated areas) compared to related sexuals (geographical parthenogenesis; Vandel, 1928; Hörandl, 2008; Cosendai & Hörandl, 2010; Hörandl, 2011). This can be explained, among other things, by the advantage of pollinator-independent uniparental reproduction for colonization, shortening of the reproductive pathway and faster seed development in shorter vegetation periods, higher abundance of seeds, more efficient niche exploitation and greater physiological and ecological tolerance due to multiple gene copies (Hörandl, 2006; Hörandl, Cosendai & Temsch, 2008). In the Alps, the highest percentage of apomicts seems to grow in the moderate subalpine and alpine grassland zones (Hörandl, 2011; Hörandl *et al*., 2011). High-elevation plants, in contrast, do not show a pronounced tendency towards polyploidy, so gametophytic apomixis turns out to be rare in the subnival and nival zones (Hörandl *et al*., 2011). Recent studies have supposed occasional re-stabilization of meiotic pathways in some apomictic lineages, followed by reversal to sexuality and allopatric speciation of newly formed sexual populations (expanded transition theory; Hörandl & Hojsgaard, 2012; Hojsgaard *et al*., 2014).

We selected *Hieracium intybaceum* L., a perennial herb scattered to locally common on siliceous bedrock in the subalpine and alpine belts of the Alps, and spatially isolated in the Vosges and the Schwarzwald Mountains, as our model species to examine the pattern of genetic variation in relation to karyological differentiation. Three ploidies have been reported so far, namely diploids ($2n = 18$), triploids ($2n = 27$) and tetraploids ($2n = 36$; Favarger, 1997; Chrtek *et al*., 2007). The mode of reproduction has not hitherto been examined. *Hieracium* polyploids have been proved to be exclusively agamospermous; conversely, *H. intybaceum* is a sister group to the rest of subtribe Hieraciinae (and thus could be placed in the separate genus *Schlagintweitia* Griseb.), but differences from this general pattern cannot be excluded. *Hieracium intybaceum* is an entomophilous species. Its achenes are relatively large and well adapted for dispersal over long distances.

In this study, we addressed the following questions:

(1) Is there any geographical pattern of genetic variation in *Hieracium intybaceum*?
(2) If there is a pattern, does it reflect cytotype and breeding system differentiation and morphological variation?
(3) Does this pattern agree with the concept of geographical parthenogenesis?
(4) What is the mode of reproduction of diploid and tetraploid cytotypes, and are tetraploids agamospermous as in other *Hieracium* spp. or sexual?

MATERIAL AND METHODS

Sampling

Forty-three populations of *Hieracium intybaceum* were collected throughout the Alps and in the Vosges (Table 1) in 2009–2011. Leaf material was taken from at least ten individuals per population and immediately stored in silica gel for further molecular analyses. Five plants from the same sets of individuals were transferred to the experimental garden of the Institute of Botany AS CR in Průhonice (49°59′41″N, 14°34′00″E). Herbarium specimens (a further five plants from the same sets) are deposited in the herbarium of the Faculty of Science, Charles University in Prague (vouchers are without identification number). Small pieces of fresh leaves of 50 plants per population (except for small populations BER, BAL, COL and HOH) were used for cytotype screening using flow cytometry.

Table 1. Details of 43 populations of *H. intybaceum* studied; * – population not used in molecular analyses; DW – rarity index (frequency down-weighted marker values)

Number of locality	Code	Locality name	Latitude	Longitude	Elevation	No. of plants	Ploidy	DW
Int0210	STU	Austria, Salzburg	47.08135	12.492167	1882	4	2*x*	0.588
Int0309	SAB	Austria, Steiermark	47.08135	14.577017	2060	3	2*x*	0.901
Int0310	KON	Austria, Salzburg	47.258861	12.105417	2014	3	2*x*	0.704
Int0410	JAU	Italy, Südtirol	46.837667	11.321028	2186	3	2*x*	1.086
Int0510	SEM	Italy, Südtirol	46.681472	11.712389	1939	3	2*x*	0.815
Int1009	TUR	Austria, Steiermark	46.914333	13.854833	2186	3	2*x*	0.671
Int1010	MOL	Italy, Lombardy	46.542389	10.422889	2477	3	2*x*	0.795
Int1109	OSS	Austria, Kärnten	46.693567	13.921167	1820	5	2*x*	0.769
Int1110	STE	Italy, Lombardy	46.53275	10.477194	2224	4	2*x*	1.030
Int1309	REI	Austria, Kärnten	46.904933	13.338967	2243	4	2*x*	0.693
Int1310	LAG	Switzerland, Ticino	46.47875	8.589111	2214	4	2*x*	0.680
Int1609	STR	Austria, Kärnten	46.5967	13.118367	1543	4	2*x*	0.579
Int1610	FIE	Switzerland, Ticino	46.536417	8.567833	1968	5	2*x*	0.724
Int1709	LAM	Austria, Kärnten	46.631783	12.935483	1598	5	2*x*	0.788
Int1809	EGG	Austria, Ost-tirol	46.880833	12.776233	2114	5	2*x*	0.725
Int1810	AND	Switzerland, Uri	46.656444	8.642611	2013	4	2*x*	1.048
Int1909	STA	Italy, Trento	46.889983	12.19655	1949	6	2*x*	0.978
Int1910	GLA	Switzerland, Uri	46.572694	8.395444	2128	2	2*x*	0.645
Int2009	PEN	Italy, Trento	46.818483	11.434633	2246	5	2*x*	0.864
Int2010	BRU	Switzerland, Valais	46.374694	7.977111	2125	4	2*x*	0.722
Int2109	VER	Italy, Trento	46.732817	10.815667	1761	5	2*x*	1.068
Int2209	ROM	Italy, Trento	46.885433	11.118467	1936	5	2*x*	0.875
Int2210	JAM	Austria, Tirol	46.926667	10.176944	1805	4	2*x*	0.871
Int2309	VEN	Austria, Tirol	46.8785	10.932183	2043	3	2*x*	0.718
Int2310	LUC	Austria, Tirol	47.019778	12.694444	2070	5	2*x*	0.699
Int2410	PAN	Austria, Tirol	46.998333	12.598333	2205	6	2*x*	0.842
Int2509	PEE	Austria, Tirol	47.156117	11.5634	1858	5	2*x*	0.754
Int2510	BER	Italy, Valle de Aosta	45.680083	6.885333	2219	6	2*x*	1.071
Int2609	GUR	Austria, Tirol	46.860528	11.024278	2065	4	2*x*	0.749
Int2610	COI	France, Savoy	45.66275	6.870472	2082	5	2*x*	0.673
Int2709	RET	Austria, Tirol	46.470194	11.024278	2035	4	2*x*	0.619
Int2710	ROS	France, Savoy	45.685528	6.696139	2145	4	2*x*	0.604
Int2809	MOR	Switzerland, Graubünden	46.470194	9.757472	2145	5	2*x*	0.890
Int2909	FLU	Switzerland, Graubünden	46.744361	9.982389	2155	4	2*x*	0.929
Int2910	CEZ	France, Hautes-Alpes	44.925222	6.409611	2112	7	4*x*	0.764
Int3410	BRO	France, Isére	45.03525	5.888972	2126	6	4*x*	0.940
Int3810	BAL	France, Haute-Savoie	46.025333	6.9585	2047	5	4*x*	0.902
Int38102	COL	France, Isére	45.387669	6.135403	1650	5	2*x*	0.708
Int4010	HOH	France, Vosges	48.041722	7.013111	1279	7	4*x*	0.856
Int4111	THO	France, Savoie	45.2893	6.588733	2475	2	2*x*	0.839
Int4911	CAS	France, Hautes-Alpes	44.98085	6.4352	2164	4	4*x*	0.976
Int3009*	PYR	Austria, Tirol	47.131333	10.209972	1787		2*x*	
Int2911*	TER	France, Haute-Savoie	45.949333	6.850633	2203		2*x*	

Ploidy and mode of reproduction

DNA ploidy (Suda *et al*., 2006) was determined by flow cytometry using a Partec PA II device equipped with an HBO mercury arc lamp. Sample preparation followed the two-step procedure using Otto's buffers I and II (Doležel, Greilhuber & Suda, 2007). Pooled samples for faster estimation were prepared from intact leaf tissue. Each sample, with an appropriate quantity of the internal reference standard *Bellis perenis* L. (2C = 3.37 pg, Schönswetter *et al*., 2007), was chopped with a new sharp razor blade in a Petri dish containing 1 mL ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) as the nuclear isolating

solution. The suspension was filtered through a nylon mesh (loop size 42 μm), incubated for 10 min at room temperature and mixed with 1 mL Otto II buffer (0.4 M Na_2HPO_4) supplemented with AT-selective DAPI (4′,6-diamidino-2-phenylindole) as the fluorochrome (at the concentration of 4 mg mL^{-1}), RNase IIA (50 mg mL^{-1}) and β-mercaptoethanol (2 mg mL^{-1}). Samples were stained for 5 min at room temperature prior to cytometry. Three thousand nuclei were analysed for each sample. Only histograms with coefficients of variation (CVs) for the G0/G1 < 5% were accepted.

The mode of reproduction was tested using the plants transferred to the experimental garden following Gadella (1987) and Krahulcová & Krahulec (1999). In diploids (in which sexual reproduction was expected), capitula were bagged and pollinated by hand (test for autogamy); results were compared with control capitula from the same plant in open pollination treatment. In tetraploids (in which agamospermy was expected), the upper part of the capitulum was cut off at the bud stage, capitula were bagged to prevent lost of achenes by wind and full (developed) achenes were counted.

AFLP data acquisition and scoring

Total genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek) following the manufacturer's instructions using 0.5 g of silica-dried leaf tissue. The concentration of DNA in the first elution was measured using a NanoDrop spectrophotometer, and the DNA was diluted to the initial concentration of 50 ng μL^{-1}. One hundred and eighty-two individuals (two to seven per population, Table 1) were analysed for AFLPs using the AFLP Core Reagent Kit I (Invitrogen) and AFLP Pre-Amp Primer Mix I (Invitrogen). The whole procedure (restriction, ligation, pre-amplification and selective amplification) followed Rejzková *et al*. (2008) with the following modifications: ca. 130 ng of genomic DNA were digested for 5 h and ligation was extended to 12 h. Pre-amplification and selective amplification was done using JumpStart RedTag Polymerase (Sigma), and the same volume of 10× buffer was used with JumpStart RedTag Polymerase (Sigma). The reaction conditions were an initial step of 2 min at 94 °C and 2 min at 72 °C followed by 20 cycles of 30 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C with final extension at 60 °C for 30 min. A 10 × diluted (in ddH_2O) product of pre-amplification was used for the final, selective amplification.

Based on preliminary tests, three primer combinations were used for selective amplification: *Eco*RI-AAC + *Mse*I-CTA, *Eco*RI-ACG + *Mse*I-CAC and *Eco*RI-ACA + *Mse*I-CTG (Applied Biosystems). The reaction conditions were an initial step of 2 min at 94 °C, 30 s at 65 °C and 2 min at 72 °C followed by eight cycles of 30 s at 94 °C, 30 s at 64 °C (reduced by 1 °C per cycle) and 2 min at 72 °C, which were followed by 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C, then the final extension of 30 min at 60 °C and a hold period at 10 °C. For each sample, 1 μL of each 6-FAM, HEX and NED-labelled products of selective amplification was purified and precipitated using ethanol/sodium acetate precipitation. The precipitate was resuspended in 0.25 μL GeneScan-ROX-500 size standard in 10 μL formamide and denatured for 3 min at 95 °C. Fragments were analysed on a 3100 Avant Genetic Analyzer (Laboratory of Sequencing, Faculty of Science, Charles University in Prague) and scored with GeneMarker v 1.8 (www.SoftGenetics.com). Twelve per cent of all samples were re-analysed from the first step of AFLP procedure (restriction) under normal conditions to test the reproducibility of the method and estimate the average error rate (Bonin *et al*., 2004). Fragments between 75 and 500 bp long were scored.

AFLP data analysis

Frequency down-weighted marker values (DW; Schönswetter & Tribsch, 2005) per population were calculated using the AFLPdat script (Ehrich, 2006) in R. Higher values are thought to indicate populations that have been isolated for a longer time and low values populations that diverged rather recently. The same script was used to create input files for Structure and Arlequin.

Partitioning of genetic variation within and among populations and cytotypes was tested for by analysis of molecular variance (AMOVA) using Arlequin 3.1 (Excoffier, Laval & Schneider, 2005). We performed two AMOVA analyses using two (within and among populations) and three (within and among populations, and between cytotypes) hierarchical levels. Population pairwise PhiPT (an F_{ST} analogue) values were computed and Mantel test (pairwise PhiPT values and geographical distances) was performed in GenAlEx 6.5 (Peakall & Smouse, 2012).

The genetic structure was evaluated using three different approaches:

(1) The first was a non-model-based approach; non-hierarchical K-means clustering (Hartigan & Wong, 1979) was chosen because of the presence of two ploidies using a script by Arrigo *et al*. (2010) in R. This approach assigns individuals to a defined number of genetic groups in order to maximize intergroup variance. This technique was successfully applied in the analysis of genetic structure of the AFLP datasets in polyploid complexes (Burnier *et al*., 2009; Arrigo *et al*., 2010).

(2) The second approach taken used model-based Bayesian clustering implemented in Structure 2.2 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2007). This approach is less appropriate for detecting admixture between cytotypes because it is based on the ideal Hardy–Weinberg population model minimizing linkage disequilibrium.
(3) Principal coordinate analysis (PCoA, Jaccard's similarity coefficients) performed using PAST (Hammer, Harper & Ryan, 2001) was the last approach. K-means clustering was conducted in 100 000 independent runs (i.e. starting from random points) for each assumed value of K clusters (ranging from 1 to 10), and the intergroup inertia of each run was recorded.

We followed the procedure described by Evanno, Regnaut & Goudet (2005) to select the most likely number of groups using intergroup inertia as a proxy of clustering accuracy. In the Structure analysis, the number of clusters was estimated using 10^6 iterations with a burn-in period of 10^5 iterations under an admixture model. Ten replicates for each K were analysed from K = 1 to K = 10; the outputs were processed following the approach of Evanno *et al*. (2005) implemented in Structure Harvester to determine the most likely groups of clusters (Earl & Vonholdt, 2012). The mixed-ploidy dataset was encoded into the 'polyploid' format used by Stock *et al*. (2010). For the purpose of the Structure analysis (a complete dataset), all individuals were coded as having four chromosome sets (corresponding to the highest ploidy in the dataset, i.e. 4*x*). The two chromosome sets not present in diploid individuals were marked as missing data (–9). This model was compared with the usual coding for diploid plants. Both these procedures were also applied to subsets of diploids and tetraploids (triploids were not found). The nonhierarchical K-means clustering analysis and Bayesian clustering analysis were performed for the mixed ploidy dataset ('complete dataset analysis') and for two ploidy subsets of diploids and tetraploids separately ('nested analysis').

