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Diversification of *Scrophularia* (Scrophulariaceae) in the Western Mediterranean and Macaronesia – Phylogenetic relationships, reticulate evolution and biogeographic patterns

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ABSTRACT

The flora of the Mediterranean region and Macaronesia is characterized by high levels of species diversity and endemism. We examined phylogenetic relationships of *Scrophularia* within one of its secondary centers of diversity located in the Iberian Peninsula and adjacent Macaronesia. In total, 65 ingroup accessions from 45 species, representing an almost complete sampling of the region, were analyzed using sequences from the internal transcribed spacer region (ITS) and the plastid *trnQ-rps16* intergenic spacer. Phylogenetic relationships were inferred using Bayesian inference, maximum likelihood and statistical parsimony networking. Incongruence between datasets was assessed with statistical tests and displayed by split networks. Biogeographic inferences incorporating information from both markers (despite low resolution in some parts of the trees) and all incongruent taxa were accomplished with a novel combination of methods, using trees generated with the taxon duplication approach as input for Bayesian binary MCMC (BBM) analysis as implemented in RASP.

Nuclear and chloroplast markers support a clade which comprises the majority of Iberian and Macaronesian species and consists of three subclades. Analyses of the substantial incongruence observed among markers indicate reticulate evolution and suggest that *Scrophularia* species diversity in this region is largely attributable to hybridization; a combination of both polyploidy and dysploidy in the karyotypic evolution of Western Mediterranean *Scrophularia* taxa is proposed. Our results provide support for an ancient hybridization event between two widespread lineages, which resulted in an allopolyploid ancestor of the Iberian – Macaronesian group with $2n = 58$ chromosomes. The ancestor then diverged into the three main lineages present in the Iberian Peninsula, Northern Africa and Macaronesia today. Subsequent interspecific hybridizations at different ploidy levels additionally generated new species. Presumably, hybridization and diversification within the genus in the Western Mediterranean have not been restricted to one particular event, but occurred repeatedly. It can be assumed that the topographical complexity found in the Iberian Peninsula has promoted diversification and hybrid speciation processes in *Scrophularia*, and that isolation in glacial refugia has preserved recent and ancient lineages. For the Macaronesian taxa, biogeographic analyses support several origins, by colonizations from at least four distinct lineages.

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1. Introduction

The Mediterranean basin is recognized as a global biodiversity hotspot and harbors app. 25,000 vascular plant species (Comes, 2004; Médail and Myers, 2004; Myers et al., 2000). As defined by Médail and Quézel (1997), important areas of plant endemism and floristic richness in the Western Mediterranean and Macaronesia are the High and Middle Atlas mountains, the Baetic – Rifan complex, the Maritime and Ligurian Alps, the Tyrrhenian Islands, and the Canary Islands and Madeira. One of two main centers of biodiversity in the Mediterranean basin is found in its western part and includes the Iberian Peninsula and Morocco (Médail and Quézel, 1997). It is assumed that patterns of plant speciation in the Mediterranean Basin have been shaped by climatic shifts and geological events, like the Betic Crisis and Messinian Salinity Crisis (Garcia-Castellanos et al., 2009; Krijgsman et al., 1999; Lonergan and White, 1997), the onset of the Mediterranean climate rhythm (Suc, 1984; Thompson, 2005), and the Quaternary glaciations (Suc, 1984). Additionally, its topographical complexity was suggested to promote diversification and speciation processes. Fragmentation and contraction of distribution ranges have triggered the isolation of populations during glacial periods, thereby causing allopatric speciation. Following inter- and postglacial range expansion,

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hybrid zones developed where different genetic lineages came into contact (Blanco-Pastor et al., 2012).

The genus *Scrophularia* L. (Scrophulariaceae), belonging to the order Lamiales and closely related to *Verbascum* L. (Datson et al., 2008; Olmstead et al., 2001), includes app. 270 extant species (Ortega Olivencia, 2009). Representatives of the mainly holarctic genus occur in both the Old and New World; the primary center of diversity is located in the Irano – Turanian region including the Caucasus and Central Asia (Gorschkova, 1997; Grau, 1981; Lall and Mill, 1978). One of two secondary diversity centers is found in the Iberian Peninsula and adjacent Macaronesia with 28 species, more than half of which are endemic (Dalgaard, 1979; Ortega Olivencia, 2009). The last taxonomic treatment of the genus was done by Stiefelhagen (1910), who classified the genus according to two sections: section *Anastomosantes* Stiefelhagen (= section *Scrophularia*), with two subsections *Vernales* Stiefelhagen and *Scorodoniae* (Benth.) Stiefelhagen (= subsection *Scrophularia*), and section *Tomiophyllum* Bentham, comprising subsections *Farinosae* Stiefelhagen, *Orientales* Stiefelhagen, and *Lucidae* Stiefelhagen (which largely corresponds to sect. *Canina* G.Don). Leaf venation, petal length, shape of the corolla tube, and life form were used as distinguishing characters. In a revision of the genus for the Iberian Peninsula, Ortega Olivencia and Devesa Alcaraz (1993a) confirmed the assignment of the respective species to sections *Anastomosantes* / *Scrophularia* (20 species) and *Canina* G.Don (three species); *S. tanacetifolia* was moved to section *Scrophularia* by the authors.

Species of *Scrophularia* are annual, biennial or perennial herbs, subshrubs or small shrubs with pinnate to undivided leaves of various forms. The inflorescence is a thyrse with cymose, often dichasial or monochasial partial inflorescences. The flowers are characterized by a more or less equally 5-lobed calyx, a mostly zygomorphic, distinctly 2-lipped corolla and typically a rudimentary fifth stamen of various shapes at the base of the upper lip. The fruit is a capsule with septicidal – septifragous dehiscence (Fischer, 2004). Representatives of the genus inhabit regions from coasts and lowlands to high plateaus and alpine regions, where the majority of species is found. Preferred habitats include mountain slopes and rock crevices, but also forests, scrubs and grassland as well as roadsides or disturbed areas. Species occurring in moist habitats (e.g. on river banks) are mainly restricted to section *Anastomosantes*, while section *Tomiophyllum* also contains more xerophytic elements; however, real desert plants are rare.

Karyological studies on taxa of the genus were confined to particular geographic areas (Carlbom, 1969, 1964; Dalgaard, 1979; Grau, 1976; Ortega Olivencia and Devesa Alcaraz, 1990; Shaw, 1962) or merely reported chromosome numbers (Vaarama and Hiirsalmi, 1967), which range from $2n = 22$ (e.g. in *S. divaricata* Ledeb.; Vaarama and Leikas, 1970) to $2n = 96$ (e.g. in *S. desertorum* (Munz) R.J. Shaw; Shaw, 1962). Many taxa from the Iberian Peninsula and Macaronesia are characterized by $2n = 58$ (Grau, 1976); and most of the species occurring in the New World are high polyploids with $2n = 70–96$ (Shaw, 1962). Shaw (1962) and Carlbom (1969) postulated that allopolyploid evolution increased species diversity and variability within *Scrophularia*. Indeed, hybridization is frequent in the genus (natural hybrids have been erroneously described as distinct species by Menezes, 1908, 1903), and is additionally supported by flower morphology and pollinator preferences. While some of the few large-flowered *Scrophularia* (*S. sambucifolia* L., *S. grandiflora* DC. and *S. trifoliata* L. from the Western Mediterranean, and *S. calliantha* Webb. & Berthel. from Gran Canaria) were shown to possess a mixed pollination syndrome between insects and passerine birds (and even juvenile lizards in *S. calliantha*; Ortega-Olivencia et al., 2012), wasp pollination was recently revealed to be the ancestral condition within the genus (Navarro-Pérez et al., 2013). Wasps (Vespidae) are considered the main pollinators (Faegri and van der Pijl, 1979; Ortega Olivencia and Devesa Alcaraz, 1993b; Wilson, 1878); other insects like bees (Valtueña et al., 2013) and syrphid flies (Knuth, 1909; Ortega Olivencia and Devesa Alcaraz, 1993b; Robertson, 1891) complete the pollinator spectrum, which thus mostly consists of generalist pollinators unable to distinguish between different species of *Scrophularia*. In addition, the mostly protogynous flowers are often self-compatible (although rarely self-pollinating; Ortega Olivencia and Devesa Alcaraz, 1993b, 1993c; Shaw, 1962; Trelease, 1881; Valtueña et al., 2013), and reproductive barriers among related species tend to be weak as well. Artificial cross-pollination experiments have shown the potential for hybridization: successful crossings were performed by Shaw (1962) and Carlbom (1964) on North American, Dalgaard (1979) on Macaronesian, and Goddijn and Goethart (1913) and Grau (1976) on some European species. Altogether, the above-mentioned ecological and morphological factors make interspecific hybridization, in combination with karyotype evolution including polyploidy and dysploidy, very likely to have played an important role in the diversification and speciation history of *Scrophularia*.

In the current work, which is part of an extensive molecular phylogenetic study of the genus, we specifically address the following questions: (1) What are the phylogenetic relationships among *Scrophularia* taxa in the Iberian Peninsula and in Macaronesia? (2) Is the high species diversity observed in the Iberian Peninsula the result of hybridization events or due to other factors? (3) Which parental taxa were involved in the origin of the polyploid Iberian species? Which event gave rise to the unusual chromosome number $2n = 58$ found in many taxa? (4) Which historical biogeographic processes have affected present distribution patterns of *Scrophularia* in the Iberian Peninsula? Are the species occurring on the Canary Islands and Madeira monophyletic (implying a single colonization event), or were there multiple colonizations?

To achieve this goal, we analyzed the plastid *trnQ-rps16* intergenic spacer, which has been successfully employed in a recent study on New World *Scrophularia* (Scheunert and Heubl, 2011), and the nuclear ribosomal internal transcribed spacer (ITS) region, which continues to be widely used for phylogenetic analyses on the interspecific level (Nieto Feliner and Rosselló, 2007).

2. Material and methods

2.1. Plant material, DNA extraction, sequencing

The taxon sampling strategy was designed to span the distribution range of *Scrophularia* in the Western Mediterranean (especially the Iberian Peninsula), Macaronesia and Northern Africa. The present study comprises all 22 species of the Iberian Peninsula according to Ortega Olivencia (2009), 27 out of 28 species occurring in the Western Mediterranean as a whole (e.g. Cartier, 1975; Ortega Olivencia, 2009; Zángheri, 1976; *S. heterophylla* was not sampled but in the region is confined to Istria only), 17 out of 21 species occurring in Northern Africa (Ibn Tattou, 2007; Qaiser, 1982; Quézel and Santa, 1963; Täckholm, 1974; the four absent species are either narrow endemics with close association to sampled species, or putative synonyms), and all of the nine species from Macaronesia (Dalgaard, 1979). Additionally, four species from other diversity centers and the putative center of origin (“Asian species”) were sampled; these include three Southern Asian/Eastern Asian taxa (*S. urticifolia* Wall., *S. ningpoensis* Hemsl., *S. yoshimurae* T.Yamaz.) and one species distributed in Southwestern Asia, Turkey and the Caucasus (*S. amplexicaulis* Benth.). Altogether, the sampling thus consists of 45 ingroup species. For a rough estimate of the degree of intraspecific genetic variation, 13 species were sampled with two or more accessions (see Table 1), laying

Table 1
Analyzed taxa with voucher information on collectors, localities and collection years, herbaria, and GenBank accession numbers. Abbreviated identifiers for individual accessions as used in the text are given after the respective locality (in quotes). Reference citations of previously published sequences: (1), Vargas et al. (2009); voucher information for the respective sequence as given there; (2), Kornhall and Bremer (2004); (3), Scheunert and Heubl (2011). CULT., plants grown in the greenhouses of the Botanical Garden Munich, Germany – the original locality and the supplier (in brackets) are given where known. Herb., Herbarium; acc. no., accession number; Bot. Gard., Botanical Garden, n/a, information not available.

