A glacial survivor of the alpine Mediterranean region: phylogenetic and phylogeographic insights into *Silene ciliata* Pourr. (Caryophyllaceae)

Ifigeneia Kyrkou, José María Iriondo, Alfredo García-Fernández

Silene ciliata Pourr. (Caryophyllaceae) is a species with a highly disjunct distribution that inhabits the alpine mountains of the Mediterranean Basin. We investigated the phylogeny and phylogeography of the species in an attempt to a) clarify the long suggested division of *S. ciliata* into two subspecies, b) evaluate its phylogenetic origin and c) assess whether the species´ diversification patterns were affected by the Mediterranean relief. For this purpose, we collected DNA from 25 populations of the species that inhabit the mountains of Portugal, Spain, France, Italy, FYROM, Bulgaria and Greece and studied the plastid regions *rbcL*, *rps16* and *trnL*. Major intraspecific variation was supported by all analyses, while the possibility of existence of more varieties or subspecies was not favoured. Plastid DNA evidence, especially in the cases of *rps16* and *trnL* markers, was in accordance with the division of *S. ciliata* into the two subspecies, one spreading west (Iberian Peninsula and Central Massif) and the other east of the Alps region (Italian and Balkan Peninsula). The present study proposes that this vicariance has probably derived from the Alps acting as a barrier to the species dispersal. The monophyletic origin of the species is highly supported. Plastid DNA patterns may have resulted from a combination of geographic factors providing links and barriers, climatic adversities and evolutionary processes that took place during Quaternary glaciations. The latter might include hybridization events for the western subspecies and mutational accumulation for the eastern ones.

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38 Introduction

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40 Alpine environments provide interesting frameworks for answering phylogeographic and
41 phylogenetic questions that remain unresolved from a botanical perspective. Plant species in
42 mountain ecosystems face challenges for survival and adaptation to different environmental
43 conditions and fluctuations (Körner, 2003). High altitude habitats often follow an island-like
44 structure due to significant levels of isolation and fragmentation (Pawłowski, 1970), thus leading
45 to adaptive divergence and, finally, speciation events (Wiens, 2004). These inland habitat patches
46 could harbour greater species diversity compared to a seamless area of the same extent (Quinn &
47 Harrison, 1988). Nunataks and peripheral glacial refugia inside mountain ranges are thought to
48 have sheltered a wide range of biological and genetic diversity during the Pleistocene glacial-
49 interglacial periods (Hewitt, 2000; Taberlet et al., 1998).
50 Various phylogeographic and phylogenetic surveys have been conducted for floristic taxa
51 of the Alps (Schönswetter et al., 2005), while the rest of the European mountain ranges and the
52 processes occurring inside them during glaciations have generally been overlooked (Hewitt,
53 2001). Nevertheless, interest in Mediterranean mountain systems has gradually been increasing
54 (e.g. Vargas, 2003; Mas de Xaxars et al., 2015). The Mediterranean Basin has undoubtedly
55 played a crucial role in shaping the genetic and distributional patterns of many species, since it
56 provided them with sanctuary during glaciations (Médail & Diadema, 2009) and served as a
57 starting point for the recolonization of northern latitudes (Petit et al., 2003; Tzedakis et al., 2002).
58 Indeed, the Southern Mediterranean Peninsulas (i.e. Iberian, Italian and Balkan) are considered
59 important glacial refugia for many plant and animal species (e.g. Taberlet et al., 1998; Hewitt
60 2000; Hewitt, 2004), and Mediterranean mountains have been considered potential refugia for
61 alpine plants (Vargas, 2003; Hughes, Woodward & Gibbard, 2006).
62 Maternally inherited plastid DNA (hereafter cpDNA) has turned out to be an invaluable
63 tool in the phylogeography and phylogenetics of angiosperms, since it provides a conservative
64 and enduring record of plant migrational spread (McCauley, 1997; Irwin, 2002) compared to
65 biparentally inherited nuclear markers that show recombination (Petit, Kremer & Wagner, 1993;

Heuertz et al., 2004). Thus, the geographically consistent distribution of variation patterns of species chloroplast haplotypes is believed to be the result of events such as interspecific hybridization, introgression, mutation and differentiation within species inside common refugia during the last Ice Ages, early postglacial expansion, or in current areas of sympatry (e.g. Petit et al., 2002, Hathaway, Malm & Prentice, 2009).

Silene L. is a genus that has caught the attention of scientists back to Darwin (1876, 1877) and Mendel (1870) due to its many interesting attributes, making it a potential "model system" in ecology and evolution (Bernasconi et al., 2009). Yet, its phylogeny still remains perplexing and unclear (Oxelman et al., 2000; Greenberg & Donoghue, 2011). The genus has c. 700 species distributed into 44 sections, which classifies it among the largest floristic genera. Half of *Silene* species inhabit the Mediterranean Basin (Greuter, 1995) and c. 87 of them are found in latitudes above the treeline (based on Jalas & Suominen,1988 and supported by Zángheri & Brilli-Cattarini, 1976; Castroviejo et al., 1986-2001; Strid & Tan, 2002), which has its lower limit at about 1800-2000 m in the Mediterranean region (McNeill, 2002). *Silene* L. presents high levels of mitochondrial DNA variation (Sloan et al., 2008) and nuclear genome diversification (Široký et al., 2001). The majority of its species are diploid with 2n=20 or 2n=24 (Bari, 1973), while a considerable number of them are endemics (Eggens, 2006). The latest taxonomic classification can be found in Greenberg & Donoghue (2011). Many recent studies have tried to clarify the phylogeny of its tribes and sections (e.g. Oxelman et al., 2000; Rautenberg et al., 2008; Rautenberg et al., 2010).

Although *Silene* species in alpine environments have been included in phylogenetic and phylogeographic studies of the genus *Silene* (e.g. Abbott et al., 1995; Popp et al., 2005), those native to Mediterranean mountains have been understudied. *Silene ciliata* is a notable species in the genus *Silene*, because it presents a circum-mediterranean distribution around mountain ranges and above the treeline. Taxonomists have consistently divided it into two subspecies based on habit differences and disjunct geographical distribution. These are *S. ciliata* subsp. *graefferi* (referred to as the "Italian race"), which is principally found in the Italian and the Balkan Peninsula, and *S. ciliata* subsp. *ciliata*, (referred to as the "Spanish race"), which occupies the Iberian Peninsula (Blackburn, 1933). Western populations are morphologically more variable and several other subspecies or varieties have been proposed (e.g. *Silene ciliata* subsp *arvatica* Lag. in Varied .Ci. (1805), *Silene ciliata* subsp. *elegans* (Link. ex Brot.) Rivas Martínez in Brotero,

1804), although the validation of these subcategories remains unsolved with available taxonomical data (Nieto Feliner, 1985). This species also stands out for its extraordinary variability of ploidy levels in natural populations (i.e., 2n = 24, 36, 48, 72, 84, 96, 120, 144, 168, 192, 240; Blackburn, 1933; Küpfer, 1974). In particular, subsp. *ciliata* is reported to vary from diploid to 20-ploid complements, whereas in subsp. *graefferi* only diploid and tetraploid plants are described (Blackburn, 1933; Küpfer, 1974; Tutin et al., 1995).

We followed a phylogenetic and phylogeographic approach to this species to gain insight into the diversification processes that have taken place in alpine environments of Mediterranean high mountains. To our knowledge, this is the first study to cover the vast majority of the alpine Mediterranean area with the aid of molecular marker evidence. We hypothesized that: 1) in spite of its heterogeneity discussed by Blackburn in 1933, the species is of monophyletic origin; 2) this heterogeneity is reflected in great cpDNA diversification that could explain the subclassification of this species into two distinct subspecies as proposed by Blackburn (1933) and maintained by Tutin et al. (1995); 3) the patterns of differentiation are essentially determined by the geomorphology and spatial location of the Mediterranean mountain ranges.