RESULTS

Cytotype screening

We ascertained the ploidy of 2270 plants from 43 populations. Two ploidies, diploid and tetraploid, were detected. Whereas diploids are distributed all across the Alps (38 populations), tetraploids seem to be confined to the western Alps (four populations analysed come from the Savoy Alps) and the Vosges (one population). We found no triploids and no mixed-ploidy populations.

Mode of reproduction

Only empty achenes were found in bagged capitula of diploid plants (eight plants, 12 capitula). Untreated (open pollination) capitula, in contrast, produced nearly 100% of full achenes, showing allogamy in sexual diploids. A high proportion of full achenes (range 86.9–92.0%, mean 89.3%) confirming agamospermy was found in tetraploid plants with emasculated capitula (five plants, one capitulum per plant).

DNA analysis

Using two of the three AFLP primer combinations (*Eco*RI-ACG + *Mse*I-CAC and *Eco*RI-ACA + *Mse*I-CTG) together generated 152 clear polymorphic fragments. The third primer combination (*Eco*RI-AAC + *Mse*I-CTA) was not included in the final analysis because it generally yielded low-quality results. Based on 22 replicates (12% of samples), the reproducibility of the dataset was 94.5%.

The rarity index (DW) varied from 0.58 in population STR (southern Austria) to 1.068 in population JAU (northern Italy; Table 1). Populations with higher DW indices are located mainly in the southern part of the geographical range (Fig. 1). The DW values of diploid (mean 0.796) and tetraploid (mean 0.888) populations are not significantly different (two-tailed *t*-test, d.f. = 38, t = 1.369, $P = 0.178$).

The AFLP analysis revealed low variability. We found no private markers for the individual populations. After dividing the whole dataset into two groups based on ploidy, diploid populations had eight private markers and tetraploid populations only one private marker. In the AMOVA with only two levels of variation (i.e. within and among populations), 27% of the overall genetic variation was found among populations. The population pairwise PhiPT values showed the greatest differentiation between ROS and BER (PhiPT = 0.667), CEZ and ROS (0.637) and CEZ and BER (0.634) with mean PhiPT = 0.258. In our nested AMOVA analyses, the variation between cytotypes accounted for 20% of the overall variation. In the subset of diploid populations, 25% of variation was detected among populations; the highest differentiation was found between ROS and BER (PhiPT = 0.667) with mean PhiPT = 0.246. Lower inter-population differentiation was found in the subset of tetraploid populations (12% of the variation can be explained by inter-population variation); the highest differentiation was found between CAS and CEZ (PhiPT = 0.280) with mean PhiPT = 0.123.

In the among-population AMOVA of the diploid sub-dataset, populations ROS from south-eastern France and the easternmost population SAB were found to be most distinct, differing by 27% and 15% from the nearest population, respectively. In the

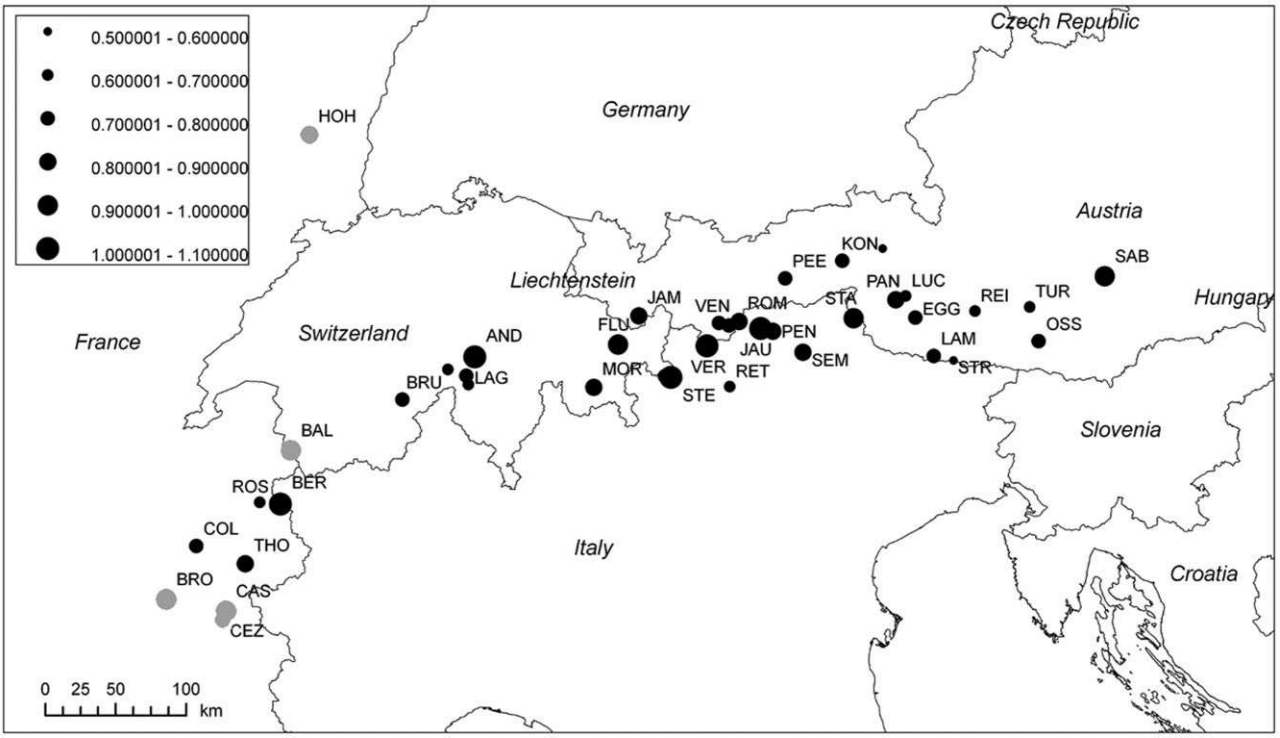

Figure 1. Cytotype distribution and rarity index of 41 populations of *H. intybaceum*. The two symbols present ploidy groups: circles – diploid populations, grey circles – tetraploid populations. The size of each symbol is proportional to the value of the rarity index.

tetraploid sub-dataset, population BAL from south-eastern France had the greatest genetic distance of 10% from the rest of tetraploid populations.

The Mantel test computed for the whole dataset revealed a low but significant correlation between genetic and geographical distances ($r_m = 0.246$, $P = 0.001$), indicating an isolation-by-distance pattern. A positive correlation was found also for diploids ($r_m = 0.193$, $P = 0.010$).

Nonhierarchical K-means clustering resulted in an optimal grouping of the complete dataset into two or four groups (Supporting Information Fig. S1). However, four groups reflect the observed cytological and geographical differentiation much better (Fig. 2A). Individuals assigned to the first genetic cluster K1 were found in diploid populations of central and eastern Alps. The individuals belonging to the genetic cluster K2 were detected in diploid populations throughout the entire distributional range of the species. Individuals falling in genetic cluster K3 were detected mainly in diploid and rarely in tetraploid populations encompassing the entire distributional range. The remnant tetraploid individuals fallen in the genetic cluster K4. Populations were mainly constituted of individuals belonging to one or two genetic clusters; rarely they shared three clusters. The K-means analysis was applied separately to the diploid and tetraploid datasets (nested analysis). It resulted in an optimal grouping into three groups for diploids and two groups for tetraploids, the latter without any geographical pattern. The classification of most individuals (except for two plants from SEM and MOL) by the nested analysis is congruent with the analysis of the complete dataset.

The Bayesian analysis of the complete dataset using Structure resulted in the best partitioning into either two (similarity coefficient 0.90) or five (similarity coefficient 0.98) groups (Supporting Information Fig. S2). In the latter case, tetraploids fall into two groups (the first one exclusively tetraploid, the second one with a small admixture of diploids) and the remaining three groups are formed exclusively by diploid plants. The first two diploid groups show a geographical pattern (east, west), whereas the third one comprises plants across the whole geographical range without any pattern. This contradicts the number of clusters suggested by the K-means clustering (four). Nested Bayesian analysis resulted in three groups of diploids and two groups of tetraploids, the latter groups without any geographical pattern, which is in agreement with results of nested K-means analysis. Classifications of plants in Bayesian

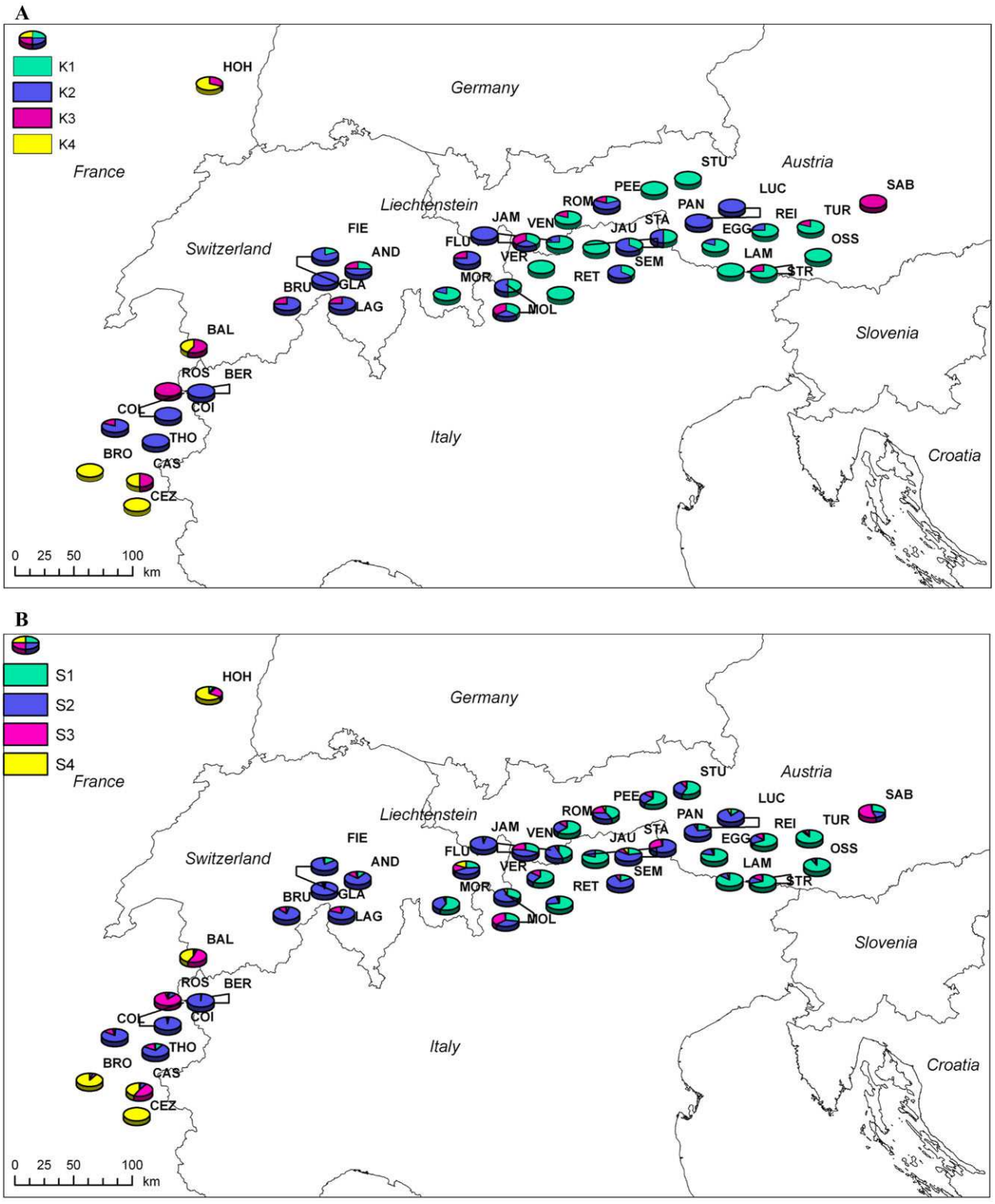

Figure 2. Phylogeographic pattern of 41 populations of *H. intybaceum* analysed. The groups are based on (A) K-means clustering; and (B) Bayesian clustering. The graphs show the proportion of individuals classified into clusters K1–K4 in the case of K-means clustering and the mean proportional presence of clusters S1–S4 in each population in the case of Bayesian clustering.

Table 2. Contingency table of numbers of individuals comparing the grouping based on Bayesian clustering (S1–S4) and K-means clustering (K1–K4)

	K1	K2	K3	K4
S1	59	2		
S2	8	63	2	
S3			23	
S4		1		22

analysis of the complete dataset (five groups) on one hand and separated sets of diploids and tetraploids (nested analysis) on the other differ considerably. Partitioning of the complete dataset into four groups, however, shows nearly the same classification of plants as the nested analyses. With these facts in mind, we decided to consider Structure clusters S1–S4 and discuss this classification further. The Structure results are (except for 13 individuals) congruent with the K-means analysis of the complete dataset (Table 2). Discrepancies between results of K-means and Bayesian clustering can be explained by the clustering method adopted; the latter takes into account possible admixture, i.e. it allows for the possibility that individuals may have mixed ancestry.

Principal coordinate analysis (PCoA, Fig. 3) confirmed the rather weak structuring of the dataset detected by the previous analyses. Similarly to K-means clustering, it divided the dataset into four groups. The first three axes explained 18.6% of the total variation. The first axis (explaining 8.7% of the total variation) separated diploid clusters (K1, K2) from tetraploids (K4). The second axis (explaining 5.5% of the total variation) separated the 'eastern diploid group' (K1) from the 'western diploid group' (K2). The third axis (explaining 5.5% of the total variation) separated the cluster K3 from the rest of the groups. Structure genetic groups (clusters S1–S4) are largely congruent with K-means clusters K1–K4 and thus also match the results of PCoA.

DISCUSSION

Cytogeography of *Hieracium intybaceum*

The present data corroborate and add details to the geographical pattern proposed by Favarger (1997), who reported tetraploids from south-western Switzerland and diploids from Switzerland, Austria and Italy. Sexual diploids occur throughout the distribution area in the Alps, whereas apomictic tetraploids are confined to the western Alps (reaching eastwards to the canton of Wallis in south-western Switzerland) and the Vosges.

We did not confirm the occurrence of triploids. Published triploid counts for *H. intybaceum* (Rosenberg, 1927; Larsen, 1954) seem doubtful even though triploid plants can generally appear at low frequencies in diploid populations. The discrepancy between our results and previous reports of triploids can be explained by misidentifications with *H. pallidiflorum* Hausm. This triploid species is morphologically similar to *H. intybaceum* and often gets confused with it in herbarium material. Alternatively and less likely, triploids of *H. intybaceum* may not have been detected due low frequencies in populations. Moreover, the geographical distribution of diploid and tetraploid populations of *H. intybaceum* does not correspond with the model of geographical parthenogenesis (van Dijk, 2003; Hörandl, 2008; Cosendai & Hörandl, 2010), a pattern in which apomictic polyploids have larger distribution ranges shifted to higher latitudes and elevations compared to related sexual diploids. Diploids of *H. intybaceum* are widely distributed across the entire range of the species (most likely except in the Vosges), mostly in previously strongly glaciated areas. Tetraploid apomictic populations, in contrast, are confined to a rather small geographical area. This pattern also differs from the trends found in other *Hieracium* spp. with ploidal differentiation in the Alps, e.g. in *H. prenanthoides* Vill. and *H. tomentosum* L. (diploids at the SW edge of the Alps; Chrtek *et al*., 2007, 2009; J. Chrtek and P. Mráz, unpubl.). We did not detect mixed-ploidy populations.

