DIPLOMARBEIT

Titel der Diplomarbeit

"Phylogenetic relationship of the genus *Scorzoneroides* (Compositae) inferred from plastid and low-copy nuclear markers, and karyological data"

Verfasser

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ERKLÄRUNG

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DANKSAGUNG

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KURZFASSUNG

Frühere Untersuchungen der molekularen Phylogenie der Gattung *Leontodon* mit Markern aus dem Chloroplastengenom und dem nukleären ribosomalen DNA-Marker ITS, eine Neuordnung der Gattung *Leontodon*. *Leontodon* subgenus *Oporinia* wurde durch diese molekularen Informationen zur Gattung *Scorzoneroides* erhoben. In unserem Projekt sollen die Chloroplasten-DNA Daten zum einen vorhergegangene Ergebnisse bestätigen, und zum anderen eine zusätzliche Informationsquelle darstellen. In den ersten Monaten unseres Projekts wurden 11 universelle Marker des Plastidengenoms an je 17 ausgewählte Spezies auf ihre Applizierbarkeit, ihren Sequenzabgleich sowie ihren Informationsgehalt untersucht. Für eine Untersuchung der gesamten Proben wurden in weiterer Folge die drei Marker *ndhF-rpl32, rpl16*-Intron und *atpH-atpI* verwendet. Sieben nukleäre Genregionen wurden untersucht, um einen möglichen hybriden Ursprung einzelner Arten der Gattung *Scorzoneroides* aufzuklären. Zwei Regionen des nukleären Genoms, *GAPDH* und *A39* konnten teilweise direkt sequenziert werden. Um die erhaltenen Sequenzen zu verbessern, wurden für beide Marker zusätzliche, interne Primer erzeugt. In beiden Regionen wurden polymorphe Sequenzen gefunden, welche durch Klonierung getrennt werden sollten. 180 Klon-Kolonien (96 für *GAPDH*, 84 für *A39*) wurden in weiterer Folge sequenziert, um die unterschiedlichen Fragmente zu erhalten. Der Sequenzabgleich von *GAPDH* ermöglichte es uns, eine Intron-Exon-Struktur des Gens im Subtribus Hypochaeridinae zu erstellen. Die Längen der einzelnen Abschnitte wurden durch manuelle Überprüfung des Alignments errechnet. Für *A39* konnte keine Struktur berechnet werden. Die erzeugten phylogenetischen Bäume beider Marker konnten keine Hybridisierungstendenzen zeigen. Ein Stammbaum der kombinierten Matritzen beider nukleärer Regionen wurde ebenso wie ein alle Marker umfassender phylogenetischer Baum, bestehend aus den beiden oben genannten und den drei nicht-kodierenden Plastiden-Abschnitten *atpH-atpI*, *ndhF-rpl32* und *rpl16*-Intron, erstellt. Zusätzlich zur DNA Sequenzanalyse wurden karyologische Untersuchungen durchgeführt. Samen von 9 Arten aus *Scorzoneroides*, welche aus allen resultierenden Gruppen der phylogenetischen Analyse stammen, wurden untersucht. Bei den Ankeimungsversuchen konnte für die annuelle Arten ein gutes Ergebnis erzielt werden, wohingegen die Samen der perennierenden Arten nicht oder nur sehr vereinzelt austrieben. Die Chromosomenzahl konnte bei den annuellen Spezies ermittelt werden. *Scorzoneroides* besitzt eine Basischromosomenzahl von x=6, bei zwei der untersuchten Arten konnten Abweichungen festgestellt werden. Hier beträgt die Chromosomenzahl x=5. Die ermittelte Genomgröße für 8 aus 9 Spezies ist relativ klein und beläuft sich auf Werte zwischen 1C=1.24pg und 1C=1.87pg.

ABSTRACT

Earlier investigations on the molecular phylogeny of the genus *Leontodon* using chloroplast regions in combination with the nuclear rDNA region ITS, led to a generic status of *Leontodon* subgenus *Oporinia*, today *Scorzoneroides*. To confirm previous results, as well as to get well-resolved phylogenetic trees, more noncoding regions of the chloroplast genome were sequenced. We were investigating the applicability, ease of alignment and informativity of 11 universal markers. The *ndhF-rpl32* intergenic spacer, the *rpl16*-Intron and *atpH-atpI* intergenic spacer regions were used to create phylogenetic trees of all samples. Seven low-copy nuclear markers were tested to detect speculated hybridization events in *Scorzoneroides*. PCR conditions were optimized for all markers. Two of the regions, namely *GAPDH* and the *A39* locus could be directly sequenced from some PCR reactions. Internal primers were designed to increase PCR reliability among the tested accessions from Hypochaeridinae. Both low-copy nuclear genes showed polymorphic sequences in some accessions, which led to cloning. We used 96 colonies from *GAPDH* and 84 colonies from *A39* to get both copies of each individual sequence that showed polymorphic sites. The alignment of *GAPDH* sequences gave information about the intron-exon structure of the amplified fragment in Hypochaeridinae. Intron and exon lengths were estimated manually by screening the alignment of all available sequence data of this study. We were not able to do the same for the *A39* marker. The resulting phylogenetic trees of both markers did not support on-going hybridization in the investigated genus. We created a combined tree of both nuclear markers from this study. The resolution of the markers' phylogenies, however, is low. This indicates that the two selected markers are not optimal for investigations in Hypochaeridinae. A combination of the two nuclear regions *GAPDH* and *A39* locus with the three plastid markers *atpH-atpI*, *ndhF-rpl32* and *rpl16*-Intron showed a well-resolved phylogenetic tree. In addition to nuclear and plastid DNA markers, karyological methods were used for the accessions where seed material was available. Chromosome counts and genome size measurements from seeds of 9 species of all resulting clades of the phylogenetic analysis were measured. Successful germination was easily obtained for annual species, whereas the germination rate of the included perennial species was very low. Chromosome counts could only be obtained for the annual plants. The basic chromosome number for *Scorzoneroides* is x=6, two species had a chromosome number of x=5. The genome size of 8 out of 9 species could be measured by flow cytometry. All species investigated possess a small genome size ranging from 1C=1.24pg to 1C=1.87pg.

1.) INTRODUCTION

1.1. Compositae

The family of Compositae, also known as Asteraceae, is one of the most successful plant families in Angiosperms. With an expected number of 25.000 to 30.000 species divided into 12 subfamilies and 43 tribes (Funk *et al.*, 2009), it comprises one tenth of all flowering plants. Compositae are distributed all over the Planet except for Antarctica, occupying almost every ecological niche one might imagine (Funk *et al.*, 2009). The age of the family is estimated to be 41-50 million years, and its origin is likely to be South America (Funk *et al.*, 2009).

The main characteristics are: a capitulum, comprising one to numerous individual flowers, the so called florets, most of the time fused anthers that are surrounding the style, and an inferior, unilocular ovary with one ovule (Bremer, 1994). The capitulum merges many flowers into one, making it less costy for plants to produce a big number of reproductive organs. The expanded shoot bearing the florets is called receptacle or disc, and shows many forms, from flat over concave and convex to columnar. It can be naked or it can carry paleae (bract-like structures). Blossoming is nearly always centripetal, i.e. flowers bloom from the outside to the center of the head. The flowering heads are surrounded by the involucre, a series of bracts reassembling the calyx of other flowers. Those so-called phyllaries are arranged either in one or more rows differing in morphology.

Six types of floret-corollas evolved in the family Compositae. The actinomorphic type has two modifications; the zygomorphic type comes up with four (see Table 1; Bremer, 1994). Most of the time actinomorphic florets occupy almost the whole disc area, making them disc-corollas. These corollas usually have five equivalent petals, more rarely four or three, and form tubes of different diameter. Zygomorphic florets very often are developed in one row in the periphery of the receptacle only. Their main task is to increase attractiveness to pollinators, thus they often do not develop anthers but only a style.

Due to the various form of the flower arrangement, different capitula-types can be defined: discoid (only actinomorphic florets, being either bisexual and fertile or functionally staminate or pistillate); radiant (discoid capitula with lobes of the outer corollas being expanded); radiate (discoid capitula with marginal florets being ray-florets, that have zygomorphic corollas consisting of a lamina terminating in only two or three, rarely in four lobes); disciform (florets being actinomorphic in corolla-shape, peripheral corollas having a tubular form, that is a narrow

actinomorphic type lacking stamen most of the time)*;* ligulate (all florets have a ligulate corolla and are bisexual) (Bremer, 1994).

Table 1: Differences of capitulum morphology in Compositae (from Bremer, 1994)

CIRCUMSCRIPTION	COROLLA TYPE	REPRODUCTIVE ORGANS	NUMBER OF LOBES	NUMBER OF FUSED LOBES	NUMBER OF LIGULES
Disc corolla	actinomorphic	both (bisexual)	([3-]4-)5	5	0
Tubular corolla	actinomorphic	usually female	5	5	0
Bilabiate	zygomorphic	both (bisexual) or male	5	3+2	3
Pseudobilabiate	zygomorphic	both (bisexual) or male	5	4+1	4
Ligulate	zygomorphic	usually both (bisexual)	5	5	5
Ray floret	zygomorphic	female or none (sterile)	5	3+2	(2-)3(-4)

Secondary pollen presentation is common in Compositae. Pollen from the already fertile anthers that are forming a narrow tube is pushed (e.g. in Asteroideae) or brushed (e.g. in Cichorieae) out by the growing style. This ensures that the capitulum is providing pollen over a long time-span by limiting the amount of pollen offered to a pollinator (e.g. Erbar & Leins, 1995).

The dry nut fruits of Compositae, referred to as cypselas or achenes, can be beaked, and usually are equipped with a hairy structure, called pappus, sitting on the distal end of the fruit. A flowering head drastically increases the gene pool of plants, as well as the pollen production per flower. One problem of that concentration of energetic compounds is that it attracts herbivores. To counterstrike this attractiveness, Compositae developed manifold secondary compounds, e.g. sesquiternpene lactones (Turner, 1977), stiff involucres, or thornes and spines.

Wind dispersal is a common strategy in Compositae. To allow the distribution of the hard and dense achenes, pappi evolved from the calyx of the florets (Carlquist, 1976). These structures allow long distance dispersal. In some cases, the pappus is used for transportation by attachment to fur or feathers of animals. In some cases, it helps to defend against herbivores, Stuessy & Garver (1996) were speculating about a possible origin of the pappus as defensive mechanism, which only later became an aid for dispersal.

Compositae, with their unique characters, have been reported very early in the history of plant taxonomy (e.g. Tournefort, 1700) and were always separated from other groups. The classification of the family was discussed by many authors (Cassini, e.g. 1816*;* Bentham, 1873*;* Hoffmann, 1890-1894*;* Carlquist, 1966, 1976*;* Cronquist, 1955, 1977, 1980*;* Turner, 1977*;* Heywood *et al.*, 1977*;* Wagenitz, 1976*;* Bremer, 1987, 1994*;* among others). Cassini laid the base to the classification in Compositae. He started his investigations with shape and variation

of the style on the whole family. Later he examined other morphological characters, like and stigmas, stamens, corollas, as well as achenes and pappus variation. He recognized 20 tribes, which are still recognized in modern classifications.

Bentham (1873), who was establishing a model of a 13-tribe classification in Compositae, noticed similarities of his work with the system of Cassini (see Bonifacino *et al.*, 2009). He regarded *Helianthae* to be the most primitive tribe in Compositae, which was widely accepted and left untouched for almost a hundred years.

Cronquist (1955) for example, set up a series of characteristics of primitive lineages of the family, and also suspected Heliantheae to be the most basal group. Carlquist, however, stated, that the ancestral lineages were rather woody than herbaceous. With the collected evidence, of Carlquist (1976), Cronquist (1977) changed his mind and supported Carlquists model (Bonifacino *et al.*, 2009).

The year 1975 had striking influence on the classification of Compositae. In this year the "Meeting on classification and evolution of the Compositae" in Reading, UK, took place. It led to a book edited by Heywood, Harbourne and Turner published in 1977 (i.e. Heywood *et al.*, 1977), which mentioned and was influenced by modern methods, like electron microscopy, new chemical approaches and biosystematics (Heywood, 2009). Electron microscopy was revolutionizing palynology, the new chemical approaches helped in the field of chemodiversity and to detect secondary metabolites. Biosystematics was used in the form of phenetics, i.e. a quantification of morphological (phenetic) data, which nowadays is replaced by the more modern cladistics approach (Hennig, 1950).

In the last decades, cladistics as well as molecular methods revolutionized the understanding of plant relationships on all taxonomic levels. Not surprisingly, numerous investigations in Compositae took place. By the late 1980s investigations by Bremer as well as Jansen and co-workers led to radical changes in the classification of Compositae.

For example, Jansen & Palmer (1987a, 1987b) utilized RFLPs (restricted fragment length polymorphism) of plastid DNA, and showed a 22 kilobase-inversion in the large single copy region of all members in Compositae, excluding subtribe Barnadesieae of Mutiseae. Further studies by Jansen *et al.* (1990, 1991), Bremer & Jansen (1992), Bremer (1994) or Kim & Jansen (1995) did confirm the previous results. Bremers cladistic approach on morphology in the whole family in 1994 was the first extensive study after Bentham (1873). He also took into account the available molecular data, and subdivided the Compositae into three subfamilies, the Barnadesioideae (Bremer & Jansen, 1992), a monotypic subfamliy, with approximately 100

species, the Asteroideae, the largest subfamily comprising about 65% of the taxa in the family, and the Cichorioideae, a group with approximately 6.000 to 7.000 species (Funk & Chan, 2009).

Kim & Jansen (1995) revealed that the Cichorioideae in contrast to the other two subfamilies were a paraphyletic group by utilizing the *ndhF* region of the chloroplast DNA. Two investigations that contributed a lot to the understanding to systematics and evolution in Compositae should be mentioned. These were Baldwin *et al.* (2002), utilizing the nuclear rDNA marker ITS, and Panero & Funk (2002), who did a more extensive approach with chloroplast DNA regions, comprising 13.380 kilobases.

Today's phylogeny of Compositae comprises 12 major lineages, these are (arranged from most derived to basal, see Figure 1): Asteroideae, Corymbioideae, Cichorioideae, Gymnarrhenoideae, Pertyoideae, Carduoideae, Heclastocleidoideae, Gochnatioideae, Wunderlichoideae, Stifftoideae, Mutisioideae, and Barnadesioideae. The following tree (Figure 1) is based on the result of a phylogenetic analysis from 10 chloroplast markers by Panero & Funk (2008).

In 2009, Funk and co-workers presented a metatree of Compositae which linked most recent molecular data from Panero & Funk (2002, 2008) with alterations from other studies (Baldwin *et al.*, 2002; Ortiz *et al.*, 2009; Funk & Chan, 2009), resulting in changes to Mutisoideae as well as the Helianthae group.

Figure 1: Phylogenetic tree of the Compositae by Panero & Funk (2008) based on 10 plastid markers. Major lineages (i.e. subfamilies) are marked by scattered boxes. (modified from Panero & Funk, 2008)
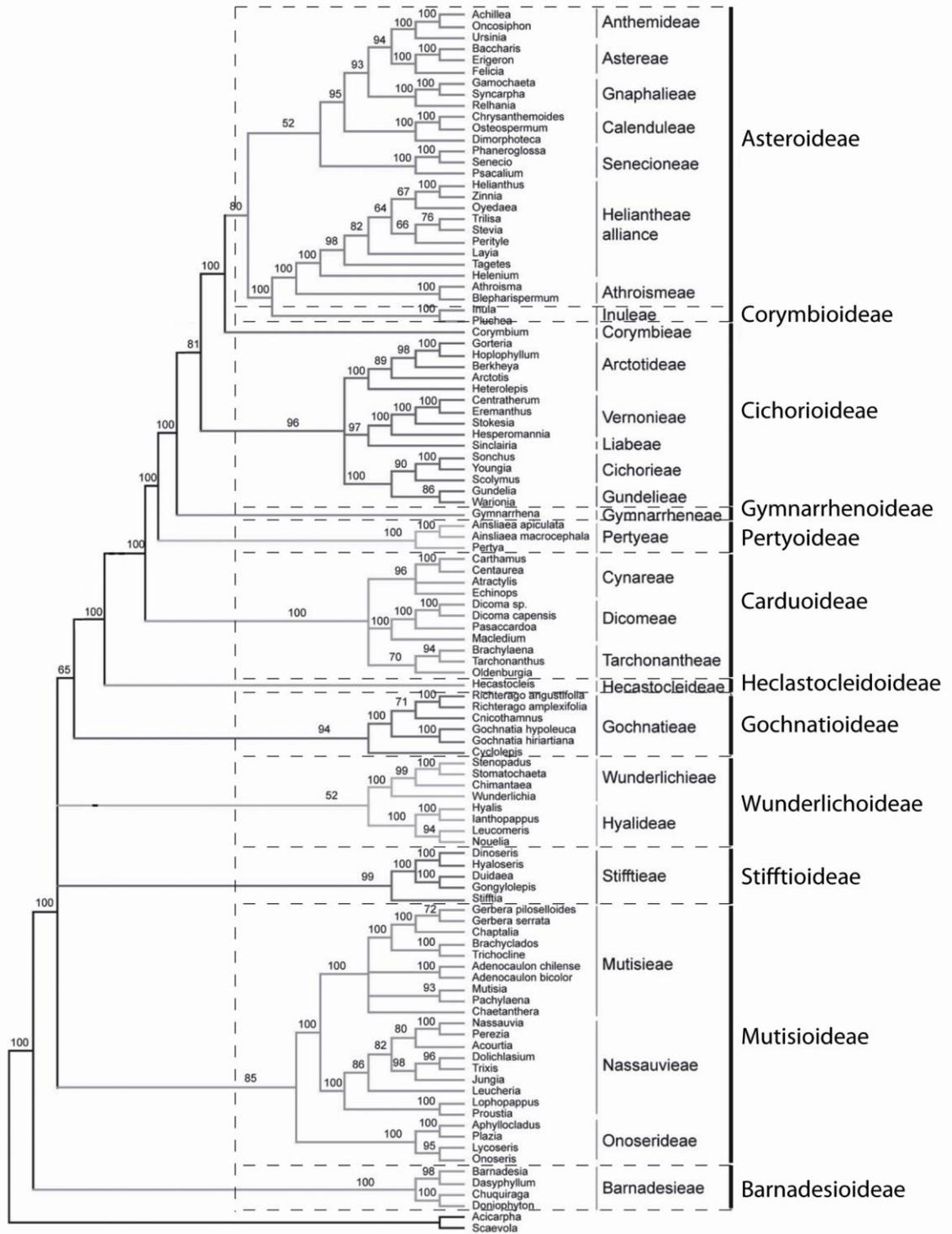
1.2. Cichorieae

Cichorieae are a tribe of the sunflower family (Compositae) in the subfamily Cichorioideae, with very unique attributes in Compositae, all members of the tribe are traditionally described to have white latex and almost always perfect 5-dentate, mostly yellow, ligulate flowers only (Hand *et al.*, 2009). The ease of distinguishing these characters led to an early recognition and description (Tournefort, 1694) of the group. This early general description (Tournefort, 1694) was widely accepted, and until today, has barely changed. All species put in this taxonomic entity by Tournefort in 1694 remained in the group, without exception he did only include species that are still found in Cichorieae today (Kilian *et al.*, 2009). Lamarck & De Candolle (1806) used the pappus as character to subdivide the tribe into four subtribes. Stebbins (1953), after several decades of work on this group with Babcock, provided a phenetic multi-evidence character approach, comprising morphological characters, ecological distribution, as well as karyological features (Kilian *et al.*, 2009). This study supported the subdivision of Cichorieae into 8 subtribes and 62 genera. Bremer (1994) recognized 11 subtribes and 98 genera.

The presence of lactiferous canals in both the subterranean and aerial plant parts seems to be an exclusive feature of the Cichorieae (Hand *et al.*, 2009). 93 genera of Cichorieae are recognized in the phylogeny (Figure 2) by Kilian *et al.* (2009). They emphasized not only on the traditional Cichorieae but also on the basal relationships of the tribe (Hand *et al.*, 2009). A special interest was laid on *Gundelia* and *Warionia*. *Gundelia* was considered to be a member of Arctoteae another tribe of Cichorioidae. Karis *et al.* (2001), however, were able to show incongruences in this placement by using the chloroplast marker *ndhF* in their study; *Gundelia* was put into Cichorieae rather than Arctoteae. An approach by Funk *et al.* (2004) based on two plastid markers and ITS confirmed the incorrect placement of *Gundelia*. The genus formed a group together with *Warionia* as sister to *Cichorium*, *Hypochaeris* and *Lactuca*. *Warionia* was either seen as a sister to the tribe (Panero & Funk, 2002; Funk *et al.*, 2004) or most basal group of the Cichorieae (Goertzen *et al.*, 2003), depending on the choice of molecular markers. While Panero & Funk (2002) and Funk *et al.* (2004) used plastid DNA, Goertzen *et al.* (2003) employedthe nrDNA marker ITS. Kilian *et al.* (2009) were using ITS data, which led to adding the subtribe Warinoniinae (Figure 2, Clade 1), composed of the monotypic genus *Warionia*, as sister to all other members of the tribe. *Gundelia* was added to subtribe Scolyminae (Figure 2, Clade 3).

Five major clades were recognized in Cichorieae with molecular data by Kilian *et al.* (2009): Clade 1 is formed by subtribe Warioniinae (*Warionia*) only. The second clade represents subtribe Scorzonerinae. Clade three is formed by subtribe Scolyminae. Clade 4 (Figure 3)

comprises two thirds of the species (if *Hieracium*, *Pilosella* and *Taraxacum* are exluded) in Cichorieae, and includes five subtribes, namely Chrondrillinae, Crepidinae, Hyoseridinae, Hypochaeridinae and Lactucinae. Clade 5 is formed by 3 subtribes, Cichoriinae, Hieraciinae and Microserdinae.

Figure 2: Scheme of a molecular phylogeny of Cichorieae by Kilian *et al.* (2009) based on the nuclear rDNA marker region ITS (ITS1, 5.8S rDNA, ITS2). Clade 4 and 5 are only schematic, Clade 4 is described in detail by Figure 3. (modified from Kilian *et al.*, 2009).
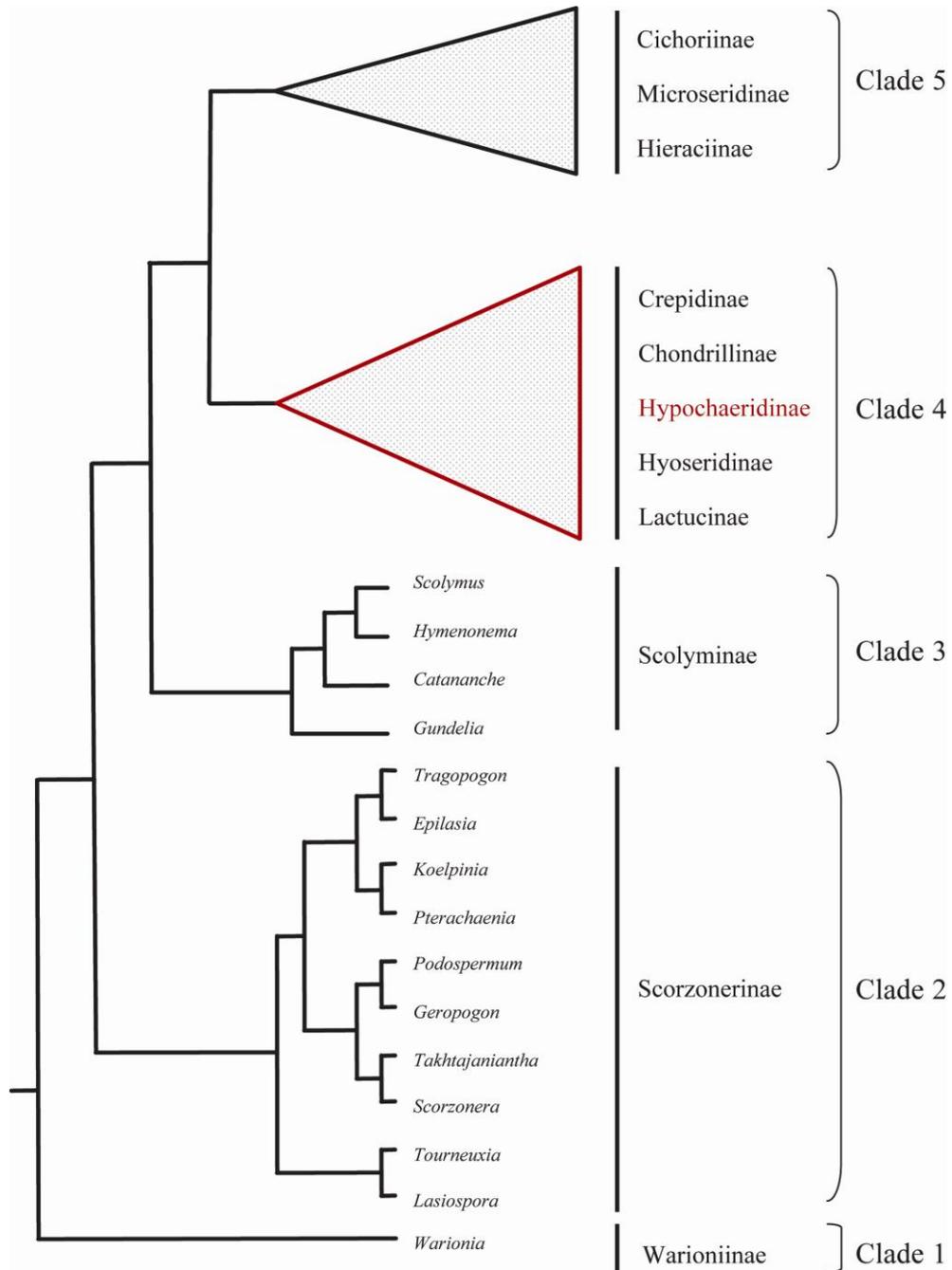
Figure 3: Scheme of the molecular phylogeny by Kilian *et al.* (2009) of one Clade (Clade 4) of Cichorieae based on the nuclear rDNA marker ITS (modified from Kilian *et al.*, 2009)
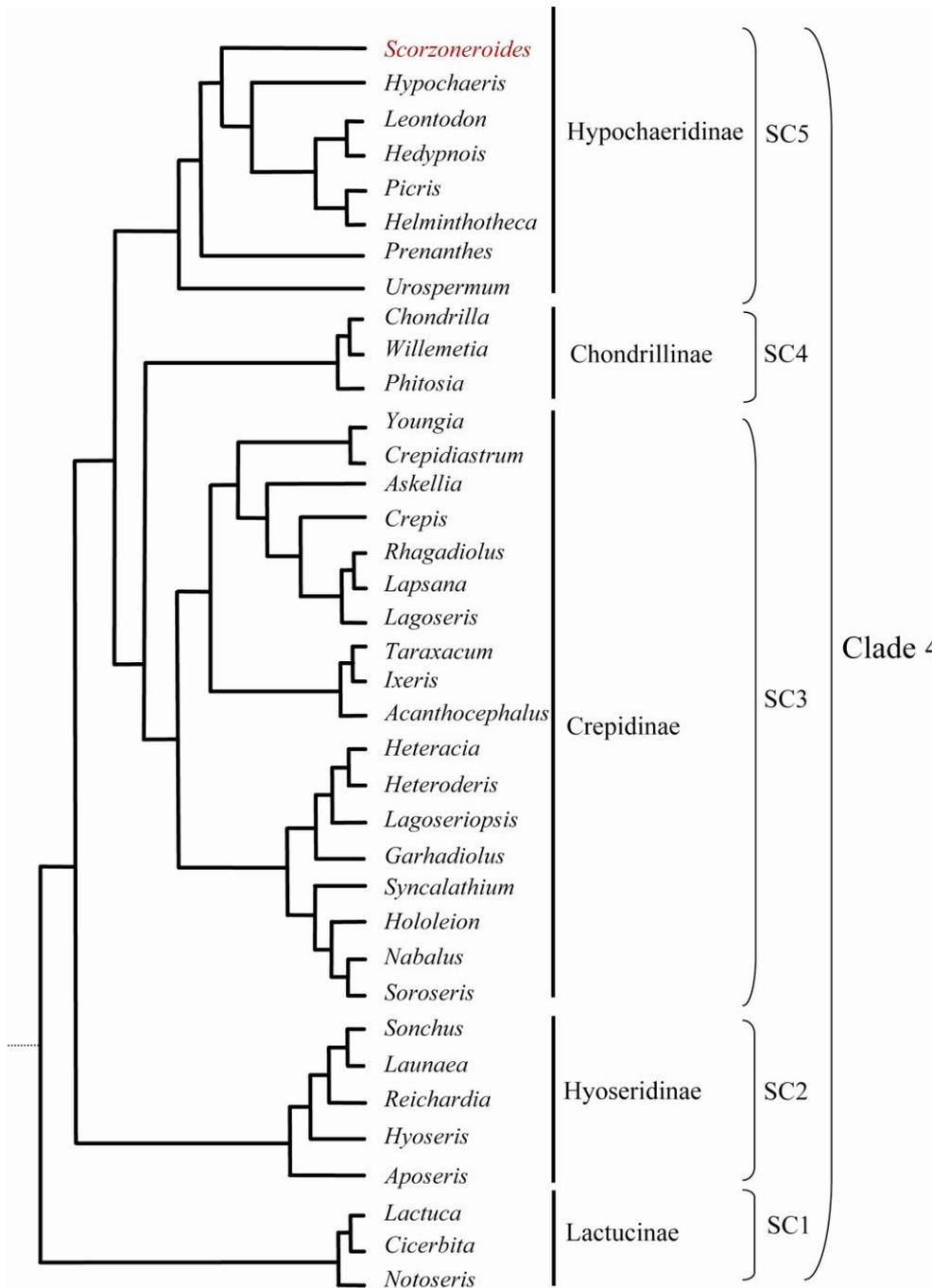
1.3. Hypochaeridinae

The subtribe Hypochaeridinae includes six genera, *Hypochaeris*, *Leontodon*, *Picris*, *Helminthotheca*, *Hedypnois*, and *Scorzoneroides* (Hand *et al.*, 2009). *Urospermum* was added as sister to the rest of the subtribe in 2009 by Kilian and co-workers. All of the genera are found in Europe, around the Mediterranean Sea and to some extent, in Central Asia, except for *Hypochaeris*, which is also found in South America. The distribution of this genus has led to speculation on its origin and dispersal, which made *Hypochaeris* target of several studies based on molecular markers and karyology (Cerbah *et al.*, 1998; Samuel *et al.*, 2003, 2006; Weiss-Schneeweiss *et al.*, 2003, 2008; Tremetsberger *et al.*, 2005; Ruas *et al.*, 2005). The results by Samuel *et al.* (2003) and Tremetsberger *et al.*, (2005) revealed that the origin of this genus lies in the Mediterranean, and that long-distance dispersal from North western Africa is likely to have given rise to the South American species of *Hypochaeris*, which, by rapid radiation could become very successful in various niches. *Leontodon* was reported to be diphyletic by Samuel *et al.* (2006), and was separated into *Leontodon* (former *L.* subgenus *Leontodon*) and *Scorzoneroides* (*L.* subgenus *Oporinia*) by Greuter *et al.* (2006). *Leontodon* s.str. comprises approximately 40 species, their distribution is centred in the Mediterranean area and extending to Northern Europe and South Western Asia (Hand *et al.*, 2009), three sections are recognized (Widder, 1975; Samuel *et al.*, 2006). *Picris* and *Helminthotheca* are closely related; Samuel *et al.* (2006) placed them as monophyletic sisters to *Leontodon* s.str. in their study. All genera in this subtribe except for *Hedypnois* possess a plumose pappus. *Hedypnois* in contrast has a scabrid pappus. This may indicate reverse development of the plumose pappus, which is plesiomorphic in the Cichorieae, back to a scabrid pappus (Hand *et al.*, 2009).