Material and Methods

Study species

Silene ciliata Pourr. (subsect. *Fruticulosae*, Caryophyllaceae) is endemic to Europe and inhabits the main Mediterranean mountain ranges in the northern half of Mediterranean Basin countries spreading along the Iberian Peninsula, the Central Massif, the Apennines and the Balkan Peninsula (Tutin et al., 1995). It is an alpine, chamaephytic, perennial, cushion plant which typically forms pulviniform rosettes of up to 2 cm in height and 15 cm in diameter with high variability in size. Each plant has an average of 13 ± 11 (mean ± SD) flowering stems that reach 15 cm in height and bear 1-5 flowers (Giménez-Benavides, Escudero & Iriondo, 2007a). Hand-crossing pollination experiments indicate that *S. ciliata* is potentially self-compatible (Giménez-Benavides, 2006; García-Fernández, Iriondo & Escudero, 2012). Nevertheless, passive autogamy is restricted by a pronounced protandry (García-Fernández, Iriondo & Escudero, 2012). *S. ciliata* is pollinated at night by *Hadena consparcatoides* Schawerda, but pollination by diurnal insects is

129 also reported (Giménez-Benavides et al., 2007b). Seed dispersal is essentially barochorous, since
130 seeds lack any specialized structure to promote dispersal and, thus, most seeds are dispersed at
131 very short distances (Lara-Romero et al., 2014).
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133 DNA extraction, amplification and alignment
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135 Twenty-five specimens of *S. ciliata* populations covering the species distribution range were
136 sampled for this study (Fig. 1). Plant material was obtained from herbarium specimens or directly
137 from the field and stored as silica gel-dried material (Table 1). All field studies made by the
138 authors were conducted with the permission of "Junta de Castilla y León" and "Comunidad de
139 Madrid" (approval numbers: 20144360000894 and 10/117476.9/14, respectively). For DNA
140 extraction, approximately 20 mg of dried leaf tissue of each plant sample were weighed.
141 Extractions were performed following the protocol of Qiagen Plant DNA extraction kit
142 (QIAGEN Inc., CA, USA) with some modifications. The DNA extraction samples were checked
143 in a 1% agarose gel stained with REDGEL (Biotium Inc., CA, USA) and stored at -20º C until
144 use. Each of the 25 extracted DNA samples was amplified for the *rbcL*, *rps16* and *trnL*
145 polymorphic cpDNA regions. These regions were selected out of the 12 regions, which were
146 described to showing major variation and the best amplification profile (Shaw et al., 2005; Shaw
147 et al., 2007). To assess possible intrapopulation cpDNA variation, DNA from four additional
148 individuals of the Cen3 population was also extracted and amplified. The primers used and the
149 PCR conditions applied for each marker, as well as the primer sequences and references, are
150 listed in Table S2. The PCR mix was prepared using PureTaq Ready-To-Go PCR beads (GE
151 Healthcare, Uppsala, Sweden). The amplified PCR products were cleaned up with ExoProStar 1-
152 Step enzyme (GE Healthcare) following the suggested protocol and then sequenced using a 3730
153 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) in the Parque Científico de Madrid
154 (Universidad Complutense, Madrid, Spain). Sequencing results were evaluated and corrected
155 manually and then subjected to multiple alignment. Contigs were assembled and edited with
156 Sequencher 4.1.4 (Gene Codes Corp., MI, USA) Bioedit (Hall, 1999) and ClustalW (Thompson,
157 Higgins & Gibson, 1994).
158 For the estimation of the polymorphic cpDNA region phylogeny, eight additional species
159 of genus *Silene*, tentatively close phylogenetically to *Silene ciliata*, were included in the study.

These species were selected based on the existing bibliography (Sloan et al., 2009; Greenberg & Donoghue, 2011) and the availability of the required polymorphic cpDNA regions. The search was performed in GenBank sequence database, and the species selected as outgroups were *S. latifolia* Poiret, *S. uniflora* Roth, *S. vulgaris* (Moench) Garcke and -phylogenetically closer to *S. ciliata*- *S. acaulis* (L.) Jacq, *S. otites* (L.) Wibel, *S. nutans* L., *S. paradoxa* L. and *S. schafta* S. G. Gmel. ex Hohen. The accession numbers of all outgroup-regions are listed in Table S3.

Genetic analyses: diversity, dendograms, networks and spatial clustering

The number of variation and informative sites of our aligned sequences was determined using DnaSP v.5.10.01 (Librado & Rozas, 2009). The phylogenetic analyses were performed using two different statistical approaches ("Bayesian inference" and "Maximum likelihood") for verification reasons. In the Bayesian analysis, sequence data were first introduced to jModeltest (Posada, 2008) to determine the best fitting evolutionary model according to the AIC criterion. This process was followed to generate a dendrogram for each polymorphic cpDNA region, plus one dendrogram that included all polymorphic cpDNA regions together. The suggested model for *rbcL* was [HKY], for *rps16* [GTR+ G], for *trnL* [HKY+ I] and for the tree including all markers [GTR+ G]. These models were then inserted into MrBayes 3.1.2 (Huelsenbeck et al., 2001) and posterior probabilities (hereafter PP) were estimated using the Markov chain Monte Carlo (MCMC) method. Four Markov chains were run in parallel for 10,000,000 generations and sampled every 100 generations. The first 100 generations were set as the "burn-in" period, while the rest were used to calculate the 50% majority rule consensus phylogeny and posterior probability. The resulting dendrogram archives were revised with FigTree v. 1.3.1 (Rambaut, 2006). A maximum likelihood dendrogram including all the polymorphic cpDNA regions together was also generated with PhyML 3.0 (Guindon et al., 2010) under the same evolutionary model used for the Bayesian analysis. The reliability of the branches was calculated through bootstrapping, after producing 1000 bootstrapped data sets. All outputs were compared and analysed to infer the evolutionary history of our study species.

Next, each group of polymorphic cpDNA region sequences was analysed with TCS 1.2.1 (Clement, Posada & Crandall, 2000) and classified according to statistically parsimonious haplotype groups. The haplotype groups were linked by the program, constructing a network of

mutation steps, which visualized the genetic distance between them. For the construction of the haplotype networks, deletions were not treated as polymorphic sites, while the analysis was performed under the default of 95% connection limit. Three haplotype networks, one for each marker, were created with this method. Neighbour-net analyses networks were also designed for each region (*trnL*, *rbcL* and *rps16*) using Splits Tree v. 4.13.1 (Huson & Bryant, 2006) and following the uncorrected p-distance between individuals. The support for each branch was tested using the bootstrapping method with 1000 replicates. Lastly, one more test was performed with BAPS 6 (Corander et al., 2008). Using BAPS, population structure can be assessed with a Bayesian analysis which considers analytical and stochastic methods to estimate the optimal (with the highest probability) grouping partition. The resulting clusters were portrayed with reference to a Voronoi tessellation that covers the samples' distribution in which genetically differentiated populations are distinguished. In order to infer the best genetic structure, we inserted the coordinates of each population and ran a test of spatial clustering of individuals.

Results

Chloroplast haplotype and intrapopulation variation

After multiple alignment evaluation of the three polymorphic cpDNA regions, the final length of the study region resulted in 564 nucleotides for *rbcL*, 756 nucleotides for *rps16* and 509 nucleotides for *trnL*. Thus, the length of the combined matrix of an "all-marker" region was 1829 nucleotides. The number of variable sites among chloroplast markers ranged from 4 to 25, while that of parsimony informative sites ranged from 3 to 16 (Table 2). Sequences were submitted to GenBank (accession numbers are available in Table S4).

The intrapopulation study showed no divergence for *rbcL* and inconsistent polymorphisms (i.e., only present in one individual and probably associated to sequencing errors) in one and two bases for *rps16* and *trnL*, respectively. Therefore, we considered that the evidence for intrapopulation variation was not strong enough to require further testing.

Phylogeny, genetic distance analyses and population structure

The resulting "all-marker" dendrogram from the Bayesian analysis (Fig. 2) revealed two distinct groups, one including all individuals in the western region (i.e., the Iberian Peninsula and France) and another one including all individuals in the eastern region (i.e., the Italian and Balkan Peninsulas). However, the calculated 65% PP for the "eastern group" did not provide a significant difference between the two groups. On the other hand, significant differentiation (100% PP) was found between *S. ciliata* individuals and the outgroups. Strikingly, two *S. ciliata* individuals, Pyr1 and Pyr4, were located between the outgroups and the rest of *S. ciliata*, and were significantly different from them as well as from each other. Both Pyr1 and Pyr4 branches were long, implying high diversification rates. One overarching clade was observed (99% PP) in the "eastern group", and the Din population was the only one branching off this clade. The "western group" consisted of one clade (78 % PP), but also had many separate individual branches. The maximum likelihood dendrogram obtained with the bootstrapping method did not differ, either in formation or in significance of branches support, from the Bayesian dendrogram.

