Evolution of the genus *Aristolochia* (Aristolochiaceae) in the Eastern Mediterranean including the Near East and Caucasia

Dissertation

zur Erlangung des akademischen Grades

Doctor rerum naturalium
(Dr. rer. nat.)

vorgelegt

der Mathematisch-Naturwissenschaftlichen Fakultät
der Technischen Universität Dresden

von

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geboren am 10. Dezember 1973 in Lattakia, Syrien

Eingereicht am 25.11.2009
Verteidigt am: 09.02.2010

Die Dissertation wurde in der Zeit vom 08/2005 bis 11/2009
am Institut für Botanik angefertigt

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Acknowledgement

Working as a Ph.D. student in a molecular phylogenetic laboratory was a magnificent as well as challenging experience to me. In these four years, many people were instrumental directly or indirectly in shaping this major part of my academic career. It was hardly possible for me to accomplish my doctoral thesis without the exhaustless support from these personalities to whom this tribute is addressed.

First of all, I would like to express my deep and sincere gratitude to my supervisor, Prof. Dr. Christoph Neinhuis for always being available with valuable help, and for his scientific advice and knowledge and many insightful discussions and suggestion, as well as for financial support of the lap work during the last years. I have to apologize because it is not sufficient to express my gratitude with only a few words.

I am very grateful to Dr. Stefan Wanke for his great help and support throughout the thesis. Starting from the first steps we made together in the field in Syria to the corrections of the last page of my thesis. His wide knowledge and his logical way of thinking have been of great value to me. His understanding, encouraging and personal guidance have provided a good basis for the present thesis. He also provided insightful discussions throughout the thesis. I will forever be grateful to him.

I also express my gratitude to Prof. Dr. Kai Müller for taking over the co-reference of my thesis.

I would like to thank my co-authors with whom the different chapters of my thesis will be published together (in alphabetic order):
Claude dePamphilis (Pennsylvania State University, USA), Hulusi Malyer (University of Uludag, Turkey), Kai F. Müller (University of Münster, Germany), Christoph Neinhuis (TU Dresden, Germany), Birgit Oelschlägel (TU Dresden, Germany), Aycan Tosunoglu (University of Uludag, Turkey) and Stefan Wanke (TU Dresden, Germany).

Am also grateful to Markus Günther (TU Dresden, Germany) for his technical assistance in using the scanning electron microscope as well as to the research group Plant Phylogenetics and Phylogenomics at the Institut of Botany, TU Dresden,

for providing support and friendship that I needed and largely contributed to making the period of my PhD thesis a wonderful experience.

I would like to express my gratitude to the staff of the Botanical Garden Dresden, and in particular Dr. Barbara Ditsch, for making the collection of living plants available.

I thank the followed herbaria for providing materials: The American University of Beirut, Lebanon (BEI), the Ankara University, Turkey (ANK), the Anadolu University, Turkey (ESSE), the Botanic Garden and Botanical Museum Berlin-Dahlem, Germany (B), the Gazi University, Turkey (GAZI), the Hebrew University of Jerusalem, Israel (HUJ), the Herbarium of the Friedrich Schiller University, Jena, Germany (JE), the Royal Botanic Garden Edinburgh, Great Britain (E), the Uludag University, Turkey (BULU), and the Yuzuncu Yıl University, Turkey (VANF), as well as numerous colleagues who provided plant material for the thesis.

The study was partly supported, by a scholarship from the Syrian Ministry of Higher Education, and a grant from the DAAD (Deutscher Akademischer Austauschdienst).

I am forever indebted to my parents, who teached me, since I was baby, to love and enjoy nature, from where I have achieved the energy during the years of the doctoral project. Also I express my sincere thanks to my parents in law, and to my sisters, brothers and their families for their loving support.

Last but not least, I owe my loving thanks to my wife (Maha) and to my small angels (Raghad and Rafih). They have lost a lot due to my research abroad. Their love and support without any complain or regret has enabled me to finish this work. I owe every achievement to them.

Introduction

The *Aristolochiaceae* are one of the largest angiosperm families, the family has been divided into two subfamilies: *Asaroideae*, which include *Asarum* and *Saruma*, and *Aristolochioideae*, which includes *Thottea* sensu lato and *Aristolochia* sensu lato (Kelly and Gonzales, 2003). *Aristolochia* sensu lato comprise between 450 and 600 species, distributed throughout the world with centers of diversities in the tropical and subtropical regions (Neinhuis et al., 2005, Wanke et al., 2006a, 2007).

However, the extended Mediterranean region including Turkey, the Caucasus and the Near East is likely to be the only diversity hotspot of the genus *Aristolochia* in the northern hemisphere were up to 60 species and subspecies could be observed (Wanke 2007). Most important contributions to the knowledge of these species were published by Nardi (1984, 1988, 1991, 1993) and Davis & Khan (1961, 1964, 1982), all of these studies were based on morphological characters only. In recent years, with the progress of molecular techniques and in light of the systematic chaos, a detailed study was needed to unravel the evolutionary history prior to a taxonomic revision of this group. The first chapter of my thesis should be regarded as the starting point for more detailed investigation on population level.

Preliminary molecular phylogenitic analysis recovered the Mediterranean *Aristolochia* species as monophyletic (de Groot et al 2006). However, only very few members were included in that study. The latest phylogenetic study by Wanke (2007) dealed with west Mediterranean *Aristolochia* species and sampled also few members belonging to the east Mediterranean and Caucasian species (3 from Greece, 2 from Georgia and 1 from Turkey). This study reported the Mediterranean *Aristolochia* species as two molecular and morphologically well supported clades, which were sister to each other. Furthermore, the two closely related species *A. sempervirens* and *A. baetica* which have an east west vicariance and are known as *Aristolochia sempervirens* complex has been recovered as sister group to the remaining west Mediterranean species. A detailed investigation of the evolutionary history of this group is the topic of the second chapter of my thesis (Chapter 2). The *Aristolochia sempervirens* complex is characterized by an unusual growth form and has a circum Mediterranean distribution. The investigation of these species complex seem to be of great importance to understand speciation and colonization of the Mediterranean by

the genus *Aristolochia* and might shade light in historical evolutionary processes of other plant lineages in the Mediterranean. Furthermore, I test applicability and phylogenetic power of a nuclear single copy gene (nSCG) region to reconstruct well resolved and highly supported gene genealogies as a prerequisite to study evolutionary biology questions in general.

Furthermore, a comprehensive overview of leaf epicuticular waxes, hairs and trichomes of 54 species from the old and new world taxa of the genus *Aristolochia* were investigated using scanning electron microscopy (SEM) to clarify taxonomic status of theses species in contrast to their molecular position. Also this study which is the third chapter of this thesis (Chapter 3), has a strong focus on Mediterranean *Aristolochia* and tries to provide additional support for molecular findings based on epicuticular waxes and to test them as synapomorphies.

Each chapter has its own introduction and abstract resulting in a short general introduction here.

Chapter 1

Detecting hybridization and incomplete lineage sorting, blurred traditional species identification – A first step towards unravelling evolution of the Near East Aristolochia species (Aristolochiaceae).

Abstract

This study provides the first molecular phylogeny of the East Mediterranean and Near East *Aristolochia* species including the Caucasian region. This group of species has been recovered as monophyletic in earlier studies based on a small sampling regarding both molecular and morphological data. All eastern Mediterranean *Aristolochia* species are endemic to the area and belong to the subsection *Aristolochia*, which share a sistergroup relationship with subsection *Podanthemum*, that together represent the Old World clade of subgenus *Aristolochia*. The present study is based on multiple coding and non-coding chloroplast datasets (*trnK* intron, *matK* gene, *trnK-psbA* spacer), and a nuclear single copy gene (nSCG) region containing multiple introns and exons for a representative sub-sampling. The study site is well known for it species diversity in general and is supposed to be the biodiversity hotspot of the Old World *Aristolochia*. We sampled all species (except *A. samsunensis*) belonging to this group including multiple populations representing the distribution area of each species, The systematic relationships within this group have not been addressed in the last decades and previous morphological and taxonomic treatments indicated the need for a thorough reinvestigation. We report the monophyly as well as the para-and polyphyly of several species indicating massive hybridization and incomplete lineage sorting. The signal obtained from chloroplast data usually supports geographic relationships of populations belonging to different morphological species, whereas nuclear data often supports traditional relationships based on phenotypic information. We proof the hypothesis that *A. maurorum* and *A. paecilantha* are producing natural hybrids employing a nSCG region in a pilot study. In addition updated distribution maps for each species are presented which provides more evidence for the Anatolian Diagonal hypothesis as a floristic break.

Introduction

The genus *Aristolochia* has a limited diversity in areas with seasonal climate of the northern hemisphere (Neinhuis et al. 2005, Wanke et al. 2006a). In North America only few species can be found (~5), whereas in Asia several more species had been reported (e.g. China 45 species, 33 of them endemic (Brach & Song 2006, Huang et al. 2009)). The highest rate of endemism coupled with species richness in the northern hemisphere with a rate of 100% endemism is confined to the extended Mediterranean region (Wanke 2007). Recent molecular based phylogenies recovered the Mediterranean *Aristolochia* species as monophyletic (Neinhuis et al. 2005, deGroot et al. 2006, Wanke 2007). However, only very few members were included in each of these studies. The Mediterranean species of the genus *Aristolochia* are part of *Aristolochia* s. str. (Neinhuis et al. 2005, Wanke et al. 2006a), an Old World clade probably nested within or sister to neotropical clades (Wanke et al. 2006a). *Aristolochia* s. str. has been subdivided into two lineages based on morphological characters: a) subsection *Podanthemum* (unilabiate flowers with a stiped utricle), and b) subsection *Aristolochia* (unilabiate or bilabiate flowers with a sessile utricle) (Gonzalez & Stevenson 2002), each recovered as monophyletic by molecular based phylogenies (Ohi-Thoma et al. 2006). Furthermore, subsection *Aristolochia* can be subdivided into two groups representing biogeography as well as morphology a) the West Mediterranean species mostly showing a non-auriculate flower, straight or U curved tube and a small perianth, and b) the Eastern Mediterranean species treated here including Turkey, the Caucasian region and the Near East showing mostly biauriculate flowers, U-curved or sigmoid tubes and a bigger perianth.

From the Western Mediterranean clade only 16 species are known whereas the Eastern Mediterranean clade and adjacent Near East region harbours about 26 species (Table 1). The largest number of *Aristolochia* species occur in Anatolia (Turkey) of which 15 are endemic to this area. All of the endemics are very local except *A. hirta* L., *A. bottae* Jaub & Spach and *A. maurorum* L. (Davis & Khan 1961, Davis 1982).

The Near East species of *Aristolochia* have not been studied since almost 20-30 years and more detailed studies and collections date back to 1960 and earlier (e.g. Duchartre 1864, Boisser 1879, Sosnowsky 1939, Davis and Khan 1961, Davis and Khan 1964, Zohary 1966, Davis 1982, Nardi 1991, Erken and Malyer 1998). All of these studies are exclusively dealing with morphological characters.

However, some of the Eastern Mediterranean species have been grouped together based on morphological similarities or sharing distribution patterns, such as the *A. maurorum* species complex (*A. maurorum*, *A. paecilantha* Boiss., *A. scabridula* Boiss, *A. bottae*) or the *A. hirta* group which represents a large number of species from Greece and the north-western part of Turkey such as *A. hirta*, *A. guichardii* Davis & Khan, *A. poluninii* Davis & Khan, *A. baseri* Malyer & Erken, *A. incisa* Duchartre (e.g. Nardi 1991, Davis & Khan 1961). Hybridization was assumed based on morphology especially for widespread representatives of these two species complexes. Possible hybridization and a consequence of unclear species boundary delimitation caused severe taxonomic problems in the past and might be symptomatic for all species belonging to the East Mediterranean.

To address some of these problems we conducted this study to provide a first molecular phylogeny of the East Mediterranean *Aristolochia* species. We generated phylogenetic hypotheses based on multiple coding and non-coding chloroplast loci (*trnK* intron, *matK* gene, *trnK* exon, *trnK-psbA* spacer), and a nuclear single copy gene region (nSCG) containing multiple introns and exons for a representative sub-sampling. The study clearly focuses on reconstructing the maternal lineage of the East Mediterranean *Aristolochia* species to evaluate groupings previously proposed on morphology and geography. Furthermore, we evaluate if previously hypothesised hybridization, based on phenotypic evidence in the *A. maurorum* group can be substantiated or rejected. This is done by testing the performance of a nSCG in a pilot study comparing the bi-parental inherited phylogenetic signal with the reconstructed maternal lineage. For the chloroplast dataset multiple accessions of each species have been sequenced representing the geographical range of each species. Furthermore, updated distribution maps are presented for each species. This study is the starting point for more detailed investigations to unravel natural hybridization, delimitate species boundaries, which finally will lead to a revision reflecting natural relationships.

Table 1 Distribution of the *Aristolochia* species in the East Mediterranean, Near East and Caucasia. All species are endemic to this region and often occur only in a restricted area of the respective country.