Taxon	Year	Locality (country, province/district)	Collector	Collector no.	Herb.	Acc. no. trnQ-rps16	Acc. no. ITS
Antirrhinum majus L.	1999	Spain, Cordoba	M. Nydegger	36531	MSB	KF447311	FJ487615[1]
Russelia verticillata Kunth	1990	Costa Rica, Guanacaste	P. Döbbeler	3795	M	–	HQ130062[3]
Hemimeris centrodes Hiern	1976	South Africa, Cape	P. Goldblatt	4033	M	HQ130033[3]	HQ130063[3]
Nemesia cheiranthus E.Mey. ex Benth.	1974	South Africa, Cape	P. Goldblatt	2534	M	KF447312	KF447249
Selago corymbosa L.		South Africa, Beaufort West	Vlok	2514	S	–	AJ550603[2]
Verbascum arcturus L.	1962	Crete, Chanía	D. Phitos	603	M	KF447313	KF447250
Verbascum nigrum L.	1998	Germany, Bavaria	H. Wunder		M	HQ130034[3]	HQ130064[3]
S. amplexicaulis Benth.	1977	Iran, Tehran	K.H. Rechinger	57228	M	KF447315	KF447252
S. auriculata L.	1993	Morocco, Tétouan	H. Förther	7104	M	KF447247	KF447287
S. auriculata "balbisii Hornem."	2008	CULT., orig.: Spain, Cantabria (J. Grau)	A. Scheunert	005/1-1	MSB	KF447248	KF447291
S. alpestris J.Gay ex Benth.	1998	France, Pyrénées-Atlantiques	D. Podlech	55135	MSB	KF447332	KF447269
S. arguta Sol.	1905	Fuerteventura, Puerto del Rosario	C.J. Pitard		M	KF447368	KF447308
S. arguta Sol.	2010	CULT., orig.: Lanzarote, Teguise (M. Erben)	A. Scheunert	015/1-1	MSB	KF447367	KF447307
S. arguta Sol.	1995	Morocco, Tiznit	D. Podlech	52494	MSB	KF447366	KF447306
S. bourgaeana Lange	1994	Spain, Salamanca	M. Martinez Ortega	(MA 631819)	MA	KF447333	KF447270
S. calliantha Webb. & Berthel.	2011	CULT., orig.: Gran Canaria, n/a	A. Scheunert	010/1-1	MSB	KF447362	KF447302
S. canina ssp. canina L.	1995	Morocco, Tiznit	D. Podlech	52525	MSB	KF447320	KF447257
S. canina ssp. ramosissima (Loisel.) P.Fourn.	1976	Sardinia, Oristano	U. Hecker	I 774 (Hec. 1560)	MJG	KF447323	KF447260
S. crithmifolia Boiss.	1983	Spain, Malaga	E. Bayer, J. Grau & G. López González		M	KF447321	KF447258
S. deserti Delile	1991	Egypt, Sinai Peninsula	D. Podlech	49719a	MSB	KF447325	KF447262
S. eriocalyx Emb. & Maire	1933	Morocco, Rif-Atlas	Sennen (& Mauricio)	8461	W	KF447351	KF447289
S. frutescens L.	1973	Spain, Cadiz	H. Merxmüller & W. Gleißner	29073	M	KF447322	KF447259
S. glabrata Aiton	1988	La Palma, Fuencaliente; "pal"	M. Nydegger	25415	M	KF447360	KF447300
S. glabrata Aiton	2010	CULT., orig.: Tenerife, Monte del Cuchillo; "ten"	A. Scheunert	011/1-1	MSB	KF447361	KF447301
S. grandiflora DC.	2010	CULT., orig.: n/a (Bot. Gard. Erlangen 218/2007)	A. Scheunert	004/1-1	MSB	KF447348	KF447285
S. herminii Hoffmanns. & Link	2009	CULT., orig.: n/a (Bot. Gard. Madrid 262-80); "na"	A. Scheunert	016/1-1	MSB	KF447370	KF447278
S. herminii Hoffmanns. & Link	1987	Spain, Zamora; "za"		(MA 510365)	MA	KF447342	KF447279
S. hirta Lowe	2010	CULT., orig.: Madeira, n/a; "mad. (1)"	A. Scheunert	009/1-1	MSB	KF447364	KF447304
S. hirta Lowe	2010	CULT., orig.: Madeira, n/a; "mad. (2)"	A. Scheunert	013/1-1	MSB	KF447365	KF447305
S. hirta Lowe	2010	CULT., orig.: Madeira, Pico de Ruivo (M. Erben); "mad. (r)"	A. Scheunert	014/1-1	MSB	KF447363	KF447303
S. hispida Desf.	1989	Morocco, Tadla-Azilal	W. Lippert	25095	M	KF447353	KF447292
S. laxiflora Lange	1995	Spain, Cadiz, Algeciras; "alg"	M. Nydegger	33671	MSB	KF447337	KF447274
S. laxiflora Lange	1996	Spain, Cadiz, Los Barrios; "bar"	M.A. Carrasco, S. Castroviejo & M. Velayos	13801SC	MA	KF447336	KF447273
S. libanotica Boiss.	1957	Iraq, Sulaymaniyyah (Kurdistan)	K.H. Rechinger	10358	M	KF447329	KF447266
S. lowei Dalgaard	2010	CULT., orig.: Madeira, n/a (Bot. Gard. Madeira 61/2009)	A. Scheunert	007/1-1	MSB	KF447369	KF447309
S. lucida L.	1967	Greece, Attica	J. Grau		M	KF447319	KF447256
S. lyrata Willd.	1971	Crete, Chanía	G. & W. Sauer	12546	M	KF447350	KF447288
S. macrorrhyncha (Humbert, Litard. & Maire) Ibn Tattou	2006	Morocco, n/a	M. Staudinger	7444	W	KF447334	KF447271
S. ningpoensis Hemsl.	1991	CULT., orig.: n/a	J. Jutila	769	GH	HQ130041[3]	HQ130071[3]
S. nodosa L.	2003	Armenia, Lori	G. Fayvush, K. Tamanyan, H. Ter-Voskanian & E. Vitek	03-0549	MSB	HQ130038[3]	HQ130068[3]
S. nodosa L.	1999	Germany, Bavaria	D. Podlech	(MSB 116671)	MSB	HQ130037[3]	HQ130067[3]
S. oxyrhyncha Coincy	1995	Spain, Badajoz	J.L. Perez Chiscano	(MA 560760)	MA	KF447343	KF447280
S. peregrina L.	1987	Croatia, Dalmatia	E. & M. Mayer	12049	M	KF447317	KF447254
S. pyrenaica Benth.	1971	France, Pyrénées-Atlantiques	H. Merxmüller & B. Zollitsch	27178	M	KF447335	KF447272
S. racemosa Lowe	2011	CULT., orig.: Madeira, n/a (Bot. Gard. Madeira 47/2009)	A. Scheunert	006/1-1	MSB	KF447354	KF447293
S. reuteri Daveau	1974	CULT., orig.: Spain, Avila (J. Grau)	J. Grau	Sc-143	M	KF447344	KF447281
S. sambucifolia L.	1996	Spain, Cadiz	D. Podlech	54066	M	KF447352	KF447290
S. scopolii var. scopolii Hoppe ex Pers.	1967	CULT., orig.: Poland, Małopolskie (Bot. Gard. Wroclaw)	J. Grau	Sc-63	M	KF447330	KF447267
S. scopolii var. grandidentata	1965	Italy, L'Aquila	H. Merxmüller & J. Grau	20788	M	KF447331	KF447268

Table 1 (*continued*)

Taxon	Year	Locality (country, province/district)	Collector	Collector no.	Herb.	Acc. no. trnQ-rps16	Acc. no. ITS
(Ten.) Boiss.							
S. scorodonia L.	1986	Madeira, Funchal; "mad"	H. Hertel	33369	M	KF447338	KF447275
S. scorodonia L.	2011	CULT., orig.: Tenerife, Anaga mountains (M. Erben); "ten"	A. Scheunert	017/1-1	MSB	KF447339	KF447276
S. smithii ssp. smithii Hornem.	2010	CULT., orig.: Tenerife, Chamorga (M. Erben); "cha"	A. Scheunert	018/1-1	MSB	KF447355	KF447295
S. smithii ssp. smithii Hornem.	2010	CULT., orig.: Tenerife, n/a; "ten. (1)"	A. Scheunert	019/1-1	MSB	KF447310	KF447294
S. smithii ssp. smithii Hornem.	1971	Tenerife, Taganana; "tag"			M	KF447356	KF447296
S. smithii ssp. langeana (Bolle) Dalgaard	2010	CULT., orig.: Tenerife, Aguamansa; "ag"	A. Scheunert	008/1-1	MSB	KF447357	KF447297
S. smithii ssp. langeana (Bolle) Dalgaard	2007	Tenerife, Los Erjos; "erj"	W. Nezadal		M	KF447358	KF447298
S. smithii ssp. langeana (Bolle) Dalgaard	2010	CULT., orig.: Tenerife, Los Silos; "sil"	A. Scheunert	012/1-1	MSB	KF447359	KF447299
S. sublyrata Brot.	1986	Portugal, Estremadura	E. Bayón & R. Vogt	4564	M	KF447345	KF447282
S. syriaca Benth.	1992	Israel, Negev; "isr"	K. Tielbörger		M	KF447326	KF447263
S. syriaca Benth.	1980	Tunisia, Gafsa; "tun"	D. Podlech	34195	MSB	KF447324	KF447261
S. syriaca "hypericifolia Wydler"	1957	Iraq, Al Ramadi	K.H. Rechinger	9514	M	KF447327	KF447264
S. tanacetifolia Willd.	1983	Spain, Valencia	A. Aguilella & I. Mateu	15531	M	KF447340	KF447371
S. tenuipes Coss. & Durieu	1986	Algeria, Skikda	A. Dubuis, H. Maurel & R. Rhamoun	18440	M	KF447318	KF447255
S. trifoliata L.	1977	Corsica, Cap Corse	H. Merxmüller & W. Lippert	31405	M	KF447349	KF447286
S. umbrosa Dumort.	2003	Iran, Chaharmahal & Bakhtiyari	M.R. Parishani	14232	M	HQ130035[3]	HQ130065[3]
S. urticifolia Wall.		n/a; voucher specimen: LP0908740	JL	17	HU/HZU	KF447314	KF447251
S. valdesii Ortega Oliv. & Devesa	1982	Spain, Salamanca	J.L. Fernández Alonso	(MA 519560)	MA	KF447341	KF447277
S. vernalis ssp. clausii (Boiss. & Buhse) Grau	1974	Iran, Azerbaijan	W. Rechinger & J. Renz	49744	M	KF447316	KF447253
S. viciosoi Ortega Oliv. & Devesa	2002	Spain, Malaga, Antequera; "ant"	B. Cabezudo	(MA 789425)	MA	KF447346	KF447283
S. viciosoi Ortega Oliv. & Devesa	1973	Spain, Malaga, El Torcal; "tor"	H. Merxmüller & W. Gleißner	29144	M	KF447347	KF447284
S. xanthoglossa Boiss.	1992	Israel, Negev	K. Tielbörger		M	KF447328	KF447265
S. yoshimurae T.Yamaz.	1992	Taiwan, Nantou Hsien	C.-C. Liao	718	A	HQ130042[3]	HQ130072[3]

particular emphasis on Macaronesian taxa. Samples do not represent the whole distribution range of the respective species as this would have gone beyond the scope of this study; therefore, as no accessions from the Azores (*S. auriculata*) and the Cape Verdes (*S. arguta*) were available, biogeographic conclusions regarding Macaronesia were restricted to the Canary Islands and Madeira. Seven outgroup species were chosen from the Scrophulariaceae (*Verbascum nigrum* L., *Verbascum arcturus* L., *Selago corymbosa* L., *Hemimeris centrodes* Hiern, *Nemesia cheiranthus* E.Mey. ex Benth.) and Plantaginaceae (*Russelia verticillata* Kunth, *Antirrhinum majus* L.) based on results by Olmstead et al. (2001), Datson et al. (2008) and Scheunert and Heubl (2011). Information on voucher specimens as well as accession numbers is provided in Table 1. Chromosome numbers for sampled ingroup taxa were obtained from the database of Index to Plant Chromosome Numbers (IPCN; http://www.tropicos.org/Project/IPCN; last accessed on 10.05.2013) and from the literature, especially Grau (1976) and Ortega Olivencia and Devesa Alcaraz (1990).

Leaf material for DNA sequencing was obtained from herbarium specimens (55 accessions from collections in A, GH, HU/HZU, M, MA, MJG, MSB, nd W), and from plants cultivated in the greenhouses of the Botanical Garden in Munich (16 accessions, vouchers deposited in MSB; See Table 1). Seeds for cultivation were acquired from seed banks, economic providers or collected during field trips; to avoid confounding effects of uncontrolled hybridization in the greenhouses, plants were grown in isolation; furthermore, generally no F1 plants were sampled for this study. Specimens were checked for correct species identification whenever possible.

One non-coding chloroplast (cp) region (the *trnQ-rps16* intergenic spacer) and one nuclear ribosomal (nr) region (the internal transcribed spacer region, ITS) were chosen for phylogenetic analyses. Total genomic DNA was extracted from dried leaf material using the NucleoSpin Plant Kit (Macherey–Nagel, Düren, Germany) following the manufacturer's standard protocol, while applying an additional phenol/chloroform extraction step to remove proteins and potentially interfering secondary compounds. PCR reactions as well as subsequent purifications and sequencing reactions were performed according to the procedure described in Scheunert and Heubl (2011; no ExoSap purification), using the following primers: for ITS, primers ITS1 and ITS4 (White et al., 1990), supported by ITS2 and ITS3 (White et al., 1990), aITS1 and aITS4 (Bräuchler et al., 2004) and ITSIR (Scheunert and Heubl, 2011) in problematic cases; for *trnQ-rps16*, primers 1 (trnQ-F) and E (rps16-1R) (Calviño and Downie, 2007), and SPF, SPR, SPF2 and SPR2 (Scheunert and Heubl, 2011). Primers were used for amplification and sequencing, except for aITS1 and aITS4 (PCR only), and SPF2 and SPR2 (sequencing only). Markers were sequenced bidirectionally in cases where the quality of single sequences proved insufficient.