In the haplotype network approach, each polymorphic cpDNA region showed different levels of diversification; *rbcL* was the least variable (i.e. five haplotypes), while *rps16* was the most variable (i.e. 15 haplotypes). In all analyses, no shared haplotype patterns were found between the "eastern" and the "western" groups. The *rbcL* sequences showed five haplotypes, with three haplotypes consisting solely of western region sequences and the other two corresponding to the eastern region (Fig. 3a). However, different frequencies were observed inside each haplotype. The three haplotypes of the "western group" had nine, four and one *S. ciliata* individuals, respectively, and the two haplotypes of the "eastern group" had nine and two individuals, respectively. The differentiation of haplotypes into the "western group" and the "eastern group" was more apparent in *rps16*. Of the 15 different haplotypes identified, eight were exclusively located in the "western group" and seven in the "eastern group" (Fig. 3b). In *trnL* a clear distinction was also found between eastern and western haplotypes. Sequences assembled into 13 haplotypes, with six haplotypes including only western region sequences and seven haplotypes including only eastern region sequences (Fig. 3c). Haplotype distribution patterns similar to *rps16* were observed. The *trnL* haplotype network indicated a hypothetical haplotype link between the "western group" and "eastern group" (marked as a star-shaped dot; see Fig. 3c), which was mostly related to the Apennines haplotype Ape1. On examining all three haplotype networks, we discerned the persistent placement of Pyr1 and Pyr2 individuals with those of the

Iberian mountain systems, while the rest of Pyrenean individuals remained together with those of the Central Massif system (Mas). The *rbcL* network was selected for visualising the geographic distribution of haplotypes, as it showed the most representative and parsimonious patterns of the three networks (Fig. 5). Cen2 and Bal 1haplotypes were prevalent in the western and eastern regions, respectively.

The neighbour-net method suggested a grouping pattern that was in accordance with the one obtained using the haplotype network approach. Besides that, it provided a chance to delve deeper into the differences among *S. ciliata* sampled populations. The *rbcL* neighbour-net (Fig. 4a) confirmed the classification of all studied populations into a western and an eastern region but was not statistically supported. On the contrary, in the case of *rps16* and *trnL* neighbour-nets, the classification into the western and eastern regions was statistically supported (Fig. 4b and Fig.4c, respectively). Furthermore, some distances inside these two networks were noteworthy. Such were the cases of the observed 86.3% difference in the distance between Cen1 and the rest of Central System populations and of the 85% difference in the distance between Ari and the Balkan populations, as indicated by the *rps16* net. These results were already implied by the *rps16* and *rbcL* haplotype networks. Another interesting result was the common clustering of the Italian Ape3 with some Balkan populations, which was indicated by both the *rbcL* and *rps16* neighbour-nets and also by the dendrograms. In the *trnL* neighbour-net, Cen3 and Din showed a near significant differentiation (94.2% and 93.5%, respectively) that was earlier suggested by the dendrograms.

The Bayesian spatial clustering of populations resulted in an optimal grouping of K=2. This supported the western-eastern region division of populations observed in previous analyses. Only the Balkan population Din deviated from this division, clustering with the western-region populations.

Discussion

Genetic diversity in the cpDNA of *S. ciliata*: a comparative approach

This study reveals high haplotype variability, especially in the case of the *trnL* and *rps16* polymorphic cpDNA regions, and therefore supports the hypothesis of high cpDNA diversification among *S. ciliata* populations. Similar results have been reported in previous

studies on other *Silene* species, such as *S. latifolia* (Ingvarsson & Taylor, 2002), *S. vulgaris* (Ingvarsson & Taylor, 2002; Štorchová & Olson, 2004) and *S. dioica* (Prentice, Malm & Hathaway, 2008; Hathaway, Malm & Prentice, 2009), among others. Yet, *S. ciliata* is ranked among the most varied. Low levels of cpDNA diversification and no diversification at all have been found in *S. hifacensis* (Prentice et al., 2003) and *S. sennenii* (López-Vinyallonga et al., 2012), respectively. A possible explanation for this could be that these two species are rare endemics (Gitzendanner & Soltis, 2000; López-Pujol et al., 2009) and consequently, a combination of narrow distribution, low population size and habitat fragmentation led to a drastic drop of genetic diversity (López-Vinyallonga et al., 2012). Considering this observation and our results as a baseline, we suggest that the variation detected in *S. ciliata* is the outcome of an ancient, wider distribution range, followed by a gradual splintering caused by a series of ice ages, as with many other high-elevation species (reviewed by Nieto Feliner, 2014). A considerable split would have come after the divergence time of the species (around 10 million years ago; Sloan et al., 2009). This interpretation is also supported by the current widespread, but fragmented, distribution of the species around the Mediterranean Basin (see Fig. 1).

Interpreting the distinction of *S. ciliata* between western and eastern regions and their origin

No evidence was found against the classification of *S. ciliata* into a western and an eastern race (Blackburn, 1933; Tutin et al., 1995). Hence, we propose maintaining the names *Silene ciliata* subsp. *ciliata* and *S .ciliata* subsp. *graefferi* to describe the noted clustering of *S. ciliata* individuals into a western and eastern group, respectively. On the other hand, both dendrograms indicated a significant difference between *S. ciliata* individuals and the outgroups, which together with the nonessential divergence between populations corroborates the monophyly of our species.

Tracing back to the species' differentiation, we hypothesize that populations of an ancestor of *S. ciliata* dominated the Mediterranean Basin. At the onset of glacial period climatic oscillations in the late Tertiary and in the Quaternary period, these ancestral populations were forced to migrate to favourable areas, while those unable to encounter a glacial refugium because of distance, time or natural barriers perished. Given that we are dealing with an alpine species, *S. ciliata* populations should have migrated following the paths that constitute links between

317 neighbouring mountains. The Alps mountain range system seems to have posed a persistent and
318 significant hurdle for this species' migration. A rigorous example supporting this theory is that
319 during Quaternary glaciations, and in contrast to the Mediterranean mountains, the Alps were
320 extensively and completely covered with ice sheets (Hughes, Woodward & Gibbard, 2006). This
321 is in accordance with previous phylogeographic studies (e.g. Taberlet et al., 1998; Hewitt, 2000)
322 and may explain why *S. ciliata* populations have not been found there. Moreover, it would
323 account for the observed disconnected distribution and division of the species into the western
324 and eastern groups, since the geographical borders formed by the two groups coincide with the
325 location of the Alps. A similar grouping pattern has been found in the Mediterranean for
326 *Androsace vitaliana* (Vargas, 2003) and *Heliosperma* (Frajman & Oxelman, 2007), genera with
327 the barrier shifting west and east of the Alps region, respectively. A connecting individual
328 between the western and eastern populations, probably inhabiting the vicinity and/or regions of
329 the Alps, was implied here by the "missing" (extinct or not found) haplotype that was indicated
330 by the *trnL* haplotype network. Disjunction in distribution, possibly resulting from the Alps and
331 distinction into two subspecies has recently been proposed in the case of *Artemisia eriantha,*
332 another alpine plant distributed along the Alps and many Mediterranean mountains (Sanz et al.,
333 2014), and comes as an additional support to our hypothesis.
334
335 Evolutionary processes and geo-climatic effects on western and eastern populations
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337 Apart from the significant difference found between eastern and western cpDNA sequences,
338 further important diversification was noticed inside each group. Polyploidization during the
339 Pleistocene is one evolutionary mechanism generating evolutionary lineages (Stebbins, 1984). *S.*
340 *ciliata* has a wide range of polyploids in both the western and eastern race, long described by
341 Blackburn (1933). Hence, we propose that -since intrapopulation polyploidization is widely
342 accepted (Lewis, 1980) - it could also explain differences within *S. ciliata* species during that
343 Era. Moreover, intrapopulation variation could partially be the result of Mediterranean refugia
344 disjunction during adverse climatic conditions, followed in some cases by elevational range shifts
345 (surviving in lowland glacial refugia) (Surina, Schönswetter & Schneeweiss, 2011) and in others
346 by *in situ* endurance (inside nunataks) (Rull, 2009). Therefore, habitat disconnection would have
347 persisted during favourable climate stages. So, the most likely explanation is that refugia

isolation resulted in slow mutational events that took place over a long period of time (Sanz et al., 2014).