Aristolochia species	Greece	Turkey	Syria & Lebanon	Israel & Palestine	Jordan	Iraq	Iran	Georgia
A. auricularia	-	+	-	-	-	-	-	-
A. baseri	-	+	-	-	-	-	-	-
A. billardieri	-	+	+	+	-	-	-	-
A. bodamae	+	+	-	-	-	-	-	-
A. bottae	-	+	+	+	+	+	+	-
A. brevilabris	-	+	-	-	-	-	-	-
A. cilicica	-	+	-	-	-	-	-	-
A. cretica	+	-	-	-	-	-	-	-
A. geniculata	-	+	-	-	-	-	-	-
A. guichardii	+	+	-	-	-	-	-	-
A. hirta	+	+	-	-	-	-	-	-
A. hyrcana	-	-	-	-	-	-	+	-
A. iberica	-	+	-	-	-	-	-	+
A. incisa	+	+	-	-	-	-	-	-
A. isaurica	-	+	-	-	-	-	-	-
A. krausei	-	+	-	-	-	-	-	-
A. lycica	-	+	-	-	-	-	-	-
A. maurorum	-	+	+	+	-	-	-	-
A. olivieri	-	-	-	-	-	+	+	-
A. paecilantha	-	+	+	+	-	-	-	-
A. poluninii		+	-	-	-	-	-	-
A. pontica	-	+	+	+	-	-	-	-
A. rechingeriana	-	+	-	-	-	-	-	-
A. samsunensis	-	+	-	-	-	-	-	-
A. stenosiphon	-	+	-	-	-	-	-	-
A. steupii	-	-	-	-	-	-	-	+

Material & Methods

Most accessions for molecular studies were collected in the field (Turkey, Greece, Syria and Jordan) or were obtained as living material from e.g. Georgia or Israel. Many species from different localities were grown in the Botanical Garden Dresden to observe morphological characters under identical conditions and character shifts under different abiotic regimes. This was required to identify morphological variation within one species and to allow proper identification of each accession. In few cases herbarium material had to be used for DNA isolation especially with respect to material from countries such as Iran, Iraq or Lebanon, but was regarded only after unquestionable identification. We investigated material of all East Mediterranean species and included all but one (*A. samsunensis* Davis, only known from the type) into the chloroplast based phylogenetic trees. Mostly multiple accessions, covering the whole distribution area of each species (Table 2) have been incorporated.

Aristolochia pistolochia L. and *Aristolochia clematitis* L. were used as outgroup. Outgroup taxa were chosen based on previous phylogenetic analyses of the family Aristolochiaceae (Neinhuis et al. 2005, Wanke et al. 2006a, 2007) and phylogenetic studies sampling more densely Old World taxa of *Aristolochia* subgenus *Aristolochia* (Wanke 2007).

Fresh leaf material was collected and preserved on silica gel. Total DNA was isolated from both silica gel dried material and herbarium specimens using a CTAB extraction method as described in Borsch et al. (2003). The chloroplast region (*trnK* intron, *matK* gene, *trnK* 3' exon and *trnK-psbA* spacer) was generally amplified in two or three parts with an overlap of 400 to 700 bp for fresh material. Smaller PCR products of ~300 bp had to be amplified in some cases when DNA was isolated from older herbarium specimens (as old as 1956) using published primers by Wanke et al. 2006a, 2006b, Wanke et al. 2007 or mentioned in the present study. Primer sequences for the nuclear single copy region have been designed for this study (Table 3). The amplification was mostly performed in two overlapping parts (~600 bp). Based on full genome and transcriptome assemblies and different blast searches, shared single copy nuclear regions were identified among *Arabidopsis*, *Populus*, *Vitis* and *Oryza* (Duarte et al. in press, BMC Evol. Biol.). One of these regions, encoding a protein belonging to the S8e family and being involved on ribosome biogenesis (following TAIR), was chosen for this pilot study orienting on systematics. Initial primers for the genus *Aristolochia* were designed using available EST data (expressed sequence tags) from basal angiosperm lineages. Comparison

of EST data and further characterization was done using PlantTribes (http://fgp.bio.psu.edu/tribedb/index.pl, Wall et al. 2008) and TIGR Plant Transcript Assemblies (http://plantta.jcvi.org, Childs et al. 2007).

The PCR settings for the chloroplast region were as follows: 25 µL reaction containing 2,5 µL of 10% Taq buffer, 1 µL of 25 mM $MgCl_2$, 4 µL of dNTP mix (each 1,25mM), 0,5 µL of each primer (20pmol/µL), and 0,2 µL taq polymerase (5 units / µL). The temperature profile for the amplification consisted of initial denaturation at 96°C (1, 5 min), 55°C annealing (1 min), and 72°C extension (2 min).

The PCR settings for the nSCG region were as follows: 50 µL reaction containing 5 µL of 10% Taq buffer, 1,5 µL of 25 mM $MgCl_2$, 8 µL of dNTP mix (each 1,25mM), 1 µL of each primer (50pmol/µL), and 0,5 µL Taq polymerase (5units / µL). The amplification profile was set to 2 min. denaturation at 94°C, elongation for 1 min. at 55°C, and extension for 2 min. at 72°C. Ingredients from PEQLAB Biotechnologie GMBH, Erlangen, Germany have been used.

The PCR products were purified in a 1.2% agarose gel. Bands were cut out and purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Direct sequencing used the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer, Massachusetts, USA) with subsequent electrophoresis on ABI automated sequencers. Alternatively, the CEQ DTCS Quick Start Kit (Beckman Coulter, California, USA) with the SEQ 8000 sequencer was used for sequencing. Standard protocols for each kit had been followed. To obtain high quality sequences of all amplicons, products had been sequenced with both the forward and the reverse primer in addition to the overlapping PCR products.

Sequence data had been edited and alignment manually using PhyDe (Müller et al. 2005), following alignment rules proposed by Borsch et al. (2003) and Löhne & Borsch (2005).

Phylogenetic analyses have been calculated using both Maximum Parsimony (MP) and Bayesian analyses (BA). Evaluation of nodes has been performed as bootstrap supports (BS) and Posterior Probabilities (PP), respectively. Maximum parsimony analyses have been performed using PRAP (Müller 2004) producing a ratchet command file for PAUP* 4.0b10 (Swofford 2002). Settings were employed as following: 10 random addition cycles of 500 iterations each with a 25% of up weighting of the characters in the iterations. The evaluation of the MP tree was performed using the Bootstrap approach (Felsenstein, 1985), conducting 1000 replicates and random addition searches with 10 iterations per cycle. For Bayesian

inference the program MrBayes v3.1 (Ronquist and Huelsenbeck 2003) was used. The GTR model of nucleotide substitution was assigned, assuming site-specific rate categories following a gamma distribution. Four runs (10^6 generations each) with four chains each were run simultaneously, starting from random trees. Chains were sampled every 100 generations and the respective trees were written to a tree file. Calculation of the consensus tree and the PP of clades was done based upon the trees sampled after the chains converged (25%). Only PP's of 95 and higher were considered significant ($\alpha = 0.05$). Trees were compiled and drawn using TreeGraph (Müller and Müller 2004).

To evaluate geographic relationships of populations a haplotype network was reconstructed using the method of Templeton et al. (1992). This method is implemented in the software TCS (version 1.21) (Clement et al. 2000). Calculation is based on identical datasets to the one mentioned before. Haplotypes are grouped according to mutational steps computing the probability of parsimony for pairwise differences (0.95). Indels were coded as missing data for this approach as genetic distance was high and a more conservative approach was chosen.

Table 2. Accessions used in the present study (only for the molecular study), including information about the origin of material (field or collection), voucher information and the herbarium where the voucher is deposited.
* (incl. lab number referring to a specific species of one population on which the voucher and the DNA sequence is based on)

Aristolochia species*	Origin	Voucher (Herbarium)	GPS and altitude (if available)
A. auricularia P138	Turkey	BG Bonn 02790, leg. Koenen, Wanke 042 (DR)	
A. auricularia P703	Turkey, Anatolia, Sillyan	Hilliand & Buett 19233 (E) April 1999	N36° 48’ 34’’, E30° 12’ 25’’, 980m
A. auricularia P1129	Turkey, Antalya, Elmali	Bilisik AB1050/04 (BULU) 14.04.2008	N36° 50’ 41’’, E30° 01’ 22’’, 1141m
A. baseri P966	Turkey, Antalya, Kazanpinar	Malyer 11592 (ESSE) 21.05.1993	N36° 27’ 38’’, E29° 38’ 07’’
A. baseri P967	Turkey, Antalya, Kazanpinar	Malyer 11591 (ESSE) 09.05.1994	N36° 27’ 38’’, E29° 38’ 07’’
A. baseri P968	Turkey, Antalya, Elmali	Malyer 9764 (ESSE) 22.05.1993	N36° 24’ 59’’, E29° 52’ 01’’
A. billardieri P860	Turkey, Bozyazi	Mahfoud 26/1 (DR) 20.04.2007	N36° 05’ 53’’, E32° 56’ 23’’, 22m
A. billardieri P1109	Turkey, Antalya, Gazipasa	Bilisik AB 1001/1 (BULU) 26.03.2008	N36° 15’ 18’’, E32° 19’ 53’’, 59m
A. billardieri P1110	Turkey, Alanya	Bilisik AB 1001/2 (BULU) 26.03.2008	N36° 18’ 21’’, E32° 17’ 16’’, 44m
A. billardieri P1111	Turkey, Mersin, Alanyali	Bilisik & Malyer AB1049/4 (BULU) 23.04.2008	N37° 07’ 46’’, E34° 32’ 55’’, 1076m
A. bodamae P941	Turkey, Bursa, Görükle	Malyer 1961 (BULU) 19.05.1978	N40° 13’ 18’’, E28° 51’ 58’’, 98m
A. bodamae P943	Turkey, Bursa, Mudanya	Malyer 12337 (BULU) 11.05.1994	N40° 15’ 50’’, E 28° 56’ 27’’, 127m
A. bodamae P944	Turkey, Bilecik, Sögüt	Malyer 12331 (ESSE) 19.05.1996	N40° 18’ 13’’, E 30° 25’ 07’’, 646m
A. bottae P257	Syria, Alzebdane	Mahfoud & Wanke 3/2 (DR) 28.03.2006	N33° 42’ 64’’, E 36° 03’ 88’’, 1127m
A. bottae P273	Jordan, Balelah	Mahfoud 29/1 (DR) 16.04.2006	N32° 30’ 36’’, E 35° 56’ 28’’, 552m
A. bottae P274	Jordan, Assareh	Mahfoud 29/1 (DR) 15.04.2006	N32° 23’ 07’’, E 35° 56’ 78’’, 764m
A. bottae P277	Syria, Bludan	Mahfoud 26/3 (DR) 11.04.2006	N33° 42’ 62’’, E36° 03’ 911’’
A. bottae P282	Syria, Damascus, Kafrehawar	Mahfoud & Wanke 4/3 (DR) 29.03.2006	N33° 20’ 95’’, E35° 57’ 69’’, 1035m
A. bottae P329	Syria, Damascus, Dorbol	Mahfoud & Wanke 5/1 (DR) 29.03.2006	N33° 21’ 79’’, E35° 54’ 438’’, 1427m
A. bottae P331	Syria, Qatana	Mahfoud & Wanke 6/1 (DR) 29.03.2006	N33° 28’ 17’’,E36° 03’ 194’’, 1029m
A. bottae P730	Iran, 24km NW-Karind	Archibald 1889 (E) 14.06.1966	1520m
A. bottae P732	Turkey, Gumushane	Stainton 8330 (E) 04.06.1960	1300m