1.4. *Scorzoneroides*

Scorzoneroides is a rather small genus with ca. 26 different species (Cruz-Mazo *et al.*, 2009), located in Compositae, subfamily Cichorioideae, tribe Cichorieae, subtribe Hypochaeridinae. Samuel *et al.* (2006) showed incongruences in the previous phylogeny by Widder (1975), who grouped *Scorzoneroides* and *Leontodon* as two subgenera, *L* .subgenus *Oporinia* and *L.* subgenus *Leontodon*, into one genus, based on morphological characters. The phylogenetic study by Samuel *et al.* (2006) showed a clear separation of the two former subgenera, with *Leontodon* s.str. as sister to *Picris* and *Helminthotheca* and not to *Oporinia*. Greuter *et al.* (2006) confirmed the generic status of *Scorzoneroides*. A later study on the genus by Cruz-Mazo *et al.* (2009), corrobated the results by Samuel *et al.* (2006), and revealed a subdivision into two major groups (Figure 11).

1.4.1. Morphology of *Scorzoneroides*

Widder (1975) classified todays genus *Scorzoneroides* as subgenus to *Leontodon*, and named it *Leontodon* subgenus *Oporinia*. The morphological characters served as a basis for his classification. These characters are simple trichomes, crenate (increasing from bottom to top), simple or branched stalk, nodding or erect flowering buds, simple Involucre, pappus diameter (10 – 21 mm). In the subgenus *Oporinia* two sections were recognized, *Oporinia* D.Don and *Kalbfussia* Sch-Bip.: Section *Oporina* was characterized by unbeaked achenes, erect flowering buds, and a pappus-diameter of 15-21mm. Within the section several series were recognized. The two sections were divided into four (*Oporinia*) or two (*Kalbfussia*) series, respectively: *Kalbfussia* has beaked and heteromorphic achenes with or without pappus, nodding buds, and a smaller pappus diameter of 10-12(18) mm. The four series of Oporinia were Boreales (Figure 4), Orientales (Figure 5), Mediani (Figure 6) and Occidentales (Figure 7), the two series of *Kalbfussia* were Perennes (Figure 8) and Annui (Figure 9).

<u>Series Boreales (Figure 4)</u>: perennial habit*;* stalk branched, few or single capitulas, peripheral florets with violet to purple straps on their backside*;* styles are darkened*;* pappus light-yellow*;* achenes glabrous below pappus*;* (*S. autumnalis*)

Figure 4: Scorzoneroides autumnalis

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Scorzoneroides autumnalis
(description from Tutin *et al.*, 1976)

"Perennial, with branched, oblique, truncate stock. Stems 1-numerous, 5-60cm*;* usually branched, glabrous or with few simple, eglandular hairs*;* bracts numerous, particularly just below the capitula. Leaves 20-200 x 3-30 mm, narrowly oblanceolate, acute, sinuate-dentate to deeply pinnatisect, the segments usually patent or recurvedm, tapered into the petiole, glabrous or with simple eglandular hairs. Capitula (1-)2-7. Involucre 7-12 x 7-11 mm*;* bracts linear-lanceolate, obtuse to acute, glabrous or with simple, eglandular hairs. Ligules deep yellow, the outer with reddish stripe on outer face. Stigmas discoloured. Achenes 3.5-7mm, reddish brown, cylindrical, slightly narrowed above, transversely muricate*;* pappus of 1 row of plumose hairs."

<u>Series Orientales (Figure 5)</u>: perennial habit*;* simple stalks*;* leaves shortened, dentate to pinnate*;* florets never violet to purple on backside*;* styles colored light*;* pappus greyish white*;* achenes glabrous below pappus*;* (*S. crocea, S. rilaensis*)

Figure 5: Scorzoneroides crocea

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Scorzoneroides crocea
(description from Tutin *et al.*, 1976)

"Perennial, with horizontal or oblique, truncate stock. Stems 1-3, 5-30cm, simple, glabrous or with pale and dark simple, eglandular hairs near the thickened apex*;* bracts 2-4*;* Leaves 25-170 x 3-20mm, denticulate, gradually narrowed to the petiole,with sparse hairs beneath. Capitulum solitary*;* Involucre 10-14mm, bracts linear-lanceolate, obtuse, with numerous dark simple eglandularhairs intermixed with shorter whitish hairs. Ligules orange-yellow, concolorous. Stigmas yellow. Achenes 5-6 mm, pale brown or pale-chestnut brown, cylindrical or narrowly fusiform, slightly narrowed on apex, weakly transversely muricate*;* pappus-hairs in 1 row, whitish, plumose."

Series Medianii (Figure 6): perennial habit*;* stalk simple*;* elongated, non petiolate leaves*;* florets never violet to purple on backside*;* styles colored light*;* pappus clean white to beige*;* (*S.melantotricha*, *S.montana*, *S.montaniformis*, *S.pseudotaraxaci*)

Figure 6: *Scorzoneroides melanotricha*

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Scorzoneroides melanotricha
(description from Tutin *et al*., 1976)

"Perennial, oblique or vertical, truncate stock*;* Stems 1-2(-4), 1-10 cm*;* simple, with numerous long simple eglandular hairs*;* bracts 0-2*;* Leaves 20-70 x 3-10mm, linear to oblanceolate, dentate to runcinate-pinnatifid, narrowed at the base to a winged petiole, with few to numerous long hairs, the terminal segment 8-20 x 3-10mm*;* Capitulum solitary*;* Involucre 12-18 x 10-14mm, with pale grey hairs*;* bracts linear-lanceolate, obtuse, with dense, long simple eglandular hairs*;* Ligules deep yellow, concolorous. Stigmas yellow*;* Achenes 6.5-7.5mm, pale brown, cylindrical or slightly fusiform, slightly narrowed at apex, weakly transversely muricate*;* pappus hairs in one row and plumose, white."

Series Occidentales (Figure 7): annual, biennal, and perennial habit*;* Stalks simple, rarely branched, rarely multiple capitulae*;* Leaves spathulate, lanceolate – petiolate to sessile, simple to cleaved*;* florets with violet to purple color on their backside*;* styles colored light*;* pappus colored greyish white to ocher*;* achenes hairy below pappus*;* (*S.pyrenaica, S.helvetica, S.cantabrica, S.microcephala, S.atlantica, S.oraria*)

Figure 7: *Scorzoneroides microcephala*

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Scorzoneroides microcephala
(description from Tutin *et al*., 1976)

"Perennial, long, slender tubes*;* stems 1-4, 3-10 cm, sometimes branched, glabrous*;* Bracts 2-numerous*;* Leaves 10-45 x 2-5mm, linear, spathulate to oblanceolate, obtuse, entire, glabrous or with a few simple eglandular hairs*;* Capitlula 1-2*;* Involucre 7-8 x 4-5 mm*;* bracts linear-lanceolate, obtuse, glabrous or with a few short simple eglandular hairs*;* Ligules yellow. Stigmas yellow. Achenes 4-5 mm, pale brown, cylindrical, weakly transversely muricate*;* pappus of 1 row of plumose hairs."

Series Perennes (Figure 8): perennial habit*;* compact rhizome, radices bold*;* achenes beaked, uniform*;* (*S.cichoracea, S.duboisii, S.carpetana, S.nevadensis*).

Figure 8: *Scorzoneroides nevadensis*

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Scorzoneroides nevadensis
(description from Tutin *et al*., 1976)

"Perennial, vertical or oblique, often branched, truncate stock*;* Stems 1-numerous, 10-45 cm, simple or branched, glabrous or with few simple eglandular hairs, bracts numerous*;* Leaves 10-120 x 1-15mm, linear or narrowly oblanceolate, obtuse to acute, sinuate-dentate to deeply pinnatisect with usually linear segment, tapered into the petiole, glabrous or with few simple hairs*;* Capitula 1-3*;* Involucre 8-10 mm*;* Involucral bracts linear-lanceolate, obtuse to acute, with numerous, long , grayish, simple, eglandular hairs intermixed with shortish ones*;* Ligules yellow, concolorous."

Series Annui (Figure 9): annual, rarely subperennial; fibrillous to spatulate radix; dimorphic achenes; (*L. muelleri, L. laciniata, L.hispidulus, L. simplex, L. salzmannii, L. garnironii*)

Figure 9: *Scorzoneroides salzmannii*

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Scorzoneroides salzmannii
(description from Tutin *et al*., 1976)

"Annual; Stems 1-3, 10-30cm, branched, glabrous, bracts 0-3; Leaves 40-110 x 10-20mm, oblong to oblanceolate, obtuse to subacute, sinuate-dentate to pinnatifid, gradually narrowed into the short petiole, glabrous; Capitula 2-3, on long peduncles.Involucre 10-12 x 8-12 mm, bracts narrowly lanceolate, obtuse, glabrous or with a few simple eglandular hairs in a median line; Ligules yellow, concolorous; Stigmas yellow.Achenes brown, strongly tuberculate-muricate, of 2 kinds; outer 2-3 mm, cylindrical, truncate at apex, without a pappus; inner 6-7mm, narrowed at apex into a long beak, with a pappus of an inner row of ~10 rigid, plumose hairs and an outer row of short scales."

1.4.2. Distribution & Ecology of *Scorzoneroides*

The distribution of this genus is vast, spanning from the Iberian Peninsula to the Black Sea (Figue 10). Also, the ecological conditions in which different species grow, is quite diverse. Some taxa, e.g. *Scorzoneroides salzmannii*, are growing in semidesertic areas, whereas others, for example *S.montana*, can be found even in altitudes of 2500 meters in the Alps. Some species in this genus are very adaptable to various ecological conditions; the best example here is *S.autumnalis*, the flagship species of *Scorzoneroides*, which can be found all over Europe, from Finland to Spain. Also, it is a successful neophyte in Eastern North America. Others are endemic to some regions, like *S.nevadensis*, distributed only in the Sierra Nevada Mountains in Spain.

Scorzoneroides' center of diversity lies in the western Mediterranean region, i.e. the Iberian Peninsula and parts of north western Africa, especially Morocco (Cruz-Mazo *et al.*, 2009; Meusel & Jäger, 1992). Also, many species are bound to mountain areas of the Sierra Nevada, Pyrenees, Alps, Carpathians, Apennine, and Balkan Mountains. Another group of taxa is growing in subdesertic, semiarid refuges on the North African borderline as well as on the shoreline of the Middle East. In *Scorzoneroides* we can roughly define two major ecological groups (Cruz-Mazo, 2009), either perennial inhabitants of continental to mountain areas or annual coast to dryland occupants.

One group occupies terrain that can be defined as semi-arid to arid or Mediterranean, geographically biased around the Mediterranean Sea. This group is formed by S.*hispidulus, S.laciniata, S.muelleri, S.kralikii, S.palisiae*, and *S.salzmannii*. Typical for that group is an

annual life cycle as well as, linked to that, a mixed dispersal strategy (heterocarpy), which is reported frequently in Compositae (Voitenko, 1989; Beneke *et al.*, 1992; Rieseberg, 1997, 2003; among others).

Heterocarpy defines the developing of two types of achenes in one flower, in the case of *Scorzoneroides* beaked achenes with pappus as type one, and beaked strongly tuberculate-muricate (wart-like structures) achenes without pappus as type two, are formed. Type one is arranged towards the middle of the capitulum, whereas type two is only found in the periphery and is retained in the flowering head (synaptospermy, see Gutterman, 1994, 2002). The pappus-developing fruits are wind-dispersed, whereas the achenes lacking pappi are tightly attached to the flowers receptacle, thus bound to the maternal plant. The second group is distributed in continental environments and mountain areas across Europe, including Sierra Nevada, Pyrenees, Alps, and the Carpathians as well as the Balkan. One species was observed in the Atlas region (*S.garnironii*). Characteristics of this group are perennial life cycle and homomorphic achenes.

Figure 10: Distribution map of *Scorzoneroides* from various sources (Meusel & Jäger, 1992; Tutin *et al.*, 1976; Euro+Med [2006-]). Darker regions indicate higher species diversity in *Scorzoneroides* (digits in map indicate number of species in this area). Numbers on the right describe species in specific areas according to Table 2. (modified from Meusel & Jäger, 1992)
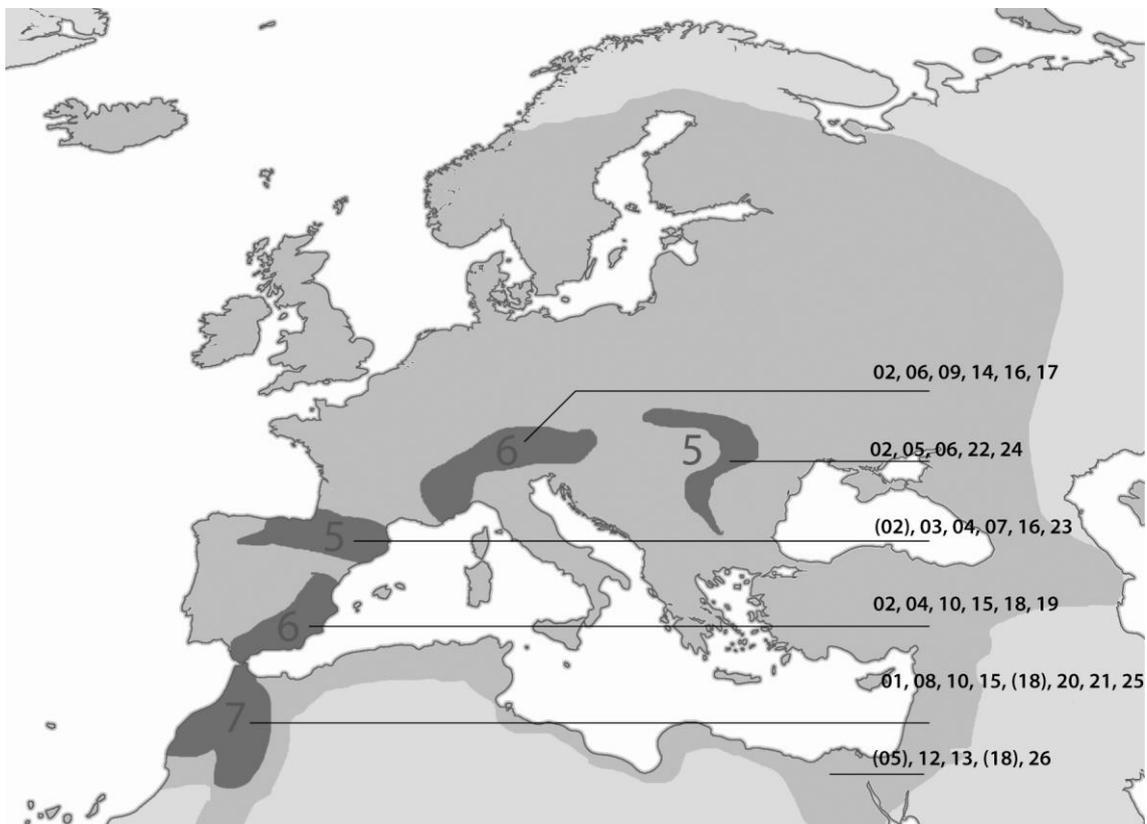
Table 2: Distribution of *Scorzoneroides* based on various sources (Meusel & Jäger, 1992; Tutin *et al.*, 1976; Euro+Med [2006-]). Numbers correspond to numbers in Figure 10.

N°	SPECIES	DISTRIBUTION
01	S. atlantica	Morocco
02	S. autumnalis	All over Europe, only scarce in southern regions
03	S. cantabrica	North West Spain and North Portugal, Pyrenees
04	S. carpetana	Mountains of Spain
05	S. cichoriacea	Central to South Italy, Balkan Peninsula, Sicily, Algeria
06	S. crocea	Eastern Alps, Eastern and Southern Carpathians
07	S. duboisii	Pyrenees and Cordillera Cantabrica
08	S. garnironii	Atlas mountains
09	S. helvetica	Southern France to Slovenia
10	S. hispidula	South Eastern Spain, Morocco, Libya, Egypt, Middle East
11	S. keretina	North Eastern Europe
12	S. kralikii	Algeria, Libya, Tunisia
13	S. laciniata	Egypt, Middle East, Turkey
14	S. melanotricha	Western and Central Alps
15	S. microcephala	Sierra Nevada, Northern Morocco
16	S. montana	Pyrenees, Central and Western Alps
17	S. montaniformis	Central and Eastern Alps, Central Appenin, West Balkan
18	S. muelleri	Southern Spain, Sardegna, Sicila, Algeria, Tunisia, Libya
19	S. nevadensis	Sierra Nevada
20	S. oraria	Morocco
21	S. palisiae	Southern Spain, Morocco
22	S. pseudotaraxaci	Carpathians
23	S. pyrenaica	Pyrenees
24	S. rilaensis	Southern Carpathians, Bulgaria
25	S. salzmannii	Morocco
26	S. simplex	Egypt, Libya

1.4.3. Recent changes in the classification of *Scorzoneroides*

A phylogenetic research based on both cpDNA and ITS by Samuel *et al.* (2006) revealed that *Leontodon*, as described by Widder (1975), is diphyletic. *Leontodon* subgenus *Leontodon*, today *Leontodon* sensu stricto is related more closely to *Helminthotheca* and *Picris*, than to former subgenus *Oporinia*. Greuter *et al.* (2006) confirmed that the former *L.* subgenus *Oporinia* has to be treated as a separate genus, *Scorzoneroides*, sister to *Leontodon*, *Helminthotheca*, *Picris* and *Hypochaeris*. A new investigation on *Scorzoneroides* (Cruz-Mazo *et al.*, 2009) based on molecular data as well as morphological (achene shape) and ecological characters (predictable or unpredictable water supply, life cycle), from 21 out of 26 recognized taxa followed that of Samuel *et al.* (2006). The results confirmed the generic status of *Scorzoneroides* and also

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showed a clear seperation of two group*s*. Widder (1975) divided *L.* subgenus *Oporinia* into two sections, section *Oporinia* (D.Don) and section *Kalbfussia* (Schultz-Bip.). However, the results by Cruz-Mazo *et al.* (2009) did refute Widder (1975), as the two resolved clades were of species of both of the postulated section*s*. The support for *Scorzoneroides* as monophyletic genus was stated by Samuel *et al.* (2003), confirmed by Samuel *et al.*(2006), and Cruz-Mazo *et al.* (2009) with molecular data. The analysis by Cruz-Mazo *et al.* (2009) formed a system of two clades within *Scorzoneroides,* Clade I and Clade II (see Figure 11).

Clade I is split again, into two subclades, A and B. Subclade A comprises five species (*S.muelleri*, *S.garnironii*, *S.oraria*, *S.palisiae, S.salzmannii*), which grow in Mediterranean to semidesertic areas, whereas four species (*S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.nevadensis*) from European mountain regions group together in subclade B. Clade II is formed by European mountain taxa (*S.cantabrica*, *S.cichoracea*, *S.crocea*, *S.helvetica*, *S.montana*, *S.melantotricha*, *S.montaniformis*, *S.microcephala*, *S.pseudotaraxaci*, *S.pyrenaica* and *S.rilaensis*), with a very low divergence, thus forming a polytomy with ITS data only. The combined dataset shows low resolution in the whole Clade, with the highest divergence values being between geographically close *S.pyrenaica* and *S.cantabrica*.

Two taxa (*S.kralikii*, *S.laciniata*) which in terms of morphology as well as ecology (subdesertic areas in Libya and Egypt, or the Middle East, respectively) should group with species of Clade I subclade A, fall into subclade B with the datasetof Cruz-Mazo *et al*. (2009). Although the support for this topology is very weak (BS 52), Cruz-Mazo *et al.* (2009) speculated possible hybrid origin for *S.laciniata*, due to incongruences with phylogenies obtained from the plastid DNA dataset. *Scorzoneroides kralikii* and *S.laciniata* are sister species to *S.muelleri* in subclade A. *Scorzoneroides muelleri* shows outbreeding tendencies and its seeds are fit for long distance dispersal. In addition the habitat of this species lies in vicinity to the natural habitat of *S.kralikii*. This may suggest that *S.laciniata* represents a hybrid of the latter two taxa. Other ambiguities in the phylogenies by Cruz-Mazo *et al.* (2009) are the relationships of *S.nevadensis* and *S.carpetana*, as well as *S.palisiae* and *S.garnironii*. Low sequence divergence in both ITS and cpDNA markers in these cases raises the question whether there may be ongoing hybridization or introgression tendencies.

Figure 11: Phylogenetic tree of *Scorzoneroides* by Cruz-Mazo *et al.* (2009) based on two noncoding plastid markers, namely *ndhF-rpL32* and *rpL32-trnL,* and the nrDNA marker ITS. Posterior probabilities and bootstrap values are given above and below branches, respectively (modified from Cruz-Mazo *et al.*, 2009).
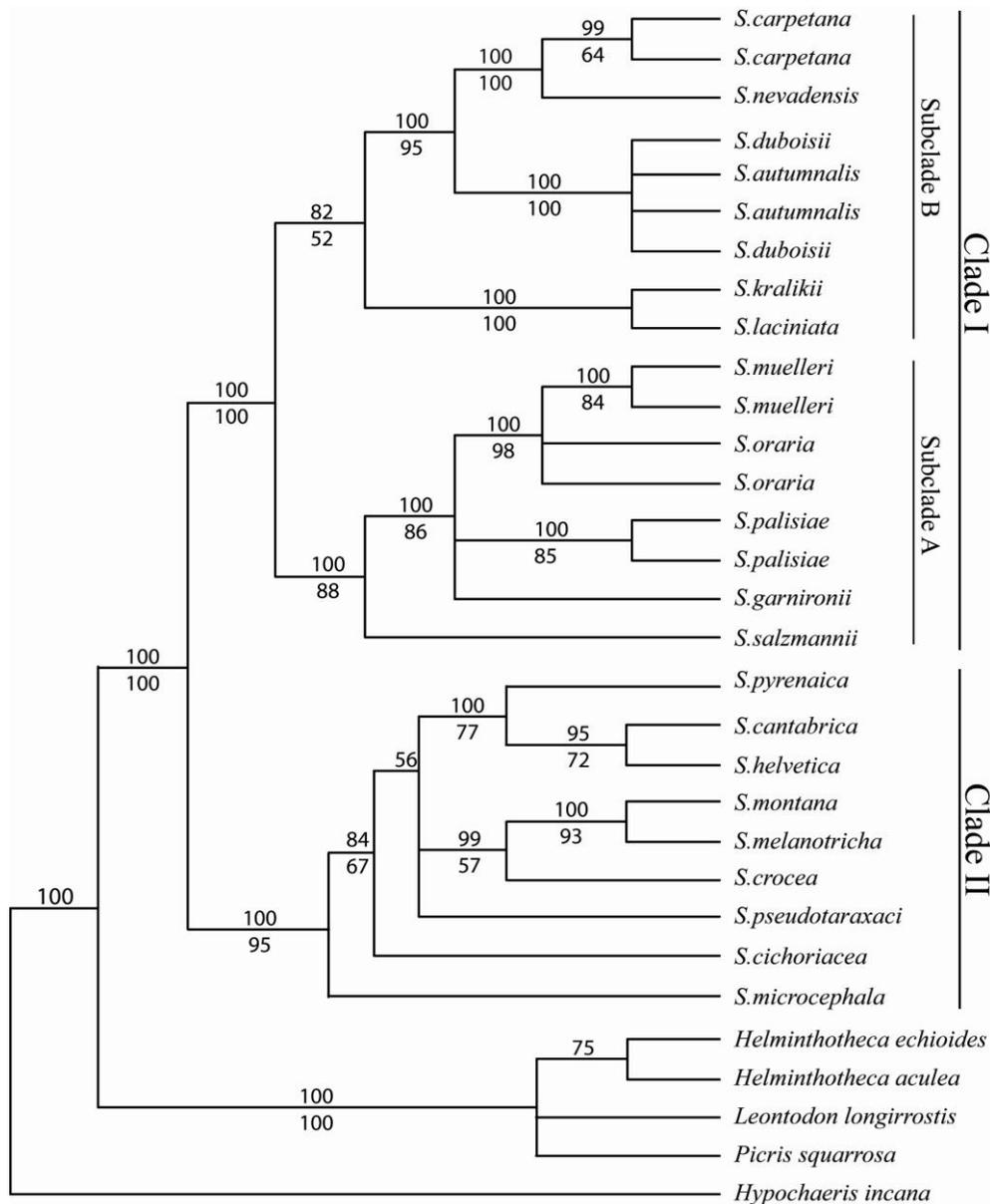
1.5. Molecular Markers

Since the 1980's, a whole new set of phylogenetic tools evolved. Morphological characters and sometimes chromosome counts were now added a new set of information stemming from genomic sequences. Three different kinds of genomic DNA can be distinguished: plastid DNA, nuclear DNA and mitochondrial DNA. All of them are useful for evolutionary biologists and

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systematists in many ways. For plant scientists, chloroplast or plastid as well as nuclear markers have proven more useful than mitochondrial DNA markers.

1.5.1. Plastid markers

The first whole chloroplast genome of Angiosperms to be sequenced was *Nicotiana tabacum* (Shinozaki *et al.*, 1986), and it revealed the circular arrangement – although there are some exceptions of this, for more information, see Lilly *et al.*, 2001 – of the plastid DNA (Figure 12). Four functional units could be observed, a large and a small single-copy unit, the LSC and SSC, respectively, and two identical inverted repeats (IR) between those regions where reported. The plastid genome of *Nicotiana* has a length of roughly 156 kilobases (kb), 87kb coding for the LSC, 18kb for the SSC and 25kb for each of the IRs. Usually, a genome size of 120-160 kb is expected; however, exceptions from 70 kb (Wolfe *et al.*, 1992: *Epifagus virginiana*) to 217 kb (*Chumley et al., 2006: Pelargonium × hortorum)* were reported, with the highest variability found in parasitic and non-photosynthetic plants (Borsch & Quandt, 2009). Most of this variability is found in the IR regions, which are 5-76 kilobases in length (Palmer, 1991; Borsch & Quandt, 2009). Setting these exceptions apart, the chloroplast genome shows a conserved arrangement in its genes and structure in Angiosperms (Downie & Palmer, 1992).

Mutational changes are affecting the LSC and the SSC in a much higher ratio than the IRs, two- to fourfold higher substitution rates were reported (Curtis & Clegg, 1984*;* Wolfe *et al.*, 1987*;* Wolfe 1991*;* Gaut 1998*;* Perry & Wolfe, 2002). The chloroplast region of Angiosperms is divided in coding and non-coding DNA. About 60 percent of the plastids nucleotides are within coding regions, being tRNA, rRNA, and protein coding genes, only 40 percent are reportedly noncoding, either introns or intergenic spacers (Borsch & Quandt, 2009). Coding regions are evolving only slowly, because of functional constraints, and repair mechanisms keep mutation rates low (Clegg *et al.*, 1994). Non-coding regions do not show hindering mechanisms except for functional constraints in certain regions of introns (Clegg *et al.*, 1994*;* Downie *et al.*, 1996, 1998*;* Jordan *et al.*, 1996*;* Kelchner & Clark, 1997*;* Kelchner, 2002*;* Borsch & Quandt, 2009). Thus, these regions should accumulate point mutations, indels or inversions more freely and be of a higher variation than coding regions (Gielly & Taberlet, 1994).