Regarding the western group, genetic diversity is apparent in the Pyrenees mountain range and has led to the genetic disaffiliation of the range into a western and an eastern section of *S. ciliata* species. The same genetic break has been found in *Artemisia eriantha* (Sanz et al., 2014). A possible explanation for this bipartition could be drawn from the study by Calvet (2004), where marked asymmetry of Pyrenean glaciers is mentioned with the ice sheets of western Spanish slopes located higher than eastern French slopes. Another component of the western group diversification was introduced by the highly divergent Cen1 sequence of Serra da Estrela. This divergence may be associated with the highly dynamic borderline region of the Cerro Rebolado-Fraga das Penhas area during the Pleistocene (Vieira & Ferreira, 1998). On the other hand, the merging of Pyr1 and Pyr2 sequences with Cantabrian and Central System *S. ciliata* individuals may imply braided migrational paths of these species during glacial-interglacial events.

Interestingly, the degree of divergence recorded in the eastern group of *S. ciliata* is higher than that in the western group. This observation has also been made for temperate trees and shrub taxa (Petit et al., 2003). We believe that this high genetic diversity and the existence of more unique haplotypes, especially in the Balkan Peninsula, is the outcome of the complex orography and restricted territorial extent of existing refugia, which did not facilitate communication among populations during Pleistocene climatic oscillations and postglacially. More specifically, the various orientations of mountain chains in the Balkans may have acted as a barrier to internal migration (Tzedakis, 2004). Thus, the concomitant genetic differences in the Balkans could have emerged due to the accumulation of mutations, natural selection and stochastic events in small isolated populations at the time of climatic oscillations as well as to successive founder effects during range expansion (Ibrahim, Nichols & Hewitt, 1996; Petit et al., 1997). The individuals from the western part of the eastern groups (e.g. Ari, Bal1 and Din) showed some important differences in certain analyses (see Figs 3b and 4b). This might be related to the nature of the east Balkan slopes, which have a more gentle relief compared to the steep west mountains (Reed, Kryštufek & Eastwood, 2004), thereby fostering higher levels of isolation. On the other hand, the grouping of Bal3, Bal4, Bal5 and Bal6 is in agreement with the theory of Turrill (1929), who proposed that elevational migration of Balkan alpine plants should result in a higher resemblance

379 of neighbouring populations along the same altitude than along the same latitude. Further
380 occasional differentiation in the Din individual could be because the Dinaric Alps were much less
381 affected by glaciations than the rest of the Mediterranean mountain systems (Frajman &
382 Oxelman, 2007) resulting in the maintenance of relict populations, which might explain why the
383 Bayesian spatial analyses yielded their clustering with the western group. A possible explanation
384 for the Italian Ape3 individual merging with some Balkan individuals may be found in the
385 proposed land connection of the north Italian and the Balkan Peninsula during the early Holocene
386 (approx. 20-16 ka BP). This connection may have resulted from changes in the sea level due to
387 glacio-hydro-isostatic effects of that time period (Lambeck et al., 2004), and this could have
388 facilitated the migration or meeting of Italian and Balkan populations during interglacial cycles.
389
390 The Pyrenees case
391
392 The Bayesian and maximum likelihood analyses showed that Pyr1 and Pyr4 differed from the
393 outgroups as well as from the rest of *S. ciliata* individuals and were situated in an intermediate
394 position between them in the dendrogram (see Fig. 2). Hence, we surmised that this pattern could
395 be another example of the Pyrenees range acting as a stable hybrid zone. This has been argued in
396 many past studies, such as in *Chorthipopus parallelus* (Hewitt, 1993) and *Saxifraga* subsect.
397 *Triplinervium* (Mas de Xaxars et al., 2015). In the case of Pyr1 and Pyr4, the observed patterns
398 may have resulted from interspecific hybridization and introgression between *S. ciliata* and other
399 congeneric, sympatric species, which led to haplotype sharing (Palmé et al., 2004; Heuertz et al.,
400 2006). This is very likely since the majority of *Silene* species have the same chromosome
401 number, 2n=24 (Bari, 1973), which could have facilitated a hybridization event. At any rate, the
402 rise of hybrid zones due to glaciations, and hence, the preservation of different species genomic
403 information via hybrid individuals (Harrison, 1990) are linked with high altitudes (Hewitt, 2001
404 and references therein). The alternative explanation of them being the result of random ancestral
405 alleles and paralogues extinction, i.e. lineage sorting, is not favoured (Frajman & Oxelman,
406 2007). After all, the geographical congruence of congeneric species causing chloroplast sharing
407 has been reported in several studies of tree genera and between some herbs like *S. latifolia* and *S.*
408 *dioica* (Prentice, Malm & Hathaway, 2008 and references therein), as well as in other plant
409 groups (e.g. Gardner et al., 2004; Okuyama et al., 2005).

Conclusions and future prospects

Our results confirm the monophyly of *S. ciliata* due to the differences found between the studied populations and the outgroups and reveal a clear west-to-east division of *S. ciliata* populations with the borderline set in the region of the Alps. This division validates the past classification of the species into two subspecies; *S. ciliata* subsp. *ciliata* found west of the Alps ("Spanish race", Blackburn, 1933) and *S. ciliata* subsp. *graefferi* located east of the Alps ("Italian race", Blackburn, 1933). In addition, major intraspecific variation is supported by all analyses, but none of them supports the occurrence of additional varieties or subspecies (according to Küpfer, 1974 and Castroviejo et al., 1986-2001). Evidence is also provided of the central role played by geographic and climatic factors in the evolutionary history of the species and the formation of the two subspecies. Further analyses that would include more individuals and markers are encouraged to secure conclusions of this role, as well as of the existence of unsolved-incongruent populations. Molecular clocks and increased sampling effort are necessary to resolve the remaining questions.

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Fig. 1: Distribution of sampled *S. ciliata* populations in the Mediterranean Basin. Acronyms were derived from the name of the mountain system where samples were collected: Can - Cantabrian Range, Ibe - Iberian System, Pyr - Pyrenees range, Cen - Central System, Mas - Central Massif, Ari - Aridaia range, Bal - Balkan-Rhodope mountain system, Din - Dinaric Alps and Ape - Apennines range.

Table 1: DNA samples of *Silene ciliata* used for the study. The table shows the acronym given to each sampled population («Name»), the «Country» where these populations were collected, «Altitude» and MGRS coordinates. A more detailed version of this table can be found in Table S1.

Table 2: Characteristics of the three polymorphic cpDNA regions and the "all-marker" region studied in *Silene ciliata*. The length of the products after amplification with the corresponding marker and alignment editing, and the variable and parsimony sites of each product ensued from the DnaSP analysis are shown.

Fig. 2: Bayesian consensus dendrogram of the "all-marker" cpDNA sequence of *Silene ciliata*.

Fig.3: Haplotype networks showing the relationships between the cpDNA parsimony haplotype groups found for *rbcL* (a, five haplotypes), *rps16* (b, 15 haplotypes) and *trnL* (c, 13 haplotypes) in *S. ciliata*. Rectangles and ovals depict haplotypes that belong to the western and eastern groups, respectively. In Figure 3.c, the star-shaped dot corresponds to the "missing" haplotype pattern that constitutes the link between the eastern and the western group.

Fig. 4: Neighbour-net analyses of *rbcL* (a), *rps16* (b) and *trnL* (c) based on uncorrected p-distances. Numbers denote significant bootstrapping values. The eastern and western groups of *S. ciliata* populations are indicated by grey-shaded clusters. Blue letters correspond to the eastern group and red letters to the western group.

Fig. 5: Distribution and frequency ratios of *S. ciliata* haplotypes for *rbcL* (see Fig. 4a) in the mountain systems of this study. The proportion of different haplotypes at each location is shown in the circles.