A. bottae P733	Iran, W-Kurdistan, Marivan	Jacobs 6510 (E) 06.06.1963	N35° 31', E64° 12', 1350m
A. bottae P734	Turkey, Sivas	Baytop 13394 (E) 02.04.1968	
A. bottae P735	Azerbaijan 34 km S-Razaiyeh.	Lamond 4178 (E) 15.06.1971	1350m
A. bottae P752 (pot. hyb.)	Turkey, Maras	Davis & Hedge 27289 (E) 30.04.1957	550 m
A. bottae P753 (pot. hyb.)	Turkey, Maras, Ahir Dag	Davis & Hedge 27408 (E) 02.05.1957	1300m
A. bottae P836	Syria, Damascus, Alruda	Mahfoud 18/1 (DR) 08.04.2007	N33° 42' 33'', E36° 047' 05'', 1184m
A. bottae P864	Syria, Damascus, Yafour	Mahfoud 38/1 (DR) 30.04.2007	N33° 36' 45'', E36° 01' 55'', 1200m
A. bottae P880	Israel, Namneman	Liston 306124 (HUJ) 19.02.1985	
A. bottae P961	Turkey, Sivas	Malyer 3371 (ESSE) 05.06.1983	N39° 16' 16'', E38° 01' 21'', 1350m
A. bottae P962	Turkey, Diyarbakir, Ergani	Güzel & Aslan 7182 (ESSE) 11.06.1982	
A. bottae P963	Turkey, Erzincan, Kemaliye	Malyer & Baser 3335 (ESSE) 04.06.1983	N39° 08' 33'', E38° 36' 47''
A. brevilabris P716	Turkey, Adana, 41 km E-Osmaniye	Nydegger 41089 (E) 08.04.1986	760m
A. brevilabris P1041	Turkey, Adana, Osmaniye	Malyer 6841 (ESSE) 11.05.1985	N37° 10' 12'', E36° 27' 38'', 272m
A. cilicica P964	Turkey, Adana, Pozanti	Malyer 6836 (ESSE) 09.05.1985	
A. cilicica P965	Turkey, Mersin, Tarsus	Malyer 28858 (BULU) 11.05.1985	
A. cilicica P1118	Turkey, Aladag	Bilisik & Malyer AB 1092/02 (BULU) 23.04.2008	N36° 56' 37'', E34° 29' 49'', 799m
A. cilicica P1119	Turkey, Mersin, Alanyali.	Bilisik & Malyer AB 1095/07 (BULU) 23.04.2008	N37° 07' 46'', E34° 32' 55'', 1076m
A. clematitis P142	Croatia, Liovik	W. Starmühler (DR)	
A. cretica P1122	Greece, Dodekanes, Kasos	Raeis 20.04.1983 (B)	N35° 24' 20'', E26° 54' 20'', 20m
A. cretica P1123	Greece, S.W-Agios Nikolaos	Muller 10.04.1975 (B)	N35° 32' 20'', E27° 11' 20'', 800m
A. geniculata P757	Turkey, Mersin, Magras Dag	Coode & Jones 756 (E) 11.05.1965	1200m
A. geniculata P1128	Turkey, Mersin	Bilisik AB1081/02 (BULU) 22.04.2008	N36° 41' 13'', E33° 32' 24'', 1009m
A. guichardii P145	Grecce, Rhode, Asklepio Burg	Wanke 186 (DR) 21.03.2005	N38° 04' 21'', E27° 56' 09'', 252m
A. guichardii P702	Turkey, Mugla, Marmaris	Davis 41444 (E) 19.04.1965	50m
A. guichardii P955	Turkey, Mugla, Fethiye	Malyer & Ögütveren 6313 (ESSE) 01.05.1984	

A. guichardii P956	Turkey, Mugla, Ula	Malyer & Ögütveren 6317 (ESSE) 03.05.1984	
A. guichardii P1100	Turkey, Denizli, Cameli	Bilisik AB 1239/03 (BULU) 31.05.2008	N36° 58’ 51’’, E29° 16’ 19’’, 1258m
A. guichardii P1102	Turkey, Mugla, Cetibeli	Bilisik AB 1018/02 (BULU) 29.03.2008	N36° 55’ 44’’, E28° 16’ 42’’, 42m
A. guichardii P1104	Turkey, Mugla, Ekincik	Bilisik AB 1042/02 (BULU) 12.04.2008	N36° 56’ 22’’, E28° 36’ 34’’, 20m
A. guichardii P1120	Greece, Rhodes, Glifada	Wanke 159 (DR)	N36° 11’ 11’’, E27° 46’ 09’’, 10m
A. hirta P167	Greece, Samos, Platanos	Neinhuis 134 (DR)	
A. hirta P687	Turkey, Antalya, Patara	Malyer & Ogutveren 11209 (E) 23.04.1994	350m
A. hirta P688	Turkey, Antalya, Adrasan	Malyer 6311 (E) 30.04.1984	490m
A. hirta P699	Turkey, Mugla, Fethiye	Davis & Polunin 25518 (E) 30.03.1956	30m
A. hirta P722	Turkey, Mugla, Marmaris	Davis 4127 (E) 17.04.1965	200m
A. hirta P723	Turkey, Aydin	Davis 41481 (E) 20.04.1965	400m
A. hirta P726	Turkey, Antalya, Kumluca	Pesmen 4260 (E) 23.03.1979	20-200m
A. hirta P789	Turkey, Antalya, Tekirova	Mahfoud 30/1 (DR) 22.04.2007	N36° 30’ 56’’, E30° 31’ 35’’, 34m
A. hirta P791	Turkey, Antalya, Chimaira	Mahfoud 32/1 (DR) 22.04.2007	N36° 25’ 44’’, E30° 25’ 41’’, 395m
A. hirta P793	Turkey, Antalya, Kumluca	Mahfoud 33/1 (DR) 22.04.2007	N36° 23’ 58’’, E30° 25’ 01’’, 512m
A. hirta P795	Turkey, Antalya	Mahfoud 29/1 (DR) 22.04.2007	N36° 22’ 65’’, E33° 05’ 18’’, 512m
A. hirta P797	Turkey, Antalya, Olympos	Mahfoud 31/1 (DR) 22.04.2007	N36° 25’ 39’’, E30° 25’ 40’’, 390m
A. hirta P800	Turkey, Antalya, Alanya	Mahfoud 28/1 (DR) 21.04.2007	N36° 37’ 41’’, E31° 45’ 48’’, 116m
A. hirta P1124	Turkey, Burdur, Bucak	Bilisik AB 1157/01 (BULU) 06.05.2008	N37° 29’ 07’’, E30° 33’ 57’’, 835m
A. hyrcana P745	Iran, Luristan, Scoul abad	Runemak & Lazari 26290 (E) 30.06.1977	1700m
A. iberica P107	Georgia	Lobin & Gröger BG BN 21911 (DR)	
A. iberica P950	Turkey, Rize, Findikli	Malyer 6708 (BULU) 28.05.1992	N41° 13’ 47’’, E41° 08’ 57’’, 90m
A. iberica P1125	Turkey, Rize, Hara	Bilisik AB 1221/03 (BULU) 17.05.2008	N41° 13’ 23’’, E41° 09’ 06’’, 86m
A. incisa P163	Greece, Samos, Platanos	Neinhuis 127 (DR)	
A. incisa P164	Greece, Samos, Pythagorio	Neinhuis 140 (DR)	
A. incisa P165	Greece, Samos, Pirgos	Neinhuis 142 (DR)	

A. incisa P709	Greece, Samos, E-Egean	Stamatiadou 8289 (E) 29.04.1970	
A. incisa P957	Turkey, Denizli, Honaz	Malyer 7467 (ESSE) 31.05.1986	N37° 44’ 21’’, E29° 21’ 31’’
A. incisa P1114	Turkey, Denizli, Serinhisar	Bilisik AB 1140/01 (BULU) 04.05.2008	N37° 38’ 23’’, E29° 12’ 59’’, 1131m
A. incisa P1115	Turkey, Denizli, Honaz	Bilisik AB 1057/03 (BULU) 16.04.2008	N37° 44’ 21’’, E29° 21’ 31’’, 876m
A. krausei P785	Turkey, Mersin, Erdemli	Mahfoud 23/1 (DR) 19.04.2007	N36° 28’ 30’’, E34° 10’ 23’’, 2m
A. krausei P787	Turkey, Mersin, Erdemli	Mahfoud 24/1 (DR) 19.04.2007	N36° 28’ 30’’, E34° 10’ 23’’, 2 m
A. krausei P953	Turkey, Mersin, Cambazli	Malyer & Ögütveren 6303 (ESSE) 25.04.1984	N36° 35’ 14’’, E34° 08’ 35’’, 850 m
A.krausei P954	Turkey, Mersin, Erdemli	Malyer & Ögütveren 6302 (ESSE) 24.04.1984	
A. krausei P1107	Turkey, Mersin, Limonlu	Bilisik & Malyer AB 1091/05 (BULU) 23.04.2008	N36° 35’ 30’’, E34° 06’ 21’’, 846m
A. krausei P1108	Turkey, Mersin, Limonlu	Bilisik & Malyer AB 1091/10 (BULU) 23.04.2008	N36° 35’ 30’’, E34° 06’ 21’’, 846m
A. lycica P710	Turkey, Güllüynit	Ayasligil 1425 (E) 30.04.1985	820m
A. lycica P951	Turkey, Isparta, Egirdir	Bilisik 29743 (BULU) 10.05.2007	N37° 49’ 57’’, E30° 51’ 59’’
A. lycica P952	Turkey, Antalya, Dag mevkii	Malyer & Ögütveren 5884 (BULU) 29.04.1984	N37° 11’ 22’’, E30° 29’ 21’’
A. maurorum P746	Turkey, Yozgat, Sorgun	Coode & Jones 1545 (E) 27.05.1965	1100m
A. maurorum P748	Turkey, Ankara, Kavaklidere	Guichard (TUR82/62) (E) 05.05.1962	300m
A. maurorum P755	Turkey, Elazig, Maden	Darrah 496 (E) 31.07.1969	524m
A. maurorum P758	Turkey, Ankara	Baytop 11466 (E) 12.06.1967	420m
A. maurorum P970	Turkey, Karaman	Malyer & Ögütvern 3104 (ESSE) .25.05.1983	
A. maurorum P973	Turkey, Isparta, Keciborlu	Malyer 11214 (ESSE) 06.05.1994	
A. maurorum P1074	Turkey, Isparta, Egirdir	Cochen & Avraham 8975 (HUJ) 27.06.1999	
A. maurorum P1130	Turkey, Sivas, Zara	Bilisik AB 1217/03 (BULU) 15.05.2008	N39° 27’ 33’’, E37° 41’ 34’’, 1348m
A. maurorum P1131	Turkey, Adana, Pozanti	Bilisik AB 1202/01 (BULU) 12.05.2008	N37° 31’ 49’’, E34° 33’ 10’’, 1329m
A. maurorum P1132	Turkey, Nigde	Bilisik AB 1200/09 (BULU) 12.05.2008	N38° 04’ 28’’, E34° 49’ 37’’, 990m
A. maurorum P1133	Turkey, Konya, Hadim	Bilisik AB 1184/05 (BULU) 10.05.2008	N37° 02’ 18’’, E32° 29’ 32’’, 1442m
A. maurorum x *paecilantha* P250	Turkey, Antakya, Karbeyaz	Mahfoud 49/1 (DR) 01.05.2006	N35° 55’ 06’’, E36° 06’ 88’’, 867m
A. maurorum x *paecilantha* P264	Turkey, Antakya, Karbeyaz	Mahfoud 49/2 (DR) 01.05.2006	N35° 55’ 06’’, E36° 06’ 88’’, 867m

A. maurorum x *paecilantha* P305	Turkey, Antakya	Mahfoud 47/1 (DR) 30.04.2006	N36° 07’ 65’’, E36° 09’ 71’’, 490m
A. maurorum x *paecilantha* P315	Turkey, Hasancali	Mahfoud 35/3 (DR) 26.04.2008	N35° 55’ 02’’, E36° 06’ 84’’, 804m
A. maurorum x *paecilantha* P318	Syria, Aleppo, Samman	Mahfoud 20/5 (DR) 01.04.2006	N36° 20’ 74’’, E36° 50’ 32’’, 450m
A. maurorum x *paecilantha* P337	Turkey, Antakya, Kirikhan	Mahfoud 36/1 (DR) 27.04.2007	N35° 44’ 12’’, E36° 12’ 75’’, 855m
A. maurorum x *paecilantha* P340	Turkey, Yayladag to Senkoy	Mahfoud 45/1 (DR) 30.04.2006	N35° 55’ 02’’, E36° 06’ 88’’, 809m
A. maurorum x *paecilantha* P342	Turkey, Senkoy	Mahfoud 43/1 (DR) 30.04.2006	N36° 04’ 49’’, E36° 09’ 23’’, 544m
A. maurorum x *paecilantha* P815	Syria, Aleppo, Aldana	Mahfoud 9/1 (DR) 05.04.2007	N36° 19’ 11’’, E36° 50’ 36’’, 470m
A. maurorum x *paecilantha* P819	Turkey, Samandag	Mahfoud 36/1 (DR) 27.04.2007	N36° 00’ 07’’, E36° 02’ 59’’, 657m
A. maurorum x *paecilantha* P823	Syria, Aleppo	Mahfoud 10/1 (DR) 05.05.2007	N36° 19’ 78’’, E36° 50’ 32’’, 462m
A. maurorum x *paecilantha* P825	Turkey, Harbiye	Mahfoud 20/1 (DR) 17.04.2007	N36° 02’ 41’’, E36° 08’ 30’’, 794m
A. maurorum x *paecilantha* P829	Turkey, Yayladag	Mahfoud 19/1 (DR) 17.04.2007	N36° 06’ 17’’, E35° 54’ 35’’, 707m
A. olivieri P706	Iran, Luristan, 40 km W-Khorramabad	Archibald 1667 (E) 30.04.1966	1220m
A. olivieri P707	Iran, Luristan, Sheshom	Jacobs 6444 (E) 28.04.1963	N33° 06’, E47° 43’, 800-1100m
A. paecilantha P286	Syria, Hums, Alhusn Fortress	Mahfoud & Wanke 8/2 (DR) 30.03.2006	N34° 45’ 34’’, E36° 17’ 74’’, 640m
A. paecilantha P330	Syria, Hums, Alhusn Fortress	Mahfoud & Wanke 8/1 (DR) 30.03.2006	N34° 45’ 32’’, E36° 17’ 74’’, 653m
A. paecilantha P686	Syria, Latakia, Salah Alden	Mahfoud 33/1 (DR) 19.04.2006	488m
A. paecilantha P801	Syria, Msyaf to Banias	Mahfoud 16/1 (DR) 06.04.2007	N35° 05’ 12’’, E36° 17’ 20’’, 902m
A. paecilantha P802	Syria, Msyaf to Banias	Mahfoud 16/2 (DR) 06.04.2007	N35° 05’ 12’’, E36° 17’ 20’’, 902m
A. paecilantha P805	Syria, Msyaf to Banias	Mahfoud 15/1 (DR) 06.04.2007	N35° 05’ 18’’, E36° 17’ 22’’, 890m
A. paecilantha P807	Syria, Msyaf to Banias	Mahfoud 14/1 (DR) 06.04.2007	N35° 05’ 19’’, E36° 17’ 22’’, 892m
A. paecilantha P812	Syria, Al zebdane	Mahfoud 39/1 (DR) 30.04.2007	N33° 42’ 60’’, E36° 03’ 95’’, 1150m
A. paecilantha P813	Syria, Hums, Marmarita	Mahfoud 41/1 (DR) 30.04.2007	N34° 45’ 18’’, E36° 17’ 56’’, 1152m
A. paecilantha P814	Syria, Hums, Alhusn Fortress	Mahfoud 42/1 (DR) 30.04.2007	N34° 45’ 32’’, E36° 17’ 74’’, 653m
A. paecilantha P879	Israel, Hermon	Cohen 306116 (HUJ) 07.05.1993	1620m
A. paecilantha x *maurorum* P256	Turkey, Yayladagi to Senkoy	Mahfoud 44/2 (DR) 30.04.2006	N35° 55’ 02’’, E36° 06’ 88’’, 809m