2.2. Phylogenetic analyses

Alignments were generated with MAFFT v.6 (Katoh and Toh, 2008; Katoh et al., 2002) using the slow iterative refinement FFT-nS-I algorithm, 1PAM/κ = 2 as scoring matrix, a gap opening penalty of 1.5 and an offset value of 0.0. All alignments were refined manually using BioEdit v.7.1.11 (Hall, 1999); mononucleotide repeats and ambiguously aligned regions were excluded from further analyses. ITS sequences were checked for potential pseudogenes (Bailey et al., 2003; Hershkovitz and Zimmer, 1996; Jobes and Thien, 1997; Liu and Schardl, 1994). In order to assess their phylogenetic information content before incorporating them into the

final dataset, nuclear and chloroplast indels were tentatively added or removed from the single marker phylogenetic analyses and the results compared. Ingroup indels were coded as binary states (discarding excluded alignment regions) using the simple indel coding method by Simmons and Ochoterena (2000) as implemented in SeqState v.1.4.1 (Müller, 2005).

The two markers were first analyzed separately using both a Bayesian inference (BI) and maximum likelihood (ML) approach. MrModelTest v.2.3 (Nylander, 2004) suggested the GTR + Γ substitution model as best fit to the data, with a proportion of invariant characters for the nuclear matrix only (Akaike information criterion). For Bayesian analyses including indel data, a mixed dataset was defined, using the model settings recommended in Ronquist et al. (2009) for the binary partition. Bayes runs were performed with MrBayes v.3.2 for 64 bit systems (Ronquist et al., 2012), using one cold and three heated Markov Chain Monte Carlo (MCMC) chains with temperature $t = 0.10$ for ITS and $t = 0.05$ for *trnQ-rps16*. For each of two independent runs per marker, 10×10^6 generations were completed, sampling every 2000th generation. The first 10% trees of each run were discarded as burn-in and the remaining 9002 trees used for computation of the majority-rule consensus tree.

ML analyses were performed with RAxML v.7.2.8 (Stamatakis et al., 2008) using raxmlGUI v.0.95 (Silvestro and Michalak, 2012). Pairs of accessions with completely identical sequences were represented by only one sequence in the analysis; the removed accession was then manually added to the final tree. A rapid bootstrap run with 10,000 replicates was followed by an ML optimization, defining *Antirrhinum* as outgroup and using the same models as in Bayesian analyses for DNA data partitions, and BINGAMMA for the binary indel partition. Each analysis yielded one fully resolved best-scoring ML tree.

2.3. Sequence divergence and statistical parsimony network analysis

To obtain more detailed information about clades lacking internal resolution in Bayesian and ML analyses, levels of nucleotide divergence among sequences (uncorrected ("*p*") distances and numbers of total character differences) were determined separately for each partition using the "pairwise distance" option in PAUP v.4.0b10 (Swofford, 2003). Calculations were performed based on the sequence data taken into account for Bayesian and ML analyses. In addition, the number of plastid haplotypes and their relationships were inferred for a subset of 36 accessions (corresponding to the 23 species of the "IPM" clade, definition see Section 3.2.) by generating a statistical parsimony network (Templeton et al., 1992), using the median joining algorithm with subsequent MP calculation as implemented in TCS v.1.21 (Clement et al., 2000). Generally, gaps were regarded as missing data, while coded indels used in Bayesian and ML analyses were added to the sequence matrix. The number of mutations among haplotypes was calculated with a maximum parsimony connection limit of 95% (=14 steps), using equal weights and setting epsilon to zero.

2.4. Identification and testing of incongruence

Patterns of phylogenetic incongruence were explored using several methods as suggested by Hipp et al. (2004). First, the phylogenies yielded from single marker analyses were visually examined and compared for incongruent placements of individual accessions or whole clades; congruence was rejected if support values for the contradictory placements exceeded or equalled 70% bootstrap support (BS) ("hard incongruence", Mason-Gamer and Kellogg, 1996), a cutoff which has successfully been used in several studies (e.g., Maureira-Butler et al., 2008; Moline et al., 2007; Scheunert et al., 2012). A Bayesian posterior probability (PP) of $\geqslant 0.95$ was additionally defined as sign of hard incongruence. The respective accessions or clades were then subjected to the Incongruence Length Difference (ILD) test (Farris et al., 1995) implemented in PAUP as Partition Homogeneity Test; accessions within incongruent clades were additionally tested alone. Applying an approach also used by van der Niet and Linder (2008), all sequences were first pruned from the dataset and then re-added and tested separately. Significant accessions were excluded; from those yielding insignificant results ($p > 0.05$), a combination of as many as possible was re-included into the dataset. All excluded accessions were then duplicated for the ancestral area reconstruction (see Section 2.5); these are referred to as "conflicting taxa/accessions" in the text. Tests were run with 1000 replicates, maxtrees set to 100 and heuristic searches with 50 random addition sequence replicates. Constant characters were removed from the data matrix prior to the test (following Cunningham, 1997; Lee, 2001).

As the ILD test has been shown to have certain weaknesses, e.g. a high false positive rate (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002), all hard incongruence accessions were additionally subjected to Templeton's significantly less parsimonious test (SLP test; Templeton, 1983; "nonparametric pairwise test" in PAUP); ILD-significant accessions or clades were only allowed for exclusion/duplication if the SLP test was significant for at least one of the two datasets. Furthermore, in order to assess whether partly (i.e., in one of the markers) unresolved or weakly supported positions represented insufficient information or a distinct phylogenetic hypothesis, several of such groups/species (especially present in the chloroplast tree) were also tested. These included the Macaronesia clade (sister to the Auriculata clade in ITS only), the Asian species (well supported as sister to *S. scopolii* and the IPM clade in ITS only), and *S. grandiflora*, *S. trifoliata* and *S. tanacetifolia* within the IPM clade (part of the Auriculata/Scorodonia clade in ITS only). Constraint topologies were simplified from clades in the Bayesian consensus tree of the other marker; the clades are indicated by the respective constraint numbers in Fig. 1 (exact constraint topologies are provided in Supplementary Fig. S1). Searches were done with maxtrees set to 5000, 50 replicates with five trees held at each cycle of the stepwise addition procedure, and the number of trees retained in each random-addition sequence replicate limited to 100. Significance was set at <0.05 following Templeton (1983).

Finally, to illustrate incongruities between the individual gene trees (Holland et al., 2004), a tree-based filtered super network (FSN; algorithm by Huson et al., 2006) was constructed based on 1501 trees from the posterior distribution of the first run of each single marker Bayesian analysis. Calculations were performed with SplitsTree v.4.12.3 (Huson and Bryant, 2006), applying the equal angle splits transformation and the Convex Hull algorithm, with no filtering of selected splits. Construction of the FSN was done using default settings, with edge weights displayed as "tree size weighted mean", and with the minimum number of trees set to 751 (corresponding to a 25% trees threshold any split must be present in to be displayed in the FSN).

2.5. Ancestral area reconstruction

Recently developed models for ancestral state inference are able to account for the uncertainty present in phylogenetic reconstructions. This is particularly important in topologies with polytomies and weakly supported nodes (Ronquist, 2004). For biogeographic analyses in this study, we used the Bayesian Binary Markov Chain Monte Carlo (MCMC) algorithm as implemented in RASP v.2.0b (Yu et al., 2011), which takes trees from the posterior distribution of a Bayesian analysis as input and infers ancestral distributions using a full hierarchical Bayesian approach. Another essential requirement for obtaining reliable results in biogeographic reconstructions is that all available data are included; to enable simultaneous analy-

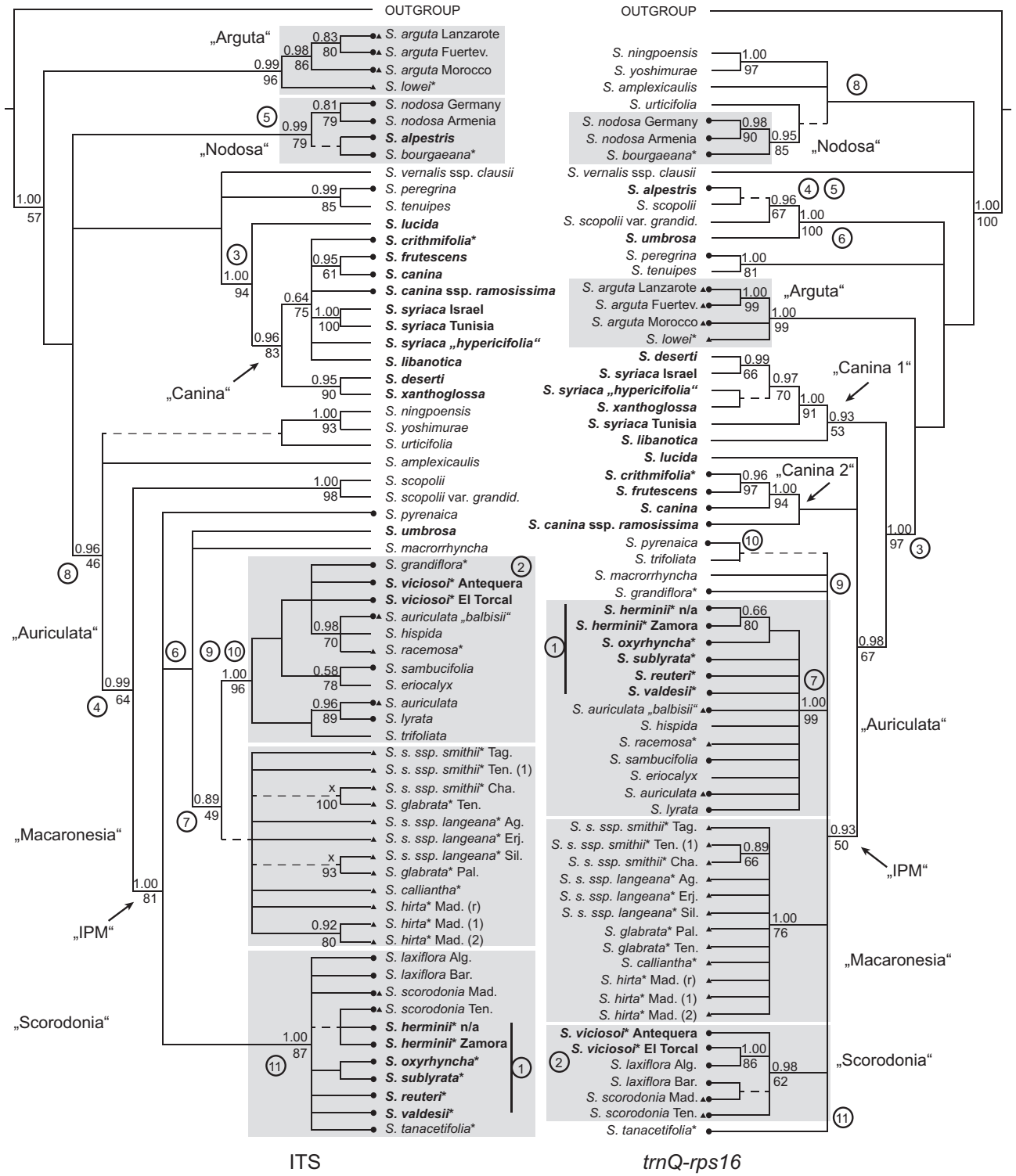

Fig. 1. Bayesian majority-rule consensus trees (cladograms) from the nuclear internal transcribed spacer (ITS) and the plastid *trnQ-rps16* intergenic spacer, with outgroup taxa reduced to a single branch. Posterior probabilities (PP) are given above each node; results from Maximum Likelihood (ML) analyses were plotted onto the cladograms, with bootstrap support values (BS) given below each node. Branches supported by at least 50% BS in the best-scoring ML trees but lacking in the Bayes consensus trees are represented by dashed lines. Both Bayesian and ML support values are given only if support at the node equals or exceeds either 0.85 PP or 75% BS ("*x*" denotes cases where the node was not present in the fully resolved tree of the respective analysis). Major clades are indicated by boxes, divergence of the IPM clade and the Canina clade is marked by arrows. Conflicting taxa (definition see Section 2.4.) are highlighted in bold. Solid dots (Iberian Peninsula) and triangles (Macaronesia) on terminal branches indicate the distribution of the respective (sub)species; endemic taxa are additionally marked by asterisks. Circled numbers refer to constraint topologies used in the SLP test. Fuertev., Fuerteventura; grandid., *grandidentata*; n/a, information not available; other abbreviations according to Table 1.

sis of all relevant data in a combined dataset containing several cases of hard incongruence and potential hybrid taxa, we constructed a combined *trnQ-rps16*/ITS data matrix following the "taxon duplication approach" by Pirie et al. (2009; 2008; also applied in e.g. Linder et al., 2013). All conflicting accessions (definition see Section 2.4.) were included twice, once as *trnQ-rps16*-only-sequence (with nuclear characters coded as missing), and once as ITS-only-sequence. The modified matrix which thus

comprised 93 sequences was used for a "duplicated analysis" using MrBayes with the same settings as for single marker analyses, except for the temperature being set to $t = 0.0001$ to permit conversion of the chains without having to enforce topological constraints. A ML analysis (using settings from the chloroplast marker calculations) was done for comparison purposes.