The relative small genome size of plastids and the availability of whole genome sequences (Shinozaki *et al.*, 1986*;* Ohyama, 1986*;* Hiratsuka, 1989*;* among others) in distant lineages allowed the development of universal primers for Angiosperms (Chase *et al.*, 1993: *rbcL;* Johnson & Soltis, 1994: *matK;* Olmstead & Sweere, 1994: *ndhF;* among others). The first studies focused on suprageneric phylogenies and utilized mainly coding regions, as these are

evolving slowly enough to enable alignment of seqences of distant groups, but also show sufficient variation. Depending on the selected marker, there are differences in the informativity of these regions (for further details refer to Shaw *et al.*, 2005). Although the potential of noncoding chloroplast DNA markers were known, only a handful universal primers were used by most studies in the late 1990's and early 2000's (Shaw *et al.*, 2005). With the revolutionary papers of Shaw and co-workers in 2005 and 2007, universal primers for many noncoding regions in all Angiosperms became manageable and easily available.

Figure 12: Full chlorplast genome of *Helianthus* and *Lactuca*. Regions highlighted in red and blue were successfully amplified in this study, blue markers were used only in primer trials, red ones were fully applied. (modified from Timme *et al.,* 2007)

One prominent example for the use of plastid markers at high taxonomic levelsis the Angiosperm Phylogeny Group: Chase *et al.* (1993) utilized the *rbcL* (RuBisCO large subunit)

gene situated in the LSC of the chloroplast genome. This study was the first molecular approach that established a phylogenetic tree of almost all important Angiosperm lineages. The *matK* (megakaryocyte-associated tyrosine kinase) gene was used in a similar investigation in Angiosperms by Hilu *et al.* (2003). Bremer (2002) utilized coding and noncoding markers for an investigation on asterids. In this and other cases (e.g. Löhne *et al.*, 2006: Nympheales; Soejima & Wen, 2006: Vitaceae), noncoding markers prove to be more useful than coding markers even at higher taxonomic levels. Most investigations at family levels employ both kind of markers (for example: Asmussen *et al.*, 2006: Arecaceae; Panero & Funk, 2008: Compositae).

1.5.2. Nuclear markers

As plastid DNA has several drawbacks in phylogenetic analysis, for example uniparental inheritance or linked single-locus genes, new markers were needed in evolutionary biology. There are two types of useful nuclear markers, being nuclear rDNA markers like ITS (internal transcribed spacer region) and low-copy nuclear gene markers. Both have in common a biparental inheritance, highly-conserved coding-regions and fast-evolving non-coding regions. This structure helps in primer design and allows investigating even intraspecific variation.

a) Nuclear ribosomal DNA (nrDNA)

Only one nuclear marker so far is widely used in many phylogenetic investigations, the internal transcribed spacer region of the 18S-26S nuclear ribosomal DNA, also known as ITS (Figure 13; Alvarez & Wendel, 2003). 18S-26S nrDNA is a so called multigene family, with many hundreds to thousands of copies, arranged in repeats on multiple loci on one or often more chromosomes. ITS is divided into two subunits, ITS1 and ITS2. ITS1 is located between the 18S subunit at the 5' end and the 5.8S subunit at the 3' end, ITS2 between the 5.8S subunit at the 5' end and the 26S subunit at 3' end (White *et al.* 1990, Baldwin, 1992). The length of ITS is approximately 500-700bp in Angiosperms (Baldwin *et al.*, 1995) up to 1500-3700bp in Gymnosperms (Bobola *et al.*, 1992; Liston *et al.*, 1996; Maggini *et al.*, 1998).

ITS is the only nuclear marker which has been used frequently in scientific approaches involving phylogenetic comparisons at generic level and below. Two thirds of the published papers from 1997-2002 (Alvarez & Wendel, 2003) used ITS sequence data. One third of all published papers including phylogenetic topologies solely relied on ITS, without utilizing any other marker (Alvarez & Wendel, 2003).

Figure 13: Organization of the ITS region in Angiosperms. Arrows indicate primer direction and primer site. Primer names from White *et al.* (1990). (modified from Baldwin (1992))
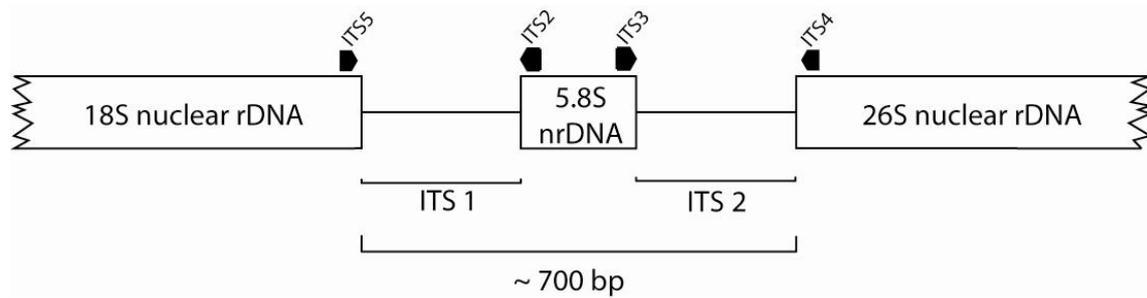
Universal primers for ITS were made available by White *et al.* (1990). These primers were designed in the flanking regions that are highly conserved. This allows the use of the universal primers which were primarily designed for an Ascomycote, (White *et al.*, 1990: *Pyrenophora*) in fungi as well as in plants. A high copy number of nrDNA regions eases the isolation and amplification of ITS. In addition, the short length makes it an ideal marker to use even for herbarium specimen, although Liston *et al.* (1996) states that this small fragment may reduce the phylogenetic utility. The variability of ITS is very high, which makes it interesting especially at specific and generic level. Difficulties in aligning the spacer sequences, or homoplasy, prohibit the use of ITS (Baldwin, 1992, Baldwin *et al.,* 1995) at higher taxonomic level.

The ITS marker is biparental inherited, which should make it useful for detection of reticulation, hybrid speciation or parentage of polyploids (Kim & Jansen, 1994*;* Wendel *et al.*, 1995). However, concerted evolution makes ITS and nrDNA in general an unreliable marker for the detection of parental lineages in a hybrid. Concerted evolution is a homogenization of repeated copies with different nucleotide sequences, theoretically resulting in a homogenized sequence for all copies of this region, i.e. biased homogenization towards one parental copy. The event of sequence homogenization may not be complete, which could be due to multiple unlinked loci (nrDNA on different chromosomes) or very high nucleotide substitution rates, disrupting the homogenization of all sequences. Main facilitators for concerted evolution are gene conversion and unequal crossing-over. (Zimmer *et al.*, 1980; Wendel *et al.*, 1995*;* Buckler *et al.,* 1996).To compensate for this unreliability, a broad sampling may help (Baldwin *et al.*, 1995*;* Alvarez & Wendel, 2003). Incomplete concerted evolution can also be caused by the degeneration of some nrDNA repeats to pseudogenes (Alvarez & Wendel, 2003), that may evolve at different rates than the functional regions.

Using the universal primers by White *et al.* (1990) bears different risks. Organic contamination, e.g. by fungi spores, might lead to amplification of the wrong ITS sequence. Pseudogenes are

more likely to be amplified with universal primers than with specific primers for the investigated group (Alvarez & Wendel, 2003; Mayol & Rosselló, 2001). Concerted evolution, as stated above, may not be complete, and may fail to affect all copies of the region (Baldwin *et al.*, 1995; Alvarez & Wendel, 2003). If an individual that did undergo incomplete concerted evolution in a nrDNA region subject to a phylogenetic investigation, it might cause incongruences in a phylogeny (Sanderson & Doyle, 1992; Baldwin *et al.*, 1995; Alvarez & Wendel, 2003). To avoid those risks, cloning or southern blotting might be necessary to distinguish the paralogous copies of such individuals (Baldwin *et al.*, 1995; Ritland *et al.*, 1993), or to rely on the conserved regions of the nrDNA genes, because they evolve much more slowly compared to the ITS or other noncoding regions of the nuclear genome.

b) Low-copy nuclear markers

Nuclear markers, though superior to plastid and nuclear ribosomal DNA markers in variability, have not been used until recently. We can trace this back to multiple problems, including gene duplication or deletion events, lineage sorting and concerted evolution. Gene duplication events create paralogues that can evolve at different rates, and over time, these previously identical copies might diverge from each other to take over different tasks in an organism. Also, the high variability often makes primer design efforting if not impossible. Universal markers up to now are scarce and not always applicable to all families. Low-copy nuclear genes evolve at different rates which complicates selection of the right region for an individual investigation. Depending on the level of investigation, different markers might give different results (Sang, 2002). One of the main utilities of nuclear genes is the reconstruction of hybridization. The biparental inheritance of chromosomes can help to find parents to a hybrid organism. A large set of genes equals a high number of multiple unlinked loci that can be used for phylogenetic analyses (Doyle, 1992; Emshwiller & Doyle, 1999). The structure of a typical functional gene shows an arrangement of one or more highly conserved coding regions, exons, together with variable noncoding regions, introns. This allows the design of specific primers for the desired regions, and, depending on the taxonomic level, makes it possible to influence the variability of a region. At higher levels, the coding regions seem preferable, whereas, at lower taxonomic levels intron regions are advantageous (Strand *et al.*, 1997; Hare, 2001; Small *et al.*, 2004). As there are no universal markers available, the utility of low-copy nuclear markers in phylogenetic investigations was limited until recently. Driving factors for this increase in use is the growing number of sequences available in open nucleotide databases, like GenBank. Schlüter *et al.* (2005) describe a number of ways to employ low-copy nuclear markers. Fingerprinting techniques are described e.g. by McLenachan *et al.* (2000) and Bailey *et al.* (2004).

2.) PLASTID MARKERS IN *SCORZONEROIDES*

2.1. Introduction

The analysis of the plastid genome has contributed tremendously to the understanding of relationships in plants. In the early 1990s, only a few plastid markers were used, e.g. *trnT-trnL-trnL-trnF* (Taberlet *et al.*; 1991), *rbcL* (Chase *et al.*, 1993), *matK* (Johnson & Soltis, 1994, 1995; Steele & Vilgalys, 1994; Hilu *et al.*, 2003) because the availability of universal primers was limited. With the beginning of the 2000s, all these laborious steps were automated, and the number of available universal primers for a range of markers developed (Shaw *et al.*, 2005, 2007). By that time an exponential increase in the use of molecular methods arose, with an emphasis on plastid and nuclear rDNA markers like ITS.

The advantages of plastid markers are a) their single copy origin, i.e. they are inherited strictly maternal in Angiosperms, and thus there is only one possible nucleotide sequence available. b) A high density of plastids in leaves reduces the needed amount of plant material to gain reasonable sequences, followed by only a low chance of degenerated DNA in all of the plastids. This enables the use of old herbarium specimen with these markers. c) Universal primers are available that can be used in Angiosperms with no or only little adjustments needed. With these primers come universal protocols for methods like PCR. The strictly maternal inheritance of plastids in angiosperms reveals only one side of evolutionary changes, thus detection of hybridization events is impossible. A low mutation rate compared to nuclear markers as well as a single locus origin, decrease the suitability of these regions at lower levels of classification.

Scorzoneroides was target of several molecular analyses (Samuel *et al.*, 2003, 2006; Cruz-Mazo *et al.*, 2009), which were based on plastid and ITS markers. While Samuel *et al.* (2006) employed coding and noncoding markers, namely *matK*, *trnL-Intron*, *trnL-trnF* as well as *ITS*, Cruz-Mazo *et al.* (2009) were focussing on the nrDNA marker *ITS* an noncoding regions available from Shaw *et al.*(2007), namely *ndhF-rpl32* and *rpl32-trnL*. The study by Samuel *et al.* (2006) showed incongruences with previous studies on *Leontodon*, based mainly on morphology (Widder, 1931, 1975) and chromosome counts (Pittoni, 1974; Izuzquiza, 1991; Izuzquiza & Nieto Feliner, 1991). The phylogenetic tree of chloroplast regions revealed *Leontodon* to be diphyletic, consequently lifting *Leontodon* subgenus *Oporinia* to a genus of its own, *Scorzoneroides* (Greuter *et al.*, 2006). Cruz-Mazo *et al.* (2009) confirmed the monophyly of *Scorzoneroides* and furthermore discovered a split into two groups in *Scorzoneroides*.

2.2. Material & Methods

2.2.1. Sampling

Most of the material used in this study was extracted from silica gel dried accessions from various collectors. Some extracts were made from herbarium specimens. 19 species of *Scorzoneroides* as well as 8 accessions from 7 species of *Leontodon*, one accession from 3 species of *Hypochaeris* and *Helminthotheca* each were used for the study. For further details, refer to Table 3.

2.2.2. DNA Extraction

DNA extraction was performed using a modified version of the standard-CTAB DNA extraction protocol by Doyle & Doyle (1987). Approximately 25 mg of leaf tissue were used from each voucher.

2.2.3. Primer selection

Universal primers, provided by Shaw *et al.* (2007) were employed in this study (Table 4). We ran primer trials to compare applicability as well as quality of the sequences (see Figure 14) and variation in *Scorzoneroides* of each selected marker.

Table 3: List of studied material indicating taxa name, project ID given in thesis and followed by locality information and voucher specimens. Sequenced regions are marked with x, unsuccessful sequencing marked by ---.

Species	ID	Origin	Collector(s) and number	Voucher location or reference	ndhF-rpl32	rpl16-Intron	atpH-atpI	GAPDH	A39-locus
Helminthotheca echioides (L.) Holub	L68	Austria, Niederösterreich	Tremetsberger & Stuessy s. n.	WU	x	x	x	---	x
Hypochaeris angustifolia (Litard. & Maire) Maire	H1	Morocco, Fontaine de Larais	Terrab & Talavera 73/06	SEV	---	---	---	---	---
Hypochaeris salzmanniana DC.	H2	Spain, Cádiz: Conil de la Frontera,	Talavera, Stuessy et al. TS5-1	WU	x	---	x	---	x
Leontodon cf. atlanticus	L32	France	Talavera, Stuessy et al. TS/KT 59	WU	x	x	x	---	x
Leontodon crispus Vill.	L5		Schönswetter & Tribsch 8909	WU	---	x	x	---	---
Leontodon hispidus L.	L57	Georgia	Ehrendorfer, s.n.	WU	x	x	x	---	x
Leontodon hispidus L.	L60		WG 37779	GU	---	x	---	---	---
Leontodon incanus Schrank	L36	Austria, Niederösterreich, Leobersdorf	WG 37630	GU	x	x	x	---	x
Leontodon maroccanus (Pers.) Ball	L39	Spain (Malaga): near Villanueva del Rosario	Spitaler & Zidorn CZ-20030421B-1	WU	x	x	x	---	x
Leontodon saxatilis Lam.	L3	Austria	WG 37572	WU	x	x	x	---	x
Leontodon villarsii (Willd.) Loisel.	L45	Austria, Tirol, Innsbruck	s.n.	WU	x	x	x	---	x
Scorzoneroides autumnalis (L.) Moench	S1	France	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides autumnalis (L.) Moench	S2	France	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides autumnalis (L.) Moench	S3	Spain	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides autumnalis (L.) Moench	S4	Spain, Huesca, Pirineos	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides autumnalis (L.) Moench	S71	Spain, Huesca, Pirineos	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides autumnalis (L.) Moench	S84	Spain, Huesca, Pirineos	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides autumnalis (L.) Moench	L2	Romania, n.a.	Greinler, s.n.	WU	x	x	x	---	x
Scorzoneroides cantabrica (Widder) Holub	S44	Spain, Palencia, Aguijas de Cardaño	Cruz-Mazo & Escudero 62	UPOS	x	x	---	---	x
Scorzoneroides cantabrica (Widder) Holub	S45	Spain, Palencia, Aguijas de Cardaño	Cruz-Mazo & Escudero 62	UPOS	x	x	x	---	x
Scorzoneroides cantabrica (Widder) Holub	S57	Spain, Palencia, Alto Campoo	Cruz-Mazo & Escudero 60	UPOS	x	x	---	---	---
Scorzoneroides cantabrica (Widder) Holub	S58	Spain, Palencia, Aguilar de Campoo	Cruz-Mazo & Escudero 63	UPOS	x	x	x	---	x
Scorzoneroides carpetana (Lange) Greuter	S9	Spain, Avila, Circo de Gredos	Cruz-Mazo & Fernández 23	UPOS	x	x	---	---	---
Scorzoneroides carpetana (Lange) Greuter	S10	Spain, Segovia, Puerto de la Cruz de Hierro	Cruz-Mazo & Fernández 26	UPOS	x	x	x	---	x
Scorzoneroides carpetana (Lange) Greuter	S66	Spain, Avila, Circo de credos	Cruz-Mazo & Fernández 22	UPOS	x	x	---	---	---
Scorzoneroides carpetana (Lange) Greuter	S74	Spain, Avila, Circo de credos	Cruz-Mazo & Fernández 23	UPOS	---	x	x	---	x
Scorzoneroides carpetana (Lange) Greuter	S75	Spain, Avila, Hoyocasero	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	---
Scorzoneroides cichoriacea (Ten) Greuter	1	Italy, Apulien	WG 36221	GU	x	x	x	---	x
Scorzoneroides cichoriacea (Ten) Greuter	2	Greece, Pindos, Koziakas	WG 35155	GU	x	x	---	---	x
Scorzoneroides crocea (Haenke) Holub	Gu	Austria, Kärnten, Lavanttaler Alpen	WG 26211	GU	x	x	x	---	x
Scorzoneroides crocea (Haenke) Holub	L11	Austria, Kärnten, Koralpen	Hö 2695	WU	x	x	x	---	x
Scorzoneroides duboisii (Sennen) Greuter	S12	Spain, Palencia, Aguijas de Cardaño	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides duboisii (Sennen) Greuter	S13	Spain, Palencia, Aguijas de Cardaño	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides duboisii (Sennen) Greuter	S15	Spain, Huesca	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	---
Scorzoneroides duboisii (Sennen) Greuter	S16	Spain, Palencia, Alto Campoo	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides duboisii (Sennen) Greuter	S18	Spain, Huesca	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides duboisii (Sennen) Greuter	S19	Spain, Lérida, Pirineos, Puerto de la Bonaigua	Cruz-Mazo & Escudero 39	UPOS	x	x	x	---	---
Scorzoneroides duboisii (Sennen) Greuter	S20	Spain, Lérida, Pirineos, P.N. Aigües Tortes	Cruz-Mazo & Escudero 40	UPOS	x	x	---	---	---
Scorzoneroides duboisii (Sennen) Greuter	S21	Spain, Lerida	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides garnironii (Emb. & Maire) Greuter	S22	Morocco	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides garnironii (Emb. & Maire) Greuter	S23	Morocco	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides garnironii (Emb. & Maire) Greuter	S64	Morocco, Antiatlas	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides garnironii (Emb. & Maire) Greuter	S65	Morocco, Antiatlas, Tafraoute- Tifnite	Talavera et al.,178/03 M	SEV	x	x	x	---	x
Scorzoneroides garnironii (Emb. & Maire) Greuter	L40	Morocco, Antiatlas. Tafraoute- Tifnite	Talavera et al.178/03 M	SEV	x	x	x	---	x
Scorzoneroides helvetica (Mérat) Holub	L9	Austria, Kärnten, Koralpe, Großes Kar	WG 26233	GU	x	x	x	---	x

WU = University of Vienna, Herbarium
UPOS = University Pablo de Olavide, Herbarium
GU = Herbarium Gutermann, Vienna
SEV = University of Sevilla, Herbarium

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Table 3 continued

Species	ID	Origin	Collector(s) and number	Voucher location or reference	ndhF-rpl32	rpl16-Intron	atpH-atpI	GAPDH	A39-locus
Scorzoneroides helvetica (Mérat) Holub	Gu	Austria, Vorarlberg, Allgäuer Alpen	WG 38110	GU	x	x	x	x	x
Scorzoneroides lacinata (Bertol.) Greuter	L53	Syria, Syria, Resata	Ehrendorfer s.n.	WU	x	x	x	x	x
Scorzoneroides montana subsp. *melanotricha* (Vierh.) Gutermann	Gu1	Italy, Julische Alpen	WG 38219	GU	x	x	x	x	x
Scorzoneroides montana subsp. *melanotricha* (Vierh.) Gutermann	Gu2	Italy	WG 38182	GU	x	x	x	x	x
Scorzoneroides microcephala (DC.) Holub	S24	Spain, Granada, Sierra Nevada	Cruz-Mazo et al., s.n.	UPOS	x	x	x	x	x
Scorzoneroides microcephala (DC.) Holub	S59	Spain, Sierra Nevada, Laguna de aguas verdes	Cruz-Mazo & Escudero 59	UPOS	x	x	x	x	x
Scorzoneroides montana (Lam.) Holub subsp. *montana*	L23	Austria, Steiermark, goßer Priel	Hö 1498	WU	x	x	x	x	---
Scorzoneroides montana (Lam.) Holub subsp. *montana*	3	Italy, Bozen (Wolfendorn)	WG 26331	GU	x	x	x	x	x
Scorzoneroides muelleri subsp. *austromaroccana* (Maire) Greuter	S50	Morocco, Gran Atlas, Taroudant-Marrakesh	Cruz-Mazo et al., s.n.	UPOS	x	x	x	x	x
Scorzoneroides muelleri subsp. *austromaroccana* (Maire) Greuter	S51	Morocco, Gran Atlas, Taroudant-Marrakesh	Cruz-Mazo et al., s.n.	UPOS	x	---	x	x	x
Scorzoneroides muelleri subsp. *austromaroccana* (Maire) Greuter	S52	Morocco Kenitra, Mediya	Cruz-Mazo et al., s.n.	UPOS	x	x	x	x	x
Scorzoneroides nevadensis (Lange) Greuter	S5	Spain, Granada, Sierra Nevada	Cruz-Mazo et al., s.n.	UPOS	x	x	x	x	---
Scorzoneroides nevadensis (Lange) Greuter	S7	Spain, Granada, Trevélez, Sierra Nevada	Cruz-Mazo & De Vega 30	UPOS	x	x	x	x	x
Scorzoneroides nevadensis (Lange) Greuter	S86	Spain, Granada, Sierra Nevada, Puerto de la Ragua	WG 37214	GU	x	---	x	---	x
Scorzoneroides nevadensis (Lange) Greuter	S87	Spain, Granada, Sierra Nevada, Hoyo de la Mora	Cruz-Mazo & De Vega 28	UPOS	---	---	x	---	x
Scorzoneroides oraria (Maire) Greuter & Talavera	S55	Morocco, Playa Tifinite	Cruz-Mazo et al., 56	UPOS	---	---	---	---	x
Scorzoneroides oraria (Maire) Greuter & Talavera	4	Morocco, Playa Tifinite	Cruz-Mazo et al., s.n.	UPOS	x	x	x	x	x
Scorzoneroides oraria (Maire) Greuter & Talavera	7	Morocco, Playa Tifinite	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides palisiae (Izug.) Greuter & Talavera	S36	Spain, Sevilla, Villaverde del Rio	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides palisiae (Izug.) Greuter & Talavera	S37	Spain, Sevilla, Puebla del Rio	Cruz-Mazo et al., s.n.	UPOS	x	---	---	---	x
Scorzoneroides palisiae (Izug.) Greuter & Talavera	S39	Spain, Sevilla, Villaverde del Rio	Cruz-Mazo et al., s.n.	UPOS	x	---	---	---	x
Scorzoneroides palisiae (Izug.) Greuter & Talavera	S80	Spain, Sevilla, Huelva	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides palisiae (Izug.) Greuter & Talavera	S82	Spain, Spain, Córdoba, Almodóvar del rio, Pantano de Breña	Cruz-Mazo et al. 13	UPOS	x	x	x	---	x
Scorzoneroides palisiae (Izug.) Greuter & Talavera	S83	Spain, Spain, Córdoba, Almodóvar del rio, Pantano de Breña	Cruz-Mazo et al. 13	UPOS	x	x	x	x	x
Scorzoneroides pyrenaica (Gouan) Holub	S41	Spain, Huesca	Cruz-Mazo et al., s.n.	UPOS	x	x	x	---	x
Scorzoneroides pyrenaica (Gouan) Holub	S42	Spain, Huesca	Cruz-Mazo et al., s.n.	UPOS	x	---	---	---	x
Scorzoneroides pyrenaica (Gouan) Holub	S43	Spain, Huesca	Cruz-Mazo et al., s.n.	UPOS	x	x	---	---	x
Scorzoneroides pyrenaica (Gouan) Holub	S63	Spain, Huesca, Benasque, Pirineos, ibón Gorgutes,	Cruz-Mazo & Escudero 41	UPOS	x	x	x	---	x
Scorzoneroides rilaensis (Hayek) Holub	L29	Romania, Sibiu, Mtii Fagarasului	Dobner & Zidorn 98-00084	WU	x	x	x	---	x
Scorzoneroides salzmannii (Sch. Bip) Greuter & Talavera	S54	Morocco, Morocco, Kenitra, Mamora forest	Cruz-Mazo et al. 55	UPOS	---	x	---	---	x
Scorzoneroides salzmannii (Sch. Bip) Greuter & Talavera	S90	Morocco, Morocco, Kenitra, Mamora forest	Cruz-Mazo et al. 55	UPOS	x	x	x	---	x
Scorzoneroides salzmannii (Sch. Bip) Greuter & Talavera	L44	Morocco	TS, Herb 17/04/03	WU	x	x	x	x	x

WU = University of Vienna, Herbarium
UPOS = University Pablo de Olavide, Herbarium
GU = Herbarium Gutermann, Vienna
SEV = University of Sevilla, Herbarium

Figure 14: Sequences from this study, examples for good, bad and polymorphic sequences as well as cloned sequences. (screenshots from DNA Lasergene 7.1 SeqMan)

good sequence

TACCAACTTCGAATTCAAGTAATAACATTCTTGAATCATCAAAACGA

bad sequence

ATTACTACCCCCTTTACCACTTTATTCGTATATGGAGTCGGGGATA

polymorphic sequence

CCTAAAGGTAACGGGTCCACCAAGGTAATGGTGGAATTTGTTTGCCT
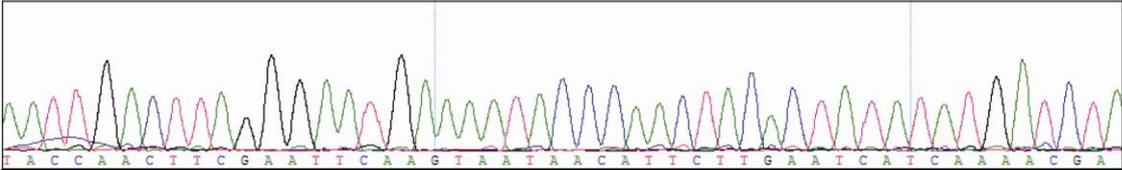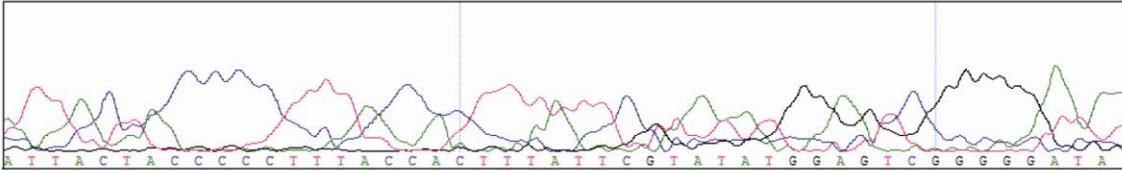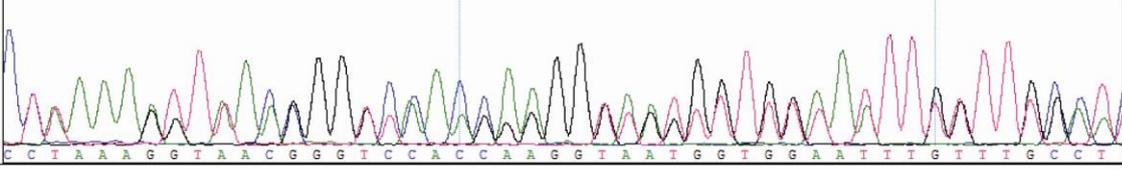
sequences obtained by cloning

CCTAAAAGTT---------AATGGGTCAACGAGGGTAGTGTTGTAAT

CCTAAAAGTT---------AATGGGTCAACGAGGGTAGTGTTGTAAT

CCTAAAAGTTAACGGGTCAAATGGGTCAACGAGGGTAGTGTTGTAAT

CCTAAAAGTTAACGGGTCAAATGGGTCAACGAGGGTAGTGTTGTAAT
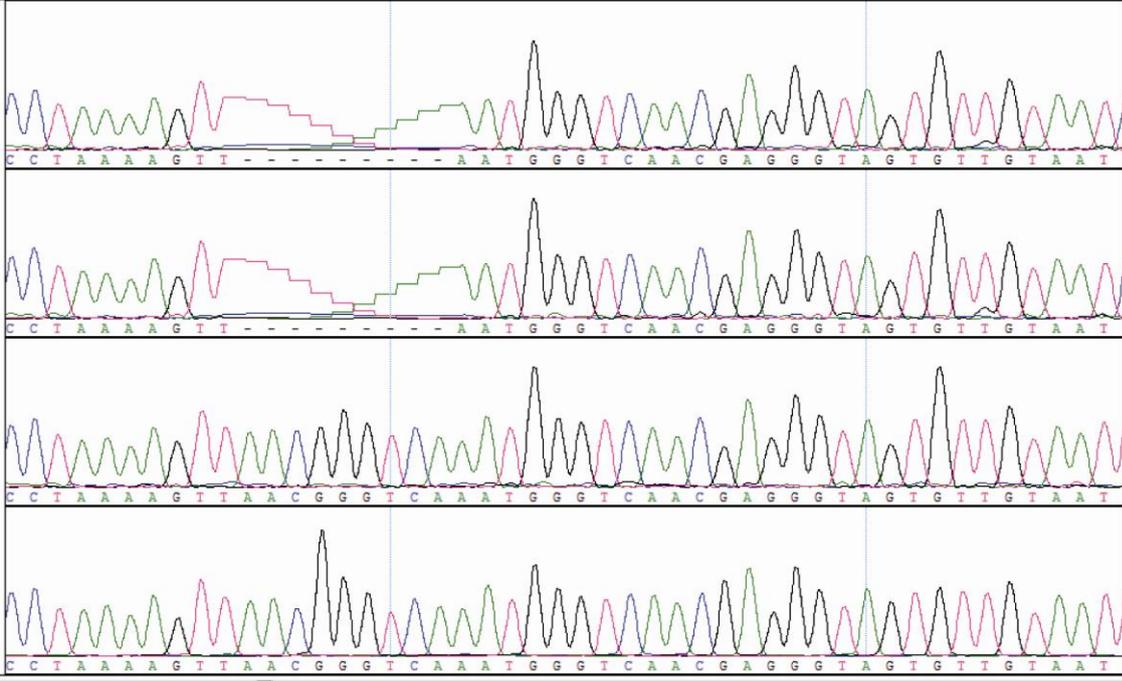
Table 4: Primers used for chloroplast markers in this study.