A. paecilantha x *maurorum* P258	Turkey, Antakya	Mahfoud 46/1 (DR) 30.04.2006	N36° 07’ 72’’, E36° 09’ 74’’, 414m
A. paecilantha x *maurorum* P260	Turkey, Antakya to Mersin	Mahfoud 41/4 (DR) 29.04.2006	N36° 64’ 71’’, E36° 45’ 78’’, 512m
A. paecilantha x *maurorum* P267	Turkey, Antakya	Mahfoud 46/4 (DR) 30.04.2006	N36° 07’ 65’’, E36° 09’ 71’’, 490m
A. paecilantha x *maurorum* P270	Turkey, Antakya	Mahfoud 48/1 (DR) 30.04.2006	N36° 07’ 72’’, E36° 09’ 74’’, 414m
A. paecilantha x *maurorum* P296	Turkey, Harbiye	Mahfoud 39/1 (DR) 29.04.2006	N36° 02’ 57’’, E36° 07’ 48’’, 888m
A. paecilantha x *maurorum* P300	Turkey, Sinanli	Mahfoud 51/1 (DR) 01.05.2006	N35° 39’ 07’’, E36° 01’ 25’’, 521m
A. paecilantha x *maurorum* P344	Turkey, Karbeyaz	Mahfoud 50/1 (DR) 01.05.2006	N35° 55’ 00’’, E36° 06’ 88’’, 867m
A. paecilantha x *maurorum* P380	Turkey, Harbiye	Mahfoud 39/3 (DR) 29.04.2006	N36° 02’ 57’’, E36° 07’ 48’’, 888m
A. paecilantha x *maurorum* P811	Turkey, Harbiye	Mahfoud 21/1 (DR) 17.04.2007	N36° 02’ 41’’, E36° 08’ 30’’, 794m
A. pistolochia P136	France, Cassis	Wanke 025372 (DR)	
A. poluninii P958	Turkey, Mugla, Fethiye	Duman & Koyunu 2971 (BULU) 30.04.1996	N36° 29’ 50’’, E29° 07’ 35’’, 200m
A. poluninii P1097	Turkey, Denizli, Cameli	Bilisik AB 1240/01 (BULU) 31.05.2008	N36° 58’ 51’’, E29° 16’ 19’’, 1258m
A. poluninii P1098	Turkey, Denizli, Cameli	Bilisik AB 1240/02 (BULU)31.05.2008	N36° 58’ 51’’, E29° 16’ 19’’, 1258m
A. poluninii P1099	Turkey, Mugla, Faralye	Bilisik AB 1045/04 (BULU)13.04.2008	N36° 58’ 51’’, E29° 16’ 19’’, 1258m
A. pontica P947	Turkey, Bursa, Mezitler	Erken 9771 (ESSE) 14.05.1992	
A. pontica P948	Turkey, Samsun	Malyer 6810 (ESSE) 05.07.1986	
A. pontica P1126	Turkey, Samsun	Bilisik AB 1226/02 (BULU) 21.05.2008	N41° 23’ 05’’, E36° 07’ 18’’
A. stenosiphon P1042	Turkey, Mersin, Silifke	Malyer 7470 (ESSE) 12.04.1987	N36° 34’ 57’’, E33° 56’ 13’’
A. stenosiphon P1043	Turkey, Mersin, Silifke	Malyer 5885 (BULU) 26.04.1984	N36° 34’ 57’’, E33° 56’ 13’’
A. steupii P1044	Georgia	BG Bonn	

Table 3 Primer sequences used for PCR amplification and sequencing of the nSCG region and the respective exon each primer has been placed in (see Figure 1). Primer sequences have been designed for this study.

Primer	Primer sequence	placement
AR-5443-1010R	GCATTTATACARCCATCATTCTCTGG	Exon IX
AR-5443-600F	GCGGGTAAATGGGAGGTTCC	Exon V
AR-5443-980R	GAACATCTCATCTTCAGCAACAGGTCTC	Exon VI
AR-5443-1230F	CAAGCARTGGAAAAGGATGGTGACC	Exon VII
AR-5443-980F	GAGACCTGTWGCTGAAGATGARATGTT	Exon VI
AR-5443-1230R	GGTYACCATCCTTTTCCAYTGCTT	Exon VII

Results

Variability of the datasets & microstructural variation

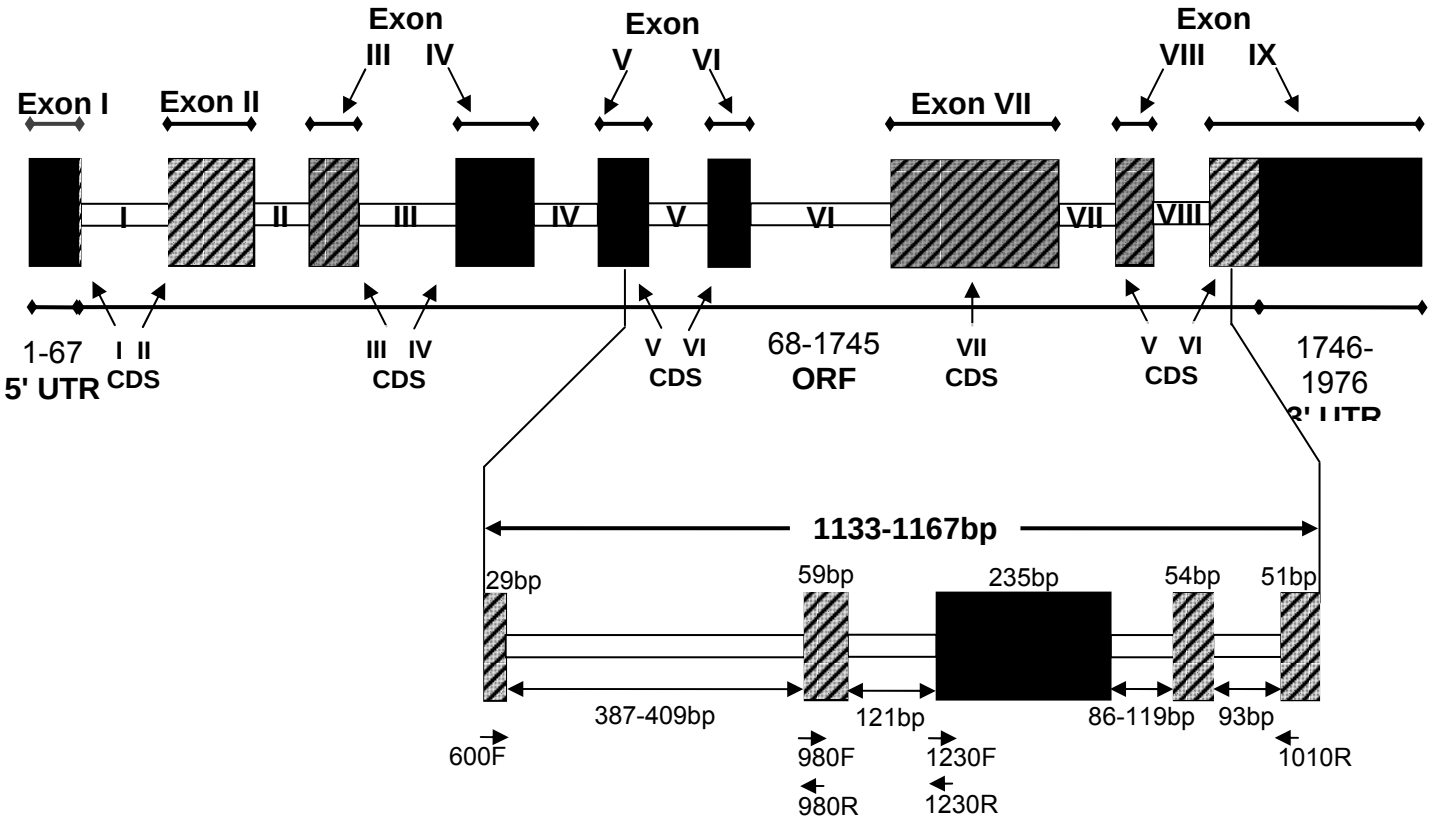
Among ingroup taxa, the total alignment length of the *trnK* intron, *matK* gene, *trnK* 3' exon and *trnK-psbA* spacer comprised 4858 characters (Table 4). Six highly variable regions had to be excluded due to ambiguous homology assessment (Table 4). In the *trnK* 5' intron, two of these microsatellites where of cryptic simple nature (Wanke et al. 2006a) and had a maximum bp length of 976 and 480, respectively (Table 4). All other satellites showed A or T mononucleotide repeats typical for the chloroplast genome (Table 4). Although, we excluded highly variable regions from the *trnK* intron for this study we believe that for further, more detailed investigations on population level, investigating only one or few species, they are likely to be useful if results from secondary structure analyses will be taken into account but this is beyond the scope of this study. In total, 150 parsimony informative characters (PIC) have been found in the combined chloroplast dataset. In addition 104 insertions and deletions (indels) have been coded (not used for the haplotype network). Indel coding was performed using the simple indel coding procedure published by Simmons & Ochoterena (2000) as implemented in Seqstate (Müller 2005). More details on the characterization of the chloroplast datasets can be obtained from Table 4.

The nSCG dataset were comparably small, containing less than ¼ (1216 characters) of the alignment length of the combined chloroplast dataset. It was alignable without ambiguous areas, thus no highly variable regions had to be excluded. The full nSCG region contains nine exons and eight introns in *Arabidopsis thaliana* (Figure 1). The flanking primers, designed for the present study were placed in exon V and exon IX, amplifying three full exons and two exons in part as well as four introns (Figure 1). Two of these introns show length variation (intron V, intron VII) of 14 and 4 indels, respectively. Although, the size was much smaller than the chloroplast region it contained 49 PICs, which is $^1/_3$ of the combined chloroplast dataset with less than ¼ of the sequencing effort.

Table 4 Alignment characteristics of the chloroplast regions on which the phylogenetic tree reconstruction are based on (original dataset). Annotations of intron, exon and spacer boundaries are based on the full chloroplast genome of *Piper cenocladum* (DQ887677). Dataset length used for final calculations, excluding highly variable regions (homology assessment questionable) is provided in brackets as well as the type of microsatellite due to which a region was excluded and the spans in bp is provided in a separate column. PIC are the parsimony informative characters found in the respective loci (excluding the highly variable regions).

loci	position	characters	length range	type of satellite	PIC
trnK 5' intron	1-2458 (1-934)	2458 (934)	815-1892 (684-734)	AT rich repeat (85-976), A mononucleotide repeat (5-8), AT rich repeat (25-480)	40
matK gene	2459-4022 (935-2481)	1564 (1547)	1521-1539 (1512-1524)	T mononucleotide repeat (8-18)	77
trnK 3' intron	4023-4336 (2482-2783)	314 (302)	259-277 (252-267)	A mononucleotide repeat (5-12)	13
trnK 3' exon	4337-4371 (2784-2818)	35 (35)	35 (35)	-	1
trnK-psbA spacer	4372-4858 (2819-3288)	487 (470)	281-311 (268-297)	T mononucleotide repeat (8-17)	19

Figure 1 Characterizing diagram of the nSCG region in *Aristolochia incisa* (P164 Greece, Samos) (lower model) compared to *Arabidopsis thaliana* (AT5G06360.1) (upper model). The CDS encodes a protein of the S8e family (ribosome biogenesis). Length of the region is presented proportional based on the situation found in the respective accession. Untranslated regions are shown as large black boxes and exons as large hatched boxes. Introns are shown as small blank boxes. Labelling of each region follows the *Arabidopsis thaliana* accession (AT5G06360.1) from "The Arabidopsis Information Resource" (TAIR) (www.arabidopsis.org). The size in bp mentioned is the range of all *Aristolochia* accessions fully sequenced for each region whereas for *Arabidopsis* only the absolute span is provided. Homology of each region is based on the alignment of the exons as the introns were not alignable between *Aristolochia* and *Arabidopsis* accessions. Proportional locations of primers used in the present study are shown below the *Aristolochia* model and the sequences of each primer can be obtained from Table 3.