Distributions of species were assigned to 15 areas, subspecies were given their own respective distribution; to avoid erroneous inferences due to human influence, only native occurrences were taken into account. Single accessions from the same species were coded with the distribution of the respective species rather than their individual origin, to avoid mistakes caused by taxa not sampled across their whole distribution range. An exception is *S. auriculata "balbisii"* (see Table 1); as the distribution of *S. balbisii* Hornem. is difficult to infer (due to naming confusions and conspecificity with *S. auriculata* L. according to Ortega Olivencia, 2009), but cannot necessarily be assumed to be identical to that of *S. auriculata* L. given different phylogenetic positions in the ITS tree (Fig. 1), this accession was coded with its geographical origin. The chosen areas are mainly areas of endemism (here defined as a geographic region inhabited by two or more species displaying congruent distributions; Harold and Moor, 1994) based on present-day natural distributions of *Scrophularia* taxa; some of those were further subdivided according to palaeogeographic or climatic characteristics. In detail, areas were defined as follows: A, Azores; B, Canary Islands (including Cape Verde Islands); C, Madeira; D, Western Mediterranean (from Portugal to Italy including Sicily); E, Eastern Mediterranean (from Slovenia and Croatia to Crete and Cyprus including the Balkan Peninsula); F, Western North Africa (Morocco to Tunisia); G, Eastern Africa (Libya to Somalia); H, W-/N-/C-Europe (from the British Isles to Norway and Austria, excluding France); I, E-Europe and Western ("European") Russia (from Czech Republic eastwards, Baltic States, Russia as far as Ural and Pechora rivers); J, Lebanon/Syria/Israel s.l.; K, Southwestern Asia (Arabian Peninsula, Iran and Iraq); L, Turkey and the Caucasus (including the Talysh Mountains); M, Southern/Southeastern Asia (India to Myanmar, including Afghanistan and Pakistan); N, Central Asia and Siberia (Kazakhstan and southwards, Southern Siberia (Russia), northwards as far as species of the genus occur); O, Eastern Asia, Mongolia and "Russian Far East" (China to Japan and Taiwan, southeasternmost Russia from Sakhalin to the Zeya River).

The outgroup taxa, as well as the virtual outgroup, were assigned a wide distribution (i.e., occurring in all defined areas), which matches the real distribution of *Verbascum*, the closest relative of *Scrophularia*. Ancestral area distributions were estimated for ingroup nodes only. The maximum number of ancestral areas inferred at each node was constrained as recommended by Ronquist (1997): assuming that ancestral ranges were similar to those of present-day descendants (Sanmartín, 2003), "maxareas" was set to five as the majority of species (39 out of 45) now occur in no more than five areas. Five independent runs of the Bayesian Binary MCMC were conducted with one million generations each, sampling every 100th tree and discarding 1/5th of the trees as burn-in. State frequencies were estimated (F81 model) with a Dirichlet distribution of 1.0, and among-site rate variation was modeled across a gamma distribution as suggested by MrModeltest. An additional run with identical settings but maxareas set to two yielded congruent results.

3. Results

3.1. Sequence variation

Between *Scrophularia* and outgroup, *p* distances from chloroplast DNA sequences were smallest between *Verbascum nigrum* and *S. nodosa* L. from Germany (0.04074) and largest between *Hemimeris* and *S. hispida* Desf. (0.16982). In the nuclear dataset, values ranged from 0.05060 between *Verbascum nigrum* and *S. arguta* Sol. from Morocco, to 0.19403 between *Antirrhinum* and *S. nodosa* from Armenia. Among ingroup taxa, chloroplast and nuclear DNA distances varied from 0.00000 to 0.03515 in *trnQ-rps16* (between *S. vernalis* L. and *S. hispida*), and from 0.00000 to 0.05602 in ITS (between *S. syriaca* Benth. from Tunisia and *S. tanacetifolia* Willd.). Completely identical sequences in *trnQ-rps16* as well as ITS were found in several cases, especially within the Auriculata, Scorodonia and Macaronesia clades (definitions see Section 3.2). All uncorrected distances and total pairwise character differences are provided in Supplementary Table S1.

3.2. Phylogenetic analyses

ITS sequences showed no length changes in conserved parts indicative of pseudogenes, and G + C contents (see Table 2) were similar to those previously published for the genus (Scheunert and Heubl, 2011), so all sequences were regarded as derived from functional copies. Altogether, the sampling covers 72 accessions, of which new sequences were generated for 71 accessions; the ITS sequences of two outgroup taxa (*Antirrhinum*, *Selago*) were obtained from GenBank (NCBI). As sequencing of the *trnQ-rps16* intergenic spacer failed for *Russelia* and *Selago*, and no sequence was available in GenBank, the species were coded as missing for the respective marker. The aligned *trnQ-rps16* matrix thus consisted of 70 accessions and 1345 characters and the ITS matrix of 72 accessions and 617 characters. Detailed information on average lengths of sequences and further alignment characteristics including parsimony – informative characters is given in Table 2.

Thirty-four ingroup indels were coded for the trnQ-rps16 dataset and 17 for the ITS dataset. Analyzing the trnQ-rps16 dataset in MrBayes with and without indels showed that support values increased in 12 cases (decrease in six cases) when using indels, and that four new nodes were supported (results not shown). Using indels with the ITS dataset (results not shown) generally did not alter support values severely; however, disregarding them resulted in one additional ingroup node and three considerably increased support values. Consequently, indels were only coded for the trnQ-rps16 dataset in the final calculations.

MrBayes runs on the single marker datasets had reached convergence after 10,000,000 generations (standard deviation of split

Table 2
Sequence and alignment characteristics, and statistics from maximum likelihood (ML) analysis for *trnQ-rps16* intergenic spacer and ITS. Percentage of parsimony – informative characters referable to non-excluded characters; lengths and G + C content calculated based on the sequences as present in the alignment without any exclusions (aligned length). Sequence divergence values based on calculation of *p* distances (dissimilarity distances). alpha, the alpha value of the gamma shape parameter as inferred by ML calculations; SD, sequence divergence; avg., average; bp, basepairs; No., number.

	trnQ-rps16	ITS
No. of taxa (including outgroups)	70	72
Sequence length (avg.)	622–1126 bp (1022 bp)	506–587 bp (556 bp)
Aligned length	1345 bp	617 bp
Non-excluded characters	1306 bp	591 bp
Parsimony-informative characters	133 bp (10.18%)	129 bp (21.83%)
Average G + C content	26.65%	61.40%
Min – max SD Outgroup–Ingroup	4.07–16.98%	5.06–19.40%
Min – max SD Ingroup	0.00–3.52%	0.00–5.60%
ML tree score	−4512.522	−3554.120
ML tree length	0.626	2.145
Alpha	1.173	0.371

frequencies 0.004 and 0.003, respectively). Log-likelihood curves, acceptance rates, chain swap frequencies and potential scale reduction factors suggested effective mixing and stationarity of the chains. The majority-rule consensus trees for the single marker datasets are shown in Fig. 1. Information about likelihoods and tree lengths in ML analyses is given in Table 2; the alpha parameter was estimated at 1.173 for the chloroplast DNA partition and 17.947 for the chloroplast binary indel data partition, and at 0.371 for the nuclear dataset, while the proportion of invariant characters was 0.135. Results obtained by single marker ML analyses were consistent with those from Bayesian phylogenetic inference, so the Bayesian majority consensus trees are provided with both Bayesian posterior probabilities (above) as well as ML bootstrap supports (below) where either PP $\geqslant$ 0.85 or BS $\geqslant$ 75. Nodes supported in ML but absent in the majority-rule BI tree were added, applying a 50% BS threshold for displaying branches.

Large parts of the chloroplast and nuclear tree topologies are incongruent; however, there is support for a clade (cp PP: 0.93, BS: 50/nr PP: 1.00, BS: 81) containing the majority of the Iberian as well as the Macaronesian species (“Iberian Peninsula – Macaronesia” = “IPM” clade, Fig. 1). Within the IPM clade, three major subclades can be distinguished: one includes *S. scorodonia* L. (“Scorodonia” clade) and is highly to weakly supported by both analyses (cp PP: 0.98, BS: 62/nr PP: 1.00, BS: 87). Another comprises several species alongside *S. auriculata* (“Auriculata” clade) and receives high support (cp PP: 1.00, BS: 99/nr PP: 1.00, BS: 96). However, while these two clades as a whole are supported by cp and nr analyses, their composition is slightly different among the datasets: three taxa remain unresolved in *trnQ-rps16* (*S. tanacetifolia*, *S. grandiflora*, *S. trifoliata*) which are part of the Auriculata or Scorodonia clades in ITS. A third subclade forming a largely unresolved polytomy contains all but one of the Macaronesian perennial endemics (“Macaronesia” clade; does not contain the Madeiran *S. racemosa* Lowe) and is sufficiently supported by the chloroplast tree only (PP: 1.00, BS: 76). Relationships within as well as among the Scorodonia, Auriculata and Macaronesia clades are only poorly resolved; the Auriculata clade is subtended by an exceptionally long branch in both analyses, while the Scorodonia clade features a long branch in ITS only (see Supplementary Fig. S2). Altogether, the IPM clade comprises 68% of all Iberian/Macaronesian species and 13 of the 16 species endemic to the two regions. Apart from the IPM clade, two smaller clades were identified by both analyses: a “Nodosa” clade containing the two accessions of the holarctic *S. nodosa* as well as the Iberian endemic *S. bourgaeana* Lange (cp PP: 0.95, BS: 85/nr PP: 0.99, BS: 79), and an “Arguta” clade consisting of the three accessions of the mainly Northern African and Southwestern Asian *S. arguta* and the Madeiran endemic *S. lowei* Dalgaard (cp PP: 1.00, BS: 99/nr PP: 0.99, BS: 96). From the species included with more than one accession, only *S. nodosa* is revealed as monophyletic in both analyses (cp PP: 0.98, BS: 90/nr PP: 0.81, BS: 79).

3.3. Phylogenetic Incongruence

While several major clades identified in the nuclear phylogeny are also present in the cp tree topology, large degrees of incongruence are indicated between the two markers, on the level of single accessions as well as whole clades regarding their composition and relationships. Focusing only on cases of well-supported, hard incongruence ($\geqslant$70% BS/$\geqslant$0.95 PP as defined above), this is true for instance for the species of the “Canina group” (*S. canina* L. and other species from subsection *Lucidae* Stiefelhagen) and *S. lucida* L.: while in the chloroplast phylogeny they are part of two separate clades with moderate support (Fig. 1, cp tree, “Canina 1”, PP: 0.93, BS: 53; “Canina 2”, PP: 1.00, BS: 94) and *S. lucida* remains unresolved, they are merged into one well supported clade in the nuclear tree (Fig. 1, nr tree, “Canina”, PP: 0.96, BS: 83), with *S. lucida* highly supported as sister, and with no trace of subclades corresponding to the clades of the cp phylogeny. Another example is the IPM clade, which is sister to *S. scopolii* Hoppe ex Pers. in the ITS phylogeny (Fig. 1, nr tree; PP: 0.99, BS: 64), but to the Canina 2 clade and *S. lucida* in *trnQ-rps16* (Fig. 1, cp tree; PP: 0.98, BS: 67).