Genetic and ploidal pattern

We detected a rather low level of genetic variation in *H. intybaceum*. The relatively low contribution of the among-population component and low genetic distances (Fig. 3) can indicate gene flow (post-glacial genetic erosion) and/or rather recent divergence, especially in clusters S1 and S2. We preferred the result obtained by Bayesian clustering which identified four clusters, three of them more or less corresponding with the observed ploidal differentiation. The first two genetic groups (clusters S1 and S2) dominate across the whole distribution range and show east-west geographical separation (Fig. 2B).

The observed genetic and ploidal pattern can be explained as follows. Diploids colonized the deglaciated areas from source populations most likely located mainly in the southern part of the distribution range, occasionally also in the western Alps (see the highest DW values). Partially differentiated populations/lineages came into contact and consequently most likely further differentiation occurred. Apomictic tetraploids most likely originated in the western Alps or in the refugium at the south-western foot of the Alps. Their rather limited recent geographical range (partly

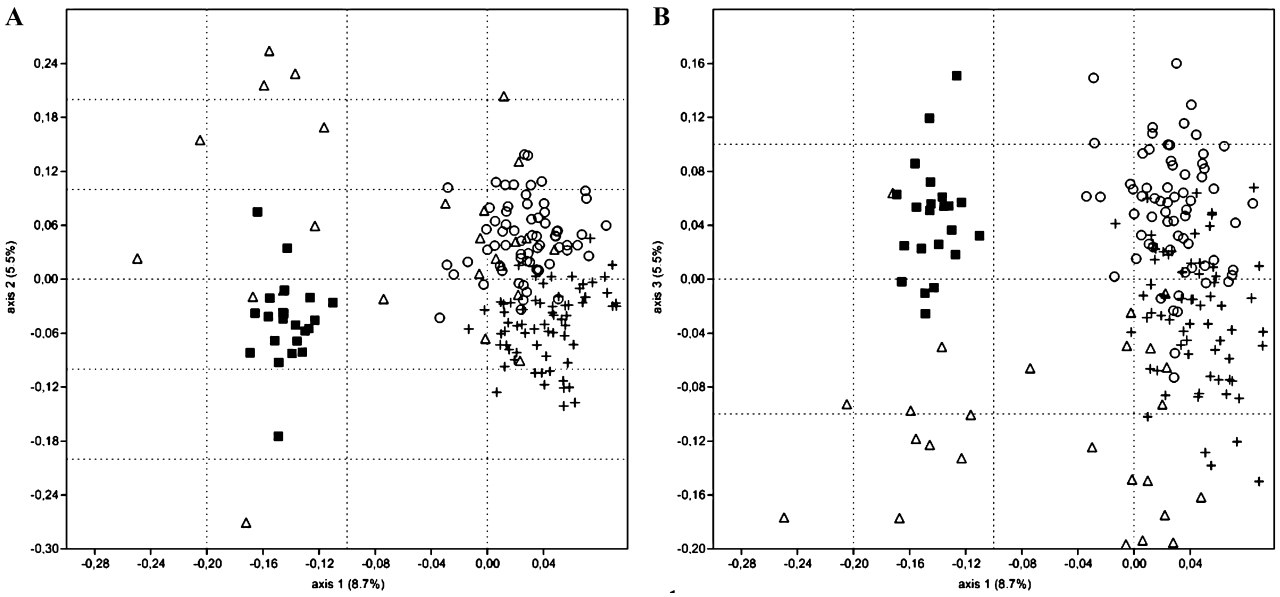

Figure 3. Principal coordinate analysis based on Jaccard similarity among AFLP phenotypes of *Hieracium intybaceum*. (A) axis 1 and axis 2; (B) axis 1 and axis 3. The four symbols represent groups produced by K-means analysis, which are congruent with those produced by Bayesian clustering; crosses – K1, white circles – K2, white triangles – K3, black squares – K4.

contrasting with the theory of geographical parthenogenesis) can be explained in two ways: (1) they originated rather recently but their occurrence coupled with a most likely complete absence of diploids in the Vosges should be taken into consideration, a more plausible explanation; or (2) they had/have a lower fitness than diploid plants. However, the latter is in a contradiction with putative general advantage of uniparental reproduction for colonization (plants reproduce independently of foreign pollen and do not suffer from low activity of pollinators in extreme habitats; van Dijk, 2003; Hörandl, 2009a). We did not carry out a detailed comparison of the fitness of diploids and tetraploids; however, seed production in tetraploids, for example, has proved to be at a similarly high level as in other successful apomictic hawkweeds (J. Zahradníček & J. Chrtek, unpubl.). Thus, the latter hypothesis seems to be much less plausible.

The diffuse borderline between clusters S1 and S2 runs from eastern Switzerland to the eastern part of North Tyrol. The geographical pattern in this genetic group is nearly, but not completely, identical to that detected in a set of silicicolous alpine species exhibiting differentiation along the main break zone (the transition between the western and eastern Alps), which runs roughly from Lake Garda to Innsbruck (Brenner zone; Thiel-Egenter *et al*., 2011).

The third genetic group (cluster S3) lacks a defined geographical range; it comprises mainly plants from two diploid populations from the opposite (west and east) margins of the distribution range and a few plants from both diploid and tetraploid populations across the distribution range in the Alps. Principal component analysis separated only one population from the south-western Alps (ROS) and tetraploid individuals along the first and second axis, respectively; other diploid individuals of the third genetic group are distributed in populations assigned to clusters S1 and S2. We therefore regard part of the distribution of genetic group 3 as an artefact of conflicts between approaches and the already mentioned low variability in the dataset. That at least one individual in each population can be assigned to cluster S3 confirms this claim.

The fourth genetic group (cluster S4) is well defined and includes only plants from tetraploid populations in the French Alps and the Vosges, and is genetically closely related to cluster S3 (most closely to population ROS). Geographically, its eastern borderline (Aosta valley and Wallis) is congruent with the main break zone detected in many alpine plants (e.g. Thiel-Egenter *et al*., 2011). The high genetic differentiation between the ROS population (cluster S3) and tetraploids (cluster S4) on the one hand and other diploids (clusters S1, S2) on the other might indicate long-term separation of these groups. We assume that genetic group 3 can be a source group of polyploid populations due to the considerably high presence of tetraploid plants (23%) in this genetic group and the genetic proximity between genetic group 3 and tetraploids.

Although certain break zones in *H. intybaceum* are more or less similar to those identified in other alpine plants, its genetic differentiation is generally lower. Four rather clearly defined groups of populations (SW, W, C and E) were found, for example, in *Ranunculus glacialis* L. (Schönswetter *et al*., 2004) and *Androsace alpina* (L.) Lam. (Schönswetter, Tribsch & Niklfeld, 2003). In both these species, the two western groups (SW and W) were genetically isolated both from each other and from the two eastern groups (C and E). Our genetic group S4 at least slightly corresponds to the SW group, S1 to the W groups and S2 approximately overlaps with the C and E groups. However, we did not detect a strong genetic divergence between the W group on the one hand and C and E groups on the other like in both *Ranunculus glacialis* and *Androsace alpina*.

To sum up, we found two cytotypes of *H. intybaceum*, namely sexual diploids prevailing throughout the Alps, and apomictic tetraploids confined to the western Alps and the Vosges. The majority of tetraploid plants forms one genetic group; diploids can be divided into three groups, but the overall genetic variation is low, most likely indicating gene flow and/or recent differentiation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Summary of K-means analysis based on AFLP data. Delta K values were counted following Evanno *et al*. (2005) and are showed for whole dataset (A), diploid dataset (B) and tetraploid dataset (C).

Figure S2. Summary of Structure analysis based on AFLP data. Delta K-values were counted following Evanno *et al*. (2005) and are showed for whole dataset (A), diploid dataset (B) and tetraploid dataset (C). Values of ln probability of the data for each number of groups (K) are plotted against the K-value for whole dataset (D), diploid dataset (E) and tetraploid dataset (F).

Genome size variation in the genus *Andryala* (Hieraciinae, Asteraceae)

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Abstract

Andryala includes perennial, annual and biennial, diploid plants distributed in the Mediterranean Basiin, Macaronesia and in one isolated outpost in the Romanian Carpathians. The aim of the present study was to analyse nuclear genome size in a phylogenetic framework and to assess relationships between genome size and life form (perennials vs. annuals/biennials) and insular vs. continental distribution. Absolute nuclear genome size of 207 plants from 67 populations corresponding to 18 species/subspecies were determined using propidium iodide flow cytometry. The evolution of genome size was investigated through the Brownian motion model with the tree scaling parameters λ, κ and δ. The mean 2C values differed up to 1,84-fold among different species (from 2,69 pg in *A. cossyrensis* to 5,01 pg in two polulations of *A. ragusina*). Little intraspecific variation was found in most species, whereas variation higher than 3,5% was detected in seven species. Chromosome numbers for six species are reported for the first time. High phylogenetic signal in genome size variation was found, which could indicate that the genome size is not strongly influenced by selection and is probably a result of neutral evolution or genetic drift. The highest C values were detected in two well-supported basal lineages corresponding to relict species *A. laevitomentosa* and *A. agardhii*. The genome size in the rest of *Andryala* species collapsed to well-supported large group named here 'Major Radiation Group' is significantly lower, except for two populations of *A. ragusina*. Genome size in perennial species is significantly lower compared with annual/biennial species. DNA amount in insular species is higher in comparison wit continental ones in the complete data set (all accession), but the relation is less clear in a reduced data set, including close related species of the 'Major Radiation Group'.

Keywords: *Andryala*, Asteraceae, genome size, Macaronesia, Mediterranean Basin, phylogeny

Introduction

One of the most important current topics in plant evolutionary biology is the functional significance of a tremendous (2000-fold) variation in genome size among angiosperms, caused by different proportions of non-coding repetitive DNA (Leitch and Bennett 2007; Wendel et al. 2016). While usually more or less constant within species or evolutionary units, genome size often considerably varies between species (even closely related ones) and can thus inform taxonomies in homoploid groups (Greilhuber 1998, 2005; Bennett et al. 2000; Murray 2005; Loureiro et al. 2010). Differences in genome size can also indicate a complex pattern, sometimes not easily detectable from morphology, and/or different evolutionary histories in morphologically delimited taxonomic units (Obermayer and Greilhuber 2005; Leong-Škorničková et al. 2007; Suda et al. 2007a, b; Slovák et al. 2009). Although there are studies that relate DNA amount to phylogeny (e. g., Albach and Greilhuber 2004; Garnatje et al. 2007; Weiss-Schneeweiss et al. 2006; Suda et al. 2007a; Chrtek et al. 2009; Dušková et al. 2010; Andrés-Sánchez et al. 2013; Frajman et al. 2015; Mandák et al. 2016), the evolutionary significance of genome size is still not well understood.

Associations between DNA amount and life cycle, mode of reproduction and ecogeographical or other factors have been also widely studied (Knight et al. 2005). Annuals and biennials have generally low genome size in comparison with perennials (Bennett 1972), the same holds for selfers when compared with outcrossers (Price 1976; Govindaraju and Cullis, 1991). A positive correlation between genome size and alpine habitats was found in e.g. *Veronica* (Albach and Greilhuber 2004), *Centaurea* s. str. (Bancheva and Greilhuber 2006) and in some grasses (Bennett 1976; Laurie and Bennet 1985; Rayburn and August 1990). This can be explained by a higher capacity for growth at low temperatures and frost resistance in larger genomes (MacGillivray and Grime 1995) or by the more phosphate-rich soils at higher altitudes (Körner 1989). In contrast, smaller genomes often occur under stressful conditions caused by the presence of heavy metals, impairment of nutrient uptake, water stress and temperature stress (Price et al. 1981; Knight et al. 2005; Šmarda et al. 2008; Pustahija et al. 2013; Lazarević et al. 2015). However, other studies have revealed other patterns, and the picture seems to be more complex depending on the group studied, and lacking any general trend (Knight et al. 2005; Loureiro et al. 2010). Nonetheless, correlation between DNA amount and insularity has been repeatedly documented and seems to be a general rule (Suda et al. 2005; Kapralov and Filatov 2011), island species possessing lower genomes in comparison with their continental counterparts.

The genus *Andryala* L. belongs to subtribe *Hieraciinae*, together with *Hieracium* L. (incl. *Stenotheca* Monnier), *Pilosella* Hill and *Hispidella* Barnadez ex Lam. In its present circumscription (including *Paua* Caball., *Rothia* Schreb. and *Pietrosia* Nyár. ex Sennikov) it includes ca. 17 perennial, biennial or annual species distributed mainly in the Mediterranean Basin and Macaronesia with centres of diversity in northwest Africa, the Iberian Peninsula and the Canary Islands, and with one stenoendemic species in the Eastern Carpathians (Greuter 2006-; Blanca 2011; Ferreira et al. 2014a, b, 2015). The genus includes the relict species *A. laevitometosa* (Nyár. ex Sennikov) Greuter with only a handful of microlocalities at two neighbouring peaks in the Eastern Carpathians (Witkowski et al. 2003; Negrea and Pricop 2009) and *A. agardhii* DC. with a few localites in Spain and one in Morocco (Blanca et al. 1998) as well as widely distributed and sometimes invasive species like *A. integrifolia* L. In contrast to a high frequency of polyploidy and apomixis in the sister genera *Hieracium* and *Pilosella*, only se-

xual diploids have been reported in *Andryala* (Goldblatt and Johnson 1979-; Ferreira et al., 2015). Phylogenetic analyses based on nuclear ribosomal DNA and the single copy gene squalene synthase (*sqs*) showed a monophyletic origin of *Andryala*, while cpDNA analysis indicated an ancestral chloroplast capture event with the sister genus *Pilosella* (Fehrer et al. 2007; Ferreira et al. 2015). Intrageneric relationships are still partly unclear due to the low level of nucleotide divergences of most markers used or to incomplete lineage sorting of divergent alleles (*sqs*). Phylogenetic analyses revealed two well-supported basal lineages corresponding to the relict species *A. agardhii* and *A. laevitomentosa*. The remaining taxa formed a well-supported clade ('Major Radiation Group'; Ferreira et al. 2015).

The objectives of the present study were to provide a comprehensive overview of genome size variation in *Andryala* and to relate the genome size with the phylogeny, the biogeographical framework (insular vs. continental accessions/species), and life forms (annuals and biennials vs. perennials).