Phylogenetic evidence from the combined chloroplast loci

Although, nearly 5000 bp have been sequenced from chloroplast genomes, which are generally considered useful for species and population level systematics (Shaw et al. 2005, 2007) the phylogenetic signal is not completely satisfying. The trees of the separate datasets are largely unresolved (not shown) and only the combination of all chloroplast regions could reconstruct a largely resolved maternal lineage. Here we provide the phylogenetic hypothesis generated from the combined cpDNA loci using Bayesian inference (Figure 2) and mention results from the same dataset based on parsimony (both with coded length mutations and without) in case of incongruence. The Bayesian tree for 143 ingroup populations shows three main clades, hereafter called I, II and III (Figure 2 A, B, C). Clade I and II are statistically supported, whereas clade III is not and collapses in the strict consensus MP tree when the information from coded length mutations is not included.

The first highly supported clade I (1.00 PP) represents mostly the endemic species from the Cilician part of Turkey (S-Turkey) *A. krausei* Davis*, A. brevilabris* Bornm. *and A. cilicica* Davis & Khan (Suppl. Fig. 1). *Aristolochia billardieri* Jaub & Spach is also occurring in this area but spreads through the eastern part of the Mediterranean: Syria, Lebanon and Israel (Suppl. Fig. 1). All four species are recovered as monophyletic (each 1.00 PP) (Figure 2). Clade I also contains *A. maurorum* populations from Central and western Turkey (Suppl. Fig. 2). These populations (*A. maurorum* group A) do not form a monophyletic group in any phylogenetic tree (Figure 2). *Aristolochia billardieri* and *A. krausei* are sister species (1.00 PP) and *A. brevilabris* is subsequent sister to this clade (0.92 PP). Although, *A. cilicica* is monopyletic, this clade is nested within the unresolved *A. maurorum* group A.

The second major clade (clade II) is also strongly supported (1.00 PP) and contains the majority of populations and species previously grouped in the *A. maurorum* complex (*A. maurorum*, *A. paecilantha*, *A. bottae, A. scabridula*), the latter is considered to be a synonym of *A. paecilantha* (Davis & Khan, 1961). In addition, *A. hyrcana* Coll. and *A. olivieri* Davis & Khan are part of clade II (Figure 2). Except for *A. olivieri* and *A. hyrcana* (the latter only one accession included), none of the species of clade II are recovered as monophyletic. *Aristolochia hyrcana* (Iran) is recovered as sister (1.00 PP) to *A. olivieri* (Iran, NE-Iraq) and forms a monophyletic Irano-Turanian clade together with *A. bottae* group C (0.89 PP). Although, relationships recovered in this study are often in congruence with biogeographical patterns, this is not the case for the *A. bottae* group A & B (Suppl. Fig. 3). Group A and B are morphologically

identical but biogeographically well separated. Our phylogenetic reconstructions support the morphological observations.

Populations of *A. paecilantha* as well as populations supposed to be of hybrid origin of the parents *A. maurorum* and *A. paecilantha* according to Davis & Kahn (1961) compose the remaining lineages of clade II and are supported as monophyletic (1.00 PP) (Figure 2). Individuals of these populations have previously been entitled as hybrids of *A. maurorum* and *A. paecilantha* because of intermediate phenotypes and these populations are occurring geographically in between the *A. maurorum* group A (Anatolia) and the *A. paecilantha* populations (Syria, Lebanon, Israel) (Davis & Khan, 1961; 1982) (Suppl. Fig. 2).

The third clade (clade III) gains no statistical support (0.59 PP) (Figure 2) and includes 14 species from all over the East Mediterranean. Although several subclades are highly supported, deeper nodes collaps and form a polytomy. Out of the 14 species belonging to this clade, several are recovered as para- or polyphyletic. *Aristolochia steupii* Woronov. from Georgia is represented only by one accession. In total five species sampled with two to four accessions, representing the distribution area of each species, are recovered as monophyletic with support (except *A. poluninii,* 0.67 PP). *Aristolochia cretica* Lam., *A. stenosiphon* Davis & Khan*, A. geniculata* Nardi*, A. poluninii* and *A. baseri* are the monophyletic species (Figure 2). Several other subclades receive moderate to high support, but do not reflect traditionally recognized species boundaries based on morphology. In contrast, biogeographical clustering is often observed.

The first lineages of clade III represent populations, which are exclusively occurring in the northern part of Turkey and Georgia. These two lineages again show biogeographic patterns (Suppl. Fig. 4):

a) *A. steupii* (Georgia) and *A. iberica* Fisch. & Mey. (Georgia, NE-Turkey), represent the first clade (0.97 PP),

b) *A. bodamae* Dingler (NW-Turkey) and *A. pontica* Lam. (N-Turkey) form the second (1.00 PP). Both species are paraphyletic with respect to populations of each species (Figure 2).

The next node of clade III receives only moderate support (statistically unsupported, 0.85 PP) and is formed by three clades being virtually unsupported (0.63 PP) to highly supported (1.00 PP). The populations of the monophyletic *A. cretica* from Greece (Islands of Crete, Kassos and Karpathos) form one clade (1.00 PP) (Figure 2, Suppl. Fig. 5). The two other clades are again fitting perfectly to biogeographic

distributions but for most species identification based on morphological evidence are practically incongruent to the findings reported here (Figure 2). All *Aristolochia* populations found in these clades represent species occurring in SW-Turkey, W-Turkey or the adjacent Aegean Islands. Here we summarize only findings of para- and polyphyletic species and the respective biogeographic relationships:

a) populations of *A. hirta* splits up into at least three independent clades named A, B, C (Figure 2), group A represents a biogeographic distinct area as compared to the two other groups (Suppl. Fig. 6);

b) populations of *A. incisa* form two biogeographically separated clades (Suppl. Fig. 5). The Greek populations (incisa group A) are clustering with Greek *A. hirta* populations (both from Samos, Figure 2) and the Turkish populations of *A. incisa* (*A. incisa* group B) together with Turkish *A. guichardii* (*A. guichardii* group C);

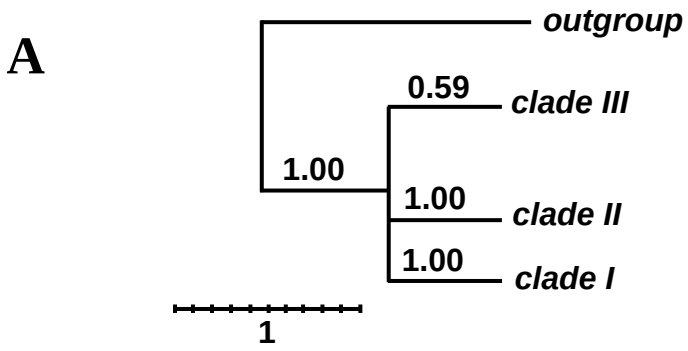
c) *Aristolochia guichardii*, which has a limited distribution in SW-Turkey and the adjacent Greek Islands (e.g. Rhode) are recovered as three independent clades. *Aristolochia guichardii* from Rhode represents one clade (*A. guichardii* group A). Two Turkish clades are recovered in which the monophyletic of *A. poluninii* clade is nested. *Aristolochia poluninii* and the two *A. guichardii* clades (group A & group B) are occurring in close neighborhood (Suppl. Fig. 5);

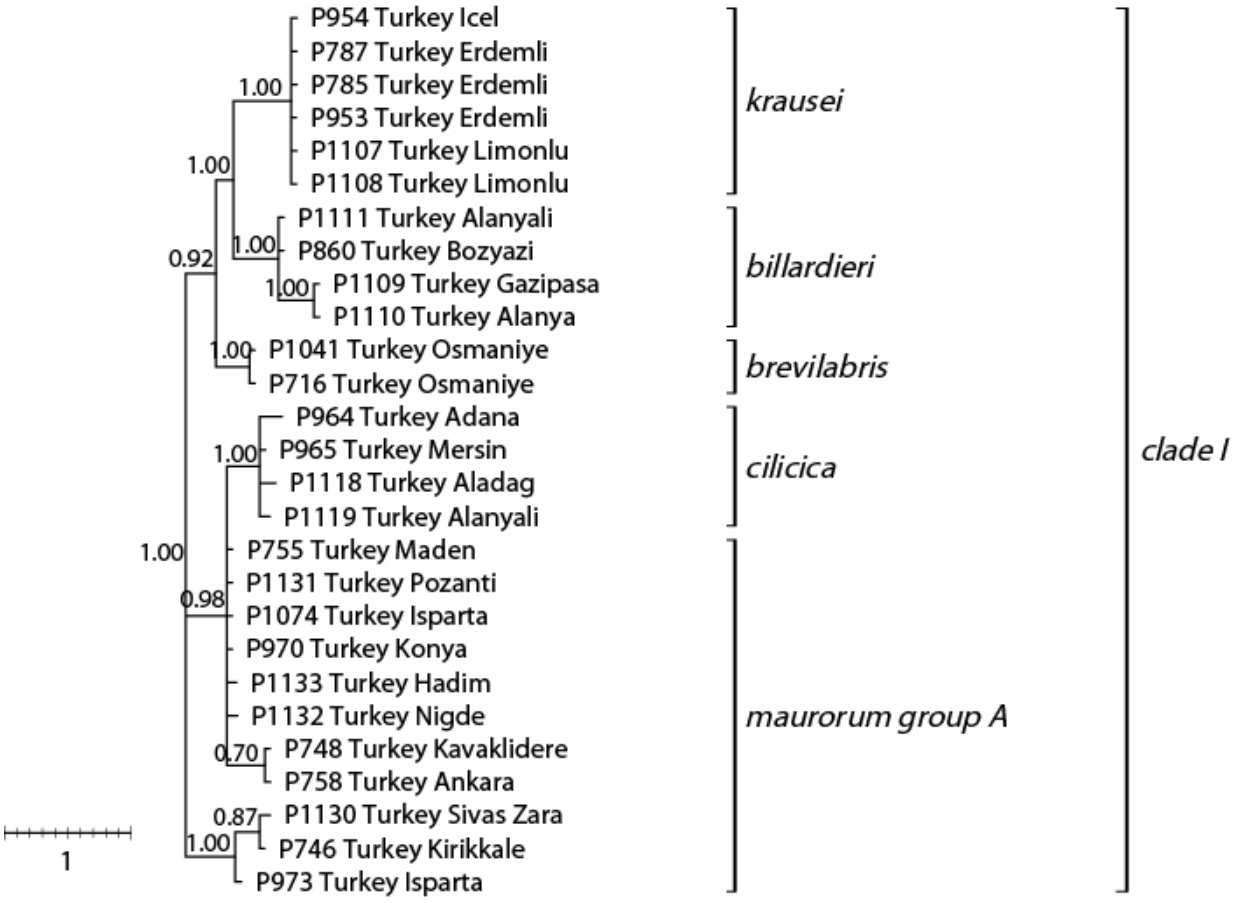
d) species traditionally belonging to the *A. auricularia* group (*A. auricularia* Boiss., *A. geniculata*, *A. isaurica* Nardi, *A. rechingeriana* Kit Tan & Sorger), from S-SW Turkey are treated here as only two species (*A. auricularia* and *A. geniculata*) based on morphological characters. Interspersed are populations of *A. hirta* occurring in the same area (*A. hirta* group C) (Suppl. Fig. 6 versus Suppl. Fig. 7). *Aristolochia auricularia* is not monophyletic as one population is clustering together with populations of *A. hirta* (group A) and the other populations of *A. auricularia* and populations of *A. geniculata* are clustering together with *A. hirta* (group C). According to the phylogenetic hypothesis as well as biogeography and morphology, *A. stenosiphon* and *A. lycica* Davis & Khan seem to be closely related to the *A. auricularia* group (Figure 2, Suppl. Fig. 7).