Region/Gene	Primer Name	Sequence	Reference
atpI - atpH	atpI	TAT TTA CAA GYG GTA TTC AAG CT	Shaw et al. (2007)
	atpH	CCA AYC CAG CAG CAA TAA C	Shaw et al. (2007)
ndhF - rpl32	ndhF	GAA AGG TAT KAT CAA YGM ATA TT	Shaw et al. (2007)
	rpl32-R	CCA ATA TCC CTT YYT TTT CCA A	Shaw et al. (2007)
petL - psbE	petL	AGT AGA AAA CCG AAA TAA CTA GTT A	Shaw et al. (2007)
	psbE	TAT CGA ATA CGT GTA ATA ATA TCA GC	Shaw et al. (2007)
psbD - trnT	psbD	CTC CGT ARC CAG TCA TCC ATA	Shaw et al. (2007)
	trnT(GGU)-R	CCC TTT TAA CTC AGT GGT AG	Shaw et al. (2007)
psbJ - petA	psbJ	ATA GGT ACT GTA RCY GGT ATT	Shaw et al. (2007)
	petA	AAC ART TYG ARA AGG TCC AAT T	Shaw et al. (2007)
rpl16 - Intron	rpl16-F71	GCT ATG CTT AGT GTG TGA CTC GGT G	Shaw et al. (2007)
	rpl16-R1516	CCC TTC ATT CTT CCT CTA TGT TG	Shaw et al. (2007)
rpoB - trnC	rpoB	CKA CAA AAY CCY TCR AAT TG	Shaw et al. (2007)
	trnC(GCAR)	CAC CCR GAT TYG AAC TGG GG	Shaw et al. (2007)
trnD - trnT	trnD(GUCF)	ACC AAT TGA ACT ACA ATC CC	Shaw et al. (2007)
	trnT(GGU)	CTA CCA CTG AGT TAA AAG GG	Shaw et al. (2007)
trnQ - rps16	5'trnQ(UUG)	GCG TGG CCA AGY GGT AAG GC	Shaw et al. (2007)
	rps16x1	GTT GCT TTY TAC CAC ATC GTT T	Shaw et al. (2007)
trnS - trnfM	trnS(UGA)	GAG AGA GAG GGA TTC GAA CC	Shaw et al. (2007)
	trnfM(CAU)	CAT AAC CTT GAG GTC ACG GG	Shaw et al. (2007)
trnV - ndhC	3'trnV(UACx2)	GTC TAC GGT TCG ART CCG TA	Shaw et al. (2007)
	ndhC	TAT TAT TAG AAA TGY CCA RAA AAT ATC ATA TTC	Shaw et al. (2007)

2.2.4. PCR amplification and sequencing

For PCR conditions we used the standard protocol provided by Shaw *et al.* (2007) with slight modifications (Table 5).

Table 5 PCR conditions for all plastid DNA primers

Step	Temperature	Time	Cycles
Initial Denaturation	80°C	5 min	1
Denaturation	94°C	30 sec	36
Primer Annealing	50°C	30 sec	36
Extension	65°C	3 min	36
Final Extension	65°C	8 min	1

Figure 15: Images from gel electrophoresis of PCR products from four different plastid DNA markers.
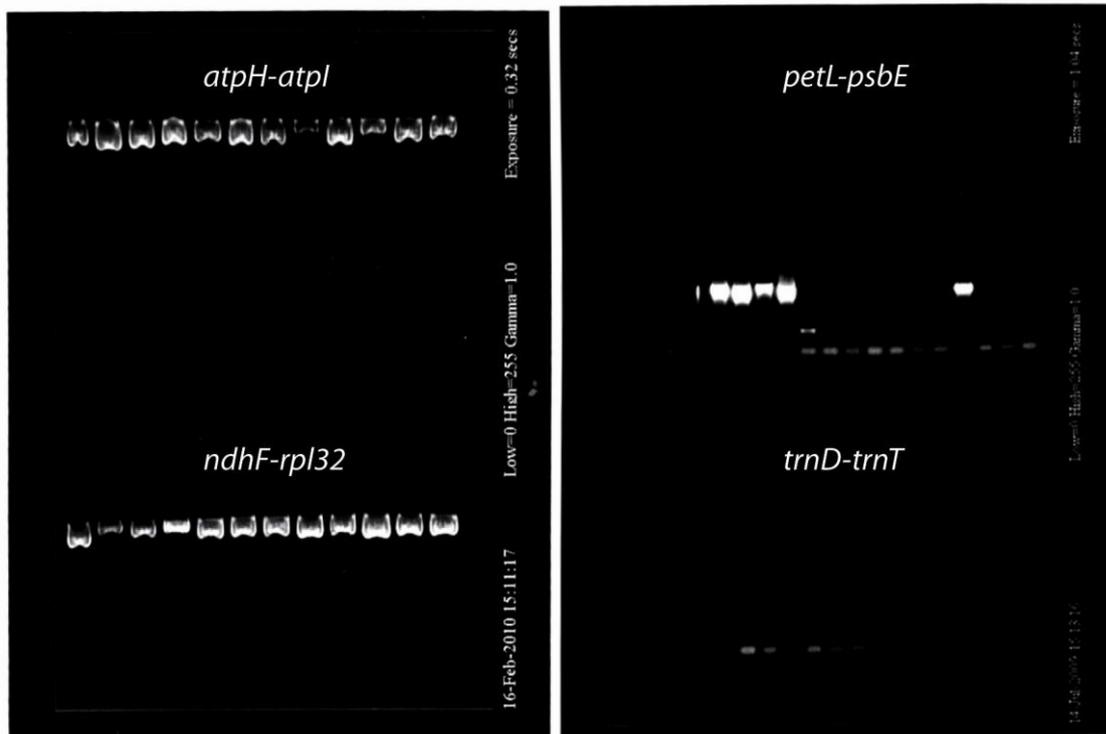
For all PCR reactions, we used a 14µl aliquot of master mix and 1µl of DNA extracts. The master mix contained 13.5µl ABGene ReddyMix PCR Master Mix1.1, 0.3µl Forward Primer (20µMol), 0.3µl Reverse Primer (20 µMol), 0.3µl BSA (Bovine Serum Albumine, 20µg/µl), 0.3µl DMSO (Dimethyl Sulfoxide). PCR products were checked on 1.5% TAE agarose gel (e.g. Figure 15), successfully amplified fragments with the expected length were purified using Exo-Fastap, containing Exonuclease I and FastAP (Fermentas), at standard conditions, 45 min incubation at 37°C, followed by 15 min denaturation at 85°C. This step eliminates most of the remaining dNTPs or unfinished fragments. Cycle Sequencing reactions were done in 10µl reactions, each containing 0.4µl Big Dye Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 1.8µl Sequencing buffer, 1.0µl Primer (4 µMol), 1.8µl dH$_2$O and 5.0µl previously cleaned PCR product, with PCR conditions given in Table 6.

Table 6: PCR conditions for cycle sequencing.

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	96°C	1 min	1
DENATURATION	96°C	5 sec	30
PRIMER ANNEALING	50°C	10 sec	30
EXTENSION	60°C	3 min	30

Sequencing was carried out on a 48-well capillary sequencer, Applied Biosystems 3130XL Genetic Analyzer following the manufacturer's protocols.

2.2.5. Phylogenetic analysis

To assemble the homologous sequences, we employed DNAStar Lasergene 7.1 Seqman, with minor changes in the assembling protocol. The minimum match percentage to create a contig was lowered from 80% to 60%, and consensus calling was set to a majority percentage of 100 instead of 75%. Alignments were created utilizing ClustalX2 as well as Muscle v3.6 with standard conditions. Every alignment was manually adjusted using BioEdit Sequence Alignment Editorv7.0.9.0.

Phylogenetic analysis was carried out with Paup4b10 (Swofford, 2002). Heuristic searches with maximum parsimony criterion tree-bisection and reconnection (TBR) branch swapping and 1000 replicates were performed, 5 (1) trees were held for each replicate. Gaps were treated as missing data; multistate taxa were interpreted as uncertainty, starting trees were obtained via random stepwise addition. No more than 25 (10) trees of score (length) greater than 25 (10) were saved in each replicate. Branches were collapsed, if maximum branch length equaled zero.

Bootstrap analysis was performed with 1000 replicates, each replicate included 10 heuristic searches with maximum parsimony criterion tree-bisection and reconnection (TBR) branch swapping attributes, 1 tree was held for each heuristic step. Gaps were treated as missing data; multistate taxa were interpreted as uncertainty, starting trees were obtained via random stepwise addition. No more than 10 trees of score (length) greater than 10 were saved in each replicate. Branches were collapsed, if maximum branch length equaled zero.

Matrices of each spacer region were analyzed individually; the three best markers were combined. For more information on these markers, refer to Table 7. Two trees, a majority rule consensus tree and a bootstrap consensus tree were created for each of the three trees as well as for the combined matrix. The majority rule consensus tree was chosen over a phylogram of one most parsimonious tree due to short branch lengths at intraspecific levels in *Scorzoneroides*.

Table 7: Detailed information to the chloroplast regions used for the full dataset in this study.

	atpH-atpI	*ndhF-rpl32*	*rpl16-Intron*	combined
No. of samples	73	76	79	60
No. of species	29	32	32	27
Length of sequences	1140 - 1251	1063-1139	993-1163	3217-3401
Length of alignment	1194	1202	1279	3675
No. of constant characters	1095	1045	1119	3342
No. of constant characters in *Scorzoneroides*	1095	1066	1184	3363
No. of variable characters	99	157	160	333
No. of variable characters in *Scorzoneroides*	99	136	95	312
No. of informative characters	58	85	79	180
No. of informative characters in *Scorzoneroides*	50	82	55	169
No. of gaps	5	16	5	37
No. of shortest trees	24	16	21750	18525
Length of shortest trees	114	193	193	415
Consistency Index (CI)	0.875	0.8531	0.9171	0.8644
Retention Index (RI)	0.9825	0.9685	0.957	0.9521

2.3. Results

2.3.1. Primer Choice

With *ndhF-rpl32* we tested a marker that was already used by Cruz-Mazo *et al.* (2009). The average length was 1020 base pairs; its ingroup-informativity was found to be the highest among the tested regions (Parsimony Informative Characters in percent of the overall Characters, further PIC, 2.84%). Despite its high variability we struggled to get good assemblies of the homologous sequences. The *rpl16-Intron*, with an average length of 1029 base pairs (bp), which was easily assembled and aligned, provided the second highest variability (PIC 2.72%). No monomeric repeats were observed, however, a microsatellite region was observed. By far lower informativity was observed in *atpH-atpI* (PIC 1.31%), which had a length of 1142bp on average. Three monomeric repeats were reported, however, those did not interfere in successful use of the marker.

The following regions were not used further in this study. 3'*trnV-ndhC* (PIC 1.2%) averaged 1022 base pairs, *trnS-trnfM* (1.07% PIC) average length of 1120 bp, *psbD-trnT* (PIC 1.04%) with 1252bp average length, showed monomeric repeats which hindered the alignment of sequences successfully. *psbJ-petA* provided us with very easily applicable fragments with no monomeric repeats but low variability (PIC 1.03%). The mean base pair number was 876.

5'trnQ-rps16 (953 base pairs in average) showed no monomeric repeats, but a low number of variable sites in our trial (PIC 0.52%). The location of the 9 regions of the chloroplast DNA successfully amplified are given in Figure 12.

Figure 16: Successfully amplified chloroplast regions based on sequence data from this study Primers used are indicated by black arrows, monomeric repeats are marked with red arrows. Regions are unscaled in length

atpI-atpH INTERGENIC SPACER

Intergenetic Spacer (avg. length 1142 bp)

0 bp   400 bp   800 bp   1200 bp
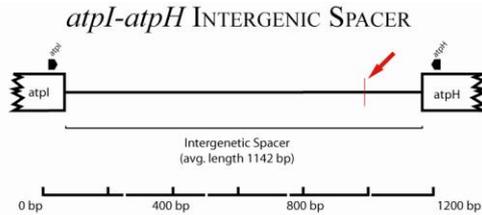
Diagram of the *atpI-atpH* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black. Poly-A/T regions are shown in red

ndhF-rpl32 INTERGENIC SPACER

Intergenetic Spacer (avg. length 1020 bp)

0 bp   400 bp   800 bp   1200 bp
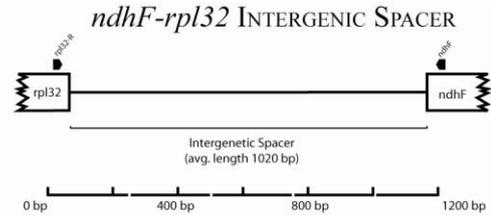
Diagram of the *ndhF-rpl32* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black.

psbD-trnT INTERGENIC SPACER

Intergenetic Spacer (avg. length 1252 bp)

0 bp   625bp   1250 bp

Diagram of the *psbD-trnT* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black. Poly-A/T regions are shown in red

psbJ-petA INTERGENIC SPACER

Intergenetic Spacer (avg. length 953 bp)

0 bp   333 bp   666 bp   1000 bp

Diagram of the *psbJ-petA* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black.

rpl16-INTRON

Intragenetic Spacer (avg. length 1029 bp)

0 bp   400 bp   800 bp   1200 bp
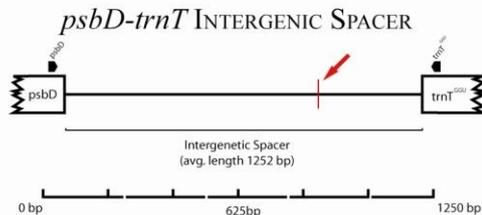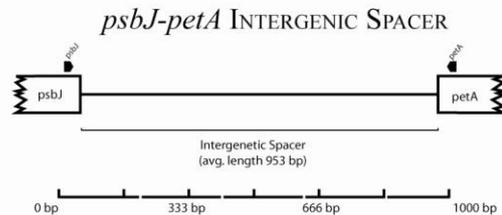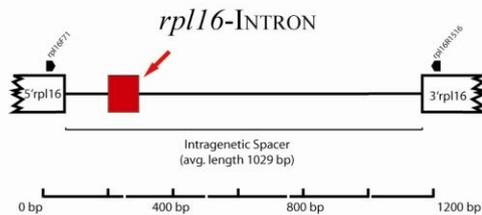
Diagram of the *rpl16* Intron region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black.Poly-A/T regions are shown in red

trnQ-rps16 INTERGENIC SPACER

Intergenetic Spacer (avg. length 953 bp)

0 bp   333 bp   666 bp   1000 bp
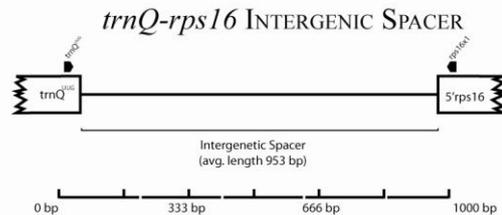
Diagram of the *trnQ-rps16* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black.

trnS-trnfM INTERGENIC SPACER

Intergenetic Spacer (avg. length 1120bp)

0 bp   400bp   800bp   1200 bp
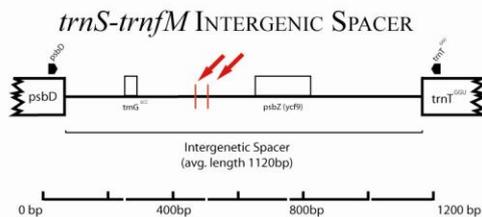
Diagram of the *psbD-trnT* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black. Poly-A/T regions are shown in red. Internal coding regions are added approximately
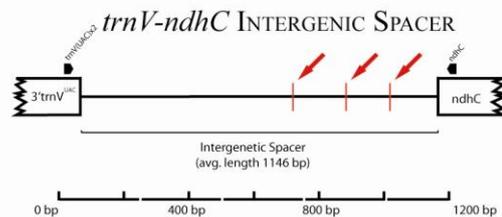
trnV-ndhC INTERGENIC SPACER

Intergenetic Spacer (avg. length 1146 bp)

0 bp   400 bp   800 bp   1200 bp

Diagram of the *psbJ-petA* intergenic Spacer region of the chloroplast DNA based on sequences of this study. Primers used for amplification are shown in black. Poly-A/T regions are shown in red

2.3.2. *rpl16*-Intron

The monophyly of *Scorzoneroides* (BS 70) is supported, having two major clades within the genus (Figure 17, 18). Clade 1 (Figure 18, BS 94) comprises all members of the ecologically

diverged group of mostly annual Mediterranean and dryland occupants (*S.laciniata*, *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii*) as well as several accessions of perennial continental and mountain inhabiting species, namely *S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.garnironii* and *S.nevadensis*. These two groups are well-supported (BS 86), clading together the annuals as well as one perennial taxon, *S.garnironii*. *Scorzoneroides garnironii* (C) is sister to two groups, one comprising *S.laciniata*, *S.muelleri* and *S.oraria* (A, BS 87), and the other containing *S.palisiae* and *S.salzmannii* (B, BS 51). The perennials with exception of *S.garnironii*, fall together in one big polytomy, the only diverged seqences we obtained were from two accessions of one population of *S.duboisii* (E, BS86) and from one accession of *S.nevadensis*. The second major group (Clade 2, BS 54) in *Scorzoneroides* comprises perennial mountain taxa, namely *S.cantabrica*, *S.cichoracea*, *S.crocea*, *S.helvetica*, *S.melanotricha*, *S.microcephala*, *S.montana*, *S.pyrenaica* and S.rilaensis. *Scorzoneroides microcephala* (H, BS 62) is sister to the rest of the group (BS 66). The sequences of *S.cichoracea* accessions (F, BS 100) and two of three accessions of *S.pyrenaica* (G, BS 83) are forming groups of their own.

The genus *Leontodon* is monophyletic with *rpl16*-Intron sequences (I, BS 84), it is showing a well-resolved phylogeny. *Helminthotheca* is sister to *Leontodon* (J, BS 55). *Hypochaeris salzmanniana* is basal, as sister to the rest of the Hypochaeridinae (K, BS 100).

2.3.3. *ndhF-rpl32* intergenic spacer

Monophyly of *Scorzoneroides* is supported (BS 100; Figure 19, 20). A split into two major clades (as in *rpl16*-intron) can be observed with this marker. The first Clade (Clade 1, Figure 20; BS 100) comprises Perennial Mountain or continental taxa (*S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.garnironii* and *S.nevadensis*) and annual inhabitants of Mediterranean and dryland areas (*S.laciniata*, *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii*). Three diverged groups are obtained in this clade. In the first clade (A; BS 65) all perennial taxa are clustered together except for *S.garnironii* (D), which is sister to groups A to C. The second separated group (B; BS 63) comprises *S.palisiae* and *S.salzmannii*. Three out of five accessions of *S.palisiae* form a subclade (BS 62) diverged from the rest of this group. The third clade (C; BS 65), is polytomous and formed by *S.laciniata*, *S.muelleri* and *S.oraria*,.

The second major clade (Clade 2; BS 69) comprises *S.cantabrica*, *S.cichoracea*, *S.crocea*, *S.helvetica*, *S.melanotricha*, *S.microcephala*, *S.montana*, *S.pyrenaica* and *S.rilaensis*. *Scorzoneroides microcephala* (F; BS 100) is sister to the main group formed by the rest of Clade 2 (E; BS 54). This polytomous clade is only resolved in two cases: both accessions of *S.melanotricha* are grouping with one of two accessions of *S.montana* (E1; BS 64), and a group of two of three accessions of *S.pyrenaica* (E2; BS 64) fall together.

All accessions of *Hypochaeris* form a highly supported group (G; BS 99), so does *Leontodon* (H1; BS 93). *Helminthotheca* (H; BS 99) is sister to this well-supported monophyletic group.

2.3.4. *atpH-atpI* intergenic spacer

Scorzoneroides' monophyly is well-supported by the results obtained with this marker (Figure 21, 22; BS 100). Two major clades are recognized as in *rpl16*-Intron and *ndhF-rpl32* phylogenies. The first clade (Clade1, Figure 22; BS 57) contains the perennial continental and mountain taxa *S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.garnironii*, *S.microcephala*, *S.nevadensis* and *S.garnironii*, as well as all accessions of the annual species *S.laciniata*, *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii*. *Scorzoneroides microcephala* is separated (B; BS 93) as sister to the rest of Clade1 (A; BS 89). The major group A is polytomous, with three divergent groups. *Scorzoneroides laciniata*, *S.muelleri*, *S.oraria* and one accession of *S.palisiae* cluster together (A1; BS 58), forming a polytomy with exception of the two accessions of *S.oraria* (A1-1; BS 80), which forms a well-supported group. One accession of each *S.autumnalis*, *S.garnironii* and *S.palisiae* fall into a separate group (A2; BS 64). Both accessions of *S.salzmannii* form a cluster on their own (A3; BS 87).

Clade 2 (BS61) is comprised by perennial mountain taxa, namely *S.cantabrica*, *S.cichoracea*, *S.crocea*, *S.helvetica*, *S.melanotricha*, *S.montana*, *S.pyrenaica* and *S.rilaensis*. In this clade, we observe two subclades, one consisting of both accessions of *S.melantotricha* and one accession of *S.crocea* and *S.montana* (C; BS 61). Within this subclade a group of one accession of each *S.melanotricha* and *S.montana* are put together (C1; BS 60). The second subclade of Clade 2 contains both accessions of *S.cichoracea* (D; BS 95).

Hypochaeris salzmannii is sister to *Scorzoneroides* with this marker (E; BS 100). *Leontodon* is a monophyletic group (F; BS 80). The one accession of *Helminthotheca* is sister to the rest of Hypochaeridinae.

2.3.5. Combined analysis

The phylogenetic tree of the combined matrix of the three plastid markers *atpH-atpI*, *ndhF-rpl32* and the *rpl16*-Intron supports the monophyletic status of the genus *Scorzoneroides* (Figure 23, 24; BS 93), with a clear separation into two major groups, clade 1 and clade 2. Clade 1 (BS 100) comprises all members of the annuals occupying dryland or Mediterranean regions, namely *S.laciniata*, *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii* as well as a group of perennials inhabiting continental or mountain areas, *S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.garnironii* and *S.nevadensis*. The combined analysis reveals two subclades of Clade1. Subclade A (A; BS 61) includes all members from the perennial group except for *S.garnironii*.

A diverged group of 3 accessions from *S.duboisii*, all 4 accessions of *S.carpetana* and one accession of *S.autumnalis* (A1; BS 60) in subclade A is observed. Subclade B (B; BS 87) consists of all annuals as well as *S.garnironii*. Two divergent groups are formed in this clade, one comprising *S.palisiae* and *S.salzmannii* (B1; BS 71) the other (B2; BS 98) *S.laciniata*, *S.muelleri*i and *S.oraria*. Three out of 5 accessions of *S.palisiae* (B1-1; BS 60) as well as both accessions of *S.salzmannii* (B1-2; BS 89) are separated in the first group, *S.oraria* accessions (BS87) were clading together.

The second major group in *Scorzoneroides* is highly supported (Clade 2; BS 93). *Scorzoneroides microcephala* (D; BS 100) is sister to a large clade (C; BS 86) comprising perennial mountain taxa, namely *S.cantabrica*, *S.cichoracea*, *S.crocea*, *S.helvetica*, *S.melanotricha*, *S.montana*, *S.pyrenaica* and *S.rilaensis*. Three diverging groups are observed. Both accessions of *S.melanotricha* and one accession of *S.crocea* and of *S.montana* form the first subclade (C1; BS 64), with *S.crocea* being sister to the other members of the group (BS 62). The accessions of *S.cichoracea* (C2; BS 100) and two out of three accessions of *S.pyrenaica* (C3; BS 96) are the other divergent clades.

The genus *Leontodon* is confirmed to be monophyletic (G; BS 100). *Helminthotheca* (H) was sister to the rest of Hypochaeridinae.

2.4. Discussion

The monophyly of *Scorzoneroides* as previously suggested (Samuel *et al.*, 2005; Cruz-Mazo *et al.*, 2009) is supported by the results obtained by each of the chloroplast markers, namely the two intergenic spacer regions *atpH-atpI* and *ndhF-rpl32* as well as the *rpl16-Intron* applied in our study as well as the combined data. We recognized two major groups, Clade 1 and Clade 2. Clade 1 always comprised all annuals, *S.laciniata*, *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii* as well as several perennials, namely *S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.garnironii*, *S.nevadensis* and *S.garnironii*.

The combined analysis strongly suggested a split of Clade 1 into two subclades, A and B. These subclades reflect the differences in ecology of the annuals (Subclade B) to the perennials (Subclade A) in the group. However, *S.garnironii,* although it shows a typical perennial life-cycle as well as a distribution in mountain areas was always grouping together with annuals of subclade B. Cruz-Mazo *et al.*(2009) suggested a secondary development of perennial lifecycle and adaptation to the distribution area.

In Subclade A, sequence divergence between different species seem very low. This might be due to recent speciation events, a close sampling range of the accessions or sampling errors.

The *atpH-atpI* analysis suggested *S.microcephala* to be a member of Clade 1, whereas *ndhF-rpl32* and *rpl16-Intron* analyses put it into Clade 2. In all cases, *S.microcephala* was never nested directly in a major group, but was sister to these.

Clade 2 shows a lower sequence divergence compared to Clade 1, a polytomy covers almost the whole group even in the combined marker. When *Scorzoneroides* was lifted to generic status, several allopatric subspecies in this Clade were treated as species. The morphological characters to distinguish between these species are very scarce (e.g. Zidorn *et al.*, 2008); sometimes hair colour becomes the only difference between these species. The consideration of separate taxa therefor might be an exaggeration if one emphasises on molecular data and leaves geographical patterns or barriers out of his speculation.

Leontodon is well-supported by our analysis of the three chloroplast regions. High sequence divergence between each included member of *Leontodon* as well as a high bootstrap support suggests a well-established genus.

The only accession of *Helminthotheca* in our study turned out to be either sister of *Leontodon* (*rpl16-Intron*, *ndhF-rpl32*) or sister to the other accessions of Hypochaeridinae (*atpI-atp*, combined matrix).

Low informativity of *atpH-atpI* in particular might have caused long-branch attraction (Sullivan & Swofford, 2001) or homoplasy. The *rpl16-Intron* suffers from possible convergent evolution because of a long polyA/T repetitive loop-fragment. Exclusion of that region did not change the phylogeny.

Overall, the generic status of *Scorzoneroides* (Greuter *et al.*, 2006) was confirmed. Likewise, we observed a monophyletic status for *Leontodon*. The available variability allowed us to reveal a separation of two clades, Clade 1 and Clade 2, in *Scorzoneroides* with a further division into subclades A and B in case of one clade.

We could, however not clearly resolve the phylogenetic relations at specific level, with one clear exception; *Scorzoneroides microcephala* is basal and showed a divergent sequence to the rest of *Scorzoneroides*. A few accessions nested in the previously mentioned Clades 1 or 2 were grouping together in all of the three markers phylogenetic trees. *Scorzoneroides palisiae* and *S.salzmannii*, as well as *S.laciniata, S.muelleri* and *S.oraria* of Clade 1 tended to fall into separated groups in all three plastid regions' sequence. In Clade 2, all of the markers separated *S.cichoracea*.

Plastid markers were only considered as a basis of information, because previous studies already showed a low sequence divergence.

Figure 17: Majority rule consensus tree of 21750 retained trees of the *rpl16*-Intron region of the plastid DNA, including 61 accessions from 19 species of *Scorzoneroides* and 11 outgroup accessions from 10 species. Majority rule percentage given above, bootstrap support and number of changes (calculated rounded median of three most parsimonious trees) below branches.