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Table 1(on next page)

Details of the sampled populations of *Silene ciliata*

Table 1: DNA samples of Silene ciliata used for the study. The table shows the acronym given to each sampled population («Name»), the «Country» where these populations were collected, «Altitude» and MGRS coordinates. A more detailed version of this table can be found in Supplemental file 1.

Name	Country	Altitude(m)	MGRS
Can1	ES	1642	29TQH4477
Can2	ES	1900	30TUN3712
Can3	ES	1881	30TUN5150
Ibe1	ES	1900	30TVM9646
Ibe2	ES	2278	30TWM0276
Pyr1	ES	1931	30TYN2920
Pyr2	ES	1350-1780	30TYN4026
Pyr3	ES	2100-2200	31T CG7967
Pyr5	ES	2161	31TDG1980
Cen2	ES	1950	30TTK7079
Cen3	ES	2340	30TVL2104
Cen1	POR	1900	29TPE1783
Mas	FR	1560	31TDL8119
Pyr4	FR	2190	31TDH3461
Ari	GR	2182	34TFL0142
Bal3	GR	1800	35TKF5580
Bal4	GR	1800	35TKF5307
Bal5	GR	1800	35TKF5586
Bal6	GR	2060	35TKF5632
Bal1	BU	1900	34TGM0365
Bal2	BU	2600	34TGM0229
Din	MAC	2480	34TEM2771
Ape1	IT	1950	33TUH8528
Ape2	IT	1366	33TUH7979
Ape3	IT	2000	33TVG2225

13
14

Table 2(on next page)

Characteristics of the polymorphic cpDNA regions

Characteristics of the three polymorphic cpDNA regions and the “all-marker” region studied in *Silene ciliata*. The length of the products after amplification with the corresponding marker and alignment editing, and the variable and parsimony sites of each product ensued from the DnaSP analysis are shown.

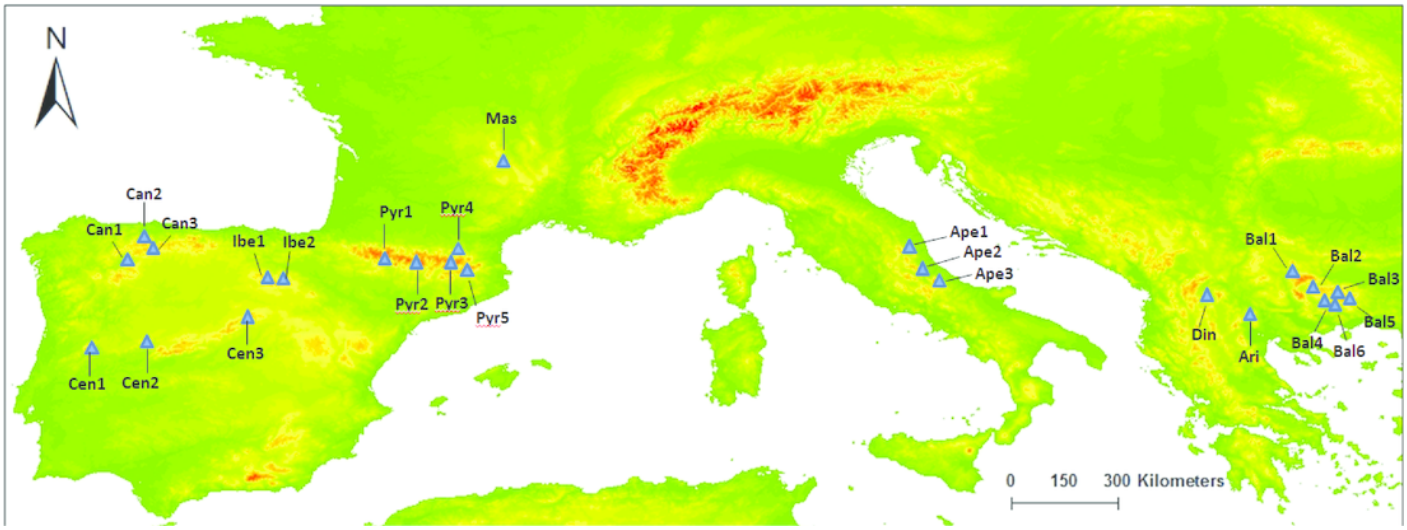
Table 2: Characteristics of the three polymorphic cpDNA regions and the "all-marker" region studied in *Silene ciliata*. The length of the products after amplification with the corresponding marker and alignment editing, and the variable and parsimony sites of each product ensued from the DnaSP analysis are shown.

Chloroplast marker	Length of selected region	Variable (polymorphic) sites	Parsimony informative sites
rbcL	564 bp	4	3
rps16	753 bp	25	16
trnL	513 bp	18	11
all	1830 bp	47	30

1

Figure1: Map of our sampled populations of *Silene ciliata*

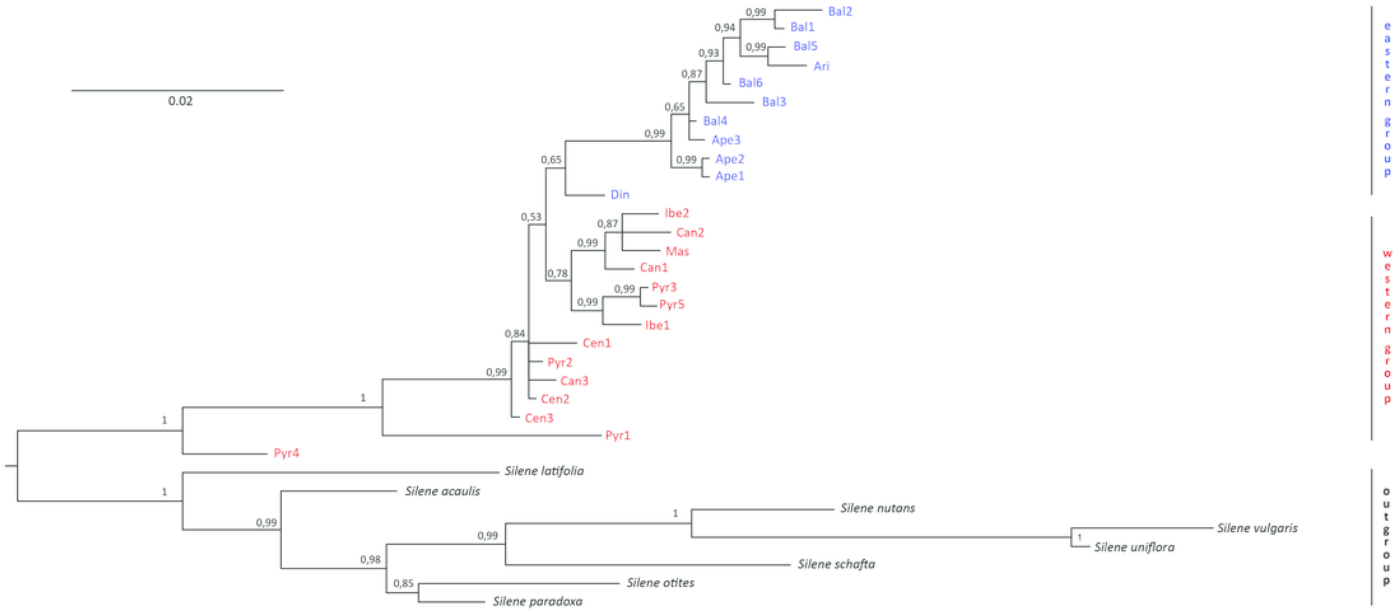
Distribution of sampled *S. ciliata* populations in the Mediterranean Basin. Acronyms were derived from the name of the mountain system where samples were collected: Can - Cantabrian Range, Ibe - Iberian System, Pyr - Pyrenees range, Cen - Central System, Mas - Central Massif, Ari - Aridaia range, Bal - Balkan-Rhodope mountain system, Din - Dinaric Alps and Ape - Apennines range.