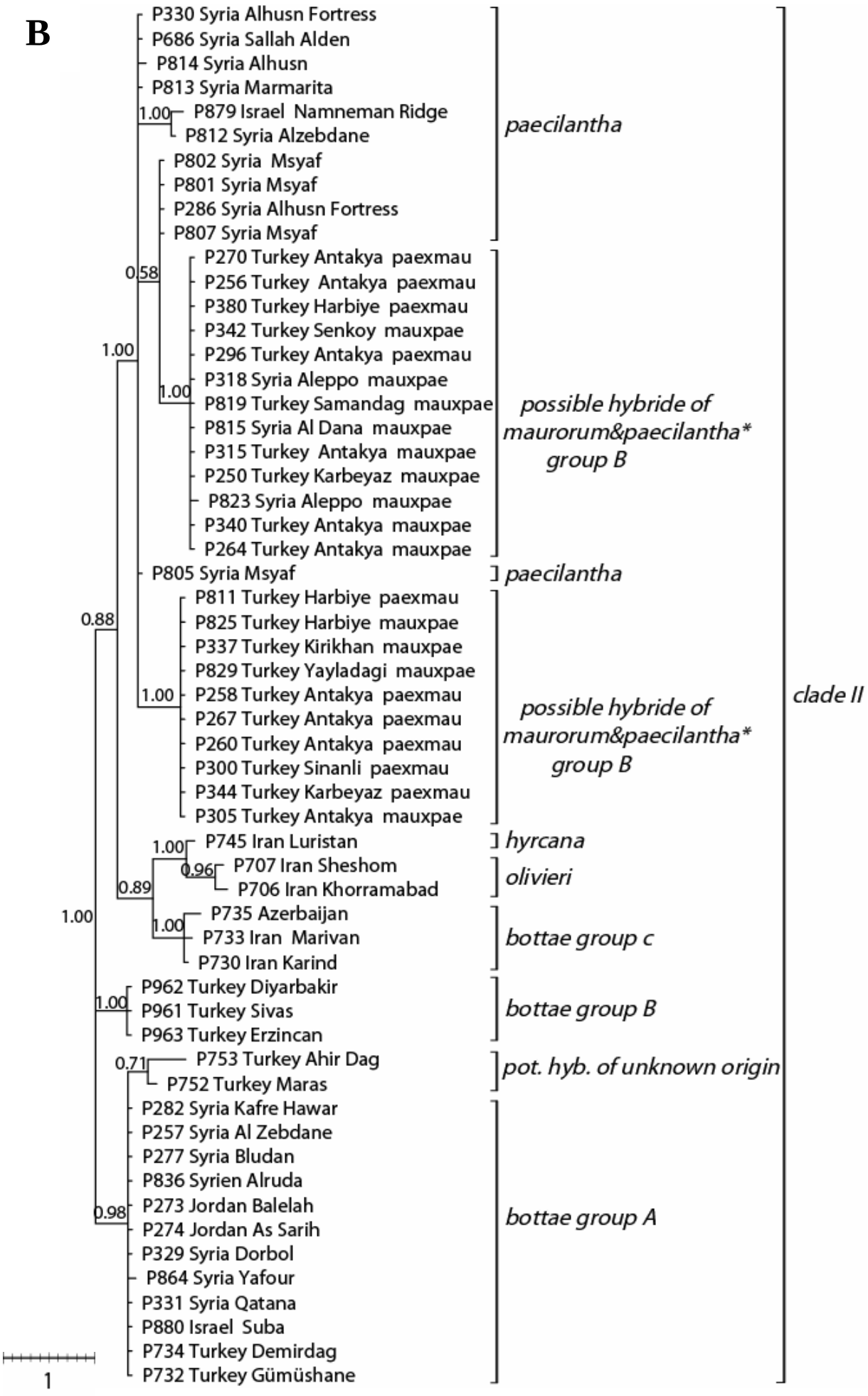
Maximum parsimony trees using substitutions only as well as those including coded length mutations disintegrate species boundaries similarly and rather fit to biogeography. Especially the MP tree disregarding coded length mutations sometimes provides less statistical support for clades, which is likely due to fewer PICs (Table 4) (trees not shown). By the exclusion of the accession of *A. auricularia* (P1129) statistical support for many related and unrelated nodes is increasing.

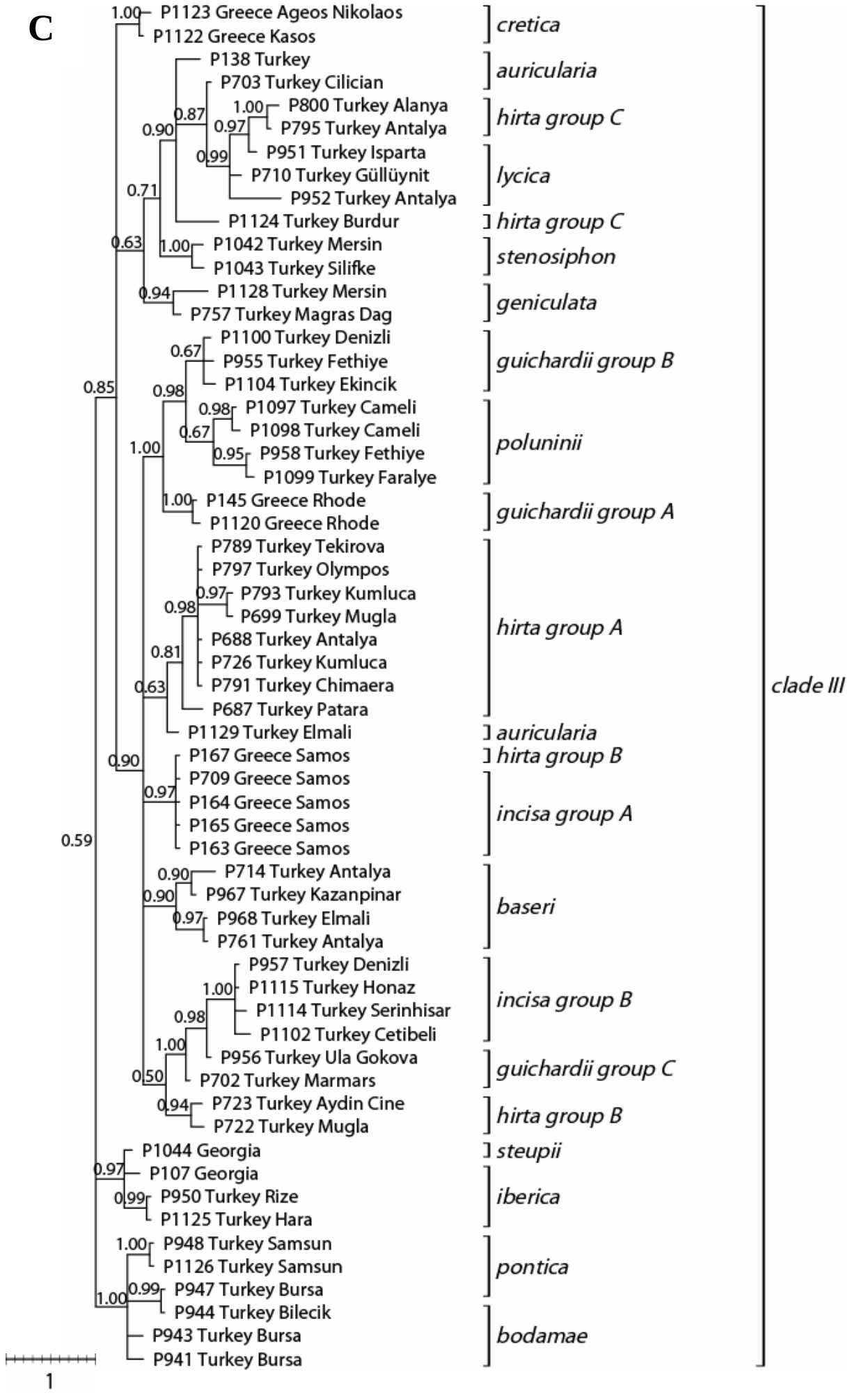
However, we stick to the completeness of our study to be able to highlight as many issues as possible for follow up studies.

Figure 2 (A, B, C) Phylogenetic hypothesis of 145 accession inferred by Bayesian analyses from the combined chloroplast dataset. Bayesian posterior probabilities (PP) greater than 50% are shown along branches. Species identifications are provided for clades and if species are polyphyletic clades have been labeled with arabic letter continuously. A) Summarized tree and clade I; B) clade II; C) clade III.






B
P330 Syria Alhusn Fortress
P686 Syria Sallah Alden
P814 Syria Alhusn
P813 Syria Marmarita
1.00
P879 Israel Namneman Ridge
P812 Syria Alzebdane
P802 Syria Msyaf
P801 Syria Msyaf
P286 Syria Alhusn Fortress
P807 Syria Msyaf
paecilantha
0.58
P270 Turkey Antakya paexmau
P256 Turkey Antakya paexmau
P380 Turkey Harbiye paexmau
P342 Turkey Senkoy mauxpae
1.00
P296 Turkey Antakya paexmau
P318 Syria Aleppo mauxpae
1.00
P819 Turkey Samandag mauxpae
P815 Syria Al Dana mauxpae
P315 Turkey Antakya mauxpae
P250 Turkey Karbeyaz mauxpae
P823 Syria Aleppo mauxpae
P340 Turkey Antakya mauxpae
P264 Turkey Antakya mauxpae
possible hybride of maurorum&paecilantha* group B
P805 Syria Msyaf
paecilantha
0.88
P811 Turkey Harbiye paexmau
P825 Turkey Harbiye mauxpae
P337 Turkey Kirikhan mauxpae
P829 Turkey Yayladagi mauxpae
1.00
P258 Turkey Antakya paexmau
P267 Turkey Antakya paexmau
P260 Turkey Antakya paexmau
P300 Turkey Sinanli paexmau
P344 Turkey Karbeyaz paexmau
P305 Turkey Antakya mauxpae
possible hybride of maurorum&paecilantha* group B
1.00
P745 Iran Luristan
hyrcana
0.96
P707 Iran Sheshom
P706 Iran Khorramabad
olivieri
0.89
1.00
P735 Azerbaijan
1.00
P733 Iran Marivan
P730 Iran Karind
bottae group c
1.00
P962 Turkey Diyarbakir
P961 Turkey Sivas
P963 Turkey Erzincan
bottae group B
0.71
P753 Turkey Ahir Dag
P752 Turkey Maras
pot. hyb. of unknown origin
P282 Syria Kafre Hawar
P257 Syria Al Zebdane
P277 Syria Bludan
P836 Syrien Alruda
0.98
P273 Jordan Balelah
P274 Jordan As Sarih
P329 Syria Dorbol
P864 Syria Yafour
P331 Syria Qatana
P880 Israel Suba
P734 Turkey Demirdag
P732 Turkey Gümüshane
bottae group A
clade II
1


C
P1123 Greece Ageos Nikolaos
P1122 Greece Kasos
cretica
P138 Turkey
P703 Turkey Cilician
auricularia
P800 Turkey Alanya
P795 Turkey Antalya
hirta group C
P951 Turkey Isparta
P710 Turkey Güllüynit
P952 Turkey Antalya
lycica
P1124 Turkey Burdur
hirta group C
P1042 Turkey Mersin
P1043 Turkey Silifke
stenosiphon
P1128 Turkey Mersin
P757 Turkey Magras Dag
geniculata
P1100 Turkey Denizli
P955 Turkey Fethiye
P1104 Turkey Ekincik
guichardii group B
P1097 Turkey Cameli
P1098 Turkey Cameli
P958 Turkey Fethiye
P1099 Turkey Faralye
poluninii
P145 Greece Rhode
P1120 Greece Rhode
guichardii group A
P789 Turkey Tekirova
P797 Turkey Olympos
P793 Turkey Kumluca
P699 Turkey Mugla
P688 Turkey Antalya
P726 Turkey Kumluca
P791 Turkey Chimaera
P687 Turkey Patara
hirta group A
P1129 Turkey Elmali
auricularia
P167 Greece Samos
hirta group B
P709 Greece Samos
P164 Greece Samos
P165 Greece Samos
P163 Greece Samos
incisa group A
P714 Turkey Antalya
P967 Turkey Kazanpinar
P968 Turkey Elmali
P761 Turkey Antalya
baseri
P957 Turkey Denizli
P1115 Turkey Honaz
P1114 Turkey Serinhisar
P1102 Turkey Cetibeli
incisa group B
P956 Turkey Ula Gokova
P702 Turkey Marmars
guichardii group C
P723 Turkey Aydin Cine
P722 Turkey Mugla
hirta group B
P1044 Georgia
steupii
P107 Georgia
P950 Turkey Rize
P1125 Turkey Hara
iberica
P948 Turkey Samsun
P1126 Turkey Samsun
pontica
P947 Turkey Bursa
P944 Turkey Bilecik
P943 Turkey Bursa
P941 Turkey Bursa
bodamae
clade III
1