Figure 18: Bootstrap consensus tree of the *rpl16* Intron region of the plastid DNA, including 61 accessions from 19 species of *Scorzoneroides* and 11 outgroup accessions from 10 species. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches
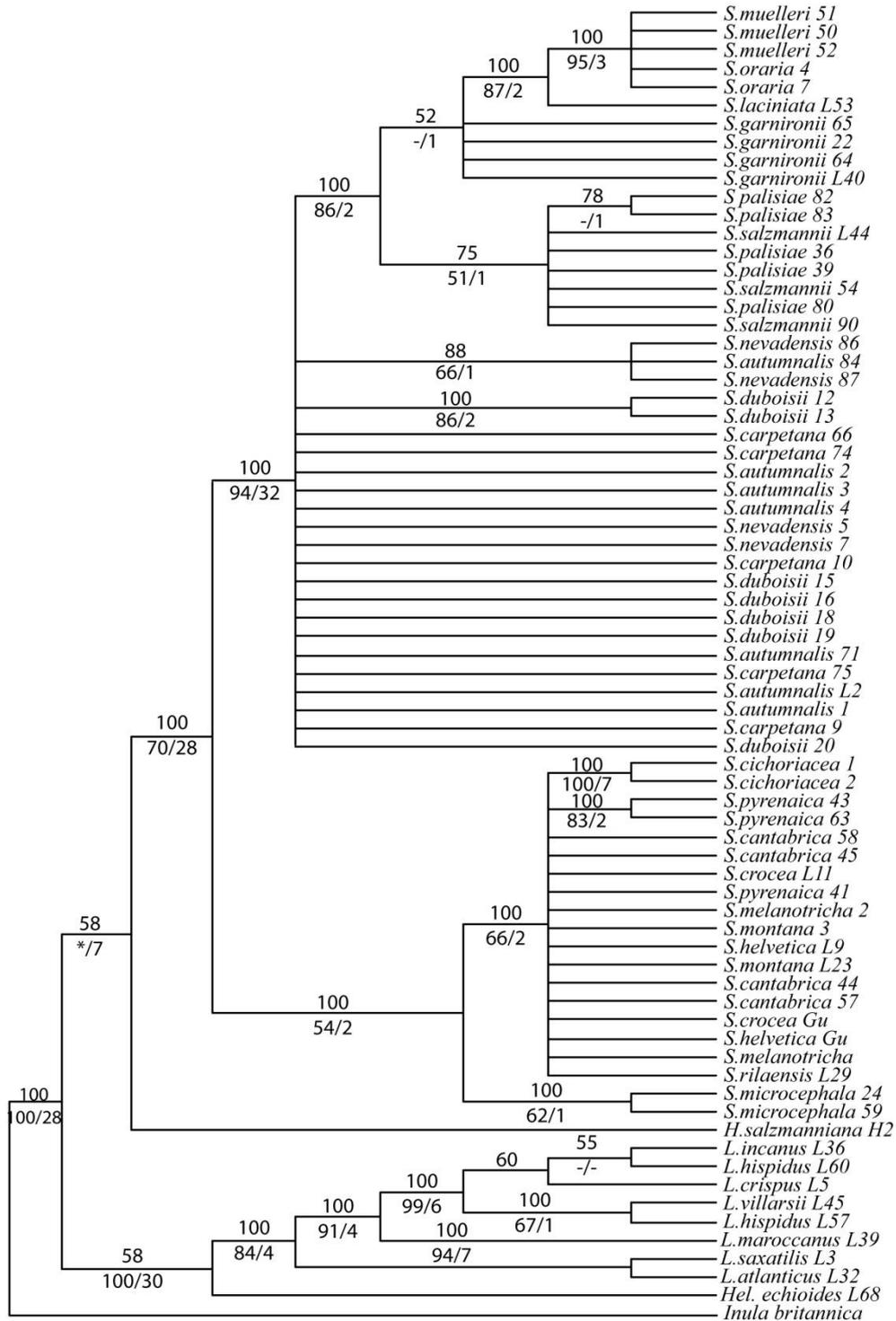
Figure 19: Majority rule consensus tree of 16 retained trees of the *ndhF-rpl32* intergenic spacer region of the plastid DNA, including 59 accessions from 19 species of *Scorzoneroides* and 15 outgroup accessions from 11 species. Majority rule percentage given above, bootstrap support and number of changes (calculated rounded median of three most parsimonious trees) below branches.

Figure 20: Bootstrap consensus tree of the ndhF-rpl32intergenic spacer region of the plastid DNA, including 59 accessions from 19 species of *Scorzoneroides* and 15 outgroup accessions from 11 species. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches.
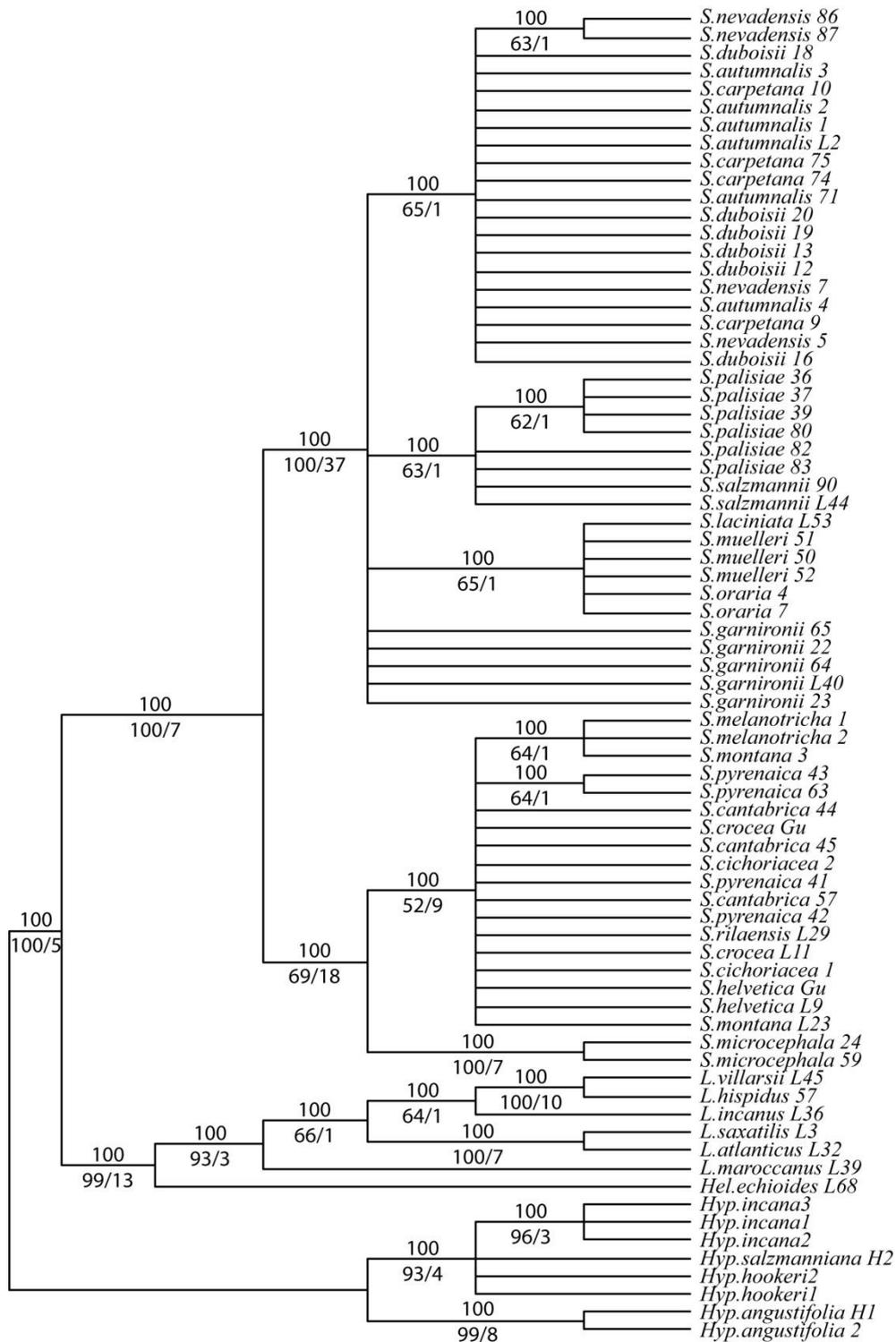
Figure 21: Majority rule consensus tree of 24 retained trees of the *atpH-atpI* intergenic spacer region of the plastid DNA, including 57 accessions from 19 species of *Scorzoneroides* and 10 outgroup accessions from 9 species. Majority rule percentage given above, bootstrap support and number of changes (calculated rounded median of three most parsimonious trees) below branches.

Figure 22: Bootstrap consensus tree of the *atpH-atpI* intergenic spacer region of the plastid DNA, including 57 accessions from 19 species of *Scorzoneroides* and 10 outgroup accessions from 9 species. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches.
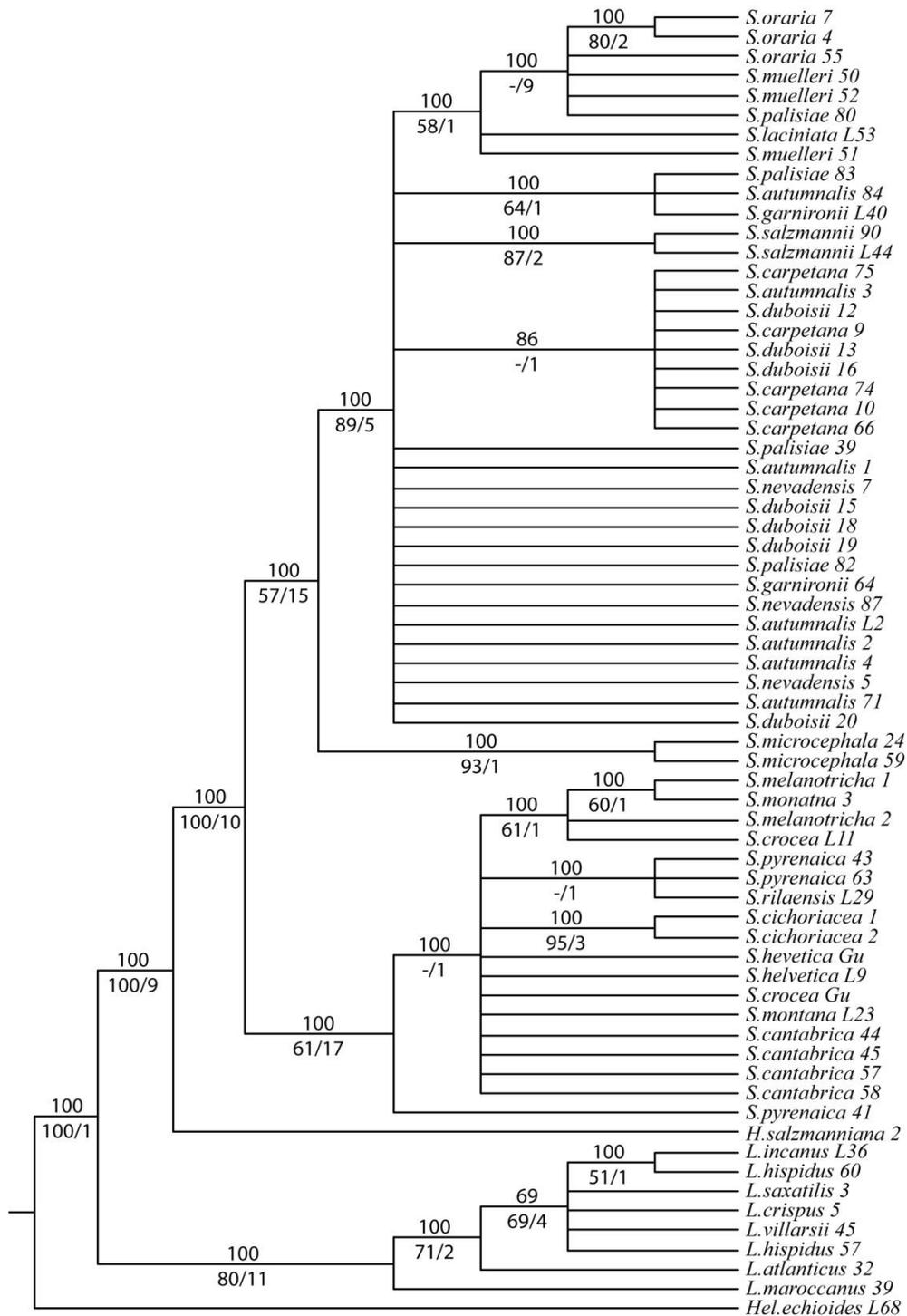
Figure 23: Majority rule consensus tree of 18525 retained trees of the matrix of the three combined chloroplast markers *rpl16*-Intron, *ndhF-rpl32* and *atpH-atpI* intergenic spacer regions of the plastid DNA, including 52 accessions from 19 species of *Scorzoneroides* and 8 outgroup accessions from 8 species. Majority rule percentage given above, bootstrap support and number of changes (calculated rounded median of three most parsimonious trees) below branches.

Figure 24: Bootstrap consensus tree of the three combined chloroplast markers *rpl16*-Intron, *ndhF-rpl32* and *atpH-atpI* intergenic spacer regions of the plastid DNA, including 52 accessions from 19 species of *Scorzoneroides* and 8 outgroup accessions from 8 species. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches.
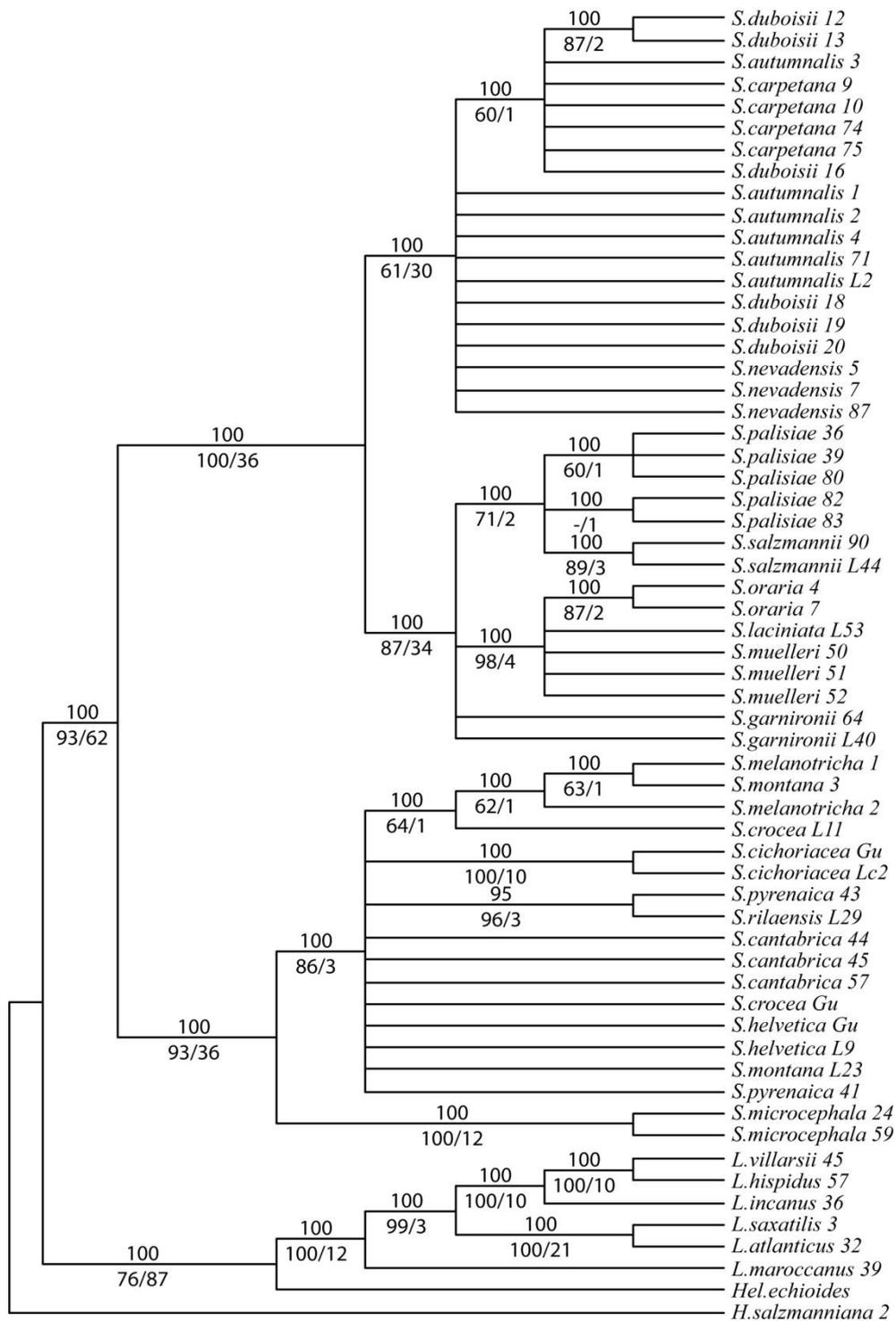
3.) NUCLEAR MARKERS

3.1. Introduction

Work on low level phylogenies in the past often suffered from insufficient resolution provided by plastid markers. Low sequence divergence makes the use of even the more informative cpDNA markers like *matK,* or the *ndhF-rpl32* intergenic spacer, ineffective for questions of interspecific relationships in *Scorzoneroides* (Samuel *et al*., 2006; Cruz-Mazo *et al.*, 2009). Maternal inheritance of plastid DNA markers masks occurring hybridization events in plants.

A phylogeny based on plastid DNA is simply not sufficient for our survey on the genus *Scorzoneroides*. Nuclear markers could help us to identify possible hybrids and to increase resolution of the phylogenetic tree.

The only region in the nuclear genome commonly used is the 18S-26S nuclear ribosomal DNA (nrDNA), with emphasis on the internal transcribed spacer region ITS (Baldwin, 1992; Baldwin *et al.*, 1995; Alvarez & Wendel, 2003). This marker provides a higher mutation rate than any region of the plastid genome (Baldwin *et al.*, 1995). ITS is available in many copies and flanking regions are highly conserved, thus, universal primers could be designed (White *et al.*, 1990; Baldwin, 1992; Baldwin *et al.*, 1995). Both internal transcribed spacer regions are rather short in Angiosperms, ranging from 300 to 700 bp, and can be amplified easily using PCR. The inheritance is biparental, which would allow the detection of hybridization events. ITS has already been used with Hypochaeridinae in general (Samuel *et al.*, 2003) and in *Leontodon* (Samuel *et al.*, 2006) as well as in *Scorzoneroides* (Cruz-Mazo *et al.*, 2009) and it gave insight to interspecific relationships. However, concerted evolution may cause homogenization of one homeolog of the parental copies (Wendel *et al.*, 1995) in ITS, and overwrite significant information on hybridization events.

Low-copy nuclear (LCN) genes have proven their value in detecting hybrid speciation events as well as in improving the resolution of phylogenies at inter- and intraspecific levels (Ford *et al.*, 1995: *Clarkia*; Gottlieb & Ford, 1996, 1997: *Clarkia*; Ford & Gottlieb, 2007: *Onagraceae*; Sang *et al.*, 1997: *Paeonia*; Small *et al.*, 1998: *Gossypium*; Emshwiller & Doyle, 1999: *Oxalis*; Olsen & Schaal, 1999: *Manihot esculenta*; Eyre-Walker *et al*, 1998: *Zea*; Russell *et al.*, 2010: *Polystachia;* among many others). The introns seem to rapidly diverge, regions suitable for primer design are easily found in exons, which, because of being coding regions for proteins have to maintain their sequence as well as their reading frame. Also, as the possibility for homoplasy is far lower than in nrDNA, these markers play an important role on detection of

(homoploid or allopolyploid) hybridization (Sang, 2002; Alvarez & Wendel, 2003). Despite their enormous potential, LCN markers are underrepresented in molecular investigations (Alvarez & Wendel, 2003). The main reasons that can be accounted for this are multiple copy numbers with the possibility of gene duplication or deletion and linked to that possible paralogs of the genes. To determine copy numbers, one might employ southern blotting (Sang, 2002). Cloning is often necessary to differentiate paralogous sequences from one individual, which might make an investigation time consuming and costly.

3.2. Material & Methods

We used accessions of 19 taxa from *Scorzoneroides* as well as outgroup taxa from *Helminthotheca*, *Hypochaeris*, and *Leontodon*. See table 3 for more information on used samples.

3.2.1. Primers and PCR conditions

Primers

Primers (Table 8) were chosen from available literature, 7 different loci were tested in our study, namely *PgiC* (*Cytosolic Phosphoglucose Isomerase*; Ford *et al.*, 1995, 2006; Gottlieb & Ford, 1996, 1997; Ford & Gottlieb, 1999, 2007; Ishikawa *et al.*, 2002; Kamiya *et al.*, 2005;), *PhyC* (*Phytochrome C*; Kolukisaoglu *et al.*,1995; Mathews & Dongoghue, 1999; Samuel *et al.*, 2005; Russel *et al.*, 2010) *GAPDH* (*Glyceraldehyde 3-Phosphate Dehydrogenase*, Olsen & Schaal, 1999; Olsen, 2002; Akinyi *et al.*, 2008; Vaezi & Brouillet, 2009), *ncpGS* (*Chloroplast-expressed glutamine synthetase,* also known as *GScp*; Emshwiller & Doyle, 1999; Perret *et al.*, 2003; Yockteng & Nadot, 2004; Chase *et al.*, 2005), *A27*, *A28* and *A39* (all three markers are conserved orthologous regions between *Arabidopsis* and *Helianthus;* Chapman *et al.*, 2007) locus. Each low-copy nuclear region was initially amplified with the available primers and modified standard conditions.

To obtain best results in our investigation, an optimization of PCR protocols to the subtribe Hypochaeridinae was necessary. A gradient PCR was done to fit annealing temperature for the primers available in the literature. In some cases, specific primers were designed for our study. The phytochrome multigene family codes for important photoreceptor proteins in plants. The main task is photoreceptive regulation of different physiological processes (photomorphogenesis), e.g. germination or development of flowers in angiosperms. *GAPDH* a protein necessary for all eukaryotic organisms as it is involved in glycolysis. (Akinyi *et al.*, 2008). *PgiC* is involved in the second step of glycolysis, which makes it a very important gene

to all eukaryotes. In different species of the genus *Clarkia*, this gene was reported to have 22 exon and 23 intron regions (Ford *et al.*, 1995).The *A27*, *A28* and *A39* loci are of an origin which is not further explained by Chapman *et al.* (2007).

Table 8: Primers used for nuclear regions in this study.

REGION/GENE	PRIMER NAME	SEQUENCE	REFERENCE
A27 locus	AT3F	CTT GCA WTG AAT GTC ATG TGG AAG	Chapman *et al.* (2007)
	AT3R	GCT CCC CAR CAT TTC A	Chapman *et al.* (2007)
A28 locus	AT1F	GYG CTC TYT TCT TTG ATK TRG ATG G	Chapman *et al.* (2007)
	AT1R	CAT CTT TGT TWT CAA GAG CAG	Chapman *et al.* (2007)
A39 locus	AT2F	ACT AGT TGG CAT YTR ATG GTA ACA	Chapman *et al.* (2007)
	AT2Fsc	RAT GGT AAM ATA TTG CAC GCT	this study
	AT2R	GCC RAC AAA ATT GAG CTG AAG ATC	Chapman *et al.* (2007)
	AT2Rsc	GCT GAA RAT CTC ACT GAT CGA	this study
GAPDH	GAPDHx3F	TTG AGG GTC TTA TGA CTA CAG T	Vaezi & Brouillet (2009)
	GAPDHx3Fsc	TCT TAT GAC TAC AGT GCA CTC AAT GAC	this study
	GAPDHx4F	AGG ACT GGA GAG GTG GAA GAG	Vaezi & Brouillet (2009)
	GAPDHx5Rsc	TAA ACT TAC TTG ATA GAC GCC TTG AT	this study
	GAPDHx6R	GGT GTA TCC CAA GAT ACC CTT GAG C	Vaezi & Brouillet (2009)
	GAPDHx6Rsc	CCT TGA GCT TGC CTT CTG AT	this study
GScp	GScp687F	GAT GCT CAC TAC AAG GCT TG	Emshwiller & Doyle (1999)
	GScp994r	AAT GTG CTC TTT GTG GCG AAG	Emshwiller & Doyle (1999)
PgiC	AA11F	TTY GCN TTY TGG GAY TGG GT	Ford *et al.* (2006)
	AA16R	CCY TTN CCR TTR CTY TCC AT	Ford *et al.* (2006)
PhyC	PhyCAFD		
	PhyCR1	CAT TCT CAC CTT ATT CTT CAA GAA AAG AAA CC	

PCR conditions

To find the best conditions for amplification or primer annealing, we used gradient PCR, as well as touchdown PCR in some cases. To achieve best results with the selected primer combinations, PCR trials were performed. Single PCR products were sequenced to adjust primers or to design internal primers.

Gradient PCR allows several different temperature settings in one PCR. The temperature gradient comes in steps of around 1°C in each column of the PCR machine. In our study, we could program 12 different temperature settings for a Eppendorf master cycler gradient 96-well PCR.

Table 9: Gradient PCR protocol for *PgiC*.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	30 sec	36
Primer Annealing	48-60°C	2 min	36
Extension	70°C	2 min	36
Final Extension	70°C	7 min	1

Figure 25: Gel electrophoresis Images of PCR products from four different samples tested with *PgiC*.
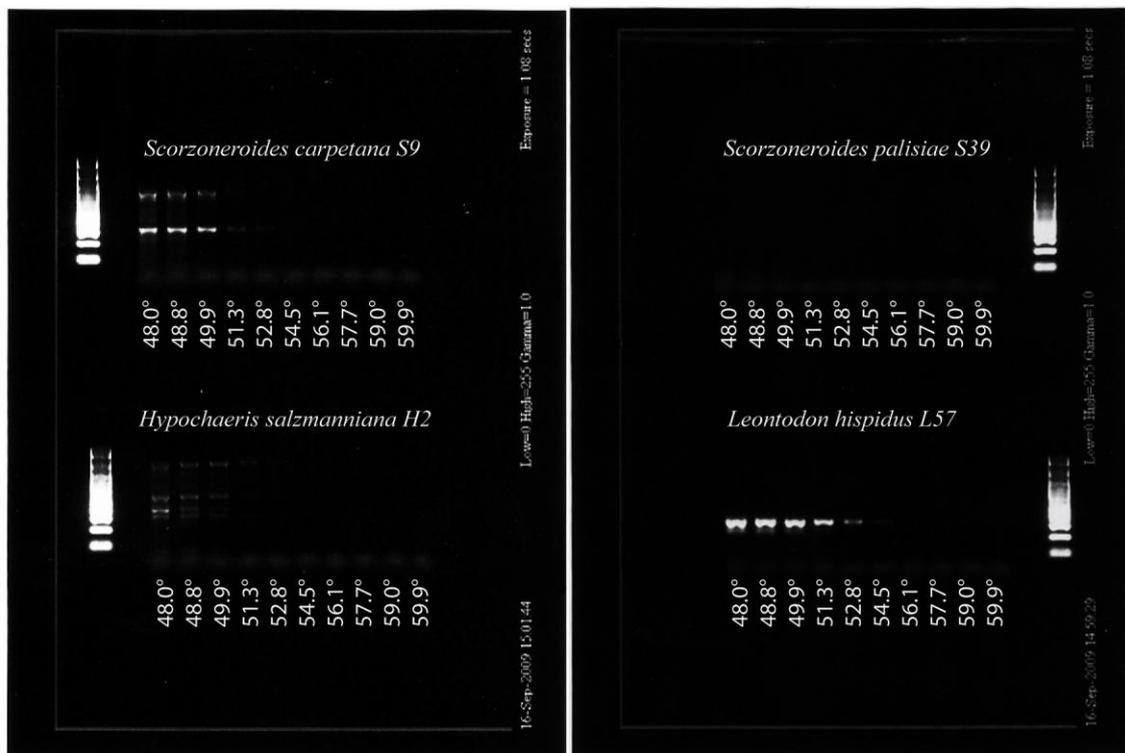
The fragments of the gradient PCR that showed the best results in gel electrophoresis, i.e. strong single bands with an expected length, were put together and directly sequenced. In the case of *PgiC* several adjustments to the PCR protocol were necessary.

A two-step PCR program with touchdown-PCR in the first cycle was used (Table 10). Though we tried to optimize PCR conditions, we realized the primer would also bind to other sites of the genomic DNA (Figure 25). Gel-purification of single PCR fragments with the expected length would have been necessary. The marker was discarded.

Table 10: Final PCR protocol for *PgiC*.

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	95°C	2 min	1
DENATURATION	95°C	30 sec	36
PRIMER ANNEALING	48-60°C	2 min	36
EXTENSION	70°C	2 min	36
FINAL EXTENSION	70°C	7 min	1

GAPDH as well as *ncpGS* were showing strong, single signals on the gel with the initial PCR protocol (Table 11, Figure 26). The resulting protocol (Table 12) was performed in two steps of 11 and 26 cycles, with a touchdown-PCR for the first step.

Table 11: Gradient PCR protocol for *GAPDH*, *PhyC* and *ncpGS*.

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	95°C	2 min	1
DENATURATION	95°C	30 sec	36
PRIMER ANNEALING	45-59°C	30 sec	36
EXTENSION	70°C	2 min	36
FINAL EXTENSION	70°C	7 min	1

The primers we used for *GAPDH* were designed for *Symphiotrichum* (Vaezi & Brouillet, 2009), a genus in Astereae (Asterales), which also anneal to the designated location on the *GAPDH* gene of the Hypochaeridinae. The first obtained alignment with four species was used to adjust primers to the Hypochaeridinae. Internal primers were created to increase amplification reliability (Figure 27).

The gene *ncpGS* came out promising. Amplification of a single product was possible with primers from Emshwiller & Doyle (1999). After PCR adjustments, 17 samples were used to test *ncpGS*, out of these 17, 8 samples showed amplification; however, no direct sequences could be obtained from these. We had to leave out this gene since we had problems to get good sequences (Figure 28).