Within the clades, considerable amounts of hard incongruence can be found as well (see Table 3 for detailed information on support values): regarding the Canina group, *S. frutescens* L. is sister to *S. crithmifolia* Boiss. in the cp tree, but sister to *S. canina* in the nr tree; *S. deserti* Delile and *S. syriaca* from Tunisia occupy incongruent positions as well. The Scorodonia and Auriculata clades display incongruence “vice versa” regarding six of their species: *Scrophularia valdesii* Ortega Oliv. & Devesa, *S. herminii* Hoffmanns. & Link, *S. oxyrhyncha* Coincy, *S. reuteri* Daveau and *S. sublyrata* Brot. (referred to as “S/A taxa”) are part of the Scorodonia clade in ITS, but belong to the Auriculata clade in *trnQ-rps16*. The same is true for the two accessions of *S. viciosoi* Ortega Oliv. & Devesa (“A/S taxon”), but in the opposite way, as they are part of the Auriculata clade in ITS and the Scorodonia clade in *trnQ-rps16*. Single taxa with hard incongruent placements also include *S. umbrosa* Dumort. (part of the IPM clade in ITS, sister to *S. scopolii* and *S. alpestris* J.Gay ex Benth. in *trnQ-rps16*), *S. alpestris* (part of the Nodosa clade in ITS, sister to *S. scopolii* in *trnQ-rps16*), and *S. scopolii* and *S. scopolii* var. *grandidentata* (Ten.) Boiss. (sister to the IPM clade in ITS, sister to *S. alpestris* in *trnQ-rps16*).

As expected, the ILD test revealed severe incongruence within the complete dataset ($p = 0.001$); the test with all incongruent clades and single accessions mentioned above removed (51 accessions altogether) resulted in $p = 0.445$. However, when those accessions of the IPM clade which are congruent within the clade were re-included (28 accessions), the result remained insignificant

Table 3
Bayesian posterior probabilities (PP), ML bootstrap support values (BS) and ILD test results for four groups and four single species displaying hard incongruence among the chloroplast (cp) and nuclear (nr) markers. Accessions/clades were added to a pruned, congruent matrix ($p = 0.144$; see Sections 2.4. and 3.3.); accessions of *S. scopolii* were only tested together. Asterisks indicate significance at the $p = 0.05$ level, accessions with insignificant results are shown in bold. No separate support values are given for accessions unresolved or weakly resolved within their respective clade/group. Explanation of “S/A taxa” and “A/S taxon” see Section 3.3.; hyp., *hypericifolia*; ramos., ssp. *ramosissima*, other abbreviations according to Table 1.

Group/accession	cp PP/BS	nr PP/BS	*P* value
S/A taxa	1.00/99	1.00/87	0.001*
S. sublyrata	–	–	0.003*
S. reuteri	–	–	0.001*
S. oxyrhyncha	–	–	0.001*
S. valdesii	–	–	0.004*
S. herminii (za)	0.66/80	–	0.001*
S. herminii (na)	0.66/80	–	0.002*
A/S taxon	0.98/62	1.00/96	0.027*
S. viciosoi (tor)	1.00/86	–	0.028*
S. viciosoi (ant)	–	–	0.027*
Canina 1	1.00/97	0.96/83	0.008*
S. libanotica	–	0.64/75	0.040*
S. deserti	0.99/66	0.95/90	**0.073**
S. xanthoglossa	0.97/70	0.95/90	0.025*
S. syriaca “hyp.”	0.97/70	0.64/75	0.019*
S. syriaca (isr)	0.99/66	1.00/100	**0.070**
S. syriaca (tun)	1.00/91	1.00/100	0.008*
Canina 2	0.98/67	0.96/83	0.018*
S. canina ramos.	–	0.64/75	0.037*
S. canina	1.00/94	0.95/61	0.033*
S. frutescens	0.96/97	0.95/61	0.009*
S. crithmifolia	0.96/97	0.64/75	0.025*
S. scopolii (x2)	0.96/67	0.99/64	**0.135**
S. alpestris	0.96/67	0.99/79	**0.051**
S. umbrosa	1.00/100	1.00/81	**0.063**
S. lucida	0.98/67	1.00/94	**0.134**

($p = 0.144$), suggesting that the incongruence observed regarding the IPM clade did concern its sister groups (*S. scopolii* and the Canina group with *S. lucida*, respectively) rather than the clade itself. Therefore, further tests were conducted using this "congruent dataset" ($p = 0.144$) with only the remaining 23 hard incongruence accessions removed. Results for all clades and accessions tentatively re-included into the dataset are shown in Table 3. Seven accessions did not render the dataset incongruent when re-added; of these, all possible pairs were tested to find suitable combinations (results not shown). The final "reduced dataset" was chosen to comprise all taxa from the congruent dataset plus both accessions of *S. scopolii*, thereby including as much data as possible while keeping congruence of the dataset as large as possible ($p = 0.135$). The remaining 21 excluded accessions (=conflicting taxa; see Table 3) represent app. 1/3 of the ingroup. The results of the SLP test are shown in Table 4. Unlike ITS, the *trnQ-rps16* dataset rejected most of the foreign topologies, in particular regarding all hard incongruence taxa but also several partly unresolved/weakly supported positions, which suggests that the latter contain explicit information and are not the mere outcome of insufficient data.

Within the FSN constructed to visualize conflicting signals between and within markers (Fig. 2A), clades are represented in a tree-like way where single marker trees are fully congruent (e.g., Arguta and Nodosa clades). However, the structure of most groups is highly networked, and their relationships among each other are entangled. The Scorodonia and Auriculata clades within the IPM clade are connected by bundles of parallel edges (which indicate different signals within the data) representing the incongruent positions of the S/A and A/S taxa switching between clades in *trnQ-rps16* and ITS. The Macaronesia clade is situated in between both clades, while the IPM clade itself is closely connected to *S. umbrosa* (Fig. 2A, "5"). *Scrophularia scopolii* ("3") represents a connection between the IPM clade and the remainder of the sampling. When the taxa within the FSN are diminished to those of the reduced dataset as defined above (Fig. 2B), the number of parallel edges decreases substantially, leaving only few reticulations. Thus, a combined analysis with conflicting taxa duplicated can be assumed to produce largely reliable results not hampered by major incongruence issues.

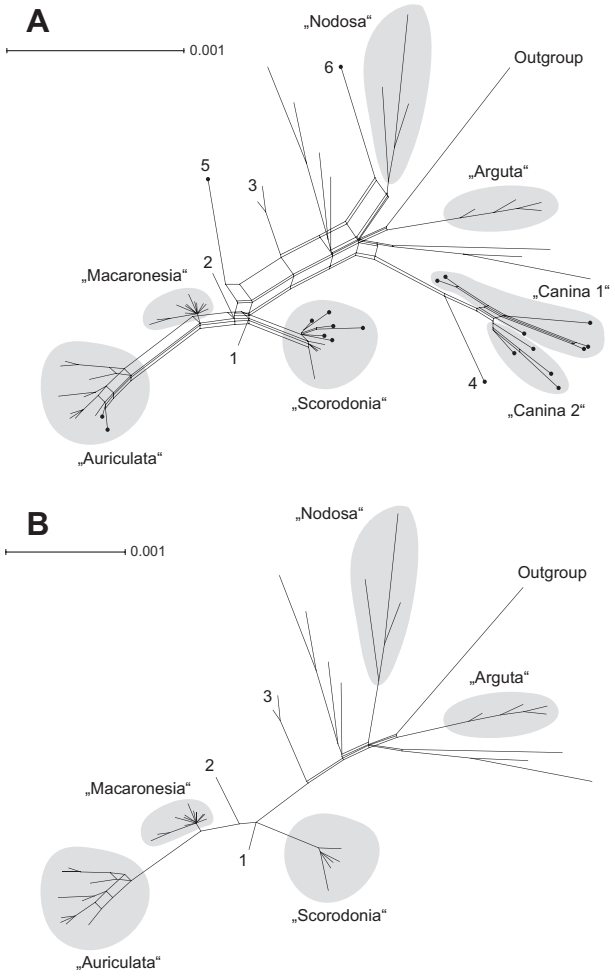

Fig. 2. Filtered super networks (split networks), based on each 1501 trees from the posterior distribution from two single marker Bayesian analyses (chloroplast *trnQ-rps16*, nuclear ITS) which yielded the consensus trees shown in Fig. 1. Scale bars represent treesize weighted mean edge weights. Major clades according to Fig. 1 are indicated in grey. Composition of the network based on (A) all 72 accessions included in the study (with seven outgroup taxa reduced to a single edge in the graphics), and (B) 21 conflicting accessions (explanation see Section 2.4.), marked by black dots on the edge tips in (A), removed using the "exclude selected taxa" option. 1, *S. pyrenaica*; 2, *S. macrorrhyncha*; 3, *S. scopolii* and *S. scopolii* var. *grandidentata*; 4, *S. lucida*; 5, *S. umbrosa*; 6, *S. alpestris*.

3.4. Plastid haplotype inference

The haplotype network analysis revealed 18 distinct haplotypes, connected by 20 missing intermediate (unsampled) haplotypes, and with no more than seven inferred mutational changes to connect sampled haplotypes. Six haplotypes are shared among up to eight accessions, and among one to five species, respectively.

Table 4
Results of SLP tests for hard incongruence taxa and partly unresolved groups/species (see Section 2.4.). Tests on chloroplast (cp, left side) and nuclear (nr, right side) dataset using 11 constrained topologies as provided in Supplementary Fig. S1. Length of most parsimonious tree(s), number of trees saved during heuristic search and minimum/maximum SLP test *P* values are given for each marker and constraint. Asterisks indicate significance at the $p = 0.05$ level, ° marks cases where only one value was significant. Constraints regarding the phylogenetic position of: 1, five "S/A taxa", explanation see Section 3.3.; 2, one "A/S taxon"; 3, the Canina/Canina 1/Canina 2 clades and *S. lucida*; 4, *S. scopolii*; 5, *S. alpestris*; 6, *S. umbrosa*; 7, the Auriculata and Macaronesia clades; 8, *S. ningpoensis*, *S. yoshimurae*, *S. urticifolia* and *S. amplexicaulis*; 9, *S. grandiflora*; 10, *S. trifoliata*; 11, *S. tanacetifolia*. No., number.

Constraint	Length (cp)	No. trees	*P* value (nr)	Length (nr)	No. trees	*P* value (cp)
None	499	5000	–	544	3800	–
1	509	5000	0.0016/0.0184*	557	3400	0.0003/0.0046*
2	509	5000	0.0016/0.0184*	557	3800	0.0003/0.0073*
3	500	5000	0.3173/0.7389	559	2800	0.0001/0.0011*
4	519	5000	<0.0001/0.0002*	549	4400	0.0956/0.2818
5	511	5000	0.0005/0.0047*	548	3800	0.1573/0.3785
6	518	5000	<0.0001/0.0003*	551	3900	0.0348/0.1488
7	507	5000	0.0047/0.0455°	546	3500	0.1573/0.5637
8	509	5000	0.0075/0.0124*	550	5000	0.1533/0.3692
9	507	5000	0.0047/0.0455°	547	3600	0.0833/0.4054
10	508	5000	0.0027/0.0290*	547	3600	0.0833/0.4054
11	501	4800	0.1573/0.5271	545	4100	0.3173/0.7630

In one case, haplotypes are shared among present-day allopatric species. Of the eight species represented by more than one accession, five feature two different haplotypes. The three groups in the network correspond to the Scorodonia, Auriculata and Macaronesia clades in the chloroplast tree (Fig. 1). Groups are separated by at least four mutational changes, while haplotypes within groups differ by two mutations at most. The "biggest outgroup probability" according to TCS, i.e., the most likely ancestral haplotype was found in eight accessions/four species of the Macaronesia clade.

3.5. Biogeographic reconstruction

The two runs from the Bayesian analysis of the duplicated dataset had converged after 10,000,000 generations (standard deviation of split frequencies 0.006). The best-scoring ML tree (results not shown) largely corroborated the Bayesian majority-rule consensus. The latter mostly reproduced the relationships of the single marker analyses, however, with reduced resolution and lower support values. Results from the RASP analyses as plotted onto the Bayesian consensus are shown in Fig. 4. Exact marginal probabilities for each ancestral range and Bayesian posterior probabilities for tree nodes are available from Supplementary Table S2.

The different RASP runs mostly yielded congruent results as well; one node (node 17) with almost identical marginal probabilities for two ancestral distributions, shifted in the different runs between including or excluding Western North Africa from the most frequent distribution. Inferred ancestral ranges had low marginal probabilities in some, especially more basal nodes; these results should therefore be treated with caution.

4. Discussion

4.1. Reticulate evolution within Scrophularia

The Nodosa, Arguta and IPM clades exclusively consist of species from section *Anastomosantes* subsection *Scorodoniae* sensu Stiefelhagen (= section *Scrophularia* subsection *Scrophularia*). Species of section *Tomiophyllum* subsection *Lucidae* sensu Stiefelhagen (or section *Canina* sensu G.Don) are restricted to the Canina clade. This is in accordance with Navarro-Pérez et al. (2013) who found a clade of semi-shrubby, sparsely foliate plants of section *Canina* embedded within section *Scrophularia* comprising mostly herbaceous representatives with numerous and often large leaves featuring clearly anastomosing nerves.