Material and methods

Plant material

Living plants or seeds of 18 *Andryala* species and subspecies (the taxonomic concept follows that in Ferreira et al. 2015) were collected in the field between 2007 and 2012 and cultivated in the Experimental garden of the Institute of Botany in Průhonice (Table 1, Appendix 1). At least three populations per species were sampled, except for very rare and geographically restricted taxa (a total of 67 populations was sampled). Three north-African species, namely *A. chevallieri* Barratte ex L. Chevall., *A. nigricans* Poir. and *A. spartioides* (Pomel ex Batt. & Trab.) Barratte, were not included due to unclear geographic range and political instability in the region. *Andryala cintrana* S. Talavera & M. Talavera, recently described from Portugal (Talavera and Talavera 2015) was also not included. Morphologically well delimited subspecies of *A. glandulosa* Lam., *A. mogadorensis* (Hook. f.) Greuter and *A. pinnatifida* Aiton were recognized here. Based on remarkable differences in the 2C values, accessions of *A. ragusina* L. were split into two groups, marked as "*A. ragusina* I" and "*A. ragusina* II". Accessions included in previously published phylogenetic study of *Andryala* (Ferreira et al. 2015) are marked in Table 1. Voucher specimens are preserved in the herbarium PRA.

Chromosome numbers

Chromosome numbers were counted for young seedlings from seeds collected in 12 populations of 11 *Andryala* taxa (Table 1). In the rest of species, there was either low seed germination or problems with cultivation of perennial species. However, chromosome numbers have been published for the rest of taxa, except for *A. atlanticola* H. Lindb., *A. crithmifolia* Aiton, *A. mogadorensis* subsp. *jahandiezii* (Maire) Greuter and *A. perezii* Ferreira et al. (cf. Goldblatt and Johnson 1979-).

The chromosome counts are based on somatic mitoses in root-tips of young seedling (all seeds originated from populations formed by only one species, hybrid origin of seeds can thus be excluded). Root-tips were pretreated with a saturated solution of α-bromonaphtalene for 3 hours at room temperature, rinsed in water and fixed in cold acetic acid-ethanol (1:3)

overnight. The fixed material was stored in 70% ethanol at 4°C until required. The maceration was carried out in 1N HCl at 60°C for 7 min. The root-tips were then rinsed in water and the cut meristems were squashed in a drop of lacto-propionic orcein (Dyer 1963).

Genome size estimation

Altogether 207 plants from 67 populations were analyzed. Genome size was determined by flow cytometry using a Partec CyFlow cytometer equipped with a green (532 nm) solid state laser. *Pisum sativum* L. 'Ctirad', 2C = 8.85 pg (Doležel et al. 1994; Suda et al. 2007a) was used as an internal standard for all species. The modified two step-procedure described by Otto (1990) was employed for sample preparation. Intact leaf tissues (approx. 1 cm^2) of the analyzed species and an appropriate quantity of the internal standard were co-chopped with a sharp razor blade in a plastic Petri dish with 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) as the nuclear isolating solution. The suspension was filtered through a 42 μm nylon filter and centrifuged at 15 g for 5 min. The supernatant was discarded and the pellet was resuspended in 100 μl fresh Otto I buffer. Samples were incubated for at least 10 min at room temperature and mixed with 1 ml Otto II buffer (0.4 M $Na_2HPO_4 * 12H_2O$) supplemented with propidium iodide (PI) as the fluorochrome, RNAse IIA (both at a concentration of 50 μl ml^{-1}) and β-mercaptoetanol (2 μl ml^{-1}). Samples were stained for 5 min at room temperature before measurement. Usually, 5000 nuclei were analyzed for each sample. Nuclear genome size was calculated as a linear relation between the ratio of 2C peaks of sample and standard. Each plant was measured at least three times on different days by the same operator to eliminate potential artifacts. If the difference between the three measurements exceeded 2%, the value was discarded and the sample was re-analyzed. Coefficients of variation (CVs) of both peaks did not exceed 5%.

Statistical analyses of genome size

Differences among species were investigated using one-way ANOVA with all but one (*A. cossyrensis*, as the species is represented by one sample only) samples included; the Scheffé test was applied to examine which species were statistically not different in their 2C values.

Evolution of genome size was investigated on a strict consensus tree based on nuclear ribosomal markers (ITS+ETS) published by Ferreira et al. (2015; Fig. 1) using the generalized least-squares method as implemented in BayesTraits (Pagel and Meade 2007). All genome size data were $\log_{10}$ transformed prior to analyses. Two models of trait evolution were compared, using likelihood ratio statistics (Huelsenbeck and Rannala 1997) or BayesFactor. Model A (drift model) corresponds to the standard constant-variance random-walk model, and model B is a directional random-walk model (Pagel 1999). The scaling parameters lambda (λ), kappa (κ) and delta (δ) were optimized for 2Cx-values. λ assesses the contribution of the phylogeny to a character, κ scales branch lengths and can be used to test punctual vs. gradual modes of trait evolution, and δ scales the overall path length in the phylogeny. Values of 1.0 correspond to the null model (tree topology and branch lengths accurately describe models A and B). To test whether a model with estimated scaling parameters is a better fit than the null model where all scaling parameters are set to 1 (i.e. a strict Brownian motion model) the likelihood ratio test or BayesFactor was used. Two different methods of analysis were used,

namely maximum likelihood and Monte Carlo Markov chain (MCMC).

To test the significance of differences in 2C-values between (i) insular and continental accessions and (ii) between perennial and annual/biennial accessions (Table 1), the t-test was used (data were $\log_{10}$ transformed). We used (i) a complete data set, and (ii) a reduced data set, restricted to species from the 'Major Radiation Group' (i.e., *A. agardhii* and *A. laevitomentosa* were excluded). However, we are aware about possible bias caused by restricted sampling of *A. dentata*, with a large Eastern Mediterranean range (we have only insular accessions) and by the fact that *A. integrifolia* in Macaronesia is most likely recently introduced. We performed thus a further test, where all *A. dentata* and *A. integrifolia* accessions were taken as continental.

Results

Chromosome numbers and genome size

Chromosome numbers were established for seedlings from 12 populations of *Andryala arenaria* (Guss. ex DC.) Boiss. & Reut., *A. dentata* Sm., *A. glandulosa* subsp. *glandulosa*, *A. integrifolia* L., *A. laevitomentosa*, *A. laxiflora* DC., *A. maroccana* (Caball.) Maire, *A. mogadorensis*, *A. pinnatifida* and *A. ragusina*, only diploids ($2n = 2x = 18$) were recorded. Flow cytometry analyses produced high-resolution histograms with CVs of G_1/G_0 peaks of *Andryala* samples from 1.29% to 6.9% (mean 2.85%) and internal reference standard *Pisum sativum* from 1.15% to 6.97% (mean 2.51%). The mean 2C values varied 1.84-fold among taxa, from 2.69 pg (1Cx =1.345 pg) in *A. dentata* to 5.01 pg in *A. ragusina* II (Table 1, Fig. 2, Fig. 3). The analysis of variance (ANOVA) found significant differences among species ($F_{17, 188} = 1593.1$, $p<0.01$) and Scheffé`s test revealed 7 groups at $\alpha = 0.01$. The same test, performed for a subset of species of the 'Major Radiation Group', separated 6 groups at $\alpha = 0.01$ (Table 2).

Within species variation

Intraspecific variation in all but one species (*A. ragusina*) was generally low, ranging from 0.15% in *A. maroccana* to 7.54% in *A. pinnatifida* (1Cx values). In *A. ragusina* (total variation 46.89%), two groups were distinguished based on genome size differences, '*A. ragusina* I' with 1.69% variation and'*A. ragusina* II' with 0.99% variation. Variation exceeding the approximate measurement inaccuracy treshold of 3.5% (following Suda et. al. 2007a in *Pilosella* and Chrtek et al. 2009 in *Hieracium*) was found in *A. agardhii*, *A. crithmifolia*, *A. dentata*, *A. glandulosa* subsp. *cheirathifolia* (L'Hér.) Greuter, *A. integrifolia*, *A. mogadorensis* (with exclusion of *A. mogadorensis* subsp. *jahandiezii*), *A. pinnatifida* (with exclusion of *A. pinnatifida* subsp. *teydensis* (Sch. Bip.) Rivas Mart. et al.) and *A. ragusina*.

Evolution of genome size

Maximum likelihood method. For the complete data set, a directional model of evolution (model B) did not result in significantly higher likelihood scores than a drift model of evolution (model A; -15.371 vs. -15.398), indicating that there is no general trend to either genome size increase or decrease. Scaling parameters leading to the highest likelihood for

1Cx values were λ = 0.929, δ = 2.179 and κ = 2.629 in model A and λ = 0.929, δ = 2.205 and κ =2.607 in model B, respectively. For both models, the values of lambda did not differ significantly from 1 (the null expectation, LR test), indicating that the phylogeny correctly predicts the pattern of covariance among species on the genome size; higher values of kappa and delta indicate accelerated rates of evolution within long branches and temporally later change (species-specific adaptation), respectively. Likelihoods of null model (with scaling parameters set to 1.0) are significantly lower (LR test) in both models, indicating that scaling parameters improve the fit of the data to the model.

MCMC method. Similarly to the maximum likelihood method, log maximum likelihoods (using harmonic means, 500000 iterations, a burn-in of 10000, and a sampling frequency of 250) in both models (A and B) did not differ significantly (-16.625 in model A, -17.376 in model B, scaling parameters set to 1.0). The model with estimated scaling parameters was a better fit than the null model (with scaling parameters set to 1.0) for both models A and B (-14.244 vs. -16.625 in model A, -14.214 vs. -17.376 in model B), showing that the scaling parameters improve the fit of the data to the model. The values of λ (0.885 and 0.886, respectively) did not differ significantly from 1.0, relatively high values of κ (1.420 and 1.427, respectively) and δ (3.179 and 3.161, respectively) indicate accelerated rates of evolution within long branches and temporally later change (species-specific adaptation), respectively.

Genome size versus insularity and life form

Genome size of the insular accessions was significantly lower in comparison with continental accessions in both complete ($t = 8.102$, d.f. = 117.12, $p<0.001$) and reduced ($t = 4.392$, d.f. = 87.579, $p<0.001$) data sets (Fig. 4A,B). However, if all *A. dentata* and *A. integrifolia* accessions are taken as continental in the reduced data set, the difference is not significant ($t = 0.723$, d.f. = 88.194, $p = 0.472$). In the complete data set, the difference remains significant ($t = 4.955$, d.f. = 111.62, $p<0.001$). Genome size was significantly lower in annual/biennial species compared to perennial species in both complete ($t = 9.125$, d.f. = 136.53, $p<0.001$) and reduced data set ($t = 7.060$, d.f. = 79.511, $p<0.001$) (Fig. 5A,B).

Discussion

Ploidy and genome size in Andryala and related species

This study provides the first genome size estimates for all but one (*A. webbii* Christ, synonymized here with *A. pinnatifida*; Suda et al. 2005) *Andryala* species and the first chromosome number records for *A. dentata*, *A. laevitomentosa*, *A. maroccana*, *A. mogadorensis*, *A. pinnatifida* and *A. ragusina*. We also confirmed previously published counts ($2n$ = 18) for *A. arenaria*, *A. glandulosa* subsp. *glandulosa*, *A. integrifolia* and *A. laxiflora* (Goldblatt & Johnson 1979-). It is likely that *Andryala* comprises only diploid species, in contrast to the closely related *Pilosella* and *Hieracium* subgen. *Hieracium* with high proportions of polyploids. The two remaining groups of the subtribe *Hieraciinae*, *Hieracium* subgen. *Chionoracium* and *Hispidella* also seem to be exclusively diploid.

The range of monoploid genome size (1Cx) in *Andryala* (from 1.35 pg to 2.51 pg, 1.84-fold

variation among species) did not substantially differ from that in *Pilosella* (from 1.72 pg to 2.16 pg, 1.23-fold variation; Suda et al. 2007a). In contrast, the DNA content in *Hieracium* subgen. *Hieracium* is approximately twofold compared with *Andryala* and *Pilosella*, ranging between 3.51 pg and 4.34 pg (varied 1.22-fold). Even bigger genome size was estimated in *Hieracium* subgen. *Chionoracium* Schultz-Bip. (Zahradníček, Krahulcová, unpubl.). The genome size of *Hispidella* has not been reported yet. The reasons for these large differences among closely related groups remains unclear. Similarly to other plant groups, accumulation of repetitive sequence elements might be one of the causes. An insight into genomes might shed light on this topic.

Evolution of genome size in Andryala

The maximum likelihood estimate of the parameter λ is very close to 1, showing high phylogenetic signal which could indicate that the genome size is not strongly influenced by selection and is probably a result of neutral evolution or genetic drift. Strong phylogenetic signals in genome size were also detected in *Orobanche* (Weiss-Schneeweiss et al. 2006), *Hieracium* (Chrtek et al. 2009), *Filago* (Andrés-Sánchéz et al. 2013) and *Primulina* (Ming Kang et al. 2014). Genome size thus might be phylogenetically conserved among closely related species in general (but see Wendel et al. 2002). Relatively high values of $\delta > 1.0$ could be a signature of accelerating evolution as time progresses, i. e. that there was an increase in the rate of genome size change in recent phases of the genus‘ evolution. Species-specific adaptations to various habitats throughout the distribution range, but especially in Macaronesia may be the most plausible explanation. A similar trend was reported in *Primulina* in China (Ming Kang et al. 2014), and *Allium* subgen. *Melanocrommyum* (Gurushidze et al. 2012). In contrast, genome size evolved more rapidly in earlier phases of evolution in e.g. *Filago* (Andrés-Sánchéz et al. 2013). High values of κ (> 1) suggest proportionally more evolution in longer branches (gradual evolution). The same mode was inferred in closely related *Hieracium* (Chrtek et al. 2009), whereas the hypothesis of punctuated genome size evolution received support in e. g. *Orobanche* (Weiss-Schneeweiss et al. 2006), Liliaceae (Leitch et al. 2007) and *Allium* subgenus *Melanocrommyum* (Gurushidze et al. 2012).

Genome size and environmental correlates and life cycles

The effect of environmental factors on genome size evolution has been debated for years and is still a matter of controversy. Here, we evaluated relations between DNA content and two factors – insularity and life form (annual/biennial vs. perennial). Genome size in insular accessions was lower in comparison with continental accessions in both complete and reduced data sets. However, the results should be interpreted carefully due to a possible bias caused by restricted sampling of *A. dentata* and status of *A. integrifolia* in Macaronesia. *Andryala dentata* is predominantly continental species with a large Eastern Mediterranean range. Unfortunately, we did not gather continental samples, and our data are based on insular accessions. *Andryala integrifolia*, common in the western and central part of the Mediterranean Basin and northwestern Africa is most likely not native in the Canary Isles.