Figure 26: Gel electrophoresis Images of PCR products for two samples of *Scorzoneroides* from three nuclear markers, namely *GAPDH*, *ncpGS* and *PhyC*
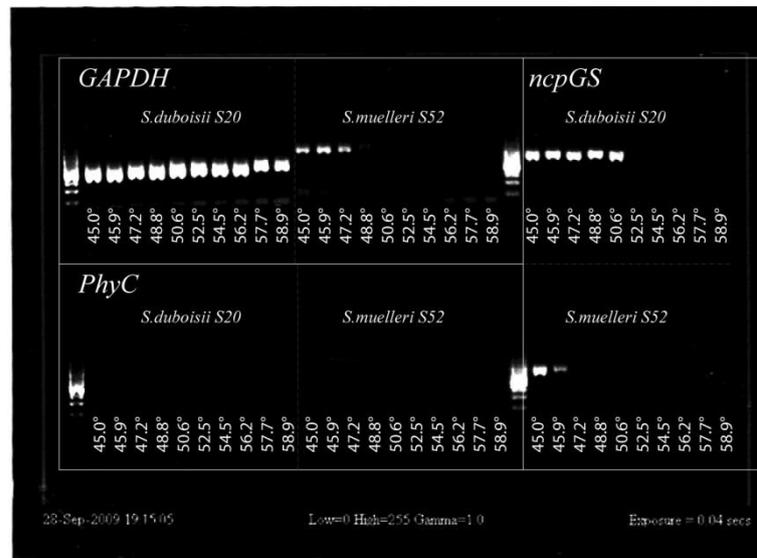
Table 12: Final PCR protocol for *GAPDH* and *ncpGS*.

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	95°C	2 min	1
DENATURATION	95°C	30 sec	11
PRIMER ANNEALING	60°C -0.5°C /cycle	30 sec	11
EXTENSION	70°C	1 min	11
DENATURATION	95°C	30 sec	26
PRIMER ANNEALING	55°C	30 sec	26
EXTENSION	70°C	1 min	26
FINAL EXTENSION	70°C	7 min	1

Figure 28: Gel electrophoresis of *GAPDH* amplified with internal primers
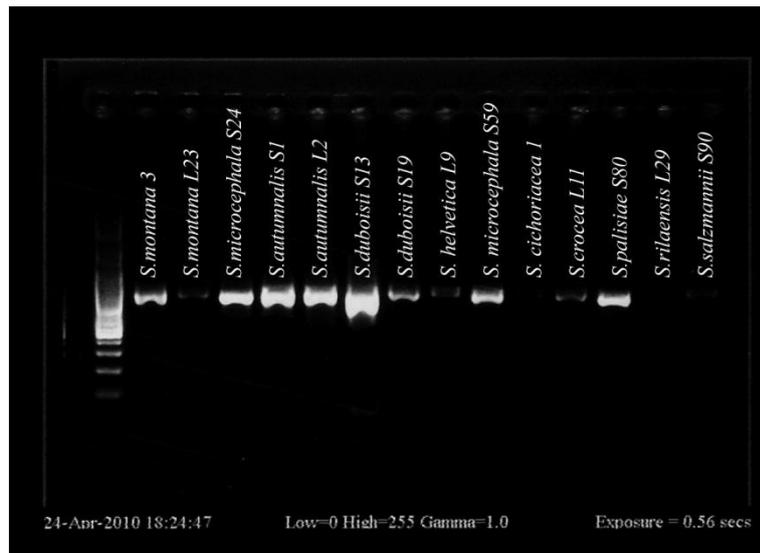
Figure 27: Gel electrophoresis of *ncpGS* amplified with standard Taq-Polymerase (right) and Titanium Taq™ (left, Clonetech, 2010).
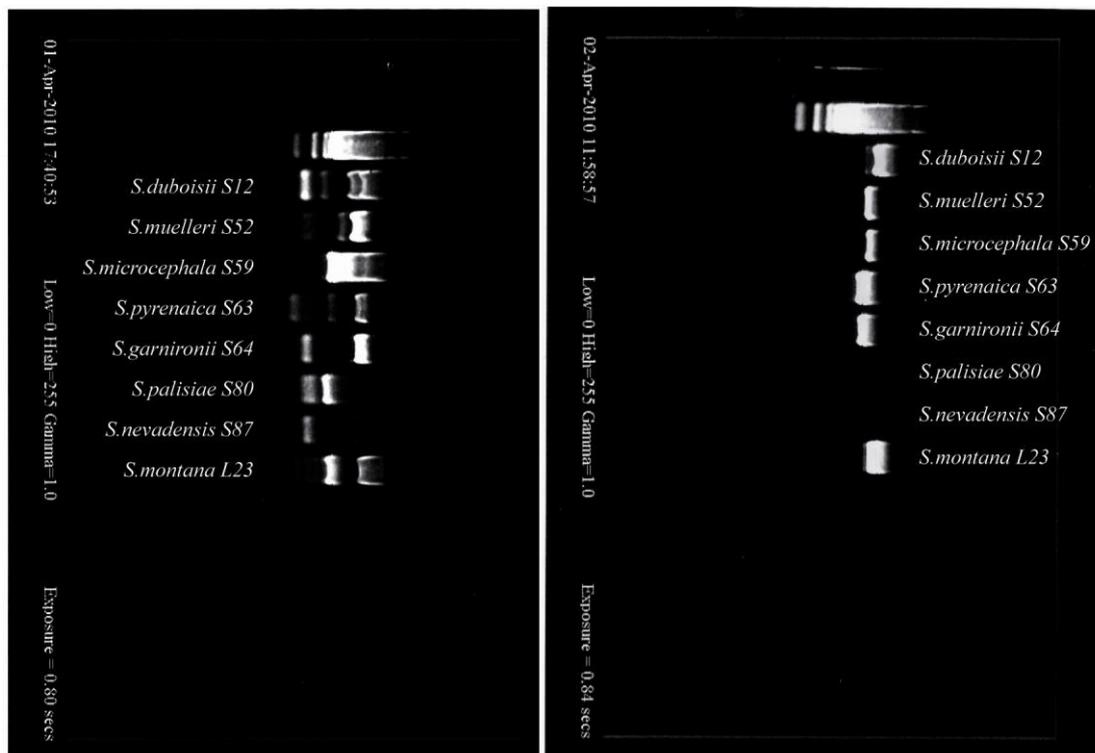
The three loci *A27*, *A28* and *A39* showed fairly different results (Figure 29, 30), with the *A39* and *A28* locus showing single bands, and *A27* locus with more than one with the initial PCR protocol (Table 13). As the *A27* locus showed strong double bands, we could not directly sequence and decided not to use it any further (Figure 29). PCR conditions were adjusted for *A28* and *A39* loci (Table 14, Figure 30).

Table 13: Gradient PCR protocol for *A27*, *A28* and *A39* loci.

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	95°C	2 min	1
DENATURATION	95°C	30 sec	36
PRIMER ANNEALING	45-59°C	30 sec	36
EXTENSION	70°C	2 min	36
FINAL EXTENSION	70°C	7 min	1

Figure 29 Gel electrophoresis Images of PCR products from *A27* and *A28* loci.
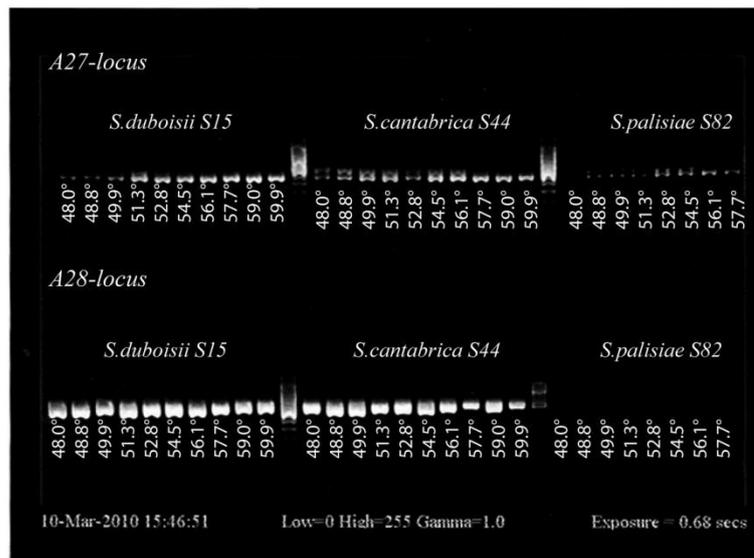
Figure 30: Gel electrophoresis Images of PCR products from *A39* locus.
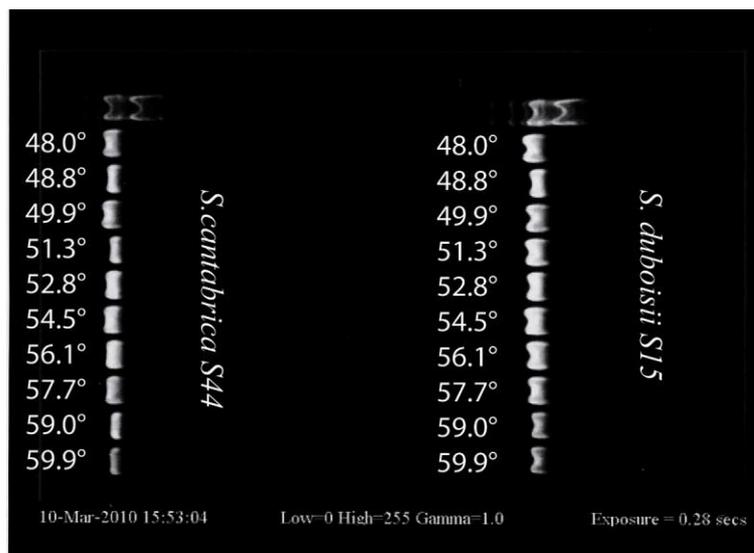
Table 14: Final PCR protocol for *A28* and *A39* loci.

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	95°C	2 min	1
DENATURATION	95°C	30 sec	11
PRIMER ANNEALING	53°C -0.5°C /cycle	30 sec	11
EXTENSION	70°C	2 min	11
DENATURATION	95°C	30 sec	25
PRIMER ANNEALING	50°C	30 sec	25
EXTENSION	70°C	2 min	25
FINAL EXTENSION	70°C	7 min	1

Figure 31 Gel electrophoresis of *A28* (top lane) and *A39* loci (bottom lane) with the adjusted PCR protocol.
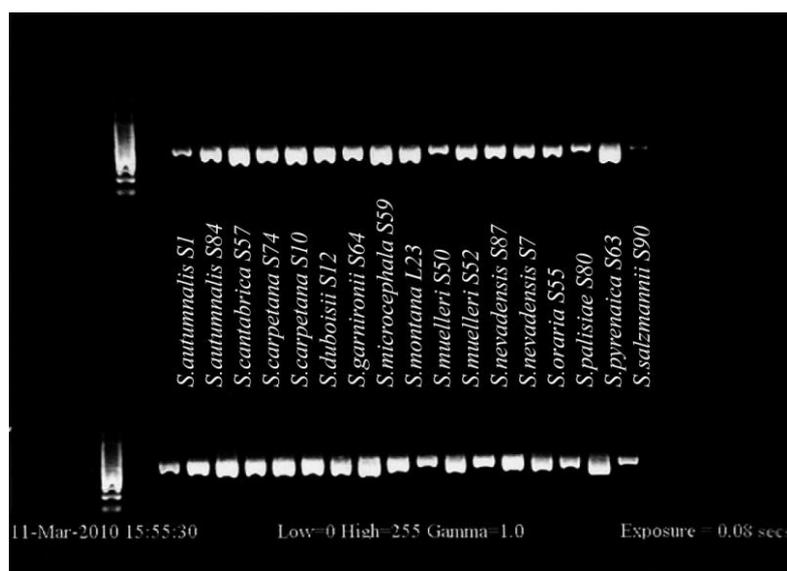
Only one marker out of the three tested universal primers by Chapman *et al.* (2007), namely the *A39* locus, could be sequenced directly using the available primers. With the adjusted primers *AT2Fsc* and *AT2Rsc*, sequencing of all tested samples was successful; however, cloning was inevitable due to two copies (two alleles of the gene were found with only slight differences) for some species. PCR results for the *A28* locus showed a double band on the gel electrophoresis, with only a small difference in sequence length. Since direct sequencing of this locus was not possible, we did not continue to use this region.

3.2.2. Primer design

If the available primers for selected markers are not binding exclusively to the specific site, or cannot amplify all of the accessions, the design of new primers becomes necessary. An overview on the topic of primer design is given in Dieffenbach *et al.* (1993). Secondary structures within a sequence might hinder Taq-polymerase to amplify the targeted region without problems or errors. Also, a high fragment length interferes with the quality of amplification (Rychlik *et al.*, 1990). Here, additional internal primers improve the results.

The search for regions suitable for primer design was done manually by screening alignments of at least two ingroup and two outgroup accessions sequences with BioEdit Sequence Alignment Editorv7.0.9.0. Sites that were conserved among all accessions were taken into account and consequently tested with Integrated DNA Technologies OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). We focused on the design of primers with similar annealing temperatures on forward and reverse primers for each region.

New primers were designed for the markers that we decided to use for our studies. *GAPDH* internal and external primers were created, for *A39* we had to adjust the available primers (see Table 8).

3.2.3. Cloning

Cloning is a very important technique in case of polymorphic sequences.

Nutrition medium for clones consists of dH$_2$O containing 1 percent Tryptone, 0.5 percent Yeast extract and NaCl each and 0.05 percent of Ampicillin. Colonies are grown on Agar-plates treated with X-Gal and the antibiotic Ampicillin. Blue colonies show a functional gene to process X-Gal whereas white colonies infer breakage of the gene and a successful insertion of the DNA fragment into the plasmid and transformation of the cells. Still some fungal spores could contaminate the growing colonies, which would result in wrong sequences or no sequence at all. Cloning Kits are expensive, and the different copies cannot be distinguished from each other prior to sequencing, except for southern-blotting. The number of clones to be sequenced depends on copy-number and on chimeras.

Cloning was done for both *GAPDH* and *A39* locus, using the pGEM-T Easy Cloning Kit (Invitrogen). No modifications were made in the manufacturers' protocol. Colonies were taken off the nutritient plate and stored in 100µl sterile H$_2$O, heated to 98°C for 10 minutes and finally spinned down 10 minutes at 10000 rpm.

We aimed to get at least 12 colonies per cloned sample, so we would be able to sequence both fragments. Altogether, we cloned 39 accessions, resulting in more than 700 cloned sequences.

For PCR, *M13* vector primers were selected; the reactions were either 15µl in *GAPDH* and in 10µl volumes in *A39*, respectively. The master mix consisted of 13.5 or 8.9µl ABGeneReddyMix PCR, 0.3 or 0.22µl *M13F*, 0.3 or 0.22µl *M13R*, 0.6 or 0.44µl DMSO (Dimethyl Sulfoxide), and 1.0 or 0.6µl DNA material. PCR conditions are given in the following table (Table 15).

Table 15: PCR protocol for *M13* primers

STEP	TEMPERATURE	TIME	CYCLES
INITIAL DENATURATION	94°C	3 min	1
DENATURATION	94°C	40 sec	36
PRIMER ANNEALING	50°C	30 sec	36
EXTENSION	72°C	2 min	36
FINAL EXTENSION	72°C	9 min	1

PCR products were checked on 1.5% TAE agarose gel, all successfully amplified fragments with the expected length were purified using Exonuclease I and Fastap at standard conditions of 37°C for 45 minutes followed by 15 minutes at 85°C.

Figure 32: Gel electrophoresis of several different cloned samples of *A39* locus.
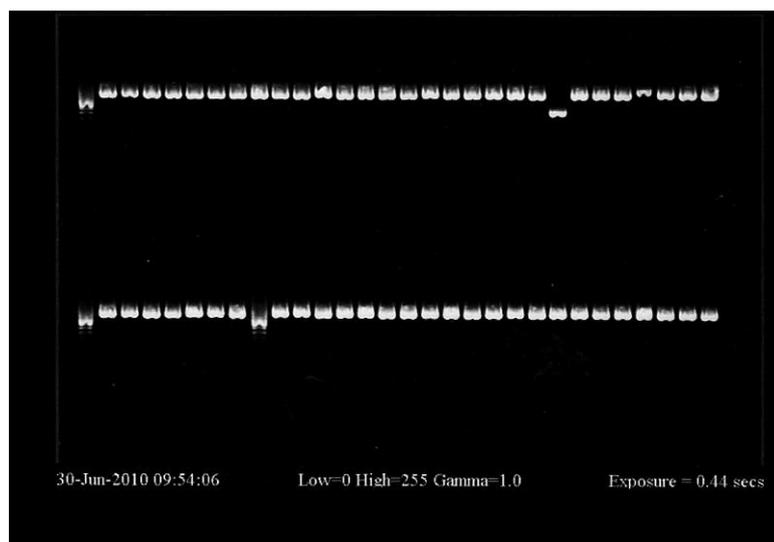
Primers for sequencing were selected after a couple of test runs with all available primers to our regions as well as the *M13* primers. The most appropriate selection for *GAPDH* was *GAPDHx3Fsc* as forward and *GAPDHx5Rsc* as reverse. For *A39* we found the *M13F* and *M13R*

primer to be best suitable for forward reactions. Forward and reverse reactions were done for *GAPDH*, only the forward reaction for *A39*.

3.2.4. Cycle Sequencing

The conditions for cycle sequencing were as follows: 10µl reactions, each containing 0.4µl Big Dye Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 1.8µl Sequencing buffer, 1.0µl Primer (4 µMol), and 6.8µl previously cleaned PCR product. For reactions, that would not give good sequences, we tried a master mix with little adjustments, as follows: 10µl reactions, each containing 1µl Big Dye Terminator v3.1 Ready Reaction Mix (Applied Biosystems), 1.8µl Sequencing buffer, 1.0µl Primer (4 µMol), 6.8µl previously cleaned PCR product. In some cases we reduced the amount of PCR product from 6.8 to 4.5µl. The standard PCR protocol was used (Table 6).

3.2.5. Phylogenetic analysis

For details on phylogenetic analysis refer to page 29, "2.2.5. Phylogenetic analysis"

Matrices of each spacer region were analyzed individually and combined. For more information on these markers, refer to Table 16. Two trees, a majority rule consensus tree and a bootstrap consensus tree were created for each of the three trees as well as for the combined matrix. The majority rule consensus tree was chosen over a phylogram of one most parsimonious tree due to short branch lengths at intraspecific levels in *Scorzoneroides*. The matrix of the two nuclear markers was combined with the matrix obtained from the three plastid markers of our study.

Table 16 Detailed information on the low-copy nuclear marker regions and combined matrices used in this study.

	GAPDH	*A39* locus	combined nuclear markers	combined nuclear and plastid markers
No. of samples	73	60	42	42
No. of species	27	26	26	26
Length of alignment	857	585	1312	4862
No. of constant characters	616	413	948	4185
No. of variable characters	241	172	364	677
No. of informative characters	134	119	186	341
No. of shortest trees	225	125	134	31
Length of shortest trees	395	321	599	1011
Consistency Index (CI)	0.7885	0.8112	0.7346	0.7626
Retention Index (RI)	0.8250	0.7724	0.8030	0.8571

3.3. Results

3.3.1. *GAPDH*

The resulting alignment (Table 16, Figure 33) gave information about the intron-exon structure of the amplified fragment of *GAPDH* with approximately 750 base pairs length. Introns and exons were estimated manually by screening the alignment of all available sequence data of this study. The resulting phylogenetic trees of *GAPDH* (Figure 35, 36) include copies of each cloned sequence, referred to as C1 and C2.

Figure 33: Structure of the amplified fragment of the gene *GAPDH* (*Glycerine Aldehyde 3-Phosphate Dehydrogenase*) based on sequences of *Scorzoneroides*, *Leontodon*, *Hypochaeris* and *Helminthotheca*. Primers used for amplification and sequencing are shown in black, additional internal primers are shown in white.
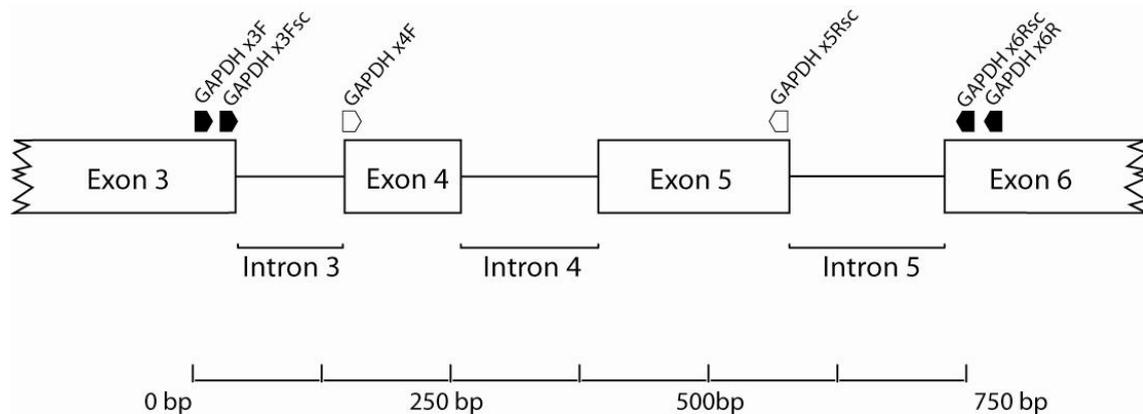
The bootstrap consensus tree of *GAPDH* supports *Scorzoneroides* monophyletic status (Figure 36, BS 86). The genus is split into seven groups with this marker.

The first group (A; BS 61) is formed by perennial continental and mountain taxa. Four out of 6 accessions of *S.autumnalis* and one *S.duboisii* clone sequence fall into this clade. Three of the *S.autumnalis* accessions clade together (BS 83). The second clade (B; BS 64) comprises all members of *S.carpetana*, one accession of *S.autumnalis* and *S.nevadensis* as well as three *S.duboisii* clones. Clade C (BS 52) is formed mainly by two species, *S.muelleri* and *S.oraria*, as well as one accession of *S.palisiae*, with clear separation of *S.muelleri* (BS 98) and one clone of both *S.oraria* individuals (BS 97). One accession of *S.muelleri* and one clone of *S.palisiae* form a group of their own (D; BS 66). A big group (E; BS 53) of perennial mountain taxa, namely *S.cantabrica*, *S.crocea*, *S.helvetica*, *S.melantotricha*, S.*montana*, and *S.pyrenaica* is observed, with a subdivision in two clades. The first clade (E1; BS 70) comprises all accessions of *S.pyrenaica*, *S.helvetica* and *S.cantabrica*, whereas the second clade (E2; BS 61) is formed by one accession of *S.montana* and two clones of two individuals od *S.melantotricha*. A polytomous clade (F; BS 67) contains sequences of *S.crocea*, *S.montana*, *S.melanotricha* and *S.rilaensis*. *Scorzoneroides microcephala* and *S.cichoracea* group together (G; BS 94) and can be well-separated (BS 100; BS 99).

Leontodon as an outgroup genus is monophyletic (H; BS 57) and shows a high divergence between the sequenced accessions. *Scorzoneroides* and *Leontodon* are forming an alliance, separating them from the rest of the Hypochaeridinae (BS 66). *Hypochaeris* and *Helminthotheca* are located in a polytomy at the base of Hypochaeridinae.

3.3.2. *A39* locus

Screening the alignment of the full matrix did not give insight in structural arrangements of introns and exons (Table 16, Figure 34). The length of the fragment was approximately 475 base pairs.

Figure 34 Diagram of the amplified fragment of the *A39* locus based on sequences of *Scorzoneroides*, *Leontodon*, *Hypochaeris* and *Helminthotheca*. Primers used for amplification and sequencing are shown in black, additional internal primers are shown in white.
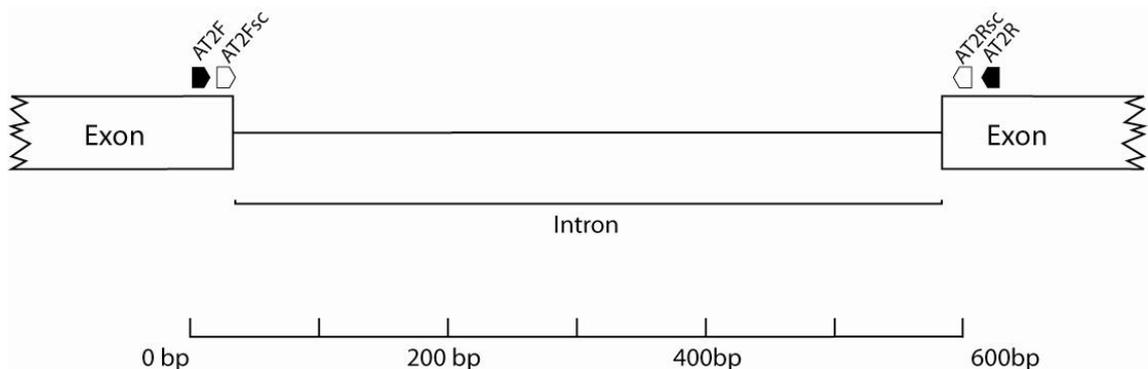
The resulting phylogenetic trees (Figure 37, 38) include copies of each cloned sequence, referred to as C1 and C2.

Monophyly of *Scorzoneroides* is not supported with this marker. A split into several clades is shown. The first Clade (Figure 38, A; BS 81) is comprised by three Perennial Mountain or continental taxa (*S.autumnalis*, *S.duboisii*, and *S.nevadensis*) and shows a polytomy except for two accessions of *S.nevadensis* forming a clade of their own (BS 84). The second clade obtained (B; BS 81) is formed by both *S.carpetana* individuals and two clones of an *S.duboisii* individual, and one clone of an accession from *S.nevadensis*. A grouping of the *S.carpetana* accessions is supported (BS 62), clading together with the two cloned copies from one *S.duboisii* individual (BS 83).

Scorzoneroides garnironii and *S.palisiae* were clading together (C, BS 64), with *S.garnironii* (BS 100). Two cloned sequences from one individual of *S.palisiae* fall together (BS 88) in this clade.

Scorzoneroides salzmannii, one of two accessions of *S.muelleri* and *S.oraria* fall into a well-supported clade (D; BS 76), all species are separated from each other, with *S.oraria* (BS 91) being sister to the group (BS 57) of *S.muelleri* and *S.salzmannii* (BS 72*)* The two cloned sequences of. *S.laciniata* fall into a clade of their own (E; BS 96).

A major clade (F; BS 99) is obtained, comprised by *S.cantabrica*, *S.crocea*, *S.helvetica*, *S.melanotricha*, *S.microcephala*, *S.montana*, *S.pyrenaica* and *S.rilaensis*. *Scorzoneroides pyrenaica*, *S.cantabrica* and *S.rilaensis* cannot be distinguished from each other; they form a polytomy at the base of the major clade. A larger group of *S.crocea*, *S.montana* and *S.melanotricha* (BS 64) is further subdivided, with two copies of one individual of *S.crocea* basal. One group includes one copy of an individual of *S.montana* and one sample of *S.crocea* (BS 63), another comprises 2 copies of two different individuals from *S.melantotricha*. A copy of an individual from *S.melantotricha* as well as a *S.montana* accession are sister to these two groups. A smaller clade of two copies from two different accessions of *S.helvetica* (BS 62) can be observed.

Scorzoneroides microcephala and *S.cichoracea* (G; BS 97) form a clade. The two species were well-seperated from each other (BS 95, BS 93).

Two clones of two accessions of *S.helvetica* and one clone of an *S.autumnalis*- individual were grouping together (H; BS 65). *S.helvetica* copies are separated (BS 100) from *S.autumnalis.*

The outgroup genera *Leontodon* and *Helminthotheca* were forming a polytomous clade (I; BS 86). *Hypochaeris* is sister to the other mentioned groups.

3.3.3. Combination of low-copy nuclear markers

The matrices of *GAPDH* (852 bp) and *A39* (585bp) were combined (see Table 16). A phylogram (Figure 39) and a bootstrap consensus tree (Figure 40) are shown.

With the combined markers, Scorzoneroides' generic status is supported (Figure 40, BS 100). It is split into several groups. Clade A (BS 98) comprised all samples from *S.cantabrica, S.crocea, S.helvetica, S.melantotricha, S.montana, S.pyrenaica and S.rilaensis.* We observed these groups with chloroplast markers before. Also, a very clear subdivision into two subclades A1 (BS 75) and A2 (BS 75), was well supported. The first group shows a polytomy with one exception of an accession of S.crocea and *S.melantotricha* were falling into one clade (BS 73). In the second group, one accession of *S.melantotricha* is sister to a group of one accession of S.*cantabrica* and *S.pyrenaica* as well as to samples of *S.helvetica* (BS90). Both individuals of *S.helvetica* fall in one clade (BS 61).

The second cluster of samples (B, BS 67) comprises all included samples of the previously observed of *S.autumnalis-S.carpetana-S.duboisii-S.nevadensis* alliance (plastid marker phylogenies of this study; Cruz-Mazo *et al*., 2009). The resolution in this group is high, separating almost every individual from the other despite of species levels.

Clade C (BS 77) includes three members of the annual species in Scorzoneroides, namely *S.muelleri*, *S.oraria* and *S.salzmannii*.

Scorzoneroides cichoracea and *S.microcephala* are forming a clade (D, BS100) and are well-seperated from each other (BS 100, BS 100). This group was also observed with the combined chloroplast marker phylogeny.