2

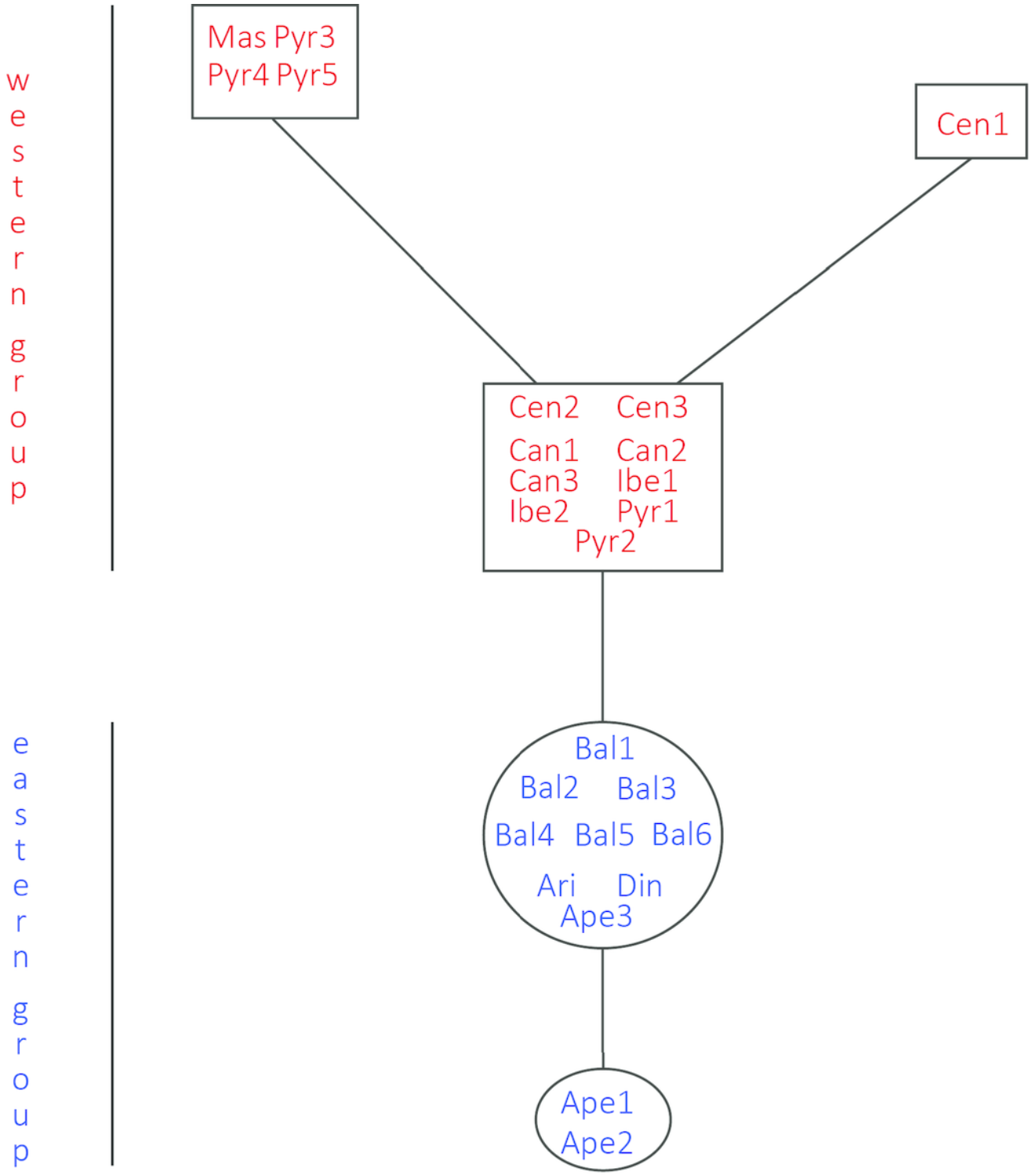
Figure 2: Bayesian dendrogram

Bayesian consensus dendrogram of the “all-marker” cpDNA sequence of *Silene ciliata.*



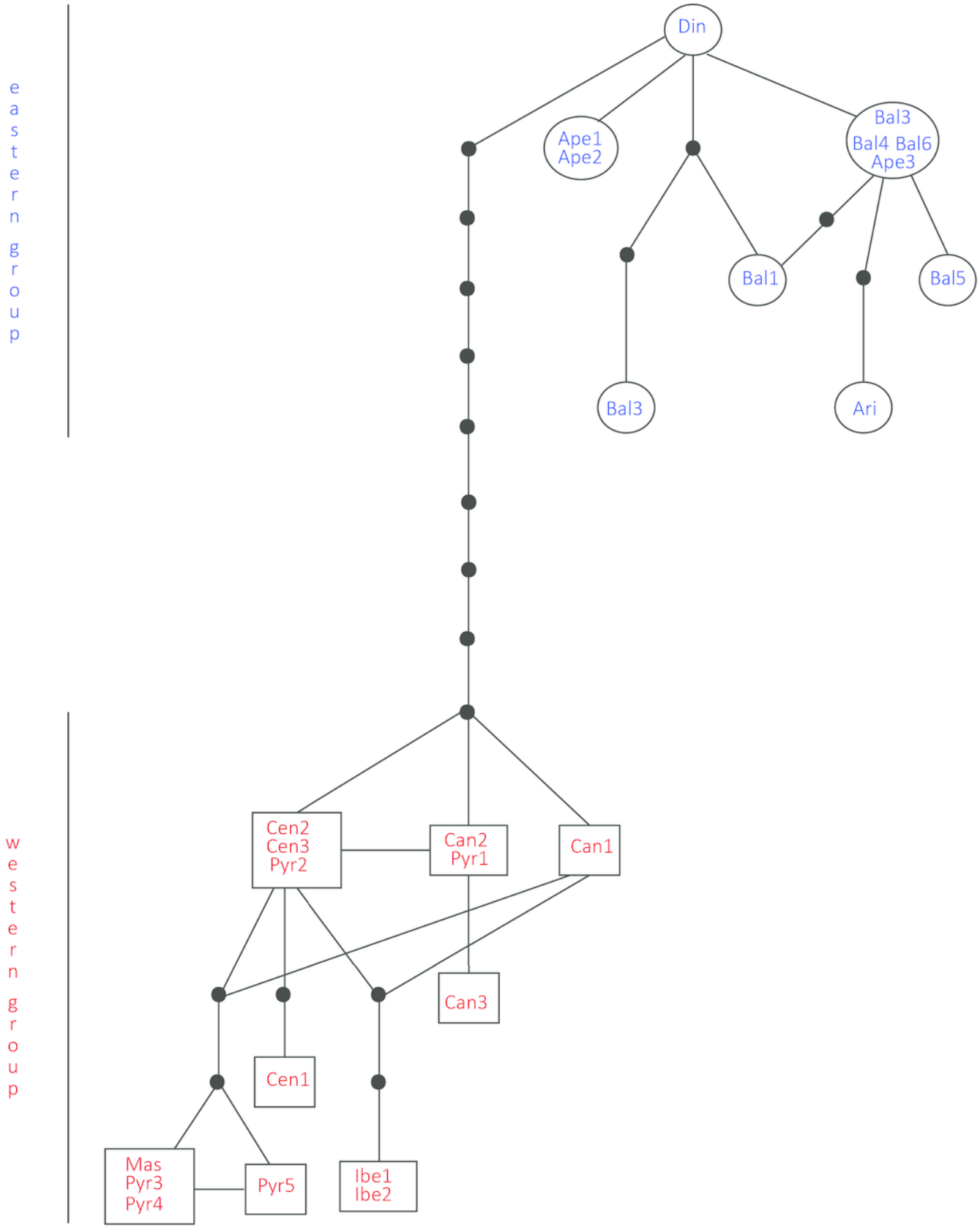
3

Figure 3A: Haplotype network of *rbcL*


western group
Mas Pyr3
Pyr4 Pyr5
Cen1
Cen2 Cen3
Can1 Can2
Can3 Ibe1
Ibe2 Pyr1
Pyr2
eastern group
Bal1
Bal2 Bal3
Bal4 Bal5 Bal6
Ari Din
Ape3
Ape1
Ape2