If these samples are taken as continental the difference between continental and insular accessions remains significant in the complete data set, but is not significant in the subset of species

of the 'Major Radiation Group'. Differences in C-values and genome size between insular and continental species were firstly reported by Suda et al. (2005), based on a detailed comparison of a set of Macaronesian vs. non-Macaronesian representatives of selected genera and families. Consequently, relatively small genome size of island endemics compared to the mainland biota was confirmed for other oceanic archipelagos and seems to be a general rule (Kapralov and Filatov 2011). The differences can be explained by a loss of DNA since the archipelago was colonized or by predominance of colonizers with small genomes or higher naturalization potential of species with small genomes (Kapralov and Filatov 2011). Another theory is based on the statement that ancestral taxa probably possessed small genomes (Leitch et al., 1998). A large set of Macaronesian plants meet this criterion, which can also support the relictual nature of at least some part of the Macaronesian flora (Bramwell 1976). However, a derived position and relatively recent origin of Macaronesian taxa has been found in e.g. *Argyranthemum* (Francisco-Ortega & al. 1997), *Echium (Mansion et al. 2009), Cheirolophus* (Garnatje et al. 2007), and *Andryala* seems to be a similar case (Ferreira et al. 2015).

Correlation between genome size and life forms (higher genome size in perennials compared to annuals) seems to be common throughout angiosperms (e.g. Bennett 1972; Bennett et al. 1998; Bennett and Leitch 2005), and was also documented in several genera of Asteraceae, e.g. in *Crepis* (Enke et al. 2011) and *Cheirolophus* (Garnatje et al. 2007). Our data match this trend, in both complete and reduced ('Major Radiation Group') data sets. However, a confounding effect of life form and mode of reproduction (selfing vs. outcrossing) must be considered, as annuals often display higher rates of selfing than perennials (Barrett et al. 1996), and the correlation might be caused by a reduction in transposable elements in selfers (deleterious recessive model; Wright and Schoen 1999; Morgan 2001). Unfortunately, we have no data about selfing rates in *Andryala* species.

Worth mentioning is a distinctly higher DNA content in the two relict species (*A. agardhii* and *A. laevitomentosa*) compared to species of the 'Major Radiation Group' (except for *A. ragusina* II). This coincides with a statement that genera with large genomes were less likely to be highly speciose, suggesting that a large genome may be disadvantageous (Knight et al. 2005). Additionally, species with higher DNA content are generally under a higher threat of extinction in comparison with species with small genomes (Vinogradov 2003), which also fits well with the rarity of both *A. laevitomentosa* and *A. agardhii*.

Taxonomic implications

Our genome size data are generally in line with proposed taxonomic rearrangements proposed by Ferreira et al. (2014a, b, 2015). Differences in genome size support the separation of northwest-African *A. mogadorensis* from Macaronesian *A. pinnatifida*, recently proposed on the basis of both molecular data (Ferreira et al. 2015) and a morphological re-evaluation (Ferreira et al. 2014b). On the other hand, accessions referring to *A. atlanticola* did not differ from those of *A. integrifolia* with respect to the DNA content, which further supports doubts about the species status of *A. atlanticola* raised by molecular and morphological data (Ferreira et al. 2015). The values of morphological traits of this species fall into the variation range of *A. integrifolia*, and molecular data also do not show any differentiation between these taxa. The species status of *A. atlanticola* thus does not seem to be justified. Species of the 'Major

Radiation Group' have distinctly lower DNA content in comparison with the two relict species, *A. aghardii* and *A. laevitomentosa* (except for two accessions of *A. ragusina*). This might doubt the inclusion of two relict species into *Andryala* and justify their separation into the genus *Pietrosia*, as proposed by Sennikov (1999). On the other hand, the monophyly of *Andryala* along with the non-monophyly *A. agardhii* and *A. laevitomentosa* strongly contradicts this concept, although also morphologically the relict taxa are more similar to each other than to the remaining *Andryala* species (Sell 1975, 1976). Differences in genome size also support the species status of *A. dentata*, which replaces *A. integrifolia* in the eastern part of the Mediterranean Basin and was synonymized with (or included in) the latter by some authors (Sell 1976; Greuter 2006-). This concept was also supported by molecular markers and distinct morphology. A contact zone of the two species is located in continental Italy and on Sicily and Pantelleria (Ferreira et al. 2015 and unpubl. data). The remarkable differences in genome size within *A. ragusina* remain a puzzle. Cryptic speciation within this morphologically rather invariable species does not seem to be a promising hypothesis because intraspecific variation for all molecular markers employed for phylogeny reconstruction was very low (Ferreira et al. 2015). Unfortunately, we do not have chromosome counts for plants with higher 2C values and thus polyploidy cannot be fully excluded. If the plants should indeed be diploid, strong bursts of repetitive elements in the genomes of the Andalusian populations might be a potential explanation. To clarify these points, living plants would have to be re-sampled and analyzed again.

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Table 1 – Accession origin, newly estimated chromosome numbers and genome size. †Life form (P = perennial, A = annual or biennial) and contitental (C) vs. insular (I) origin of accession are given in parentheses. Accessions included in Ferreira et al. (2015) are marked by an asterisk.

Taxon	Number of plants	Identifier	Locality	2n	2C (pg) mean	1Cx (pg) mean	2C population variation (%)	SD	SE	min	max	2C (pg) taxon mean	1Cx (pg) taxon mean	2C taxon variation (%)	SD tax.	SE tax.
Andryala agardhii DC. (P;C)	2	JC 2011_30	Spain, Andalusia: Calar de Santa Barbara		4,90	2,45	1,96	0,07	0,05	4,85	4,94	4,80	2,40	5,26	0,05	0,01
*	5	JC 2011_31	Spain, Andalusia: Calar del Desabezedo		4,80	2,40	5,68	0,11	0,05	4,70	4,97					
	5	JC 2011_33	Spain, Andalusia: Maria		4,77	2,38	1,41	0,03	0,01	4,73	4,80					
*	4	JZ 2011_3	Spain, Andalusia: Mágina		4,74	2,37	0,82	0,02	0,01	4,72	4,75					
	2	JZ 2211	Spain, Andalusia: Pinar Liano		4,78	2,39	0,83	0,03	0,02	4,76	4,80					
A. arenaria (Guss. ex DC.) Boiss. & Reut. (P;C)	3	JC 2011_3	Spain, Andalusia: Marbella		3,26	1,63	0,63	0,01	0,01	3,25	3,27	3,23	1,62	2,56	0,03	0,01
	2	JC 2011_7	Spain, Andalusia: Bornos	2n = 18	3,24	1,62	0,05	0,00	0,00	3,24	3,24					
	1	JC 2011_9	Spain, Andalusia: Almonte		3,19	1,60	0,00	0,00	0,00	3,19	3,19					
A. atlanticola H. Lindb. (A;C)*	1	10JZ_08	Morooco, High Atlas: Araund		3,02	1,51	0,00	0,00	0,00	3,02	3,02	3,01	1,50	1,51	0,02	0,01
	1	JC 13_V	Morocco, High Atlas: Imlil 1		2,98	1,49	0,00	0,00	0,00	2,98	2,98					
	3	Ixis	Morocco, High Atlas: Imlil 2		3,01	1,51	1,14	0,02	0,01	2,99	3,03					
A. cossyrensis Guss. (A;C)*	1	JC 3	Morocco, region Oriental: Zegzel		2,69	1,35	0,00	0,00	0,00	2,69	2,69	2,69	1,35			
A. crithmifolia Aiton (P;I)	3	260_ZF	Portugal, Madeira: Pináculo		3,30	1,65	1,15	0,02	0,01	3,28	3,32	3,27	1,63	4,45	0,05	0,00
	3	259_ZF	Portugal, Madeira: Cabo Girão		3,32	1,66	1,87	0,03	0,02	3,29	3,35					
	5	301_ZF	Portugal, Madeira: Garajau	2n = 18	3,22	1,61	0,80	0,01	0,01	3,21	3,24					
A. dentata Sm. (A;C)	3	JZ 0412	Italy, Pantelleria: Rekhale	2n = 18	2,75	1,37	1,33	0,02	0,01	2,73	2,77	2,78	1,39	6,17	0,06	0,00
*	3	JZ 0512	Italy, Pantelleria: Tracino		2,75	1,37	0,75	0,01	0,01	2,74	2,76					
*	3	JZ 0612	Italy, Pantelleria: Siba-Roncone		2,84	1,42	5,16	0,07	0,04	2,75	2,90					
A. glandulosa subsp. *cheiranthifolia* (L'Hér.) Greuter (P;I)	7	248_ZF	Portugal, Madeira: Pico do Areeiro		3,31	1,66	4,00	0,05	0,02	3,22	3,38	3,33	1,67	4,78	0,05	0,00
	2	271_ZF	Portugal, Madeira: Jardim do Mar		3,34	1,67	0,41	0,01	0,01	3,33	3,35					

	3	241_ZF	Portugal, Madeira: Encumeada		3,38	1,69	1,50	0,03	0,01	3,36	3,41						
	4	257_ZF	Portugal, Madeira: Fajã da Ovelha		3,31	1,65	1,26	0,02	0,01	3,29	3,34						
	3	269B_ZF	Portugal, Madeira: Calheta		3,33	1,67	0,05	0,02	0,01	3,32	3,35						
A. glandulosa Lam. subsp. G*landulosa* (P;I)*	1	233_ZF	Portugal, Madeira: Seixal		3,32	1,66	0,00	0,00	0,00	3,32	3,32	3,29	1,64	2,95	0,03	0,00	
*	3	Glan_ZF	Portugal, Madeira: Porto Moniz		3,29	1,64	0,18	0,00	0,00	3,28	3,29						
	4	298_ZF	Portugal, Madeira: S. Jorge		3,24	1,62	0,90	0,01	0,01	3,23	3,25						
	3	ZF_278A	Portugal, Madeira: Ponta do Pargo		3,30	1,65	1,02	0,02	0,01	3,29	3,32						
A. integrifolia L. (A;C)*	4	KV 4	Spain, Canary Islands, Gran Canaria: Valleseco		3,11	1,55	1,79	0,02	0,01	3,09	3,13	3,09	1,54	4,75	0,04	0,01	
*	3	SB_H12	Spain, Canary Islands, El Hierro: Tiñor	2n = 18	3,14	1,57	1,71	0,03	0,02	3,11	3,16						
	3	SB H15	Spain, Canary Islands, El Hierro: San Andrés I		3,06	1,53	1,31	0,02	0,01	3,05	3,09						
	3	SB H16i	Spain, Canary Islands, El Hierro: San Andrés II		3,09	1,54	0,86	0,02	0,01	3,08	3,10						
	3	ZF LM5104	Spain, Madrid: Guadalix de la Sierra		3,11	1,55	1,20	0,02	0,01	3,09	3,13						
*	3	AZ_3	Algeria, Alger: Kouba		3,05	1,53	1,87	0,03	0,02	3,02	3,07						
	2	AZ_4	Algeria, Alger: Le Caroubier		3,06	1,53	2,23	0,04	0,02	3,02	3,09						
A. laxiflora DC. (P;C)	2	JC 2011_15	Spain, Andalusia: Encinasola		3,56	1,78	0,80	0,02	0,01	3,56	3,58	3,57	1,78	1,31	0,02	0,01	
*	1	JC 2011_19	Spain, Andalusia: Hornachuelos	2n = 18	3,59	1,79	0,74	0,00	0,00	3,57	3,57						
*	3	JC 2011_20	Spain, Andalusia: Linares		3,57	1,78	0,00	0,01	0,01	3,58	3,60						
A. laevitomentosa (Nyár. ex Sennikov) P.D. Sell ex Greuter	3	A	Romania, Bukovina: Pietrosul Bogolin I	2n = 18	4,97	2,48	0,55	0,01	0,01	4,95	4,98	4,97	2,49	1,22	0,02	0,01	
	1	C	Romania, Bukovina: Pietrosul Bogolin II		5,01	2,50	0,00	0,00	0,00	5,01	5,01						
*	2	JC 2011/40/2	Romania, Bukovina: Pietrosul Brostenilor		4,95	2,47	0,00	0,00	0,00	4,95	4,95						

A. maroccana (Caball.) Maire (P;C)*	3	JZ_1711	Morocco, region Oriental: Beni Chiker	2n = 18	3,63	1,81	0,15	0,00	0,00	3,63	3,63	3,63	1,81	0,15	0,00	0,00
A. mogadorensis (Hook. f.) Greuter (P;C)*	5	JC 15	Morocco, region Sous-Masa-Drâa: Tamri		3,52	1,76	4,40	0,06	0,03	3,42	3,57	3,53	1,77	5,88	0,10	0,02
*	5	JC 18/V2	Morocco, region Doukkala-Abda: Moulay Abdallah	2n = 18	3,61	1,80	1,17	0,02	0,01	3,58	3,62					
*	5	JC 16N	Morocco, region region Marrakech-Tensift-El-Haouz: Essaouira (Mogador) I		3,54	1,77	2,72	0,04	0,02	3,48	3,58					
	1	JC 17	Morocco, region Doukkala-Abda: Sidi Rosia	2n = 18	3,54	1,77	0,00	0,00	0,00	3,54	3,54					
*	5	ZF 263A	Morocco, region region Marrakech-Tensift-El-Haouz: Essaouira (Mogador) II		3,50	1,75	3,58	0,03	0,01	3,54	3,58					
A. mogadorensis subsp. *jahandiezii* (Maire) Greuter (P;C)*	3	JC 14	Morocco, region Sous-Masa-Drâa: Inezgane		3,47	1,73	0,79	0,01	0,01	3,45	3,48	3,47	1,73	0,79	0,01	0,01
A. perezii Ferreira et al. (P;I)*	3	KV 51V	Spain: Canary Islands, Lanzarote: Haria		3,28	1,64	1,33	0,02	0,01	3,25	3,30	3,29	1,65	2,68	0,03	0,01
*	3	KV 52V	Spain, Canary Islands, Lanzarote: El Jurado		3,30	1,65	2,16	0,04	0,02	3,27	3,34					
A. pinnatifida (P;I)	3	KV 10	Spain, Canary Islands, Gran Canaria: Barranco de Guayadeque		3,26	1,63	3,13	0,05	0,03	3,21	3,31	3,26	1,63	7,54	0,05	0,01
	6	KV 16	Spain, Canary Islands, Tenerife: Cumbre de Bolico		3,26	1,63	2,73	0,03	0,01	3,20	3,29					
	3	KV 25	Spain, Canary Islands, La Gomera: Alto de Garajonay		3,29	1,64	1,70	0,03	0,02	3,27	3,33					
	3	SB G5	Spain, Canary Islands, La Gomera: Hermigua		3,33	1,66	1,37	0,03	0,01	3,30	3,34					
*	3	SB H13	Spain, Canary Islands, El Hierro: San Andrés		3,21	1,61	0,41	0,01	0,00	3,20	3,22					
*	6	SB G7b	Spain, Canary Islands, La Gomera: Arure		3,29	1,65	4,79	0,07	0,03	3,21	3,37					
*	3	SB G9	Spain, Canary Islands, La Gomera: Vallehermoso		3,28	1,64	2,17	0,04	0,02	3,24	3,31					
*	3	SB T2	Spain, Canary Islands, Tenerife: Puerto de la Cruz		3,27	1,64	0,98	0,02	0,01	3,25	3,29					
*	6	SB H14	Spain, Canary Islands, El Hierro: Sabinosa	2n = 18	3,24	1,62	0,88	0,02	0,01	3,22	3,24					
*	3	SB H16p	Spain, Canary Islands, El Hierro: San Andrés		3,19	1,60	3,53	0,06	0,03	3,13	3,24					

A. pinnatifida subsp. *teydensis* (Sch. Bip.) Rivas Mart. et al. (P;I)	1	SB T4	Spain, Canary Islands, Tenerife: Cañadas		3,30	1,65	0,00	0,00	0,00	3,30	3,30	3,30	1,65	2,03	0,03	0,01
*	3	SB T18	Spain, Canary Islands, Tenerife: Cañadas		3,27	1,64	0,54	0,01	0,01	3,27	3,29					
*	3	SB T17	Spain, Canary Islands, Tenerife: Montañas de Anaga	2n = 18	3,22	1,61	0,55	0,01	0,01	3,22	3,23					
	3	SB T19	Spain, Canary Islands, Tenerife: Cañadas		3,32	1,66	0,67	0,01	0,01	3,31	3,33					
A. ragusina L. I. (P;C)*	4	JC 2011_35	Spain: Comunitat Valenciana: Sax	2n = 18	3,42	1,71	0,13	0,00	0,00	3,42	3,43	3,43	1,72	1,69	0,02	0,01
*	3	ZF LM5103	Spain, Madrid: Guadalix de la Sierra		3,43	1,72	1,69	0,03	0,02	3,41	3,47					
A. ragusina L. II. (P;C)*	3	JC 2011_2	Spain, Andalusia: Alhaurin el Grande		5,02	2,51	0,99	0,03	0,01	4,99	5,04	5,01	2,51	0,99	0,03	0,01
*	2	JC 2011_27	Spain, Andalusia: Jérez del Marquesado		4,99	2,50	0,00	0,00	0,00	4,99	4,99					

Table 2 – Grouping of *Andryala* species according to the genome size (Scheffé´s test).