Clade E (BS 61) comprises the rest of the annual species except for *S.laciniata*, which does not fall into a group. A clear separation of *S.garnironii* (BS 100) can be observed in Clade E

3.3.4. Combination of plastid and low-copy nuclear markers

All three chloroplast markers *atpH-atpI*, *ndhF-rpl32*, *rpl16-Intron* and both low-copy nuclear markers, *GAPDH*, *A39* locus were merged into one matrix, with a length of ca. 4800 base pairs (see Table 16). The combined matrix includes 39 accessions of 19 species of *Scorzoneroides*, 5 accessions of 5 species of *Leontodon*, and one accession of one species of *Helminthotheca* and *Hypochaeris*.

The bootstrap consensus tree of the combined matrix (Figure 42) shows a well-supported monophyletic status of the genus *Scorzoneroides* (BS 100). A clear separation into two major groups is observed. Clade 1 (BS 96) comprises all members of the Annuals, occupying dryland or Mediterranean regions, namely *S.laciniata*, *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii* as well as a group of perennials inhabiting continental or mountain areas, *S.autumnalis*, *S.carpetana*, *S.duboisii*, *S.garnironii* and *S.nevadensis*. Two subclades of Clade1 were recognized.

Subclade A (BS 71) groups together all annuals as well as *S.garnironii*. *Scorzoneroides laciniata* is sister to two groups in Subclade A. One group contains S.*muelleri*, *S.oraria* and *S.salzmannii* (A1; BS 60) and shows a polytomy of *S.muelleri* and *S.salzmannii* as sister to *S.oraria* (BS 100). The other group (A2; BS 60) is formed by *S.garnironii* and *S.palisiae*, with both species separated from each other (BS 100; BS 75).

Members of the perennial group, namely *S.autumnalis, S.carpetana, S.duboisii* and *S.nevadensis*, form subclade B (BS 93). In this cluster, one big group (BS 52) comprises accessions of all four species, with exception of two individuals of *S.autumnalis* and one of *S.nevadensis*, which are sister to this clade. The crown group is formed by *S.carpetana* (BS 99).

Clade II (BS 80) is split into two major groups, C and D. Clade C (BS 67) includes *S.crocea, S.montana, S.melantotricha* and *S.rilaensis*, clade D (BS 63) comprises *S.cantabrica, S.helvetica* and *S.pyrenaica* comprises *S.microcephala* and *S.cichoracea* form a group of their own (E, BS 99) as sister to clades C and D. Both species are well-separated from each other (BS 100, BS 100).

3.3.5. Comparison with previous studies

We compared the results of our analyses to the results from Cruz-Mazo *et al.* (2009). Therefore we reconstructed the phylogenetic tree based on two chloroplast markers and ITS by using the provided matrix from this study (Figure 11) The results of both studies on the phylogeny of *Scorzoneroides* are congruent in most points, except for the placement of *S.laciniata*, which, in our study, clearly groups with other annual species of the Mediterranean, whereas with ITS, these taxa were included in the perennial group of *S.autumnalis*, *S.carpetana*, *S.duboisii* and *S.nevadensis.*

3.4. Discussion

The two selected nuclear markers are not appropriate for this genus, despite their high variability. The combined nuclear and plastid DNA data, however, shows a well-supported

monophyly (BS 100) of *Scorzoneroides*. The division into two major clades as well as the separation of one of the latter into two subclades is well supported (BS 93 and 71, see Figure 27).

A better resolution within the clade of the perennial European mountain taxa (Figure 27, Clade 2) is observed, which splits it into three subdivisions, a group of *S.pyrenaica*, *S.carpetana* and *S.helvetica*, one clade comprising *S.montana*, *S.melanotricha*, *S.rilaensis* and *S.crocea.* This result is congruent to previous studies, where these two groups were put as S.montana and S.pyrenaica variation groups (Finch & Sell, 1976), and also congruent to the morphology and ecology. *S.cichoracea* and *S.microcephala* are forming a very well-supported sister group to the rest of the perennial mountain taxa alliance around *S.pyrenaica* and *S.montana*. This is supported by nuclear as well as plastid DNA sequence analyses.

Cruz-Mazo *et al.* (2009) observed that *S.laciniata* as well as *S.kralikii* are clustering with the perennial group of Subclade B (Figure 26), although they were expected to come within the annual Subclade A. Our results do not support this ambiguity at least for *S.laciniata*.

Although a high divergence in the group around *S.autumnalis* was given with the nuclear markers, we could not separate the three species from each other, the results showed a rather high divergence between individuals than between species. Almost all of these samples were collected in Spain, some in close to intermixed areas. The phylogenetic trees from nuclear marker may indicate that this group in particular is undergoing gene flow events or reticulate evolution.

Nonetheless, the question of ongoing hybridization in *Scorzoneroides* could not be answer in this study. A use of populational studies in this genus may help to find out about gene flow, introgression and hybrid speciation. In our investigation, the number of individuals per species was low, which hindered us to utilize powerful investigative methods like AFLP to get species boundaries.

For further studies, more material is necessary. A field trip to get a high number of individuals of different populations from each species is needed to find out about critical members or non-included taxa of the genus. Also, additional low-copy nuclear markers that are better suited for *Scorzoneroides* are necessary to get good resolution at inter- and intraspecific levels.

We cannot guarantee the successful use of our primers in distant groups of Compositae as the nuclear markers used in this study are highly variable. We were not able to automatically align a

GenBank accession of *Symphyotrichum* provided by Vaezi & Brouillet (2009) to our sequence data of *GAPDH*. We were successful in the end by doing a manual alignment.

Figure 35: Majority rule consensus tree of 225 retained trees of the nuclear marker *GAPDH*, including 34 accessions from 15 species as well as 25 cloned sequences (bold) of 13 taxa of *Scorzoneroides* and 8 outgroup accessions from 8 species. Majority rule percentage given above, bootstrap support and number of changes (calculated rounded median of three most
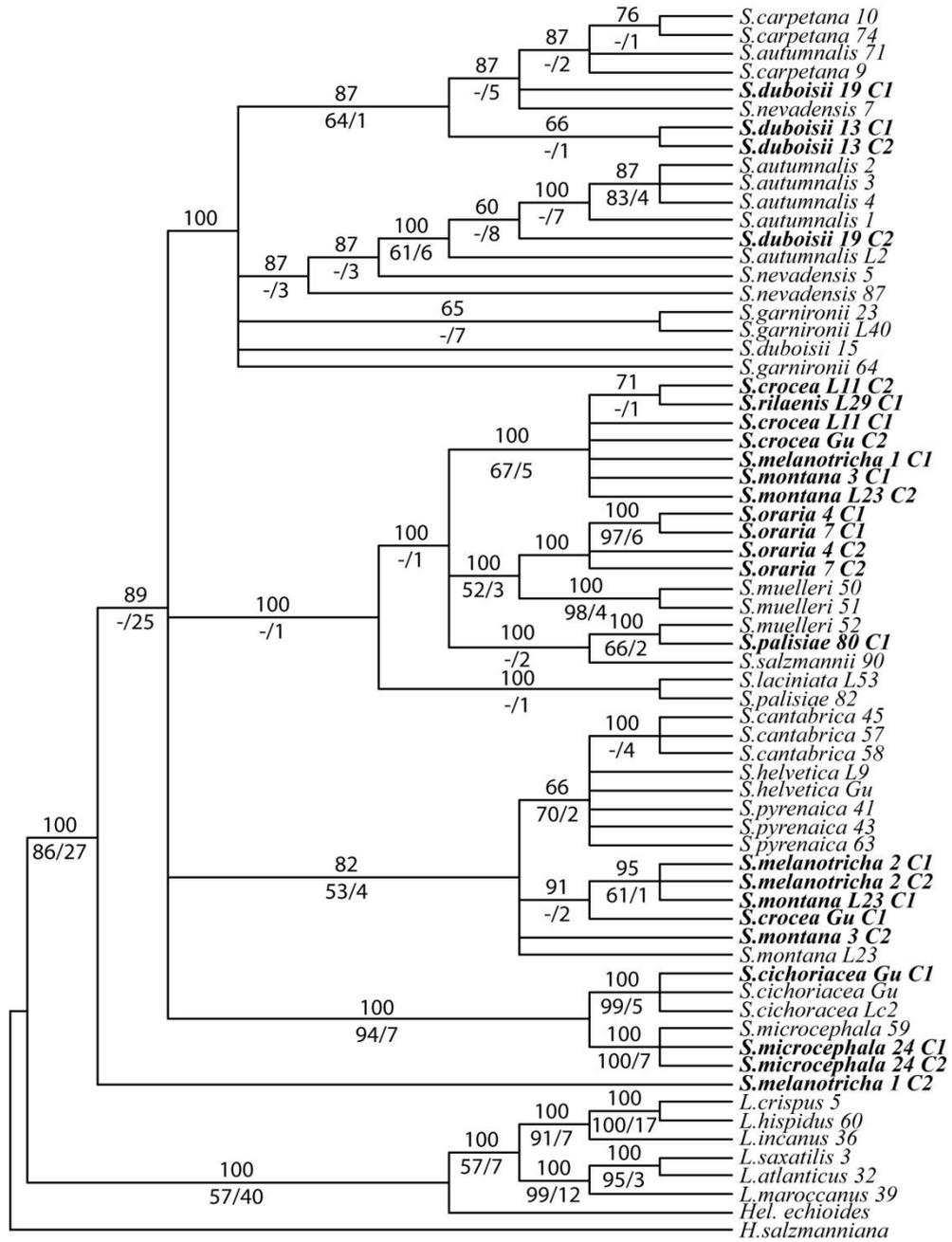
Figure 36: Bootstrap consensus tree of the nuclear marker *GAPDH*, including 34 accessions from 15 species of *Scorzoneroides* species as well as 25 cloned sequences (bold) of 13 taxa of *Scorzoneroides* and 8 outgroup accessions from 8 species. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches.
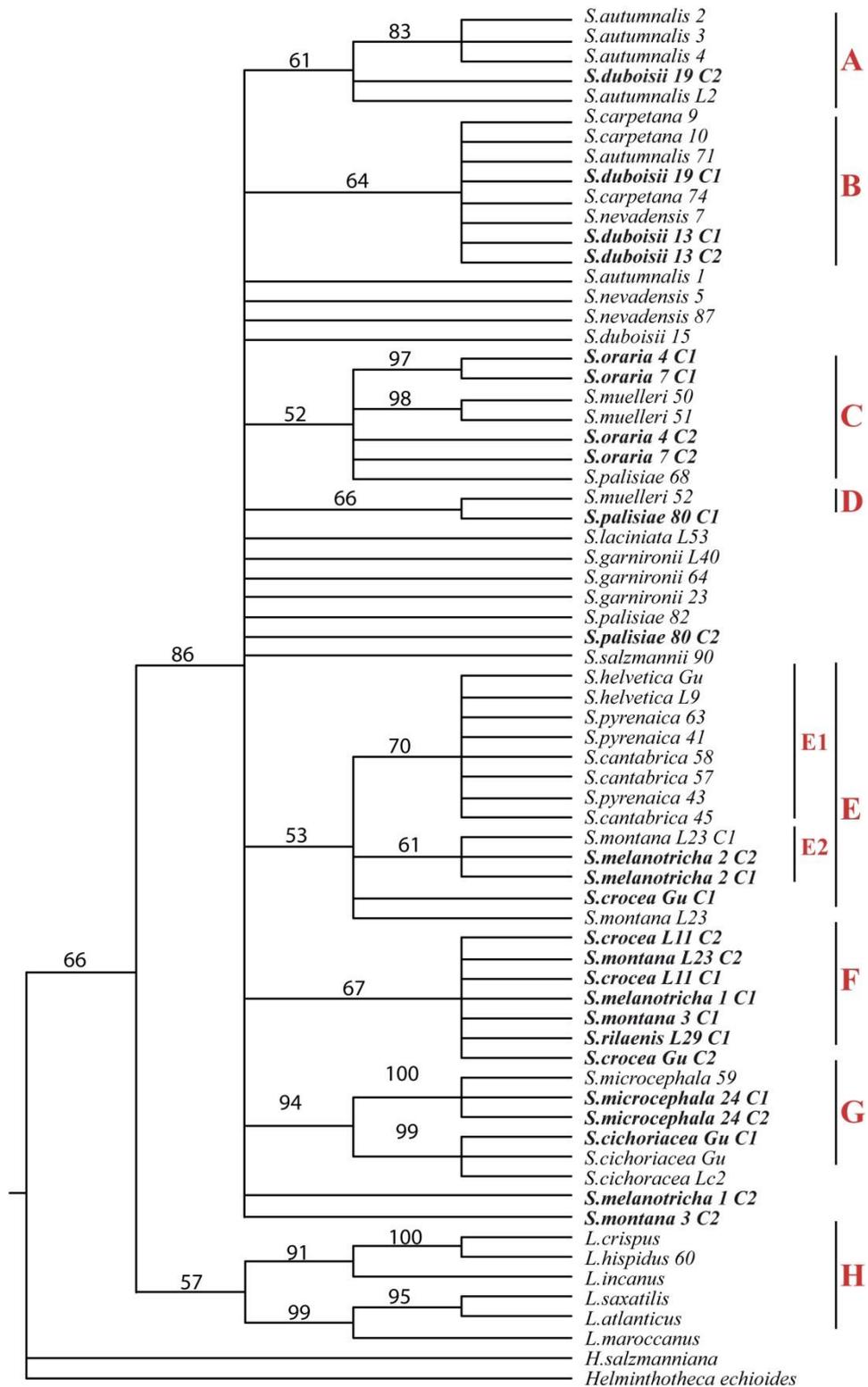
Figure 37: Majority rule consensus tree of 125 retained trees of the nuclear *A39* locus, including 34 accessions from 15 species as well as 28 cloned sequences (bold) of 13 taxa of *Scorzoneroides* and 7 outgroup accessions from 7 species as well as 3 cloned sequences of 2 taxa of *Leontodon*. Majority rule percentage given above, bootstrap support below branches.
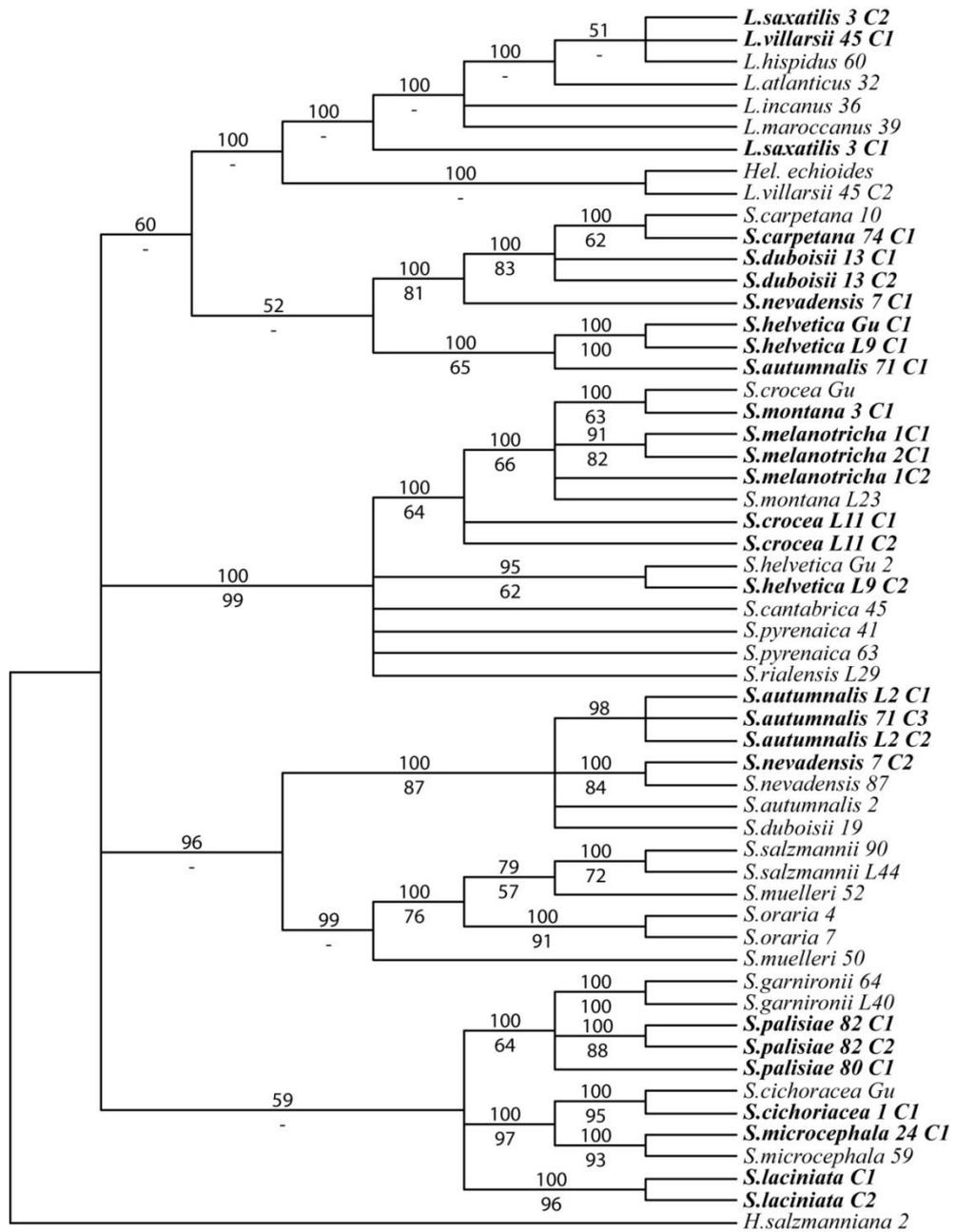
Figure 38: Bootstrap consensus tree of the nuclear *A39* locus, including 34 accessions from 15 species as well as 28 cloned sequences (bold) of 13 taxa of *Scorzoneroides* and 7 outgroup accessions from 7 species as well as 3 cloned sequences of 2 taxa of *Leontodon*. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches.
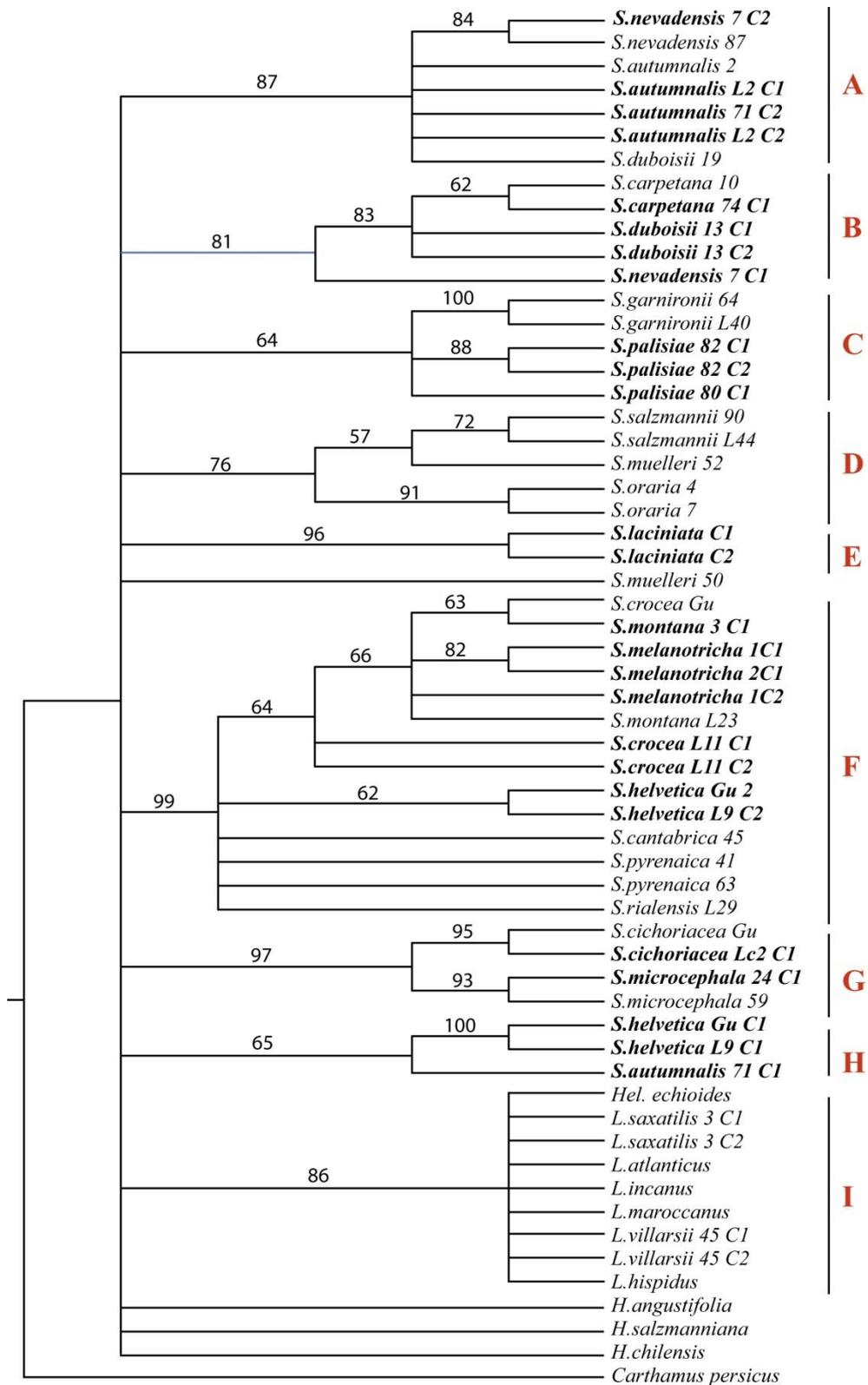
Figure 39: One out of 134 best trees of a combined matrix from both applied nuclear markers, namely *GAPDH* and *A39*, including 34 samples from 19 species of *Scorzoneroides* and 7 outgroup accessions from 7 species. Number of changes is given above, bootstrap support below branches.
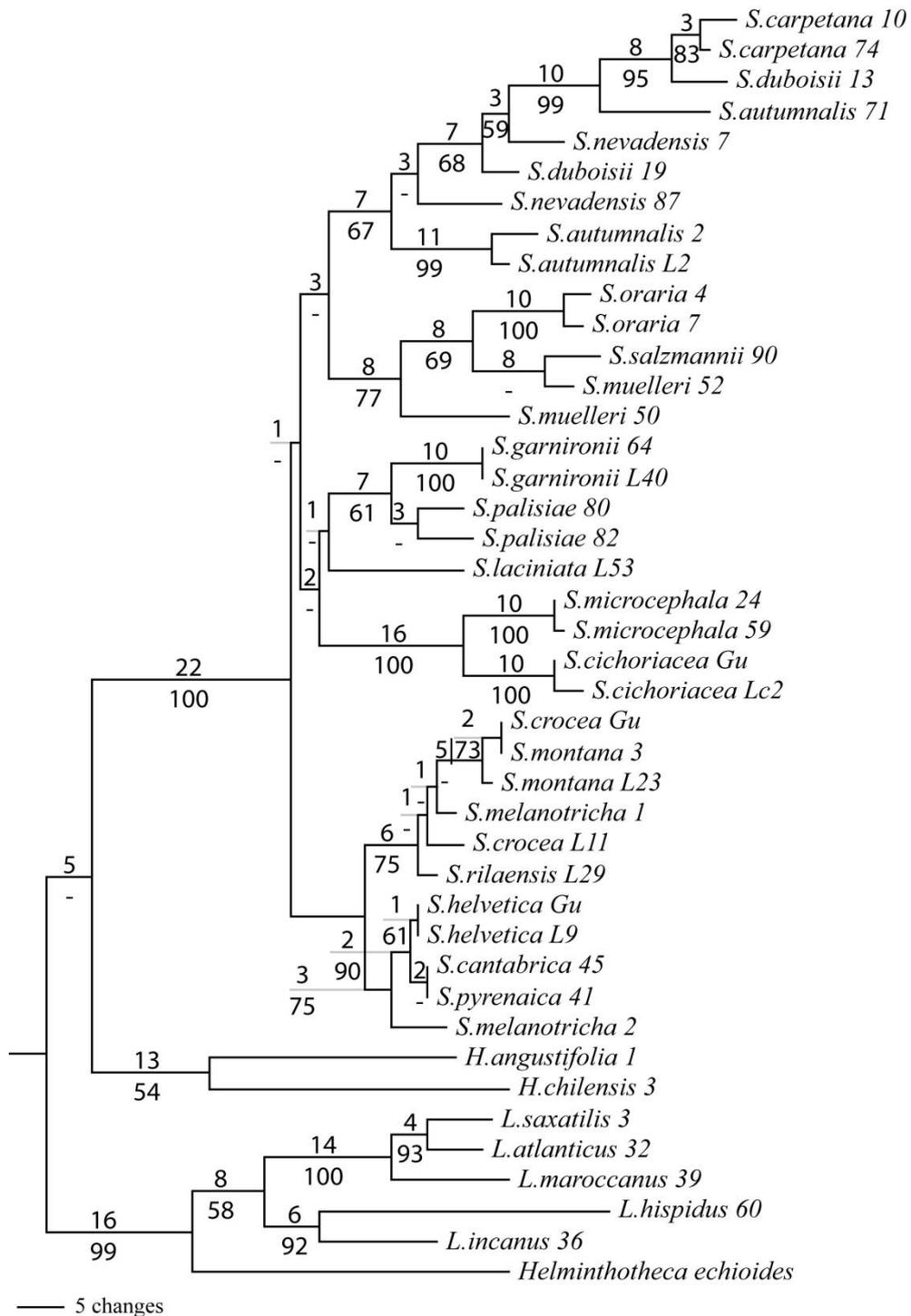
Figure 40: Bootstrap consensus tree of a combined matrix from both applied nuclear markers, namely *GAPDH* and *A39,* including 34 samples from 19 species of *Scorzoneroides* and 7 outgroup accessions from 7 species. Letters in red indicate groups referred to in the text. Bootstrap values are given above branches.
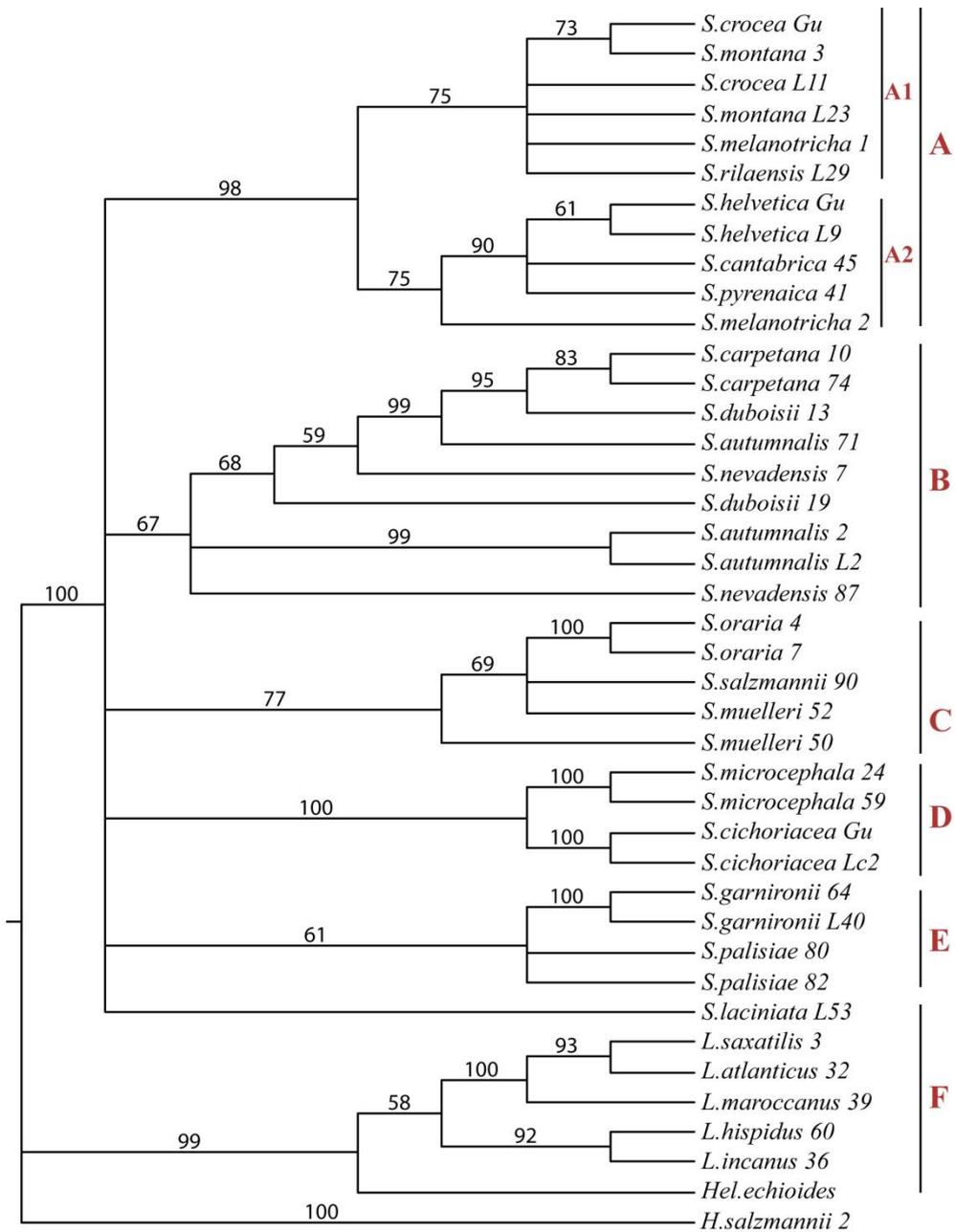
Figure 41: One out of 31 best trees of a combined matrix from the nuclear markers *GAPDH* and *A39* together with three markers of chloroplast DNA, namely *rpl16*-Intron, *ndhF-rpl32* and *atpH-atpI* intergenic spacer regions, including 35 samples from 19 species of *Scorzoneroides* and 7 outgroup accessions from 7 species. Number of changes is given above, bootstrap support below branches.
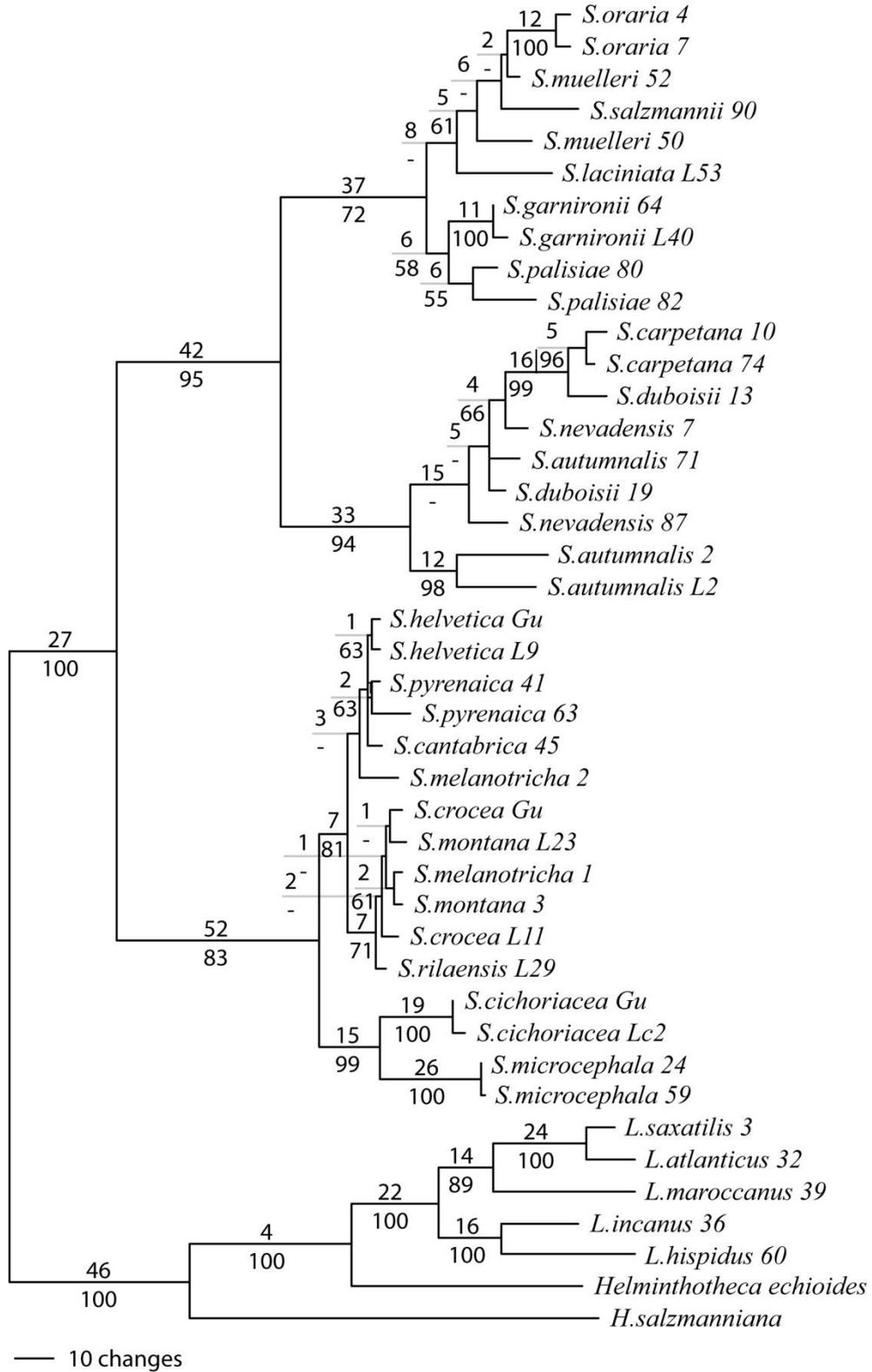
Figure 42: Bootstrap consensus tree of a combined matrix from the nuclear markers *GAPDH* and *A39* together with three markers of chloroplast DNA, namely *rpl16*-Intron, *ndhF-rpl32* and *atpH-atpI* intergenic spacer regions, including 35 samples from 19 species of *Scorzoneroides* and 7 outgroup accessions from 7 species. Number of changes is given above, bootstrap support below branches.
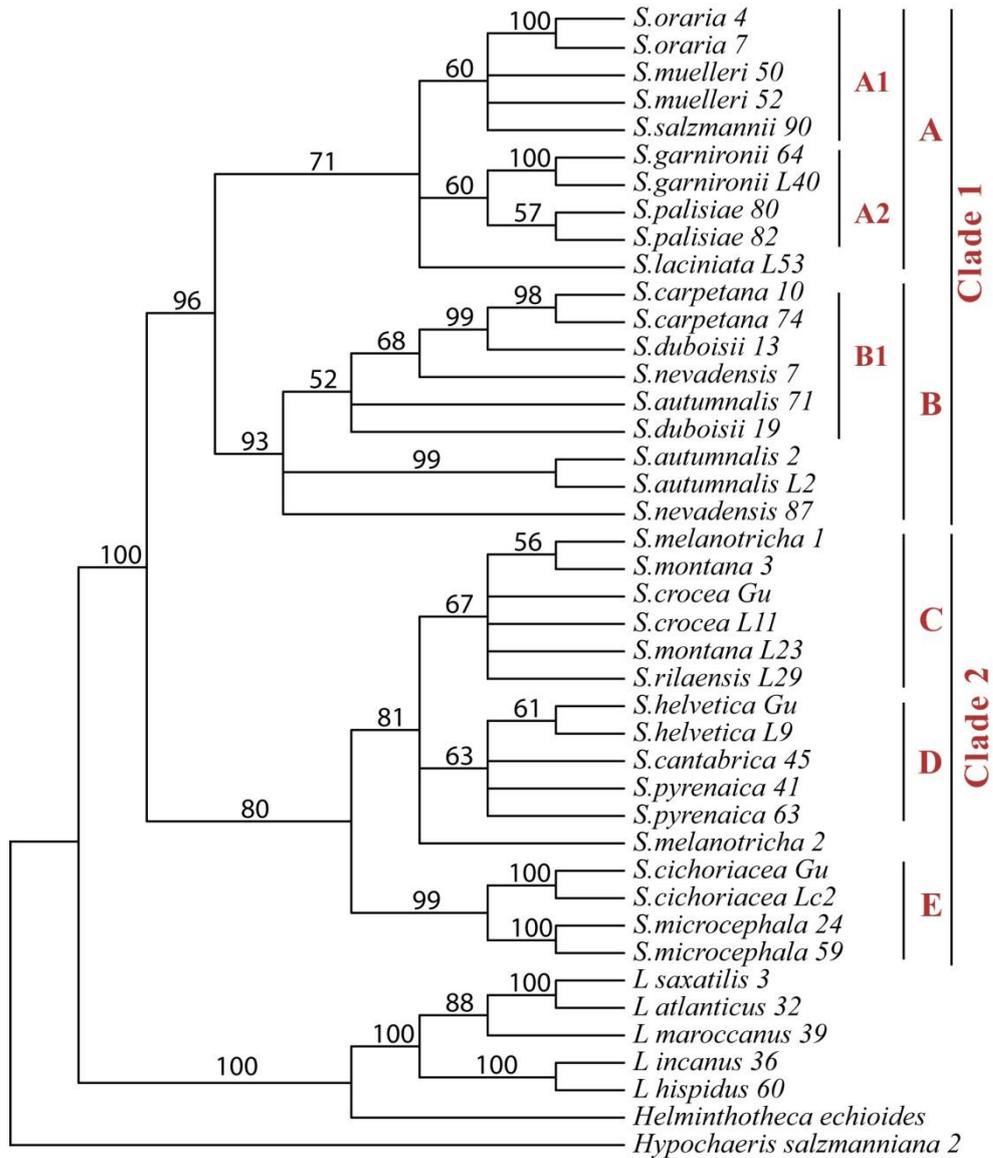
4.) CHROMOSOME COUNTS AND GENOME SIZE MEASUREMENTS