The species of the IPM clade (Fig. 1) as presented here can be characterized as subshrubs or perennial (biennial) herbs, with undivided to 3-pinnatisect, lanceolate to suborbicular leaves. A scarious margin, more or less distinct, is always present on the calyx lobes. The corolla is usually indistinctly bicolored, with the posterior part showing predominantly purple or brownish tones, while the anterior is greenish, brown, yellowish or reddish in color. The staminode is suborbicular, obovate, reniform, or transversely elliptical in shape and of considerable variability. The globose, ovoid, or subconical capsule is often apiculate, or the base of the style is persisting as mucro on mature capsules. However, none of these morphological characters can be regarded as synapomorphic for the clade. Similarly, the monophyly of the IPM clade (with or without *S. umbrosa* depending on the marker) is supported by only two nucleotide synapomorphies in the ITS sequence alignment and one in *trnQ-rps16*. In constrast, the Auriculata and Scorodonia clades mostly receive high supports in phylogenetic analyses, but also lack clear morphological synapomorphies. This is best explained by the comparatively young age of the IPM clade (inferred at around 3.7my by Navarro-Pérez et al., 2013) and is also reflected in the weakly resolved relationships among and within the IPM clade, as well as low levels of sequence divergence with several cases of identical sequences (see Supplementary Table S1).

As a consequence, especially regarding the high frequency of hybridization and polyploidy within the group, reticulate events among the closely related species studied here are to be expected, and the large amount of incongruence found between trees from nuclear and plastid markers corroborates this assumption (see Fig. 2). Similar examples within the Lamiales are known from e.g. Plantaginaceae (Albach and Chase, 2004; Blanco-Pastor et al., 2012) or Lamiaceae (Bräuchler et al., 2010), among others. Reticulation can be due to e.g. hybridization (Arnold, 1997; Rieseberg et al., 1996), introgression (Mason-Gamer, 2004; Rieseberg and Wendel, 1993) or incomplete lineage sorting (persistence of ancient polymorphism/deep coalescence; Degnan and Rosenberg, 2009; Maddison, 1997), all of which have frequently been considered as explanation for cases of well-supported topological incongruence among phylogenetic trees (hybridization: e.g. Albaladejo et al., 2005; Doyle et al., 2003; Nieto Feliner et al., 2002; see further references in Vriesendorp and Bakker, 2005; lineage sorting: e.g. Jakob and Blattner, 2006; Vilatersana et al., 2010). However, artificial factors can cause topological incongruence as well (Wendel and Doyle, 1998); such factors were excluded as far as possible in the present study. Sampling error was avoided by re-determination of nearly all accessions; species coverage is close to 100% for the main study regions, avoiding insufficient taxon sampling (Stockley et al., 2005). Methodical mistakes caused by model misspecification were ruled out by performing all analyses with two different methods. Furthermore, only robustly supported cases of incongruence were taken into account. Undetected ITS pseudogenes can also introduce topological incongruence into datasets (Álvarez and Wendel, 2003); however, no signs of sampled non-functional nrDNA copies were found in the sequences. Some sister groups with long branches in the basal (and sparsely sampled) part of the trees (see Supplementary Fig. S2) could possibly be the result of long branch attraction (Felsenstein, 1978), so no implications are made regarding these taxa.

In cases where incongruence reflects some kind of reticulate evolutionary history, forcing conflicting signals into one phylogenetic tree might blur real relationships (Bull et al., 1993; Lecointre and Deleporte, 2005); on the other hand, pruning hard incongruent taxa from a combined analysis (Huelsenbeck et al., 1996; Johnson and Soltis, 1998) will disregard much of the available data, and valuable information about possible parent species will be lost in cases where hybridization is frequent (Rieseberg and Brunsfield, 1992; see examples in Albaladejo et al., 2005; Fehrer et al., 2007; Okuyama et al., 2005; Soltis and Kuzoff, 1995). The duplication approach (Pirie et al., 2009, 2008) as used in this study provides a suitable solution to this problem.

In the case of *Scrophularia* as sampled here, the ITS phylogeny generally seems to better reflect the relationships known from morphological studies (e.g. Bentham, 1846; Ortega Olivencia, 2009; Stiefelhagen, 1910). Single accessions of the same species are more often retrieved as monophyletic (*S. scopolii*, *S. arguta*, *S. nodosa*). It has already been recognized that in many cases, the ITS topology is congruent with phylogenetic hypotheses established from morphological or biogeographical data (Baldwin et al., 1995; Fehrer et al., 2007; Kellogg et al., 1996). However, species – independent geographical structuring as described by Wolf et al. (1997) is hardly if at all present in the *trnQ-rps16* tree. Therefore, we chose to refer to the ITS topology (or the duplicated topology, Fig. 4) when inferring species relationships, and to draw additional information from the chloroplast tree where useful.

Although detailed differentiation between evolutionary mechanisms lies beyond the scope of this study, parts of the reticulation observed here are very likely to be the result of hybridization. As an example, six species within the IPM clade display hard

incongruence, changing positions between the Auriculata and the Scorodonia clades in ITS/*trnQ-rps16* (S/A and A/S taxa). Results from Navarro-Pérez et al. (2013) match this observation in at least two cases; three of the other species remain unresolved in the plastid tree, which is probably due to the choice of a different marker (*trnL-trnF*) providing weaker resolution. In contrast, the incongruence regarding the sixth of the species, *S. herminii*, as found here clearly contradicts results by Navarro-Pérez et al. (2013); unless more specimens are analyzed, we refrain from making any conclusions regarding this species here. The evolutionary split between the Auriculata and Scorodonia clades has been shown to be rather young (mid-Pliocene; Navarro-Pérez et al., 2013); consequently, lineage sorting is a possible explanation for the observed pattern (Maddison, 1997). On the other hand, if incongruent placements in the ITS tree resulted from sorting of ancestral polymorphisms in the IPM clade ancestor, one would expect, from the stochastic nature of the process, that accessions appeared at any position in the IPM clade (Buckley et al., 2006). Here, the species change positions between two clades exclusively. Furthermore, both clades are highly supported and well separated (Figs. 1 and 3, also see Navarro-Pérez et al., 2013) which makes lineage sorting a less likely cause for the incongruence (Morgan et al., 2009). Introgression by geographically close populations of the other clade cannot be excluded due to the lack of populational sampling; however, the "Sambucifolia" haplotype (Fig. 3) encloses species from very different geographic regions, which precludes introgression as the only cause for haplotype sharing and suggests common ancestry of the respective species (Gutiérrez Larena et al., 2002). On the other hand, viable hybrids between *S. scorodonia* and *S. auriculata* have already been created by Grau (1976) and Dalgaard (1979), and the natural hybrid *S.* × *moniziana* Menezes was shown to be derived from *S. scorodonia* and *S. racemosa* (belonging to the Auriculata clade; Fig. 1). Therefore, it seems more likely that *S. sublyrata*, *S. reuteri*, *S. oxyrhyncha*, *S. valdesii* and *S. viciosoi* have originated through homoploid hybrid speciation (Mallet, 2007), with ancestors or members of the Scorodonia clade being the female parent in *S. viciosoi*, and ancestors/members of the Auriculata clade acting as female donor in the remaining cases, contributing the maternally inherited plastid.

Morphologically, *S. sublyrata* shares characters with *S. sambucifolia* (Richardson, 1972), and *S. reuteri* is similar to *S. sambucifolia* (Daveau, 1892) and to some extent also *S. sublyrata* (Grau, 1976). This corresponds to the identical haplotype found in the three species (Fig. 3). Morphological similarities are also present between *S. oxyrhyncha* and *S. sublyrata* (Coincy, 1898; Stiefelhagen, 1910). Furthermore, *S. oxyrhyncha* is connected to *S. reuteri* by a distinctive long - subconical capsule (Grau, 1976); both are local endemics of Western Spain. *Scrophularia valdesii* is a threatened narrow endemic known from only 14 populations occurring in the Duero Basin in Spain and Portugal (Bernardos et al., 2006). It shares the haplotype found in *S. sambucifolia*, *S. sublyrata* and *S. reuteri* (Fig. 3) and is closely related to the latter morphologically (Ortega Olivencia and Devesa Alcaraz, 1991). *Scrophularia viciosoi* is the only hybrid with its paternal source found in (ancestors of) the Auriculata clade. Ortega Olivencia and Devesa Alcaraz (1991) relate the species to *S. grandiflora*, a local endemic of the Coimbra region in Portugal; indeed, the ITS sequence of *S. viciosoi* from Antequera is identical to that of *S. grandiflora* (Supplementary Table S1). Morphological similarities include the densely pubescent – glandular, pinnatisect leaves possessing many small intercalars, and the subsessile peduncles (Ortega Olivencia and Devesa Alcaraz, 1991).

4.2. Chromosome number evolution, origins of polyploidy and ancestral hybridization within Iberian Scrophularia

Hybridization and polyploidization are considered as driving forces in the diversification history of the genus *Scrophularia*, e.g. in the high level polyploids occurring in North America (Carlbom, 1969; Scheunert and Heubl, 2011; Shaw, 1962); they also play an important role in plant evolution and speciation in general (Hegarty and Hiscock, 2005; Leitch and Leitch, 2008; Otto and Whitton, 2000; Schubert, 2007). Besides the evidence for homoploid hybrid speciation as discussed above, our phylogenetic reconstructions support allopolyploid hybridization in several cases.

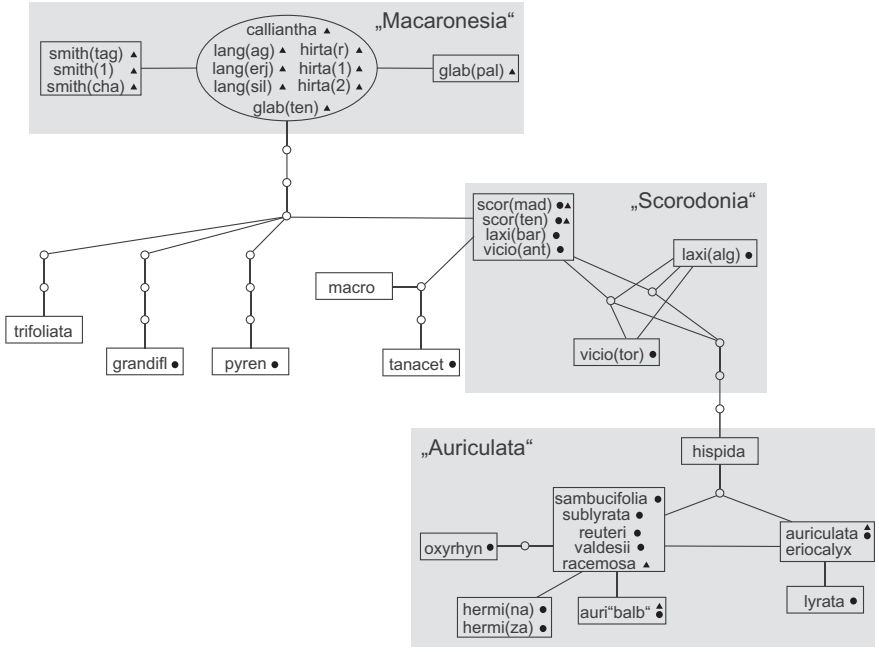

Fig. 3. Statistical parsimony network obtained from analysis of the *trnQ-rps16* intergenic spacer, limited to taxa from the IPM clade. Lines represent single mutational steps, small circles represent inferred haplotypes. The size of boxes is relative to the number of accessions possessing the respective haplotype; the oval represents the most likely ancestral haplotype as inferred by TCS. Major plastid lineages indicated by grey boxes correspond to clades in Fig. 1. Solid dots (Iberian Peninsula) and triangles (Macaronesia) indicate the distribution of the respective (sub)species. For complete taxon names and other abbreviations see Table 1.

Scrophularia alpestris, distributed in montaneous regions of Southern France and Northern Spain, with a chromosome number of $2n = 68$ (Grau, 1976), is sister to *S. scopolii* ($2n = 26$; Grau, 1976) in the *trnQ-rps16* phylogeny and, according to ML estimations, sister to *S. bourgaeana* ($2n = 42$; Ortega Olivencia and Devesa Alcaraz, 1990) in the ITS topology. Regarding the long branches of *S. alpestris* and *S. bourgaeana* in ITS, their sister relationship could theoretically be a result of long branch attraction (Supplementary Fig. S2). However, *S. alpestris* was already proposed to be an allopolyploid (with *S. scopolii* and *S. bourgaeana* as progenitors) by Grau (1976) and Ortega Olivencia and Devesa Alcaraz (1990). This hypothesis is corroborated by morphology and by molecular phylogenetic reconstructions as presented here.