Species	Mean	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
A. dentata	2,69	+					
A. atlanticola	3,01		+				
A. integrifolia	3,09		+				
A. arenaria	3,24			+			
A. pinnatifida subsp. *pinnatifida*	3,26			+			
A. crithmifolia	3,27			+			
A. glandulosa subsp. *glandulosa*	3,28			+			
A. perezii	3,29			+	+		
A. pinnatifida subsp. *teydensis*	3,30			+	+		
A. glandulosa subsp. *cheiranthifolia*	3,33			+	+		
A. mogadorensis subsp. *jahandiezii*	3,47				+	+	
A. ragusina I	3,47					+	
A. mogadorensis	3,54					+	
A. laxiflora	3,58					+	
A. maroccana	3,63					+	
A. ragusina II	5,01						+

Figure legends

Fig. 1 – Phylogenetic analysis of *Andryala* based on nuclear ribosomal markers (ITS + ETS) and genome size of particular accession. Consensus tree based on Bayesian analysis with posterior probabilities below branches. Redrawn from Ferreira et al. 2015, with permission of Taxon editorial office.

Fig. 2 – Variation in *Andryala* absolute genome size (2C). A – taxa of the 'Major Radiation Group' except for *A. ragusina* II, B – relict species *A. agardhii* and *A. laevitomentosa* and *A. ragusina* II.

Fig. 3 – Absolute genome size (2C) of species / subspecies of *Andryala*, arranged alphabetically.

Fig. 4 – Genome size (2C values) in continental (con) and insular (ins) accessions of *Andryala*. For delimitation of continental and insular accessions see Tab. 1. A – complete data set, B – reduced data set, taxa of the 'Major Radiation Group' (all taxa except for *A. agardhii* and *A. laevitomentosa*).

Fig. 5 – Genome size (2C values) perennial (per) and annual / biennial (ann) accessions of *Andryala*. For delimitation of perennial (per) and annual / biennial accessions see Tab. 1. A – complete data set, B – reduced data set, taxa of the 'Major Radiation Group' (all taxa except for *A. agardhii* and *A. laevitomentosa*).

Fig. 1

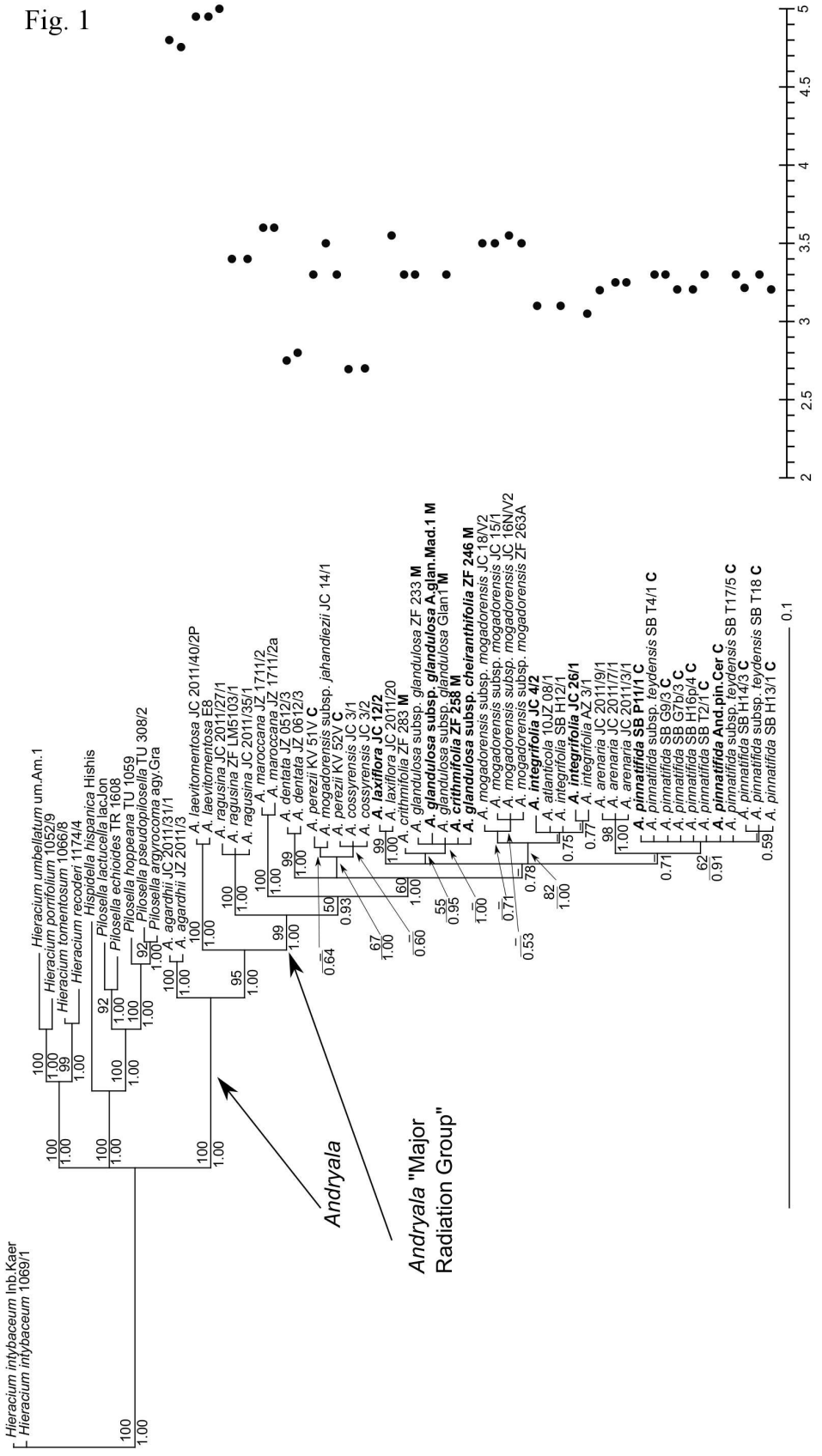

Hieracium intybaceum Inb.Kaer
Hieracium intybaceum 1069/1
Hieracium umbellatum um.Am.1
Hieracium porrifolium 1052/9
Hieracium tomentosum 1066/8
Hieracium recoderi 1174/4
Hispidella hispanica Hishis
Pilosella lactucella lacJon
Pilosella echioides TR 1608
Pilosella hoppeana TU 1059
Pilosella pseudopilosella TU 308/2
Pilosella argyrocoma agy.Gra
A. agardhii JC 2011/3 1/1
A. agardhii JZ 2011/3
A. laevitomentosa JC 2011/40/2P
A. laevitomentosa E8
A. ragusina JC 2011/27/1
A. ragusina ZF LM5103/1
A. ragusina JC 2011/35/1
A. maroccana JZ 1711/2
A. maroccana JZ 1711/2a
A. dentata JZ 0512/3
A. dentata JZ 0612/3
A. perezii KV 51V C
A. mogadorensis subsp. jahandiezii JC 14/1
A. perezii KV 52V C
A. cossyrensis JC 3/1
A. cossyrensis JC 3/2
A. laxiflora JC 12/2
A. laxiflora JC 2011/20
A. crithmifolia ZF 283 M
A. glandulosa subsp. glandulosa ZF 233 M
A. glandulosa subsp. glandulosa A.glan.Mad.1 M
A. glandulosa subsp. glandulosa Glan1 M
A. crithmifolia ZF 258 M
A. glandulosa subsp. cheiranthifolia ZF 246 M
A. mogadorensis subsp. mogadorensis JC 18/V2
A. mogadorensis subsp. mogadorensis JC 15/1
A. mogadorensis subsp. mogadorensis JC 16N/V2
A. mogadorensis subsp. mogadorensis ZF 263A
A. integrifolia JC 4/2
A. atlanticola 10JZ 08/1
A. integrifolia SB H12/1
A. integrifolia JC 26/1
A. integrifolia AZ 3/1
A. arenaria JC 2011/9/1
A. arenaria JC 2011/7/1
A. arenaria JC 2011/3/1
A. pinnatifida SB P11/1 C
A. pinnatifida subsp. teydensis SB T4/1 C
A. pinnatifida SB G9/3 C
A. pinnatifida SB G7b/3 C
A. pinnatifida SB H16p/4 C
A. pinnatifida SB T2/1 C
A. pinnatifida And.pin.Cer C
A. pinnatifida subsp. teydensis SB T17/5 C
A. pinnatifida SB H14/3 C
A. pinnatifida subsp. teydensis SB T18 C
A. pinnatifida SB H13/1 C
0.1
2
2.5
3
3.5
4
4.5
5
Andryala
Andryala "Major Radiation Group"

Fig. 2

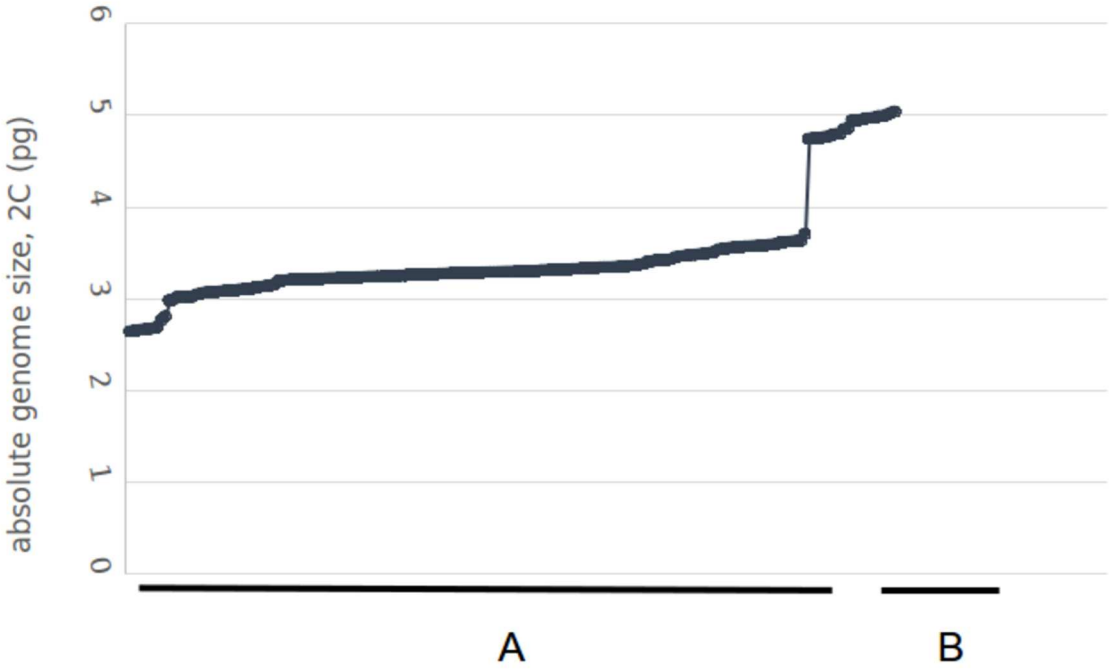

absolute genome size, 2C (pg)
0
1
2
3
4
5
6
A
B

Fig. 3

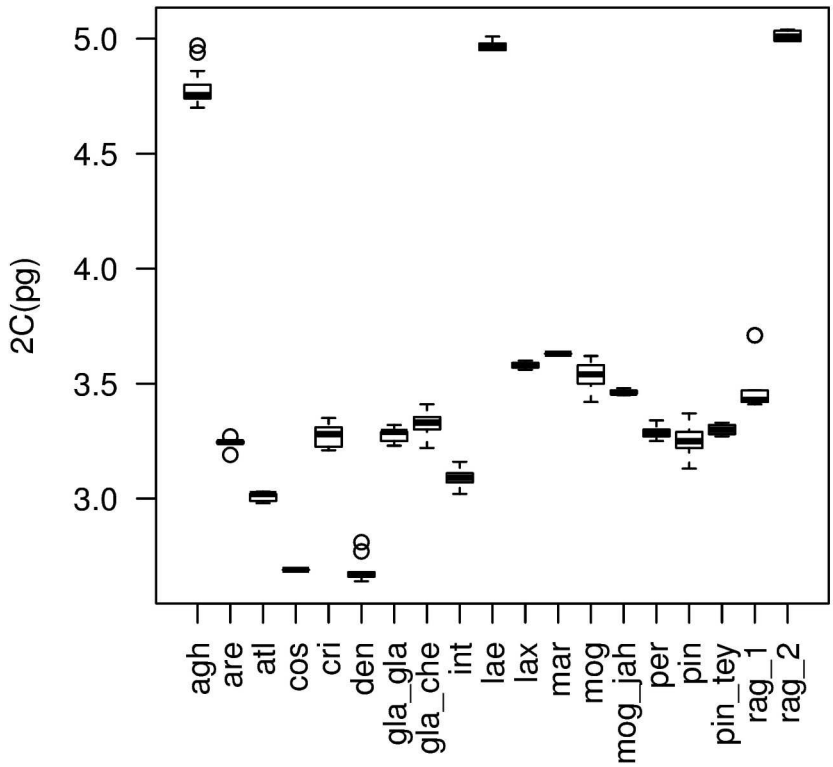

2C(pg)
5.0
4.5
4.0
3.5
3.0
agh
are
atl
cos
cri
den
gla_gla
gla_che
int
lae
lax
mar
mog
mog_jah
per
pin
pin_tey
rag_1
rag_2