Haplotype network reconstruction

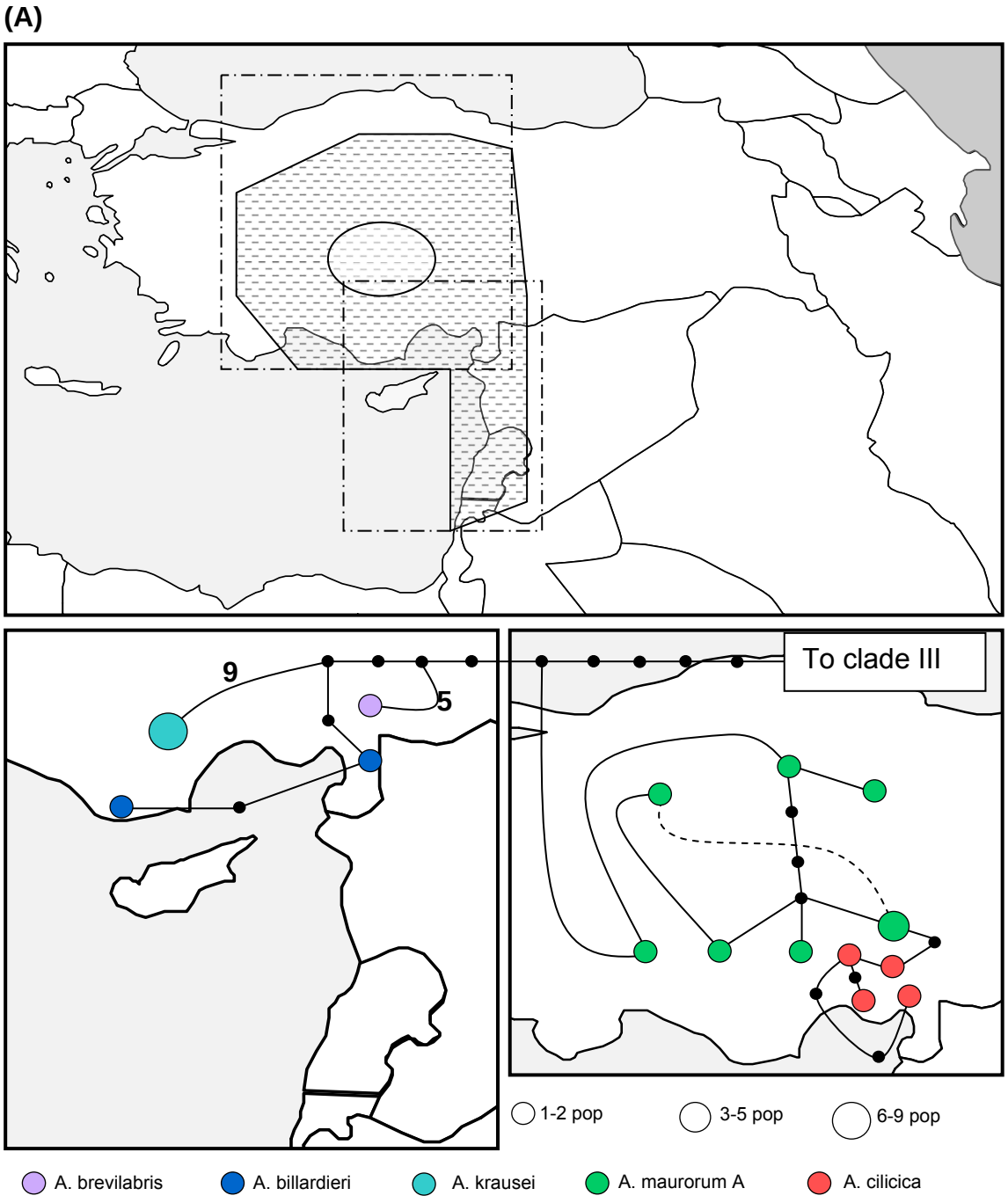
Geographic relationships of populations have been reconstructed using a haplotype network approach (Suppl. Fig. 8) and results have been plotted onto maps of the respective area for visualization (Figure 3 A, B, C). A general observation is that the haplotype diversity among a single species is often extremely high. Only populations of few species show one or few different haplotypes. Most of the populations belonging to one species show discrete haplotypes only present in one population. Also genetic distance between populations belonging to only one species is high (Figure 3, Suppl. Fig. 8) and often genetic distance within populations belonging to one species is higher then to population of the sister species (Figure 3).

A central position in the haplotype network (Suppl. Fig. 8) is hold by the unsupported clade III of the phylogeny (Figure 2). Especially populations belonging to the species from Hyrcano-Colchic area (*A. bodamae*, *A. pontica*, *A. iberica*, and *A. steupii*) are arranged in a circle, linking the remaining populations of clade III and clade I and II together. This finding is the strongest case of incomplete lineage sorting in the present study and likely the reason for the absent of support of the phylogenetic tree reconstruction. In addition four more cases of circles are observed both among populations of one species as well as populations belonging to different species (Figure 3 A, B, C; Suppl. Fig. 8). From a geographical point of view, populations belonging to clade I occur in the western and central part of Turkey and the eastern part of the Mediterranean (Syria, Lebanon, Israel and adjacent areas) (Figure 3A). Populations belonging to clade II instead occur in the eastern part of Turkey as well as Iran, Iraq and adjacent area but are also found in the eastern Mediterranean similar to populations of clade I (Figure 3B). The barrier between populations belonging to both clades in Turkey was first postulated by Davis (1971) as a hypothetical demarcation line of Anatolia which is suggested to be a distributional floristic break, running from NE Anatolia to the Anti-Taurus. This barrier is known as the Anatolian Diagonal (Davis 1971). However, populations of clade I also have a common border with populations of clade III along the southern and western Mediterranean cost and along the Black Sea (Figure 3A & C).

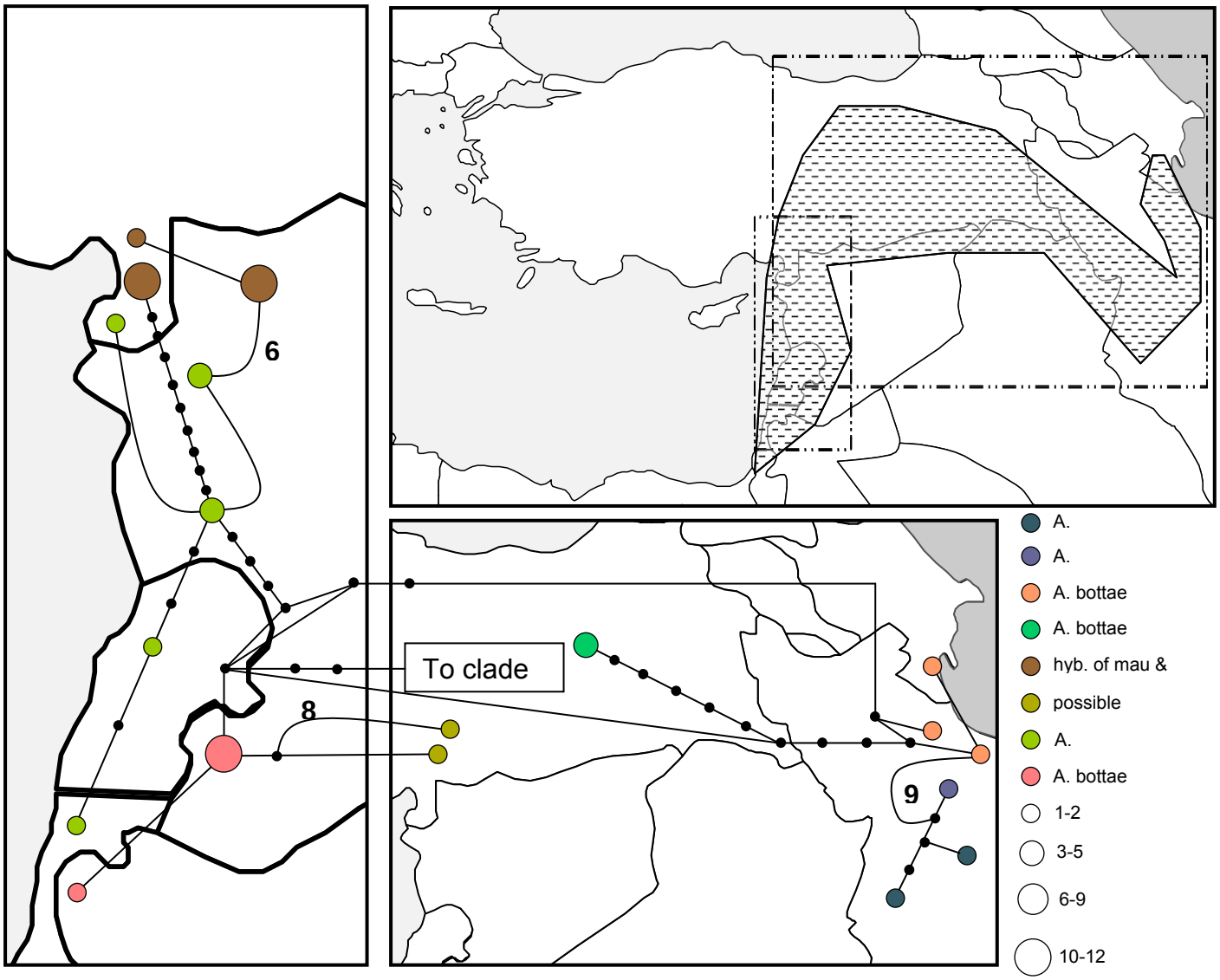
These findings clearly indicate that the populations belonging to the different clades are geographically isolated. However, this isolation can not be regarded as a complete geographic isolation but populations belonging to the different clades might share an area at the distributional borders of each clade or are even overlapping in

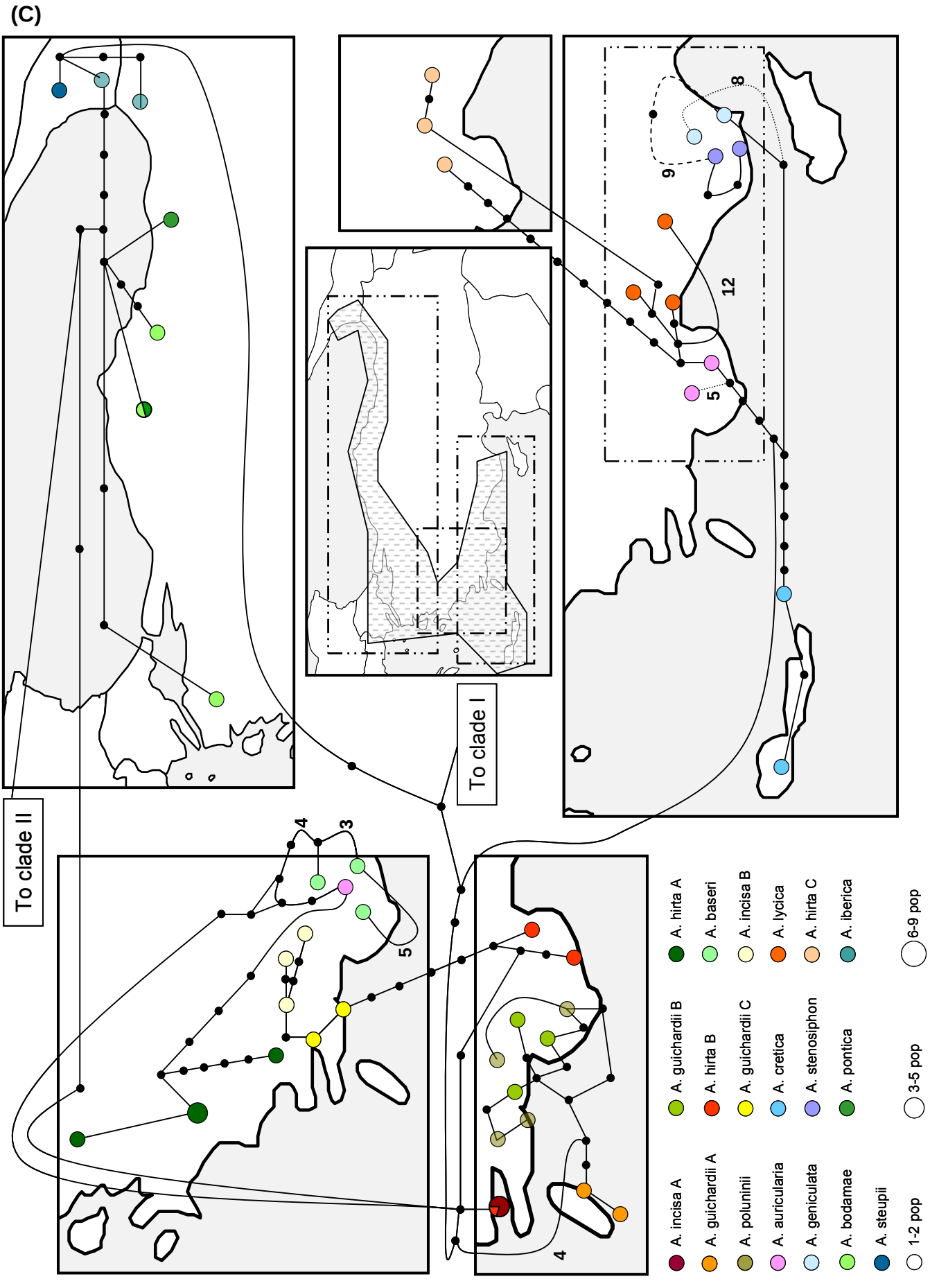
parts of their distribution (eastern Mediterranean). These geographical connections are likely the hotspots for natural hybridisation (hybrid zones) among evolutionary separated lineages when speciation hasn't leaded to reproductive isolation through e.g. genetic incompatibility.