A similar case seems to be apparent in *S. auriculata* which typically possesses $2n = 84$ (Grau, 1976) chromosomes. Grau (1979) suggested the species to result from allopolyploid hybridization between (ancestors of) *S. lyrata* Willd. ($2n = 58$; Grau, 1976) and *S. umbrosa* ($2n = 26, 52$; Vaarama and Hiirsalmi, 1967). This is supported by intermediate morphological traits connecting *S. auriculata* to its putative parents; e.g., bracts and bracteoles in *S. lyrata* are scariously margined across their whole length as opposed to the non-margined *S. umbrosa*, while the bracts of *S. auriculata* have no or narrow margins generally confined to the tip of the leaf. Furthermore, the staminode is reniform to bilobed in *S. umbrosa*, obovate to suborbicular in *S. lyrata*, and subreniform in *S. auriculata*.

A close relationship of *S. auriculata* to *S. lyrata* is evident from the ITS phylogeny in one of the two accessions only (Fig. 1); connections to *S. umbrosa* are present in neither plastid nor nuclear trees. Possibly, this unexpected result is due to fixation of the maternal ITS copy in the hybrid species through concerted evolution (Álvarez and Wendel, 2003), an event that would leave no trace of a hybrid origin as long as no additional markers are included (see e.g. Blöch et al., 2009; Joly et al., 2006; Sang et al., 1997).

The second accession, *S. auriculata "balbisii"* (originally determined as *S. balbisii* Hornem, a name synonymized with *S. auriculata* L. ssp. *auriculata* by Ortega Olivencia, 2009), does not cluster with the first specimen, but instead is sister to the Algerian - Moroccan endemic *S. hispida* (Fig. 1). This species is morphologically similar to *S. lyrata* and has the same chromosome number ($2n = 58$; Grau, 1976). With respect to the considerable variability found within *S. auriculata* (visible in e.g. *S. auriculata* ssp. *valentina* with lyrate-pinnatisect leaves, as well as several synonyms listed in Ortega Olivencia, 2009), and the great potential for hybridization, a definite conclusion about the phylogenetic position of *S. auriculata* does not seem advisable based on two inconsistently placed specimens. However, an involvement of *S. hispida* in the origin of *S. auriculata* should be considered, especially with regard to the third taxon in the respective ITS clade, the Madeiran endemic *S. racemosa*. This species has been related to *S. auriculata* and also possesses $2n = 84$ chromosomes (Dalgaard, 1979); its position is clearly separated from the remaining Macaronesian perennial endemics which are part of the Macaronesia clade.

Disregarding hybrid species with higher chromosome numbers (*S. auriculata*, *S. racemosa*) as described above, the IPM clade is characterized by the derived chromosome number $2n = 58$ (with aneuploidy in some taxa, e.g. *S. sublyrata*, *S. glabrata* Aiton) as already pointed out by Grau (1976). The number seems to be exclusive for the species of the IPM clade; this points toward a single evolutionary event, which generated a presumably allopolyploid ancestor with that particular chromosome number. Some suggestions have been made regarding its origin, but no concrete evidence was provided: Ortega Olivencia and Devesa Alcaraz (1990) hypothesized an allopolyploid taxon with $2n = 60$ derived from progenitors with $2n = 36$ (as present in *S. nodosa* and *S. peregrina*; Grau, 1976; Vaarama and Hiirsalmi, 1967) and $2n = 24$ (as present in *S. crithmifolia* and occasionally found in *S. canina*; Ortega Olivencia and Devesa Alcaraz, 1990), with subsequent chromosome number reduction to $2n = 58$. Based on the sister relationships as present in the nuclear and plastid phylogeny, our molecular data support an allopolyploid origin, by ancient hybridization involving an ancestor from the Canina group (possibly with $2n = 30$ chromosomes as occasionally found in *S. canina*; Ortega Olivencia and Devesa Alcaraz, 1990) as maternal parent. Interestingly, almost all species of the Canina group themselves yield highly significant ILD test results (Table 3), and their cp and nr sequences occupy different positions in the duplicated tree (Fig. 4; Canina clade vs. Canina 1 and 2 clades). This could be explained by assuming reticulation present in their origin as well, possibly involving groups not sampled for this study. Regarding the paternal source, a contribution by a taxon with $2n = 36$ chromosomes as proposed by Ortega Olivencia and Devesa Alcaraz (1990) is not supported; instead, an *S. umbrosa* – like species ($2n = 26$) is suggested by the duplicated tree (Fig. 4), as *S. umbrosa* is placed in a polytomy with the Canina clade and *S. lucida* with all being sister to the IPM clade. *Scrophularia umbrosa* was already mentioned as potentially involved in the origin of the group; it does not occur in the Iberian Peninsula today (westernmost populations reach Norway, the British Isles, and France), but could easily have done so in the past according to Grau (1976). A natural hybrid between *S. auriculata* and *S. umbrosa* (sub *S. alata* Gilib.) was already noted by Stiefelhagen (1910); this confirms the close association of the species. Surprisingly, when consulting the ITS tree, *S. scopolii* is sister to the IPM clade and *S. umbrosa* is nested within the latter. However, the position of *S. umbrosa* is characterized by a long branch in the phylogram (Supplementary Fig. S2); the substitutions shared with the rest of the IPM clade species could thus be homoplasious. The low alpha value of the gamma shape parameter in the ITS dataset (Table 2) supports this view. If we finally consider the fact that the chromosome number of *S. umbrosa* with $2n = 26$ would be unique within the IPM clade, we can conclude that this position as a member of, and not sister to, the IPM clade is likely artificial.

If we assume an *S. canina* – like ($2n = 30$) and an *S. umbrosa* – like species ($2n = 26$) as progenitors for the IPM clade ancestor (Fig. 4), and that subsequent chromosome doubling was necessary to enable fertility of the new hybrid, the resulting allopolyploid should have had $2n = 56$ chromosomes. Ascending aneuploidy could then have resulted in the present-day $2n = 58$ for the group; the latter process was also proposed to account for the deviant chromosome number of *S. viciosoi*, counted with $2n = 58$ as well as $2n = 64$ (Grau, 1976, sub *S. sublyrata*; Ortega Olivencia and Devesa Alcaraz, 1990).

4.3. Biogeographic implications

The primary diversity center of the genus *Scrophularia* is assumed in the Irano - Turanian region (Grau, 1981; Lall and Mill, 1978). Most of the species studied here are part of a secondary center of diversity located in the Iberian Peninsula (Ortega Olivencia, 2009; Ortega Olivencia and Devesa Alcaraz, 1990). According to ancestral area reconstructions as performed by RASP, the most recent common ancestor (MRCA) of the IPM clade was distributed in the Western Mediterranean, however with low Bayesian support for the underlying node (Fig. 4, node 31; for node supports and exact frequencies of occurrence see Supplementary Table S2). Regarding the progenitors of the IPM clade as discussed above, RASP inferred that the ancestor of the Canina group was distributed in a region ranging from Eastern Africa, Israel, Lebanon and Syria to Southwestern Asia, the Caucasus and Turkey (node 18, 27). However, the marginal probabilities for the inferred range are low (42.19, 43.23); a reconstruction with possible areas at each node restricted to two, narrows the most frequent ancestral range

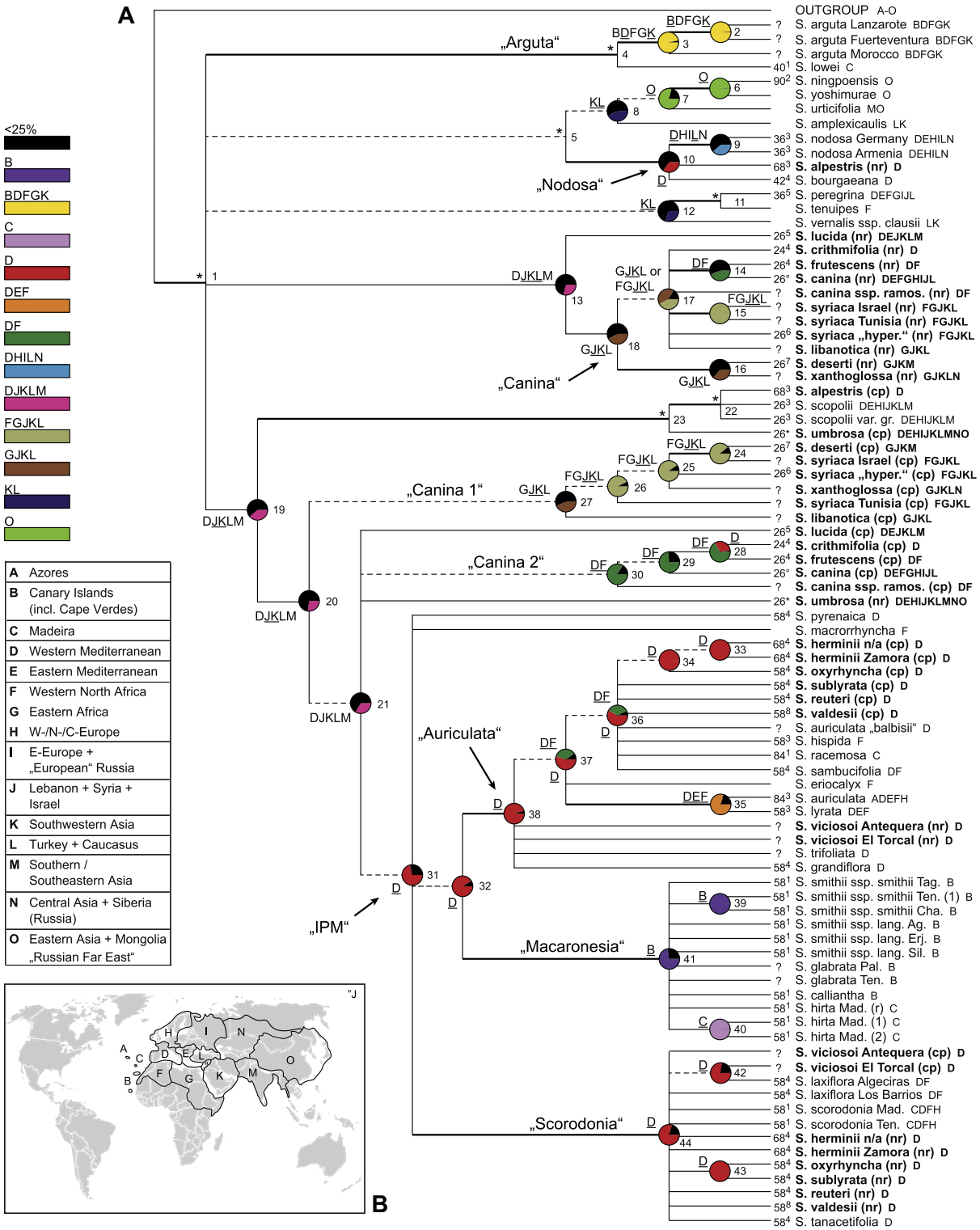

Fig. 4. (A) Biogeographical optimization as performed by RASP (maxareas = 5), using the majority-rule consensus of 9002 trees from a duplicated Bayesian analysis of the ITS region and the plastid *trnQ-rps16* intergenic spacer. Conflicting taxa duplicated for the analysis are highlighted in bold and suffixed with "nr" and "cp", for the nuclear and chloroplast sequences, respectively (see Section 2.5.). Branches with PP < 0.85 in the consensus tree are shown by dashed lines, branches with PP ⩾ 0.95 in bold. Outgroups were reduced to one branch in the diagram, clade names correspond to those in Fig. 1. Pie charts illustrate inferred distributions of MRCAs from one of five RASP runs (only ranges supported by all five runs are shown). Color – coded fractions represent the frequency of occurrence/marginal probability (⩾25) of the respective ancestral distribution over the Bayesian sample of trees. Asterisks mark nodes where no ancestral distribution reached a marginal probability of ⩾25 in all five runs. Areas additionally supported by an optimization with maxareas set to two are underlined. Contemporary distribution and known chromosome numbers are denoted next to each taxon, "?" indicate cases where counts yielded inconsistent results; °: known autopolyploidy (2n = 26, 52; Vaarama and Hiirsalmi, 1967) within *S. umbrosa*. °: apart from 2n = 26, occasionally counted numbers in *S. canina* also include 2*n* = 24 and 2*n* = 30 (Ortega Olivencia and Devesa Alcaraz, 1990; Vaarama and Leikas, 1970). [1]: Dalgaard, 1979; [2]: Ge and Li, 1989; [3]: Grau, 1976; [4]: Ortega Olivencia and Devesa Alcaraz, 1990; [5]: Vaarama and Hiirsalmi, 1967; [6]: Murin and Sheikh, 1971; [7]: Mohamed, 1997; [8]: Ortega Olivencia and Devesa Alcaraz, 1991. ramos., *ramosissima*; hyper., *hypericifolia*; gr., *grandidentata*; lang., *langeana*; other abbreviations according to Table 1. (B) Table and map showing 15 areas defined for ancestral area reconstructions, and color codes for inferred ancestral ranges.

to Israel, Lebanon, Syria and Southwestern Asia with a higher frequency of occurrence (85.96, 58.58). According to the reconstructions, east–west migrations would then have expanded the distribution range of the group to Western North Africa (nodes 14, 15, 17, and 24-26). The ancestral range inferred for the Canina 2 clade (chloroplast; node 30, marginal probability: 83.59) suggests that the ancestor of this part of the Canina group (here represented by *S. canina*, *S. frutescens* and *S. crithmifolia*) at some point reached the Iberian Peninsula via the Strait of Gibraltar and diversified in situ. This is supported by the ancestral areas inferred for nodes 14, 28 and 29 with mostly sufficient marginal probabilities and Bayesian node supports. Similar biogeographical patterns involving expansion from east to west have been recorded in several Mediterranean groups, e.g. in elements of the Spanish steppe flora (Polunin and Smithies, 1973), in Asteraceae (Font et al., 2009), Araceae (Mansion et al., 2008), Rutaceae (Salvo et al., 2011), and in insects (Sanmartín, 2003).