Fig. 4

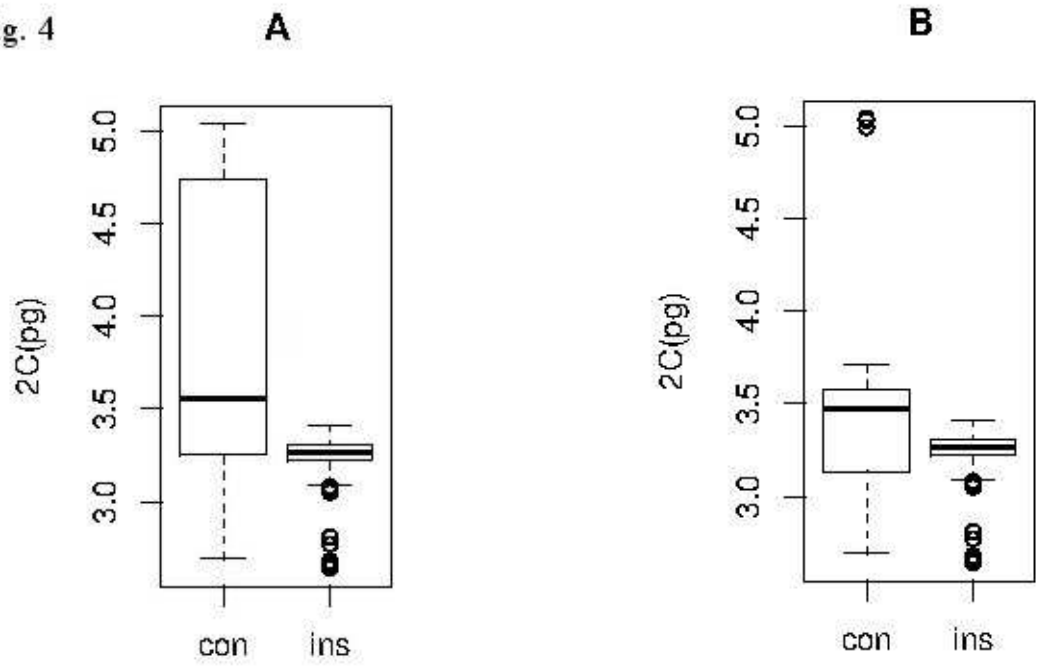
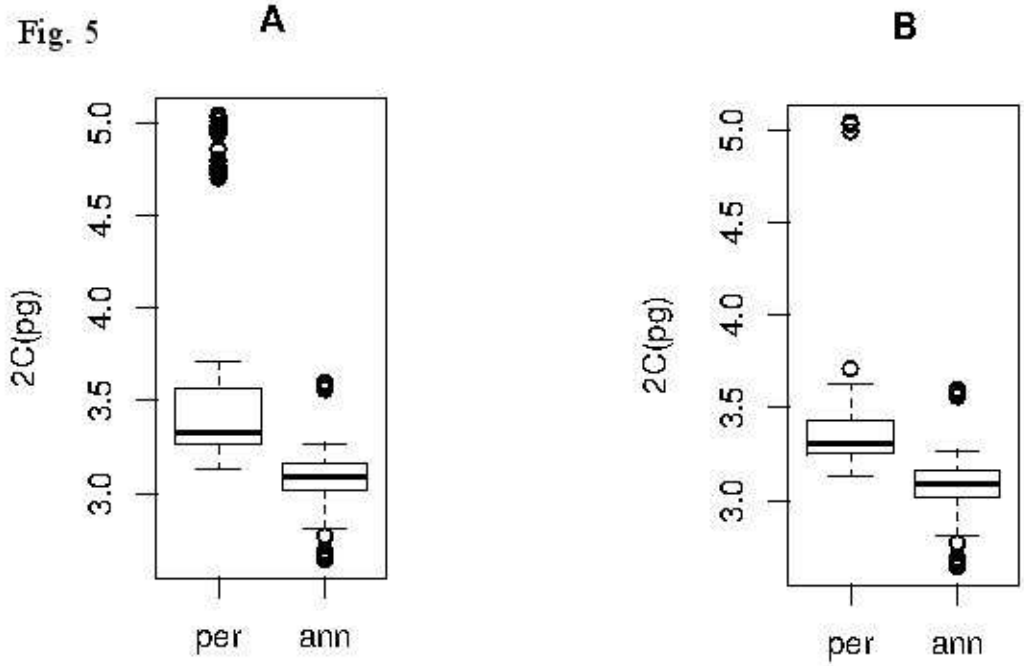


Fig. 5



Conclusions

The thesis deals with closely related genera with contrasting evolutionary histories and modes of reproduction, namely *Hieracium* (a huge polyploid complex with prevailing apomicts) and *Andryala* (a medium-size genus, with only sexual diploids) from the subtribe *Hieraciinae*. I focused on phylogenetic reconstruction using both nuclear and chloroplast markers, evolution of the genome size in phylogenetic context, biological and ecogeographical correlates of genome size and phytogeographical and evolutionary aspects of especially Mediterranean and Macaronesian flora. The second line of the thesis is devoted to cytotype distribution and genetic structure of *Hieracium intybaceum.*

Genome size was established (mostly for the first time) for 42 basic *Hieracium* species. The mean 2C values differed up to 2.37-fold among different species (from 7.03 pg in diploid to 16.67 in tetraploid accessions), the 1Cx values varied 1.22-fold (between 3.51 and 4.34 pg). Inytraspecific variation was generally low, variation higher than 3.5% was detected in only seven species, which may have a polytopic origin. Downsizing of genomes in polyploids was detected (mean 1Cx values of the 2*n*, 3*n* and 4*n* accessions differed significantly). Phylogeny was the most important factor explaining the pattern of genome size variation in *Hieracium* sensu stricto. Two main groups corresponding with species distribution (named "western" and "eastern" clade) separated in phylogenetic analyses based on nuclear ribosomal DNA marker (ETS) significantly differed in the genome size, species of western European origin having significantly lower genome size in comparison with those of eastern European origin. *Hieracium transylvanicum*, the only one exception, fell into the phylogenetically defined western lineage, but has a genome size and geographic range congruent with the 'eastern' group. Correlation with ecogeographic variables were not significant, outweighed by the divergence of the genus into two major phylogenetic lineages, except for a relations between genome size and geographical latitude for the whole data set (bot major groups).

Phylogenetic relationships in the genus *Andryala* were studied using three nuclear markers (ETS, ITS and single-copy gene sqs) and two chloroplast markers (*trn*T-*trn*L and *trn*V-*ndh*C). While cpDNA analysis confirmed a previously inferred chloroplast capture event with the sister genus *Pilosella*, nuclear markers supported the monophyletic origin of *Andryala*. None of phylogenetic analyses gave well resolution, due to low level of nucleotide divergence in case of two nuclear and two chloroplast markers and high degree of homoplasy and incomplete lineage sorting in the *sqs* marker. Only two well-supported basal lineages corresponding with relict mountains species *A. agardhii* and *A. laevitomentosa* were separated. Third well-supported group named 'Major Radiation Group' comprised the rest of *Andryala* species without resolution among them. The extremely low level of genetic divergence among most of the species, high morphological diversity and basal polytomy found in all markers, suggests their relatively recent and rapid speciation and old origin and long-time mountain separation of relict species *A. laevitomentosa* and *A. agardhii.*

Genome size was established for 18 *Andryala* species and subspecies, for 17 of them for the first time. It ranges (2C values) from 2.69 to 5.01 pg. First chromosome counts (2n = 18) were recorded for 6 species. It is likely that *Andryala* comprises only diplod species with 2n = 18, in contrast to *Hieracium* s. str. with a high frequency of polyploids. Highest C values (4.8 – 5.01 pg) were detected in basal relict species *A. laevitomentosa* and *A. agardhii* and in two

populations of *A. ragusina*; another two polulations of *A. ragusina* had distinctly lower C values. Genome size in species of the 'Major Radiation Group' ranges from 2.69 to 3.63 pg. We also found a correlation between genome size and life form (higher in perennials compared to annuals and biennials), and insularity (lower genome size in island accession compared to continental ones).

Cytogeography and phylogeography of *Hieracium intybaceum*, a perennial silicicolous species distributed in the Alps and the Vosges Mts, was studied using flow cytometry and amplified fragment length polymorphism (AFLP). Only two ploidy levels, diploid and tetraploid, were found, mixed-ploidy populations were not detected. The discrepancy between our results and previous reports of triploids can be explained by misidentifications of the plants (they might have belong to morphologically similar and triploid *H. pallidiflorum*). While diploid and sexual populations are widely distributed across the nearly whole distribution range, apomictic tetraploids seem to be rare and restricted to the western Alps and the Vosges Mts. This pattern does not correspond with the general model of geographical parthenogenesis which supposes that apomictic polyploids have larger distribution ranges shifted to higher latitudes and elevations compared to related sexual diploids. We detected an overall low level of genetic variation. Bayesian clustering identified four clusters/genetic groups, which are partly congruent with the ploidal pattern and cytogeographical distribution. We suppose that diploids colonized the deglaciated areas from source populations most likely located mainly in the southern part of the recent distribution range and probably also in the western Alps. Gene flow and further differentiation likely took place. Apomictic tetraploids most likely originated in the western Alps or in the refugium at the south-western foot of the Alps. Their limited geographical range can be explained by their rather recent origin.

Professional Curriculum vitae

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Education and employment

since 2008 Ph.D. study in Botany, Department of Botany, Faculty of Science, Charles University in Prague

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2010–2016 Institute of Botany ASCR, Průhonice, Czech Republic

Research interests

- karyology
- flow cytometry
- breeding systems
- taxonomy and evolution of *Hieracium* and *Andryala*
- molecular approaches and data analyses

SCI publication

Chrtek J. jun., Mráz P., **Zahradníček J**., Mateo G. & Szelag Z. (2007): Chromosome numbers and DNA ploidy levels of selected species of *Hieracium* s.str. (*Asteraceae*). – Folia Geobotanica 42: 411-430.

Chrtek J. jun., **Zahradníček J**., Krak K. & Fehrer J. (2009): Genome size in *Hieracium* subgenus *Hieracium* (*Asteraceae*) in strongly correlated with major phylogenetic groups. – Annals of Botany 104: 161–178.

Ferreira M. Z., **Zahradníček J**., Kadlecová J., de Sequeira M. M., Chrtek Jr., J. & Fehrer J. (2015): Tracing the evolutionary history of the little-known Mediterranean-Macaronesian genus *Andryala* (*Asteraceae*) by multigene sequencing. – Taxon 64: 535–551.

Zahradníček J. & Chrtek J. (2015): Cytotype distribution and phylogeography of *Hieracium intybaceum* (*Asteraceae*). – Botanical Journal of the Linnean Society 179: 487–498.

Chrtek J., Herben T., Rosenbaumová R., Münzbergová Z., Dočkalová Z., **Zahradníček J.**,

Krejčíková J. & Trávníček, P. (2017): Cytotype coexistence in the field cannot be explained by inter-cytotype hybridization alone: linking experiments and computer simulations in the sexual species *Pilosella echioides* (*Asteraceae*). – BMC Evolutionary Biology 17: 87.

Grant projects:

2005–2007: Molecular phylogeny and evolutionary trends in *Hieracium* (*Asteraceae*, *Lactuceae*) (GA ČR, Czech Science Foundation, GA206/05/0657, project leader J. Chrtek)

2007–2009: Interaction between cytotypes in sympatric populations of *Hieracium echioides* (GA UK, Grant Agency of Charles University in Prague, 1207/2007, project leader P. Trávníček)

2009–2011: Phylogeography and cytotype distribution of *Hieracium intybaceum* (GA UK, Grant Agency of Charles University in Prague, 72309/2009, project leader J. Zahradníček)

2010–2013: Phylogeny of subtribe *Hieraciinae* (*Asteraceae*) – a model example of contrasting evolutionary strategies in closely related lineages (GA ČR, Czech Science Foundation, GAP506/10/1363, project leader J. Chrtek)

2013–2015: Remarkable cytotype co-existence of *Pilosella echioides*: the only known sexual system with triploid dominance (GA ČR , Czech Science Foundation, 13-18610P, project leader P. Trávníček)

Supplementary files

Supplementary files to Paper 1, 2 and 3 are icluded in electonic version of the Ph.D. Thesis.

Supplementary files to Paper 4 **Genome size variation in the genus *Andryala* (*Hieraciinae*, *Asteraceae*):** List of localities of the population samples. Records are given as follows: species/subspecies (in alphabetical order), population code, locality description, geographic coordinates (WGS84), altitude, date of collection, and collector.

A. agardhii

JC 2011_30 – Spain, Andalusia, province Granada, Sierra de Baza Mts: Calar de Santa Barbara, 7,5 km NE of the village of Las Juntas, 1 960 m alt., 37º23'05"N, 02º51'03"W, 31 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_30 – Spain, Andalusia, province Granada, Sierra de Baza Mts: Calar del Desabezedo, 4,6 km E of the the village of Las Juntas, 1 995 m alt., 37º19'49"N, 02º51'20"W, 31 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_33 – Spain, Andalusia, province Almería, Sierra de María Mts: María, near a path to Maimón 3,2 km SE of the village, 1 380 m alt., 37º41'29"N, 02º08'21" W, 2 June 2011, leg. J. Chrtek and Z. Dočkalová; JZ 2011_3 – Spain, Andalusia, Sierra Mágina: north of Huelma, along the road to Mágina, 1 584 m alt., 37°42'19"N, 3°27'45.24"W, 11 June 2011, leg. J. Zahradníček, K. Krak, P. Vít, J. Kalůsková, T. Urfus and M. Urfusová; JZ 2211 – Spain, Andalusia, Sierra de las Cabras: road from Canada de la Cruz, saddle west of Mt. Pinar Liano, 1 806 m alt., 38°04'17.58"N, 2°20'02"W, 12 June 2011, leg. J. Zahradníček, K. Krak, P. Vít, J. Kalůsková, T. Urfus and M. Urfusová.

A. arenaria

JC 2011_3 – Spain, Andalusia, province Malaga, vicinity of Marbella: Artola, Urbanización Las Chapas, hill near the Avenida Uno, 25 m alt., 36º29'38"N, 04º45'36"W, 25 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_7 – Spain, Andalusia, province Cádiz: Bornos, subruderal places near the main street 2 km WNW of the town, 237 m alt., 36º49'01"N, 05º46'01"W, 26 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_9 – Spain, Andalusia, province Huelva: Almonte, near the road A-483, 5.9 km SSW of the town, 55 m alt., 37º12'35"N, 06º30'21"W, 26 May 2011, leg. J. Chrtek and Z. Dočkalová.