4.1. Introduction

Chromosome numbers, ploidy levels, and genome size (i.e. nuclear DNA content) data provide useful information in various fields of plant biology, including systematics, evolution and conservation biology (Stuessy, 2009). Most species are diploids, meaning, they have two sets of chromosomes, one set inherited from each parent. However, polyploidy is found in some organisms and is especially common in plants (Soltis & Soltis, 1999). Polyploidy can be artificially induced in plants by some chemicals, of which colchicine is the best known. The definition of polyploidy is having three, four or more sets of chromosomes instead of two present in diploids. In plants, the process of polyploidy sometimes results in new species, making it an important mechanism in evolution. Cotton, potatoes and wheat are Polyploids (Hilu, 1993; Wendel & Cronn, 2003), while maize and soybeans retain vestiges of ancient polyploidy events (Wendel *et al.*, 1989; Gaut *et al.*, 2000), called paleopolyploids. Fossil records show that over 80% of plants may be product of polyploidy, Soltis & Soltis (2009) discovered ancient polyploidy in all angiosperm lineages they included in their study except for *Amborella*.

Ploidy levels can be influenced by other factors than polyploidization. Aneuploidy stands for the loss or gains of regular individual whole chromosomes and with them all genes on that particular chromosome. In dysploidy, a whole chromosome of the haploid genome is lost, but no genetic information. This is also reflected in a more or less unchanged genome size. These events often can be traced back to wrong microtubuli-centromer interaction. (Stuessy *et al.*, 2009; Cumini & Degrassi, 2005).

Homoploid hybridization and allopolyploidy can be potential services of new species (Rieseberg, 1997), hybridization is referred to as a "ubiquitous feature of green plant evolution" (Soltis & Soltis, 1999, 2009). The success of hybrids is due to the vast increase in genetic variation, and especially allopolyploids have the advantage of heterozygosity in many alleles (Mallet, 2007). Hybrids may show traits, which are intermediate compared to the parents. Polyploids carry double the genetic material than the parents which increases the amount of alleles, and gives way for new, possibly advantageous combinations of gene variations resulting in novel traits (Levin, 1993). Diploid hybrids carry only one chromosome set of each parental species. This bottleneck in allelic variation (only one allele from one parent) increases the

possibility of a combination of extreme traits (e.g. Grant, 1981; Rieseberg 1997). In different studies, homoploid hybrids show adaptations to extreme habitats as a consequence (Heiser *et al.*, 1969; Gompert *et al*., 2006). A good example is *Helianthus* (Heiser *et al*., 1969; Rieseberg *et al*., 1991, 2003). The ecological niche changed from a broader scale for the parents – *H. annuus* grows on mesic and *H.petiolaris* grows on dry sandy soils – to very restricted habitats in the three occuring hybrid species, *H.anomalus*, *H.deserticola*, both restricted to the Great Basin desert in western US, and *H.paradoxus* , an endemic to saline and brackish marshes in Texas.

The genome size is the total amount of DNA contained within one copy of a genome. It is typically measured in terms of mass in picogram (10^{-12} of a gram) and abbreviated pg. Thousands of eukaryotes have been analysed over the past decades and genome sizes were made available via online databases, the database for plant DNA C-values (www.kew.org/genomesize/homepage.html) lists C-value data for more than 6.000 angiosperm species and is updated on a regular basis (Benett & Leitch, 2010).

Nuclear genome size is typically measured in eukaryotes using either densitometric measurements (i.e. measuring the optical density) of feulgen-stained nuclei or flow cytometry.

The term C-value (Swift; 1950), more specifically the 1C-value, refers to the amount of DNA contained within a haploid nucleus (e.g. a gamete) or half of the amount in a diploid somatic cell of a eukaryotic organism (Greilhuber *et al*., 2005). Measuring genome size is a useful tool in understanding evolution. An environmental adaptation often leads to a change in genome size. Water stress has reportedly led to, e.g., a decrease in genome size in Berbeis (Bottini *et al.*, 2000). Inter- and even intraspecific (for example in Maize, Sanmiguel & Bennetzen; 1998) alterations in genome size in context with adaptation to novel environmental influences (e.g. altitude, water availability) have been observed. The difference in genome size between closely related species can be high (Greilhuber, 1998).

Genome size increase is easily achieved by polyploidization, which often directly responds in a duplication of the genomic material (i.e. twice the chromosome set of autopolyploids) or in a value close to that level (depending on the parental lineages of allopolyploid hybrids) (Wendel, 2000). Another way to increase the 1C-level is an amplification of transposable elements, with the most effect coming from "LTR" – long terminal repeat retrotransposons (ability for "selfish" behaviour by copying itself via retrotransposition). Genome size decrease mainly is due to reduction of these multi-copy, non-coding retroelements. LTR (long terminal repeat) retrotransposons make up for a large amount of nuclear DNA (Bennetzen, 2002). Sanmiguel & Bennetzen (1998) discovered that about 70 percent of the whole genomic sequences in maize are made up by these retrotransposons. Changes in genome size and genome arrangement can

accumulate fast and may occur almost directly after a hybridization event. (Templeton, 1981; Rieseberg, 1997).

4.2. Chromosome work in Compositae

First chromosome counts in the family of Compositae dates back more than a hundred years from now (Semple & Watanabe, 2009). The basic chromosome number in Compositae is x=9 (Solbrig, 1977; Cronquist, 1981, Carr *et al.*, 1999). Semple & Watanabe (2009) summarized chromosome numbers of more than 1500 species and applied them to a metatree of Compositae from Funk *et al.* (2005). The base chromosome number in Cichorieae also is x=9 (Kilian *et al.*, 2009).

4.2.1. Chromosome work in Hypochaeridinae

In Hypochaeridinae, the number of chromosomes lies between x=9 and x=3 (Kilian *et al.*, 2009). Tis subtribe has been target of chromosome and karyotypic investigations by a number of scientists (Samuel *et al.*, 2003; Weiss-Schneeweiss *et al.*, 2003, 2007; Tremetsberger *et al.*, 2005; Ruas *et al.*, 2005, 2008). In most of these studies, emphasis was laid on *Hypochaeris*. The analyses of the chromosome number in this genus revealed a geographical pattern in the distribution of karyotypes. European and Mediterranean taxa showed different chromosome numbers (x=3,4,5,6) and types, whereas the South American group posseses only x=4 (Cerbah *et al*., 1998; Samuel *et al*., 2003). The use of karyotypic measuremens in *Hypochaeris* has given insight in evolution of the genus (Weiss-Schneeweiß *et al.*, 2003). Combined with molecular data, it led to the detection of North African origin for South American species. Rapid radiation caused more than 40 species to form after long-distance dispersal of one species from Morocco.

Chromosome counts for six species of *Leontodon* which are representatives of all three sections described for the genus, i.e. two species of the sect. *Asterothrix* (*L. crispus* and *L. incanus*), of sect. *Leontodon* (*L. hispidus* and *L. rigens*) and of sect. *Thrincia* (*L. longirostris* and *L. taraxacoides*) were done by Samuel *et al*. (unpublished). The basic chromosome numbers of *x* = 4, 6, and 7 were found in the examined species supporting a previous report for other *Leontodon* species (Pittoni, 1974).

4.2.1. Chromosome work in *Scorzoneroides*

For *Scorzoneroides*, chromosome numbers of x=6 and in some cases x=5 were reported (Pittoni, 1974; Izuzquiza, 1991; Izuzquiza & Nieto Feliner, 1991; Cruz-Mazo, pers. comm.; Ruas, pers. comm.).

4.2.2. Genome size measurements in *Scorzoneroides*

Two species of *Scorzoneroides*, namely *S.autumnalis* and *S.pyrenaica* have been measured in genome size. Their values of 1C=1.35 pg and 1C=1.43 pg, respectively could be accessed via the kew database for plant DNA C-values.

4.3. Material & Methods

4.3.1. Chromosome counts

Seeds were collected by Cruz-Mazo & Talavera from the following species: S.*muelleri*, *S.oraria*, *S.palisiae*, *S.salzmannii, S.autumnalis*, *S.cantabrica*, *S.carpetana*, *S.nevadensis* as well as *S.pyrenaica*. S.*muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii* germinated after 4 days. Of these, *S.oraria* developed cotyledons faster than the rest of this group. Five or more germinated seeds were pretreated with 2mM 8-hydroxyquinolin for 2.5 hours at room temperature, and 2.5h at 4°C, fixed in Carnoy (ethanol:acetic acid, 3:1) and stored at -20$^{\mathrm{o}}$C until used. Chromosome preparations were done using a standard Feulgen method. In brief, root tips were hydrolyzed in 5N HCl for 30 min at room temperature, washed briefly with tap water (2x), and stained with Schiff's reagent for 1h (room temperature, in darkness). Schiff's reagent is very sensitive to water and UV-light, thus the water used for washing has to be removed very careful. Root meristems were squashed in a drop of 45% acetic acid. Coverslips were removed on dry ice and the preparations were air dried and mounted in DPX. Chromosome counts were determined from at least three metaphase cells with well spread chromosomes from 3-5 plants for each species. Chromosomes were analyzed under the light microscope (Polyvar, Reichert-Jung) and photographed with Technical Pan 100 ASA film (Kodak).

4.3.2. Flow cytometry

For flow cytometry we used meristemic root tips if available or seeds if there was no germination success. Meristemic roots could be received for *S.muelleri*, *S.oraria*, *S.palisiae* and

S.salzmannii and one accession of both *S.autumnalis* and *S.carpetana*. A modified was employed, using

Isolation of the nuclei, staining and detection of the DNA was done according to a modified Propidium Iodide staining protocol for flow cytometry by Temsch (2003): From every individual, approximately 25 mg of root meristemic tissure of sample material and 20mg of the internal standard were chopped in 1.1 ml isolation buffer (pH ~1.5). The resulting suspension containing cell walls, and cell content including nuclei, ought to be filtered through a 30 micrometer nylon mesh (Sefar AG) in order to remove large debris, which might block the flow chamber. Subsequently, 50 ml of RNase A were added, the digestion of RNA was done in a water bath at 37 °C for 30 minutes. After digestion, 4ml propidium iodide (PI) solution (pH ~ 9.5) were added to the suspension, and incubated overnight in the refrigerator. A Partec PA II flow cytometer with mercury lamp was used; data management as well as histograms were conducted with FloMax. At least three runs were performed for each individual.

4.4. Results

We observed a difference in germination time success between annual and perennial species. The annuals had a very high germination rate, the average time for a germ bud to show cotyledons was 4 days, while perennial species showed germination in approximately 12 days.

Table 17: Chromosome numbers and genome size of 9 species of *Scorzoneroides*. * indicates chromosome numbers counted in different studies (Ruas, pers. comm.; Pittoni, 1974; Izuzquiza, 1991; Izuzquiza & Nieto Feliner, 1991; Cruz-Mazo *et al.*, 2009).

	Chromosome number	Genome size (mean)	Life cycle
Scorzoneroides autumnalis	2n=12*	1C=1.868 pg	perennial
Scorzoneroides cantabrica	2n=12*	n.a.	perennial
Scorzoneroides carpetana	2n=12*	1C=1.862 pg	perennial
Scorzoneroides muelleri	2n=12	1C=1.240 pg	annual
Scorzoneroides nevadensis	2n=12*	1C=1.773 pg	perennial
Scorzoneroides oraria	2n=12	1C=1.752 pg	annual
Scorzoneroides palisiae	2n=10	1C=1.787 pg	annual
Scorzoneroides pyrenaica	2n=12*	1C=1.662 pg	perennial
Scorzoneroides salzmannii	2n=10	1C=1.676 pg	annual

4.4.1. Chromosome counts

Observed chromosome numbers vary from 2n=10 to 2n=12 (Table 17). The size of the chromosomes varies from ca. 2 – 3 μm, nearly all of them are submetacentric (Figure 44)

4.4.2. Genome size

We had enough germinated material for the annual species *S.muelleri*, *S.oraria*, *S.palisiae* and *S.salzmannii*. The other five species, namely *S.autumnalis*, *S.cantabrica*, *S.carpetana*, *S.nevadensis* and *S.pyrenaica*, were at least partly measured by using ungerminated seeds. Seeds from *S.cantabrica* showed a very low quality; we could not use this species for genome size measurements. 1C-values of the investigated species vary from 1.24 to 1.87 pg (Table 15, Figure 42), and are relatively similar, with the exception of *S.muelleri*, which showed the lowest value. Most accessions showed useful, with only one case of a histogram-peak-coefficient of variation higher than 4 percent.

Figure 43: Genomic DNA 1C-values for eight species of the genus *Scorzoneroides*. Light grey indicates annual and dark grey perennial life cycle, respectively.
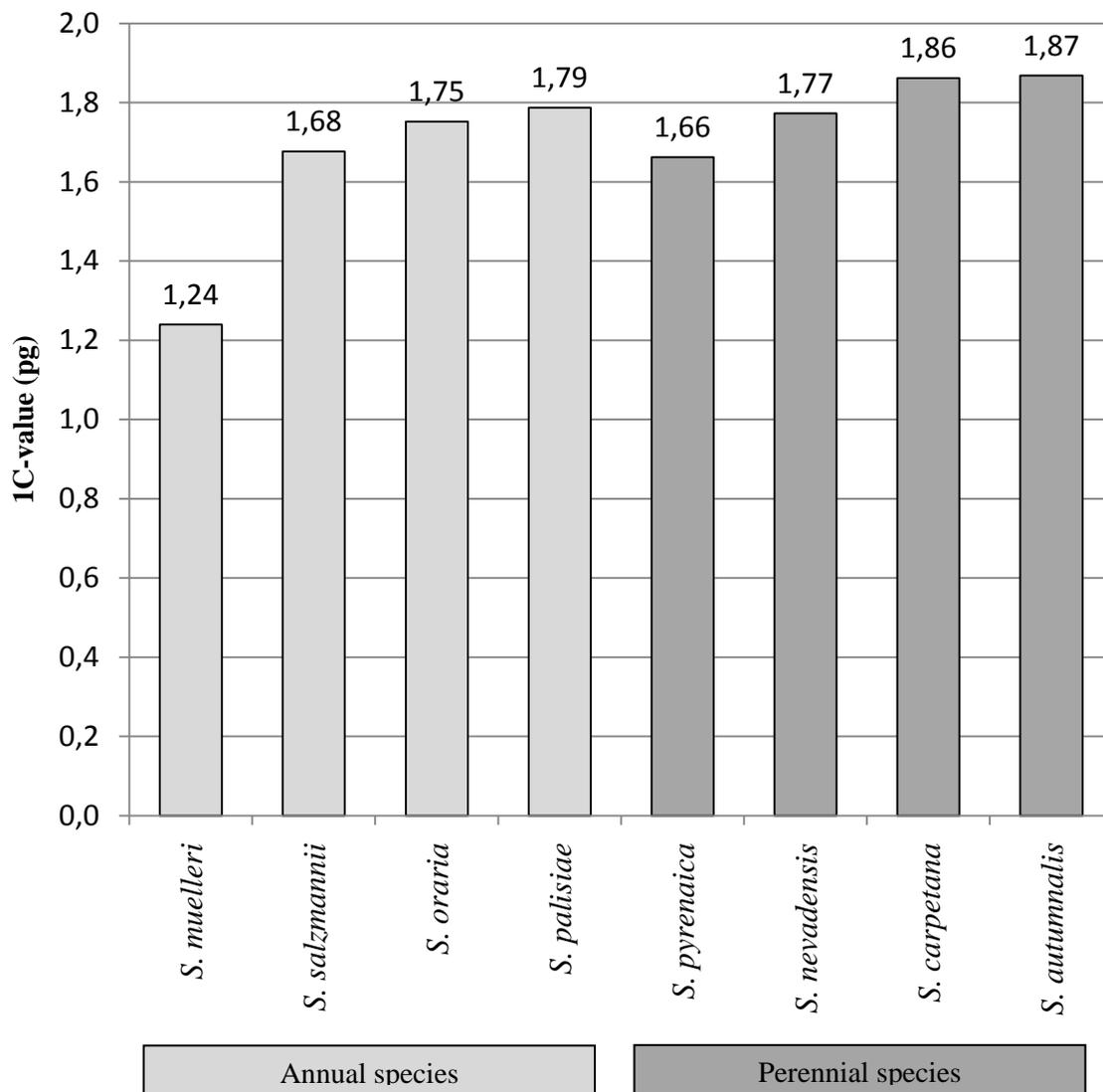
4.5. Discussion

Chromosome number of *Scorzoneroides* is reported to be 2n=12 in most of the cases, with some exceptions, where 2n=10. We merged our results with the observations from previous studies, to get an idea of chromosome evolution in *Scorzoneroides*. Observations from *S.autumnalis*, S.*montana*, *S.pyrenaica*, *S.cantabrica*, *S.carpetana* and *S.duboisii* were added to our results (Cruz-Mazo, pers.comm.; Ruas, pers.comm.)

Scorzoneroides autumnalis, *S.cantabrica*, S.*montana*, *S.duboisii*, *S.garnironii*, S.*muelleri* and *S.oraria* were reported to have a chromosome number of 2n=12 (x=6). Two annual species, namely *S.palisiae* and *S.salzmannii* show alterations in that number with 2n=10 (x=5). The change in chromosome number is likely to have occurred only after speciation in both cases. Also, chromosomes of *S.montana* are distinct from those of S.*muelleri* (Ruas, pers. comm.). The chromosomes of *S.montana* vary from metacentric to acrocentric and are larger than those of *S.muelleri* that possess all nearly submetacentric chromosomes.

The average genome size in *Scorzoneroides* is 1C=1.75 pg, annuals and perennials seem to be very similar in that context. Only one annual species, which occupies more extreme semidesertic habits, namely S.*muelleri*, has shown strong alterations towards a smaller genome size (1C=1.24 pg).

Changes in genome size and chromosome number in *Scorzoneroides* occurred explicitly in the annual taxa, which leads to the conclusion that the genus had to rearrange its genome as tribute to the environmental stress in arid zones. The genome size in perennials seems to be very stable, as it only varies between values between 1C=1.66 and 1C=1.87 pg. This is in congruence with the chromosome numbers.

Polyploidization events could not be observed in our study. Combining karyological data with phylogenetic analyses of our study, we suspect that *Scorzoneroides* originally had a perennial life-cycle, which goes along well with a previous study (Cruz-Mazo *et al*., 2009).

Figure 44: Chromosome plates of eight accessions from four species of *Scorzoneroides*, namely *S.salzmannii*, *S.oraria*, *S.palisiae* and *S.muelleri* (from top to bottom).

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CURRICULUM VITAE

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Date of Birth	20.11.1983
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EDUCATION

since 2008	Diploma Thesis under supervision of Ao.Univ.-Prof.Dr. Rosabelle Samuel at the Faculty Centre of Biodiversity, Department of Systematic and Evolutionary Botany on 'Phylogenetic relationship of genus *Scorzoneroides* [Compositae] inferred from plastid and low-copy nuclear markers, and karyological measurements in the genus'.
2003-2008	Diploma student of Biology at the University of Vienna, with specialization in Botany
06.2002	Final exam [Matura]
1994-2002	Student at Bundesrealgymnasium Schärding

PREVIOUS EMPLOYMENT

SCIENTIFIC PROJECT ASSISTANCE

06.2009-06.2011	'Detecting hybrid speciation with low copy nuclear genes and its usefulness in cultivated and invasive plants using taxa of Leontodon/Scorzoneroides [Asteraceae] as model organisms.' KIÖS P2008-05
01.2010-06.2010	'Molecular Phylogeny & Evolution of Polystachya [Orchidaceae]' FWF P19108
07.2009-09.2009	'Molecular Phylogeny & Evolution of Polystachya [Orchidaceae]' FWF P19108

PRACTICAL COURSES

DNA Barcoding course
DNA Marker und Chromosomen in Pflanzensystematik und Evolutionsforschung
DNA-Sequenzanalyse und molekulare Phylogenie
Ethnobotanical excursion to Thailand
Molecular markers in population biology [AFLP and Microsatellites]
Makromoleküle und molekulare Phylogenie in der Pflanzen-Systematik und Evolution
Metabolomics course [LC-MS]
Phytochemisches Projektpraktikum [Isolation of pure compunds, HPLC, MPLC]
Practical course on electron microscopy

LANGUAGES & SKILLS

LANGUAGES

German [native speaker], English [fluent], Italian [basic knowledge]

SOCIAL SKILLS

Teaching assistance [tutor] 'Pflanzenanatomische Übungen' for five Semesters; teaching assistance [tutor] 'DNA Marker und Chromosomen in Pflanzensystematik und Evolutionsforschung' for one Semester; technical assistant at at the "19th International Symposium 'Biodiversity and Evolutionary Biology' of the German Botanical Society [DBG]" held in Vienna from 16.-19.09.2003

TECHNICAL SKILLS

Lab working experience for more than two years; good knowledge of PCR, cycle sequencing, gel electrophoresis, cloning techniques; basic knowledge of AFLP, SSR, MPLC and HPLC techniques, primer design, chromosome counts and karyotype techniques;

COMPUTER SKILLS

Computer administration experience at the Department of Systematic and Evolutionary Botany for two years; excellent knowledge in computer hardware; good knowledge in MS Office [Excel, Word, Powerpoint], Adobe Photoshop, Adobe Illustrator, and various programs for phylogenetic analysis;

ADDITIONAL INFORMATION

PUBLICATIONS

Poster publication 'Molecular Phylogeny and detection of diploid hybrids in Scorzoneroides [Asteraceae]' at the '19th International Symposium "Biodiversity and Evolutionary Biology" of the German Botanical Society [DBG]' held in Vienna from 16.-19.09.2003