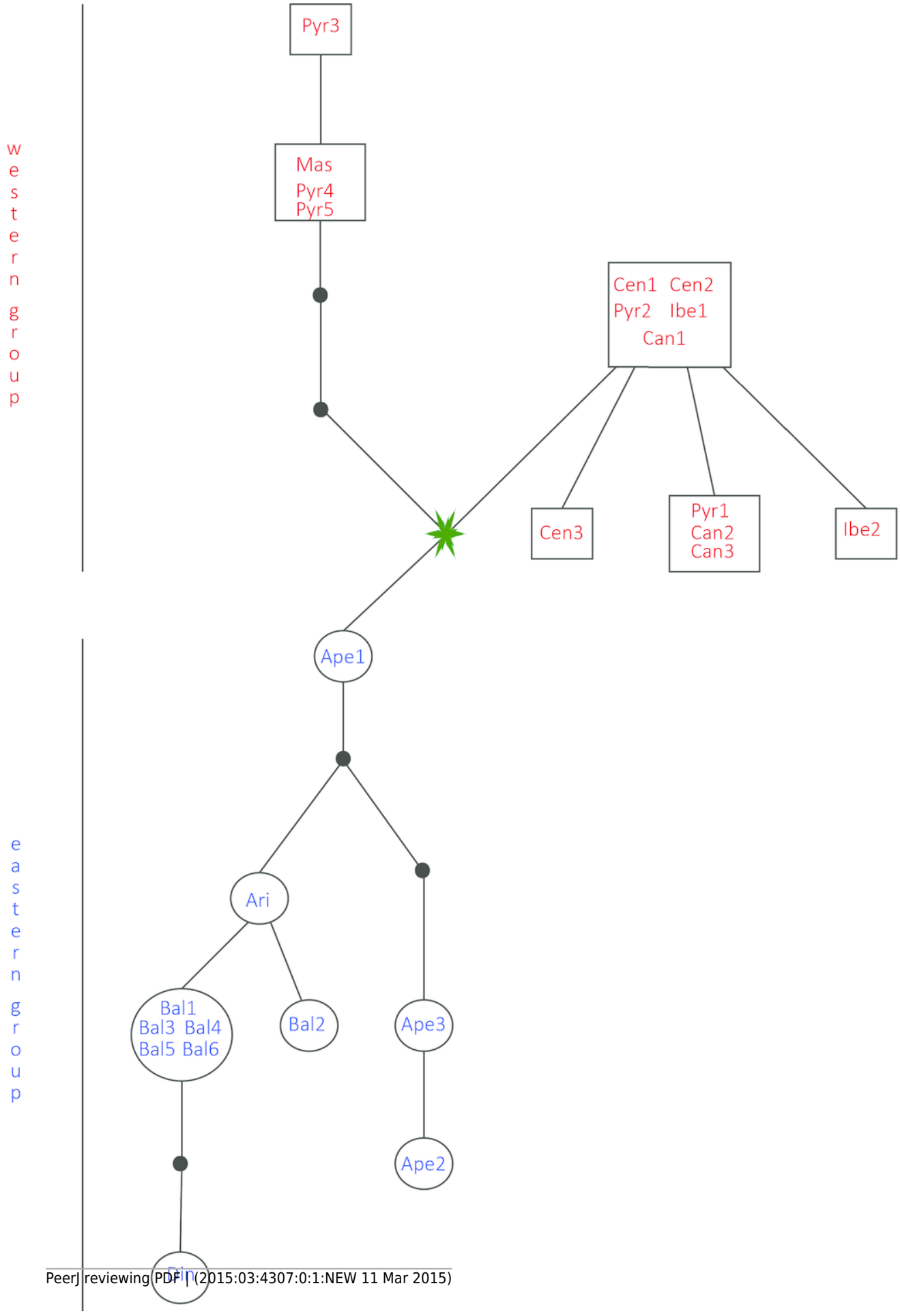
4

Figure 3B: Haplotype network of *rps16*


eastern group
western group
Din
Ape1
Ape2
Bal3
Bal4 Bal6
Ape3
Bal1
Bal5
Bal3
Ari
Cen2
Cen3
Pyr2
Can2
Pyr1
Can1
Can3
Cen1
Mas
Pyr3
Pyr4
Pyr5
Ibe1
Ibe2

5

Figure 3C: Haplotype network of *trnL*


western group
Pyr3
Mas
Pyr4
Pyr5
Cen1 Cen2
Pyr2 Ibe1
Can1
Cen3
Pyr1
Can2
Can3
Ibe2
Ape1
eastern group
Ari
Bal1
Bal3 Bal4
Bal5 Bal6
Bal2
Ape3
Ape2

6

Figure 4A: Neighbour-net analysis of *rbcL*

Neighbour-net analyses of *rbcL (a),* rps16 *(b) and* trnL *(c) based on uncorrected p-distances. Numbers denote significant bootstrapping values. The eastern and western groups of* S. ciliata *populations are indicated by grey-shaded clusters. Blue letters correspond to the eastern group and red letters to the western group.*

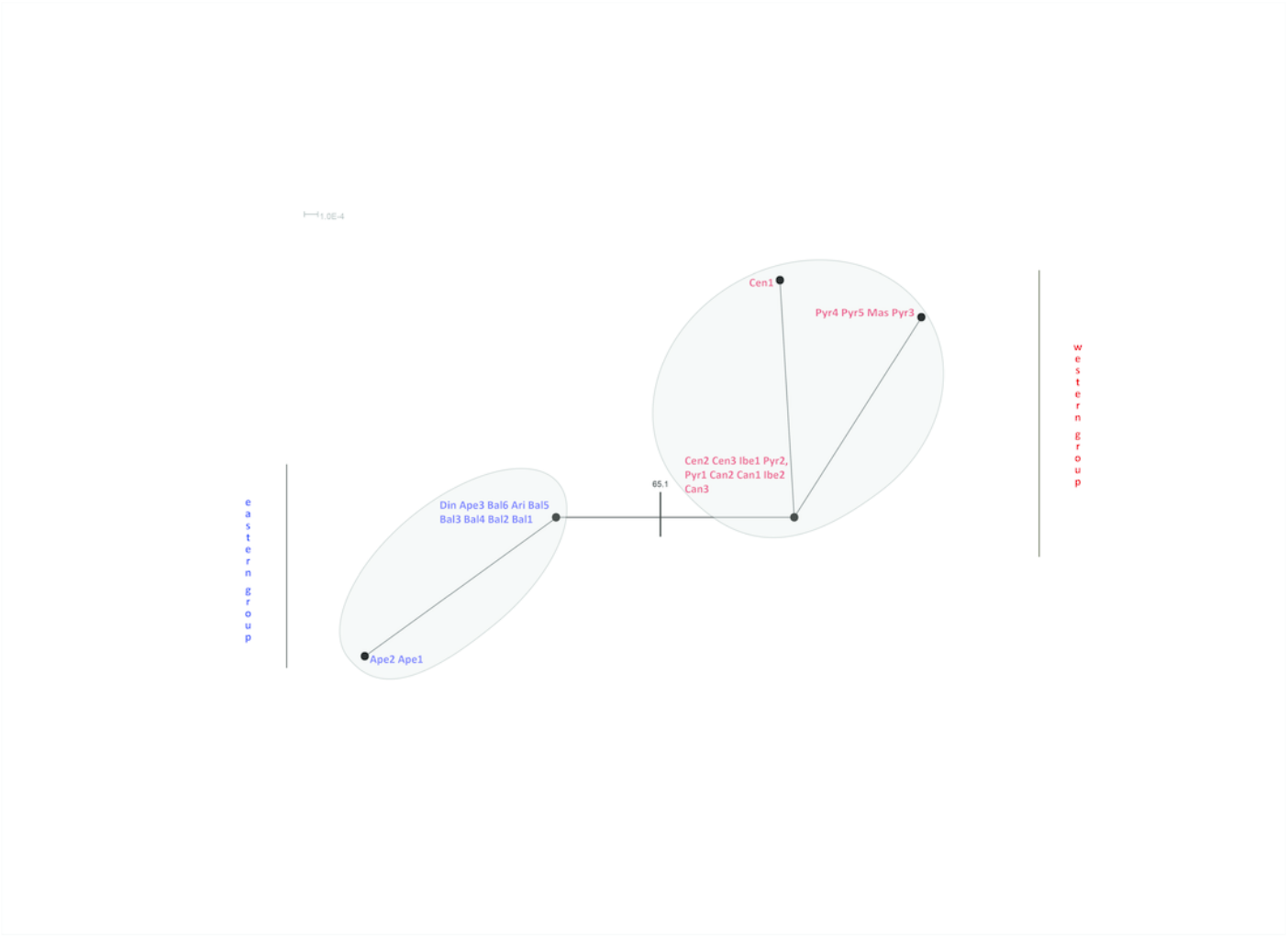


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Figure 4B: Neighbour-net analysis of *rps16*

Neighbour-net analyses of *rbcL* (a), *rps16* (b) and *trnL* (c) based on uncorrected p-distances. Numbers denote significant bootstrapping values. The eastern and western groups of *S. ciliata* populations are indicated by grey-shaded clusters. Blue letters correspond to the eastern group and red letters to the western group.