For *S. umbrosa*, the second assumed progenitor of the IPM clade, biogeographic reconstructions were ambiguous and marginal probabilities insufficient; hypotheses about the biogeography of this widespread species must remain speculative at this point. In every case, the contact of an *S. umbrosa* ancestor with a taxon from the Canina group in the Iberian Peninsula should have resulted in the hybridization event generating the allopolyploid ancestor of the IPM clade.

In the course of the diversification of the IPM clade, an early dispersal of *S. macrorrhyncha* (Humbert, Litard. & Maire) Ibn Tattou into Northern Africa is suggested by its position within the clade. This subshrub species is adapted to semi-arid conditions and is endemic to Morocco today. From the three main lineages derived from the MRCA, one dispersed to Macaronesia (Macaronesia clade, see Section 4.4. and Fig. 4, node 41); the Scorodonia clade underwent local radiation and mostly remained restricted to the Iberian Peninsula (Fig. 4, nodes 42–44). In contrast to that, RASP reconstructions show that Northern Africa might have played a larger role in the diversification of the Auriculata lineage (nodes 35, 36, and 37). Both Scorodonia and Auriculata clades also contain more widespread elements which have dispersed into Macaronesia, the Eastern Mediterranean, and Europe (*S. scorodonia*, *S. auriculata*).

Diversification of *Scrophularia* in the Western Mediterranean and especially the Iberian Peninsula as mentioned above is likely to be, to a great extent, the result of repeated hybridization as discussed in Sections 4.1 and 4.2. In addition, there is evidence that glacial refugia also played a role in promoting and preserving species diversity. Five taxa of the IPM clade remain unresolved in the chloroplast tree (Fig. 1; *S. pyrenaica* Benth., *S. macrorrhyncha*, *S. grandiflora*, *S. trifoliata*, *S. tanacetifolia*); their positions were shown to contain distinct phylogenetic signal in two of three tested cases (Table 4) and correspond to haplotypes which are isolated from the remainder of the IPM clade by up to seven steps (Fig. 3). In the duplicated tree (Fig. 4), these taxa are unresolved as well (*S. pyrenaica*, *S. macrorrhyncha*), are sister to the remainder of the Auriculata clade (*S. grandiflora*, *S. trifoliata*) or part of the Scorodonia clade (*S. tanacetifolia*). All except the latter species are restricted to only small areas, and exclusively or predominantly inhabit regions classified as refugia within the Mediterranean bioclimatic region (Médail and Diadema, 2009): the central Pre-Pyrenees and Pyrenees (*S. pyrenaica*; Ortega Olivencia, 2009), Sardinia and Corsica (*S. trifoliata*; Gamisans and Marzocchi, 1996), Beira Litoral of Western Portugal (*S. grandiflora*; Ortega Olivencia, 2009), and the High, Middle and Anti Atlas mountains of Morocco (*S. macrorrhyncha*; Ibn Tattou, 2007). Given their distinctive genetic features and their occurrence in refugia, these four species are likely to represent more ancient lineages within the IPM clade which persisted in the favorable conditions of the climatically stable refugial areas. Long isolation in restricted regions likely accumulated the genotypic changes and accounts for the long branches in the phylograms (Supplementary Fig. S2). *Scrophularia tanacetifolia* does not have a restricted distribution, but is more widespread in the eastern and southeastern parts of the Iberian Peninsula. Unlike in *S. grandiflora* and *S. trifoliata*, the SLP test was insignificant for this species (Table 4), suggesting that its position in the chloroplast tree might also be due to a lack of informative characters. However, it is sister to a clade containing *S. laxiflora* and *S. scorodonia* in the combined analysis by Navarro-Pérez et al. (2013), which corroborates its isolated position. It is also subtended by a long branch in the *trnQ-rps16* phylogram (Supplementary Fig. S2), and, like *S. macrorrhyncha*, is characterized by 2–3 pinnatisect leaves resembling those of the Canina group (confusions with *S. crithmifolia* were reported by Ortega Olivencia, 2009), a rather plesiomorphic character within the IPM clade. Possibly, this species dispersed to its present distribution area in Southeastern Spain from refugia located in the area.

Scrophularia species are distributed throughout nearly all regions of the Iberian Peninsula today, occurring from sea level up to 2500 m. Hybrid species from the Scorodonia and Auriculata clades (*S. sublyrata*, *S. reuteri*, *S. oxyrhyncha*, *S. valdesii* and *S. viciosoi*) are confined to granite or siliceous substrates; their distribution corresponds to a biogeographical pattern as shown by Moreno Saiz et al. (2013), which divides the Peninsula into two distinct distributional areas characterized by different soil conditions. Whether substrate characteristics influenced hybridization in this area remains unclear; but furthermore, the present-day distributions of these species likely reflect the influence of the varied topography in the region, also in the context of the climatic conditions during their formation. Divergence time estimations by Navarro-Pérez et al. (2013) suggest their very recent divergence in the Pleistocene; this is corroborated by identical haplotypes in three of five cases (Fig. 3) and very low levels of nuclear sequence divergence among each other and to closely related non-hybrid species (Supplementary Table S1) as shown here. Possible parental taxa and hybrid offspring are allopatrically distributed within the Peninsula in several cases; thus, it is conceivable that range shifts in the parental lineages, promoted by climate fluctuations during the Pleistocene (Hewitt, 2000), enabled hybridization in contact zones. The narrow distributions of three of the hybrids, (including one threatened species; Bernardos et al., 2006) indicate that geographic features might also have had special influence, by isolating new species e.g. on the sierras of the Cordillera Central (*S. reuteri*) and the Sierra Morena (*S. oxyrhyncha*). Isolation is regarded essential for survival of homoploid hybrids (Rieseberg and Willis, 2007) as it prevents backcrossing with the already established lineages. Likewise, the role of the sierras of Central Spain as refugia for plant and animal species has been highlighted (Crochet et al., 2004; Médail and Diadema, 2009). Isolation and range shifting in glacial refugia have promoted speciation in several genera, amongst others *Erodium* (Geraniaceae; Fiz-Palacios et al., 2010) and *Armeria* (Plumbaginaceae; Gutiérrez Larena et al., 2002).

4.4. The origin of the Macaronesian taxa

Although our sampling of the Macaronesian taxa does not allow detailed inferences on colonization pathways among the islands, some general patterns are clearly supported by the present data (Fig. 5). According to phylogenetic reconstructions, at least four distinct lineages of *Scrophularia* have colonized the Macaronesian archipelago; these roughly correspond to the three groups defined by Dalgaard (1979). Multiple independent introductions into Macaronesia have also been shown in e.g. *Asteriscus* (Asteraceae; Goertzen et al., 2002), *Ilex* (Aquifoliaceae; Cuénoud et al., 2000), *Lavatera* (Malvaceae; Fuertes-Aguilar et al., 2002), or *Plantago* (Plantaginaceae; Rønsted et al., 2002), and have been reviewed in Carine et al. (2004). Regarding *Scrophularia*, Madeira was colonized

at least four times (by members of the Scorodonia, Auriculata, Macaronesia and Arguta clades, see Figs. 4 and 5). At least two dispersals to the Canary Islands can be assumed (by members of the Arguta clade and the ancestor of the Macaronesia clade). In particular, the perennial endemics of the Canary Islands (*S. smithii* Hornem., *S. glabrata*, *S. calliantha*) are shown to be the result of a dispersal event from the Western Mediterranean mainland to the Atlantic islands (Fig. 4, node 32, marginal probability: 91.50). The dated phylogeny by Navarro-Pérez et al. (2013) places the split between mainland taxa of the Scorodonia clade and Macaronesian perennial endemics in the late Pliocene. Indeed, many species and lineages of the Macaronesian islands have been shown to be recently derived from continental ancestors, rather than being relictual elements of the flora (Barber et al., 2002; Carine et al., 2004, see also studies reviewed therein; Francisco-Ortega et al., 1997; Helfgott et al., 2000); this also seems to be the case for *Scrophularia*. The ancestors of the Madeiran perennial endemic *S. hirta* were inferred to have originated on the Canary Islands (Fig. 4, node 41, marginal probability: 71.76). Colonization routes from the Canary Islands to Madeira were also found in e.g. *Sonchus* (Asteraceae; Lee et al., 2005), *Bystropogon* (Lamiaceae; Trusty et al., 2005) or *Micromeria* (Lamiaceae; Meimberg et al., 2006). A second colonization event, from the Western Mediterranean or possibly also Western North Africa (Fig. 5), was inferred for the other perennial endemic on Madeira, *S. racemosa* (Fig. 4, node 36, marginal probabilities: 58.20 and 35.06, respectively). Two of the species occurring in Macaronesia are more widespread (*S. scorodonia, S. auriculata*); therefore, their biogeography should be examined using more specimens from different parts of their distribution range, and no conclusions are made here. The annual taxa occurring in Macaronesia today are part of a clade whose closest relatives remain unclear; accordingly, no informative ancestral distributions could be inferred.

4.5. Conclusions and perspectives

This study provides an initial framework in understanding the complex evolutionary history of *Scrophularia* lineages from the Western Mediterranean, Northern Africa and Macaronesia. Interspecific hybridization and polyploidization have significantly influenced the diversification of the genus in this area and explain the major incongruences found between nuclear and chloroplast datasets. Hybrid speciation is favored by the pollination biology of the genus and the absence of reproductive barriers among closely related taxa. The comparatively young age of the lineages might explain the lack of resolution among Macaronesian and Iberian groups as well as single species (on the other hand, haplotypes are not necessarily identical within species). This also indicates that apart from hybridization, other reticulate processes like introgression and lineage sorting may have occurred among and within species. The sampling and methods employed in the present study are not intended for assessing these topics or for disclosing inter-island colonization patterns across the Atlantic archipelagoes in detail. A different approach involving an extensive geographic and intraspecific population-level sampling and appropriate markers (e.g. SSR, ISSR, AFLP etc.), together with additional chromosome counts, would help to further unravel the evolutionary history of the genus.

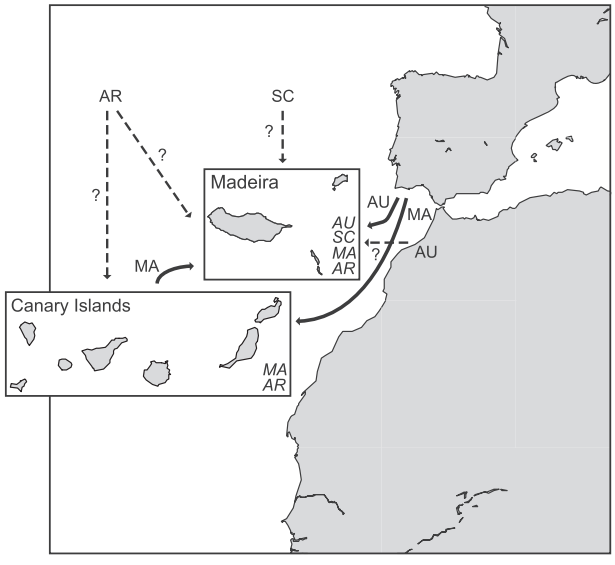

Fig. 5. Colonization of the Canary Islands and Madeira by different lineages of *Scrophularia*. Solid arrows represent dispersal events as inferred by the ancestral area reconstruction in Fig. 4 further possible or unknown migration routes are indicated by dashed arrows (see Section 4.4.). Abbreviations of *Scrophularia* lineages, corresponding to clade names in Fig. 1: AU, Auriculata; SC, Scorodonia; MA, Macaronesia; AR, Arguta. Present distributions for each archipelago are indicated in italics.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.09.023.

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