A. atlanticola

10JZ_08 – Morocco, High Atlas Mts., region Marrakech-Tensift-El-Haouz: 2 km of Around, rocks along the road from Around to Tubkal, 1 960 m alt., 31°06'59"N, 7°55'11"W, 20 June 2010, leg. J. Zahradníček and P. Schindlerová; JC 13_V – Morocco, High Atlas Mts., region Marrakech-Tensift-El-Haouz: Imlil, slopes near the road to Asni, 2.5 km NNW of the village, 1585 m alt., 31º09'29"N, 07º55'29"W, 19 May 2010, leg. J. Chrtek and Z. Dočkalová; Ixis – Morocco, High Atlas Mts., region Marrakech-Tensift-El-Haouz: Imlil, saddle above the town, 2 063 m, 31°09'56"N, 7°54'45"W, 20 June 2010, leg. J. Zahradníček and P. Schindlerová.

A. cossyrensis

JC 3 – Morocco, region Oriental, province Berkane, Monts des Beni Snassen: Berkane, Zegzal (Zegzel) valley, along a road near a small bridge over the stream, 6,4 km SW of the town, 280 m alt., 34º52'31"N, 02º21'28"W, 14 May 2010, leg. J. Chrtek and Z. Dočkalová.

A. crithmifolia

260_ZF – Portugal, Madeira, south coast: Pináculo, 12 August 2009, leg. Z. Ferreira; 259_ZF –

Portugal: Madeira, south coast: Cabo Girão, alt., 32° 39' 12.72" N, 16° 59' 36.06"W, 09 August 2009, leg. Z. Ferreira and I. Ferreira; 301_ZF – Portugal, Madeira, south coast: Garajau, 32º38'15" N 16º51'02" W, 17 July 2012, leg. Z. Ferreira, A. Pupo Correia and R. Jardim.

A. dentata

JZ 0412 – Italy, Isola di Pantelleria: Rekhale, near the road 0,9 km ENE of the village, 305 m alt., 36º45'41"N, 12º00'20"E, 17 May 2012, leg. J. Zahradníček and J. Chrtek; JZ 0512 – Italy, Isola di Pantelleria: Tracino, near the road to Casa Bono 2.4 km SSW of the village (square), disturbed place, 220 m alt., 36º46'15"N, 12º01'44" E, 17 May 2012, leg. J. Zahradníček and J. Chrtek; JZ 0612 – Italy, Isola di Pantelleria: Siba-Roncone, near the road 0,9 km SE of the village, 450 m alt., 36º46'58"N, 11º59'11"E, 17 May 2012, leg. J. Zahradníček and J. Chrtek.

A. glandulosa subsp. *cheiranthifolia*

248_ZF – Portugal: Madeira, Pico do Areeiro, 32º 43'N 16º 55'W, 26 July 2009, leg. Z. Ferreira., A. Pupo Correia and M. Sequeira; 271_ZF – Portugal, Madeira, SW coast: Jardim do Mar, 3 July 2010, leg. Z. Ferreira; 241_ZF – Portugal, Madeira: Encumeada, 32º44'N, 17º01'W, 24 July 2009, leg. Z. Ferreira., A. Pupo Correia and M. Sequeira; 257_ZF – Portugal, Madeira: Fajã da Ovelha, 29 July 2009, leg. Z. Ferreira, M. Benedito and M. Sequeira; 269B_ZF – Portugal, Madeira, south coast: Calheta, 3 July 2010, leg. Z. Ferreira.

A. glandulosa subsp. *glandulosa*

233_ZF – Portugal, Madeira, north coast: Seixal, 24 July 2009, leg. Z. Ferreira., A. Pupo and M. Sequeira; Glan_ZF – Portugal, Madeira, north coast: Porto Moniz, rocks near the road, 263 m alt., 32º51'46"N, 17º10'29"W, 23 July 2011, leg. Z. Ferreira; 298_ZF – Portugal, Madeira, north coast: S. Jorge, 32°50'N, 16°54'W, 15 July 2012, leg. Z. Ferreira; ZF_278A – Portugal, Madeira, west coast: Ponta do Pargo, 32º48'N 17º15'W, 3 July 2010, leg. Z. Ferreira.

A. integrifolia

KV 4 – Spain, Canary Islands, Gran Canaria: Valleseco, 0.3 km W of the village, along the road, 1 060 m alt., 28º02'59"N, 15º34'43"W, 15 April 2007, leg. K. Vazačová (seeds); SB_H12 – Spain: Canary Islands, El Hierro: Tiñor (3 km SW of Valverde), near the road to San Andrés, 0,2 km W of the village, 980 m alt., 27º47'18"N, 17º56'10"W, 20 May 2010, leg. S. Bräutigam and E. Bräutigam; SB H15 – Spain, Canary Islands, El Hierro: San Andrés, near a road to El Pinar, 4 km SSW of the village, 1 125 m alt., 27º44'08"N, 17º58'28"W, 22 May 2010, leg. S. Bräutigam and E. Bräutigam; SB H16i – Spain, Canary Islands, El Hierro: San Andrés, near the road to Frontera, 2 km WSW of the village, *Chamaecytisus proliferus* shrubs, 1175 m alt., 27º45'41"N, 17º58'17"W, 22 May 2010, leg. S. Bräutigam and E. Bräutigam; ZF LM5104 – Spain, Guadalix de la Sierra, rotonda de la ctra. a Miraflores, 0440371 W 4515490 N, 850 m, 19 June 2010, leg. L. Medina and M. Sequeira; AZ_3 – Algeria, Alger: towm district of Kouba, 90 m alt., 36º43'N, 03º05'E, 2 July 2011, leg. Abida Zeddam; AZ_4 – Algeria, Alger: town district of Le Caroubier, 2 m alt., 36º44'N, 03º07'E, 3 July 2011, leg. Abida Zeddam.

A. laxiflora

JC 2011_15 – Spain, Andalusia, province Huelva: Encinasola, near a road H-211 4,4 km SSE of the town, 260 m alt., 38º05'56"N, 06º51'16"W, 27 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_19 – Spain, Andalusia, province Córdoba: Hornachuelos, near a road A-2212 3,5 km NW of the town, 280 m alt., 37º51'39"N, 05º15'57" W, 28 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_20 – Spain, Andalusia, province Jaén: Linares, near a road J-6030 3 km WNW of the town center, 405 m alt., 38º06'37"N, 03º39'29"W, 28 May 2011, leg. J. Chrtek and Z. Dočkalová.

A. laevitomentosa

A – Romania, Bukovina, Suceava county, Vatra Dornei: Mt. Pietrosul Bogolin, rocks near the top (northernmost population), 1 680 m, 47º23'19"N, 25º32'1" E, 5 August 2011, leg. J. Chrtek, P. Mráz, V. Mrázová and M. Puskás; C – Romania, Bukovina, Suceava county, Vatra Dornei: Mt. Pietrosul Bogolin, rocks near the top, 1 740 m alt., 47º23'08"N, 25º32'19"E, 5 August 2011, J. Chrtek, P. Mráz, V. Mrázová and M. Puskás; JC 2011/40/2P – Romania, Bukovina, Suceava county, Vatra Dornei: Mt. Pietrosul Brostenilor, rocks in the upper part, 1 780 m alt., 47º22'31"N, 25º32'29"E, 5 August 2011, J. Chrtek, P. Mráz, V. Mrázová and M. Puskás.

A. maroccana

JZ 1711 – Morocco, region Oriental, rocks on northern seaside of peninsula near Melilla (Spain), 74 m alt., 35° 26' 4.62"N, 2° 58' 1.86"W, 9 July 2011, J. Zahradníček and K. Krak.

A. mogadorensis

JC 15 – Morocco, region Sous-Masa-Drâa: Tamri, near the main road along the Atlantic coast, 4,7 km WSW of the village, 30 m alt., 30º41'33"N, 09º52'28"W, 22 May 2010, leg. J. Chrtek and Z. Dočkalová; JC 18/V2 – Morocco, region Doukkala-Abda: Moulay Abdallah (SW of El Jadida), near the main road 13.5 km SW of the village, 12 m alt., 33º05'09"N, 08º39'16"W, 22 May 2010, leg. J. Chrtek and Z. Dočkalová; JC 16N – Morocco, region Marrakech-Tensift-El-Haouz: Essaouira (Mogador), coastal dunes at the northern margin of the town, 8 m alt., 31º31'40"N, 09º45'01"W, 22 May 2010, leg. J. Chrtek and Z. Dočkalová; JC 17 – Morocco, region Doukkala-Abda: Sidi Rosia (S of Safi), near the main road 3.4 km SSW of the village, 15 m alt., 32º11'37"N, 09º15'19"W, 22 May 2010, leg. J. Chrtek and Z. Dočkalová; ZF 263A – Morocco, region Marrakech-Tensift-El-Haouz: Essaouira (Mogador), coastal dunes at the northern margin of the town, alt. 31º32'40"N, 09º45'00"W, 22 May 2010, leg. Z. Ferreira and I. Álvarez.

A. mogadorensis subsp. *jahandiezii*

JC 14 – Morocco, region Sous-Masa-Drâa: Inezgane, near the road between the golf resort and King´s Palace, near the river of Sous, 7 m alt., 30°21'46"N, 09°34'27"W, 21 May 2010, leg. J. Chrtek and Z. Dočkalová.

A. perezii

KV 51V – Spain, Canary Islands, Lanzarote: Haria, near the road 2 km SW of the village, 380 m alt., 29°08'04" N, 13°30'41"W, leg. K. Vazačová; KV 52V – Spain, Canary Islands, Lanzarote: El Jurado, Guinate, near the road 0.7 km SW of the village, 440 m alt., 29°10'33"N, 13°29'57"W, leg. K. Vazačová.

A. pinnatifida

KV 10 – Spain, Canary Islands, Gran Canaria: Barranco de Guayadeque, 4.5 km NW of the village of Agüimes, 540 m alt., 27°55'59"N, 15°28'44"W, 13 May 2007, leg. K. Vazačová; KV 16 – Spain, Canary Islands, Tenerife: Cumbre de Bolico, near the road 2 km SE of the village of Las Portelas, 1 155 m alt., 28°18'53"N, 16° 49'48"W, 4 June 2007, leg. K. Vazačová; KV 25 – Spain, Canary Islands, La Gomera: Alto de Garajonay, 1.5 km NE of the village of Igualero, 1 300 m alt., 28°06'30"N, 17°14'30"W, 6 November 2007, leg. K. Vazačová; SB G5 – Spain, Canary Islands, La Gomera: Hermigua, El Curato, near the rocks Los Gemelos, 300 m alt., 28°08'58"N, 17°12'18"W, 9 May 2010, leg. S. Bräutigam and E. Bräutigam; SB H13 – Spain, Canary Islands, El Hierro: San Andrés, near the road to Frontera, 4 km SW of the village, shrubs with prevailing *Chamaecytisus proliferus*, 980 m alt., 27°44'46"N, 17°59'08"W, 20 May 2010, leg. S. Bräutigam and E. Bräutigam; SB G7b – Spain, Canary Islands, La Gomera: Arure, at the

path to Montaña de los Manantiales 2,5 km ENE of the village, *Erica arborea-Cistus monspeli ensis* shrubs, 940 m alt., 28°08'32"N, 17°18'06"W, leg. S. Bräutigam and E. Bräutigam; SB G9 – Spain, Canary Islands, La Gomera: Vallehermoso, 3 km SSE of the village, at the road to Las Rosas, 980 m alt., 28°09'05"N, 17°15'13"W, 11 May 2010, leg. S. Bräutigam and E. Bräutigam; SB T2 – Spain, Canary Islands, Tenerife: Puerto de la Cruz, above the village of Puerto de la Cruz, 7 km WSW of the town, disturbed places in the agricultural landscape, 780 m alt., 28°22'31"N, 16°36'37" W, 3 May 2010, leg. S. Bräutigam and E. Bräutigam; SB H14 – Spain, Canary Islands, El Hierro: Sabinosa, near the Ermita Virgen de los Reyes, 3 km SW of the village, rocky places, nearby the *Euphorbia-Kleinia* shrubs, 720 m alt., 27°43'51"N, 18°07'16"W, 21 May 2010, leg. S. Bräutigam and E. Bräutigam; SB H16p – Spain, Canary Islands, El Hierro: San Andrés, near the road to Frontera, 2 km WSW of the village, *Chamaecytisus proliferus* shrubs, 1175 m alt., 27°45'41"N, 17°58'17"W, 22 May 2010, leg. S. Bräutigam and E. Bräutigam.

A. pinnatifida subsp. *teydensis*

SB T4 – Spain, Canary Islands, Tenerife: Cañadas, at the road Arona – Vilaflor near La Escalona (km 13), 1 190 m alt., 28°07'37"N, 16°39'24"W, 7 May 2010, leg. S. Bräutigam and E. Bräutigam; SB T18 – Spain, Canary Islands, Tenerife: Cañadas, near the road below El Portilla, rocks with *Pinus canariensis* forest, 1950 m alt., 28° 18'27"N, 16°33'58"W, 27 May 2010, leg. S. Bräutigam and E. Bräutigam; SB T17 – Spain, Canary Islands, Tenerife: Montañas de Anaga, 3 km E of Cruz del Carmen near Las Casas de Cumbre, road margin, "Fayal-Brezal" (*Myrico fayae-Ericetum arboreae*) vegetation, 800 m alt., 28°32'02"N, 16°15'12"W, 26 May 2010, leg. S. Bräutigam and E. Bräutigam; SB T19 – Spain, Canary Islands, Tenerife: Cañadas, on the path 1,5 km E of Parador Nacional de la Cañadas, below Guajara, 2 200 m alt., 28°13'32"N, 16°36'46"W, 28 May 2010, leg. S. Bräutigam and E. Bräutigam.

A. ragusina I

JC 2011_35 – Spain: Comunitat Valenciana: Sax, along the road to Catalla, 5.6 km NE of the town (city centre), 610 m alt., 38°34'34"N, 00°46'59"W, 3 June 2011, leg. J. Chrtek; ZF LM5103 – Spain, province Madrid: Guadalix de la Sierra, 40°46'48"N, 3°41'49"W, 19 June 2010, leg. L. Medina and M. Sequeira.

A. ragusina II

JC 2011_2 – Spain, Andalusia, province Malaga: Alhaurin el Grande, near the old street to Alhaurin de la Torre, 5.7 km ESE of the town (near Golf Lauro), 230 m alt., 36°38'52"N, 04°37'17"W, 24 May 2011, leg. J. Chrtek and Z. Dočkalová; JC 2011_27 – Spain, Andalusia, province Granada: near the road to Jérez del Marquesado, 4.5 km of the town (city centre), 1075 m alt., 37°15'25"N, 03°08'16"W, 31 May 2011, leg. J. Chrtek and Z. Dočkalová.