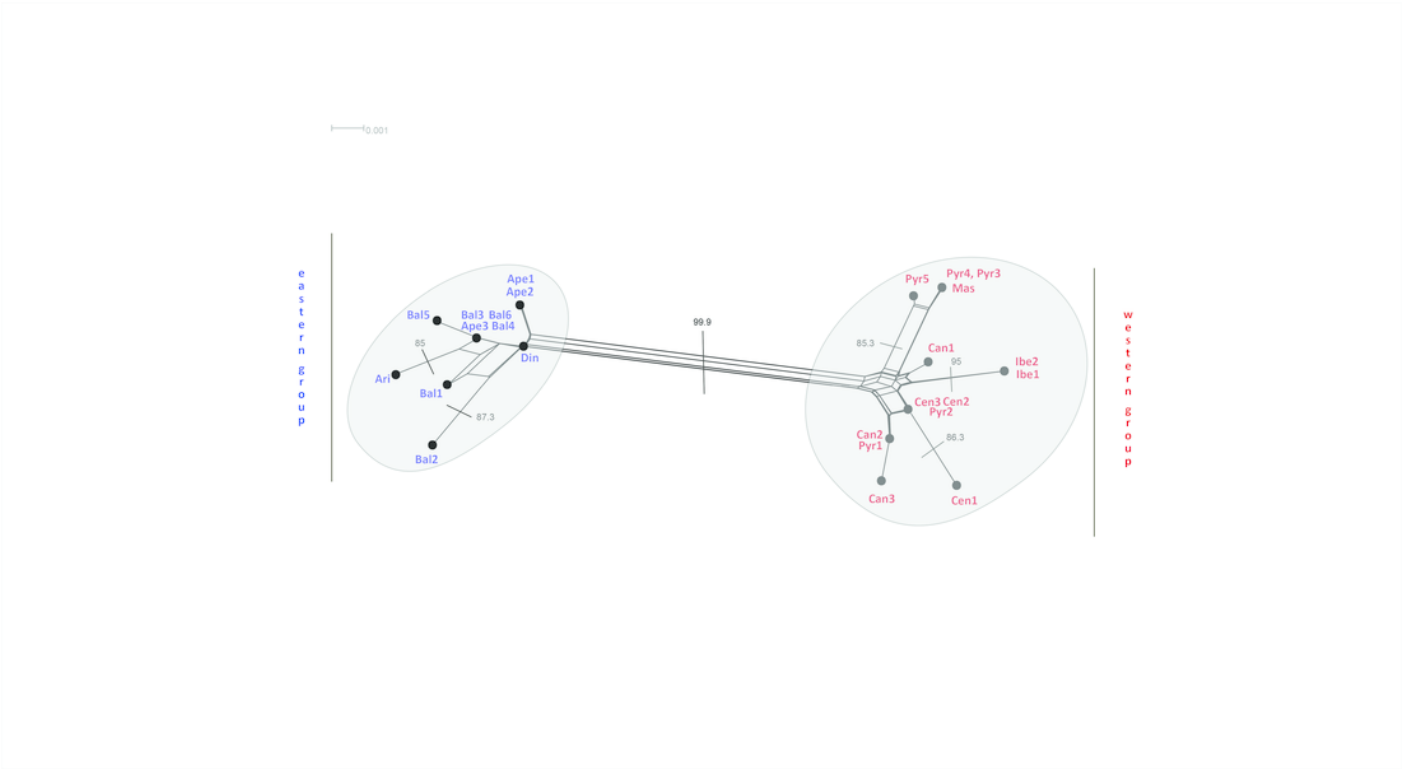

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Figure 4C: Neighbour-net analysis of *trnL*

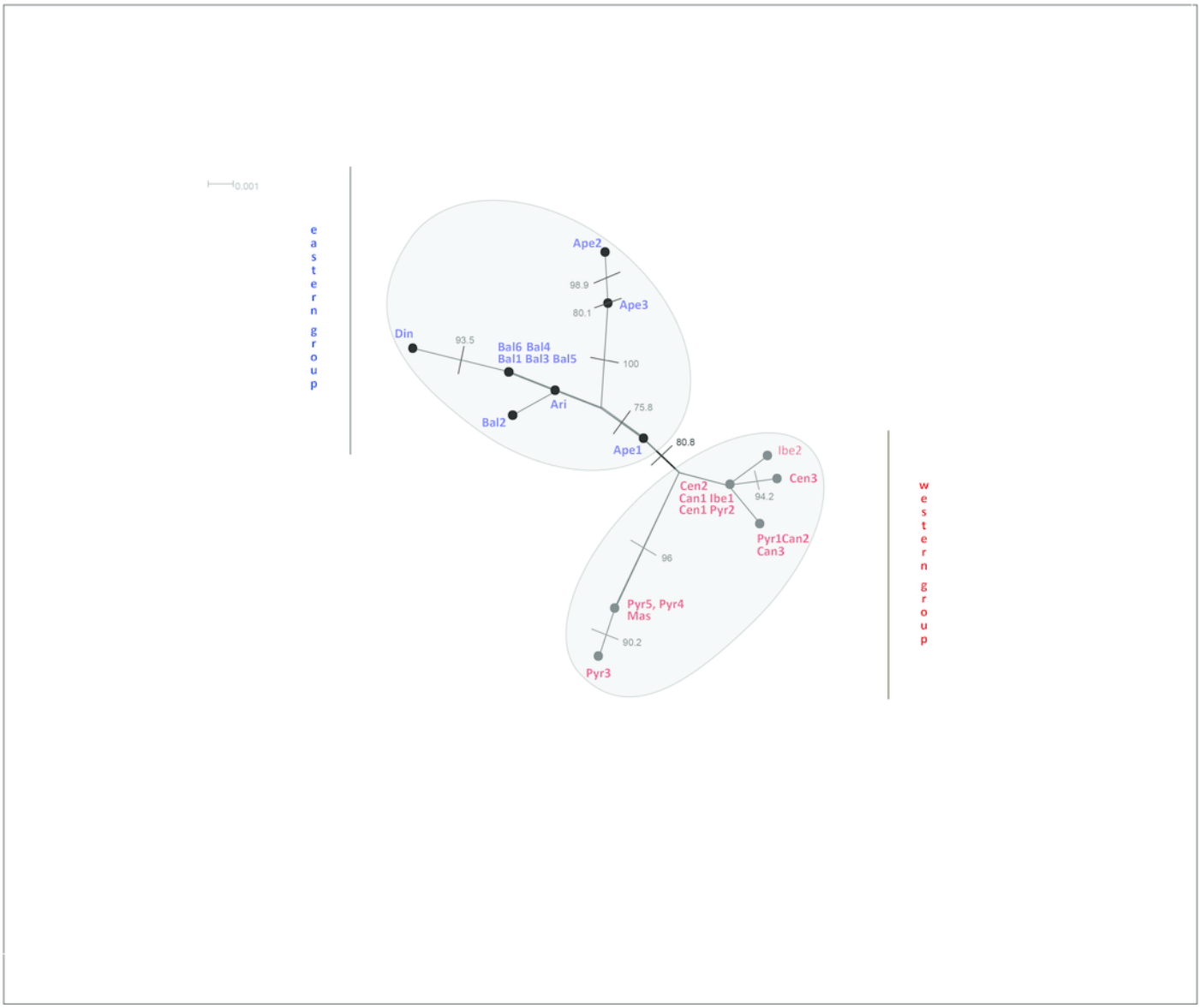

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Figure 5: Distribution and frequency ratios of *rbcL* haplotypes

Distribution and frequency ratios of *S. ciliata* haplotypes for *rbcL* (see Fig. 4a) in the mountain systems of this study. The proportion of different haplotypes at each location is shown in the circles.