Figure 3 (A, B, C) Haplotype network reconstruction plotted for visualisation onto the respective maps where populations are distributed. The original reconstruction can be found in the supplementary material (Suppl. Fig. 8). A) shows populations of clade I, B) of clade II and C) of clade III reconstructed in Figure 2. The area where all the respective populations are occurring is hatched and the rectangular area shows the closeup used for plotting the hyplotypes.



(B)


6
8
9
To clade
A.
A.
A. bottae
A. bottae
hyb. of mau &
possible
A.
A. bottae
1-2
3-5
6-9
10-12


(C)
To clade II
To clade I
4
3
5
4
9
8
12
5
A. incisa A
A. guichardii A
A. poluninii
A. auricularia
A. geniculata
A. bodamae
A. steupii
A. guichardii B
A. hirta B
A. guichardii C
A. cretica
A. stenosiphon
A. pontica
A. hirta A
A. baseri
A. incisa B
A. lycica
A. hirta C
A. iberica
1-2 pop
3-5 pop
6-9 pop

Phylogenetic evidence from the nSCG region

In this study the signal of the nSCG region revels totally different relationships, as all three main clades are falling apart (Figure 4) compared to the maternally inherited signal from the chloroplast (Figure 2). Biogeographic patterns are not important anymore, but often species are recovered which appear to be para- or polyphyletic in the cpDNA tree. This is especially the case for species of clade III, where species boundaries are chaotic (see above). Unfortunately, the phylogenetic signal of the nSCG region (i.e. 49 PIC) is not sufficient to fully resolve the East Mediterranean *Aristolochia* species (Figure 4). Therefore, we only summarize the results of the well-supported clades as well as the relationships of the *A. maurorum* complex.

One of the most interesting findings employing the nSCG region (Figure 4) is related to the *A. maurorum* complex, which is supposed to involve hybridization.

Aristolochia maurorum (group A, clade I, Figure 2) is clustering together with *A. maurorum* populations from group B, clade II (Figure 4). The latter is supposed to be of hybrid origin between *A. maurorum* and *A. paecilantha* (Davis & Khan 1961) but showing a phenotype more closely to *A. maurorum* (mau x pae, leaves and perianth) (Figure 6). Some populations of *A. bottae* are nested within and others are sister to this group (Figure 4, Figure 5). These populations are occurring in the same area (*A. bottae* group A, Suppl. Fig. 3) and are separated from the other *A. bottae* populations by a mountain range or by deserts (Suppl. Fig. 3). The other species traditionally belonging to the *A. maurorum* complex (*A. paecilantha*) is found in a totally different part of the tree (Figure 4). Here the *A. paecilantha* populations form a clade together with the *A. maurorum* x *paecilantha* hybrids depicting a phenotype more closely to the *A. paecilantha* phenotype (pae x mau, with *A. paecilantha* leaves and perianth characters mixed between *A. maurorum* and *A. paecilantha*) (Figure 5, Figure 6).

In contrast to Figure 2, a monophyletic *A. auricularia* (1.00 PP) is found to be sister to the hybrid clade depicting the A. paecilantha-like phenotype (0.88 PP, Figure 4). This species was not recovered as monophyletic in the cpDNA tree (Figure 2). A similar case is found in *A. guichardii*. The populations belonging to this species are recovered in the cpDNA tree according to biogeographical patterns together with populations of other species (group A, B, C, scattered throughout clade III in Figure 2). Here, (Figure 4) the populations form a monophyletic clade (0.92 PP).

Although, there are a lot of differing branching patterns and relationships recovered, the nSCG tree also shows congruence with respect to biogeographic clustering or the recovery of some species boundaries identical to the cpDNA tree. *Aristolochia*

geniculata for example is also recovered in Figure 4 as monophyletic (1.00 PP) and the populations of *A. incisa* and *A. hirta* (group B), which are both from the island of Samos, are clustering together (0.85 PP). Identical biogeographical patterns (Irano-Turonian elements) and the relationships of species are also found for *A. olivieri* sister to *A. hycana* and subsequent sister to this is *A. bottae* (group C) (Figure 4). One result, which is currently not explainable but receives at least some support, (0.90 PP) is the grouping of *A. steupii* (Georgia) together with *A. cilicica* (S-Turkey) and populations from A. hirta (group A) (W-Turkey) (Figure 4).

Figure 4 Phylogenetic tree reconstruction based on Bayesian analysis for the reduced sampling for the single copy gene (nSCG). Bayesian posterior probability values greater than 50% are shown above branches.

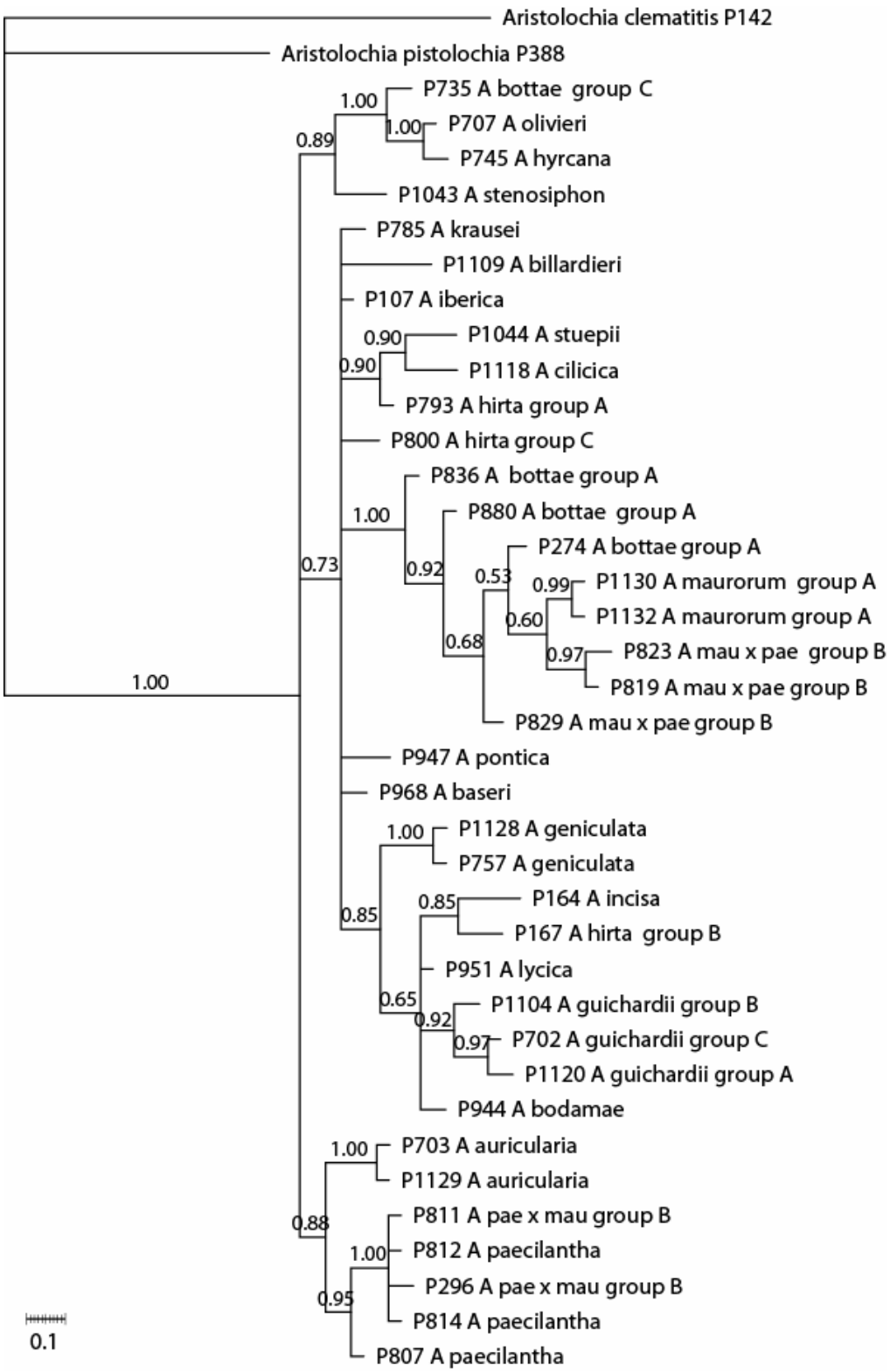


Figure 5 Reticulate evolution involving *A. maurorum* and *A. paecilantha*. Part of clade I and clade II of the chloroplast tree is shown to the left and the nSCG tree from Figure 4 is shown to the right. Hybrid populations are coloured in light blue for the hybrids showing a phenotype closer to *A. maurorum* and in orange for populations showing a phenotype closer to *A. paecilantha*. Non hybrid populations are shown in red for *A. paecilantha* and blue for *A. maurorum*. As explained in the text, *A. paecilantha* has to be the pollen acceptor and *A. maurorum* has to be the pollen donor (indicated with arrows).

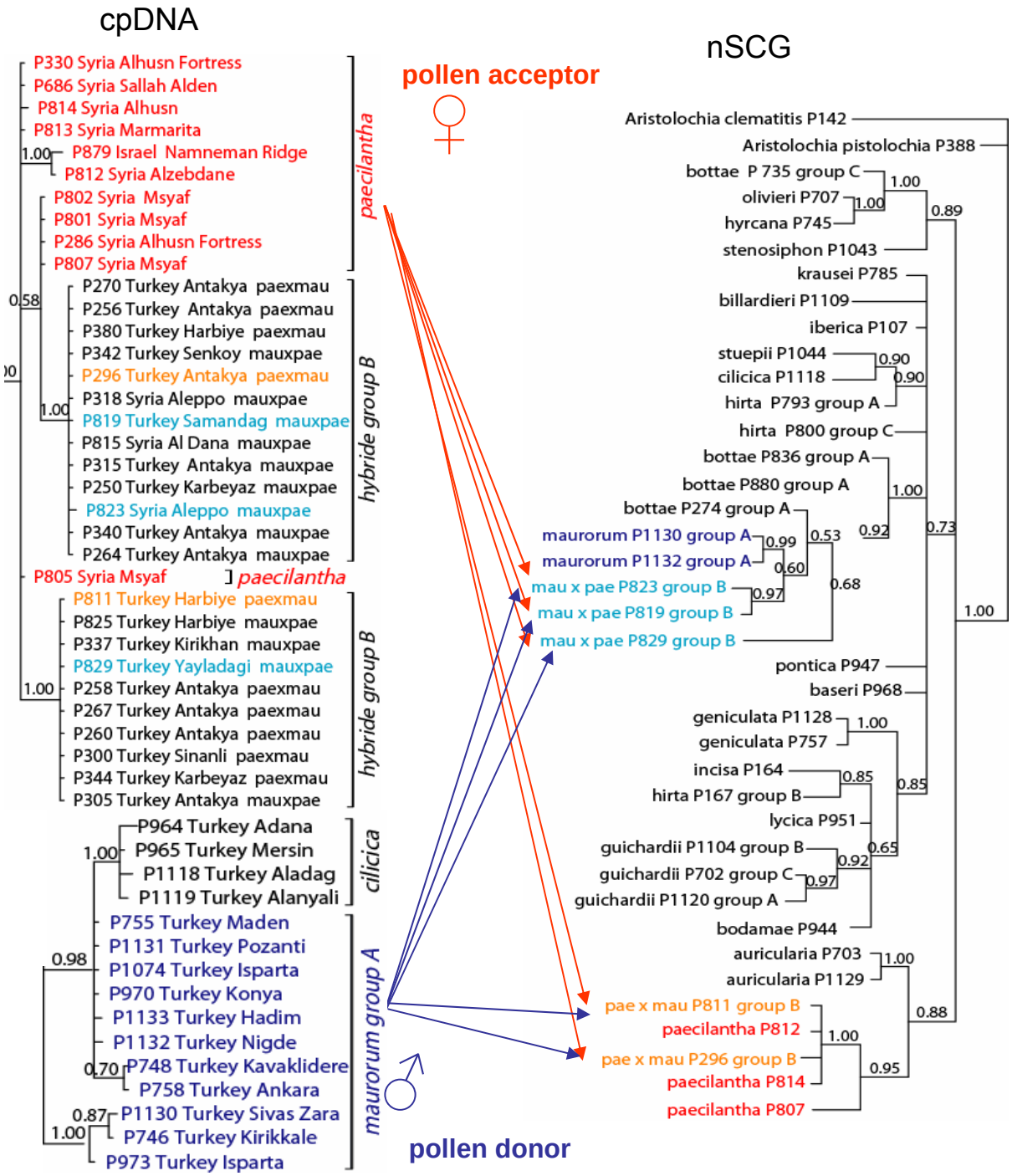
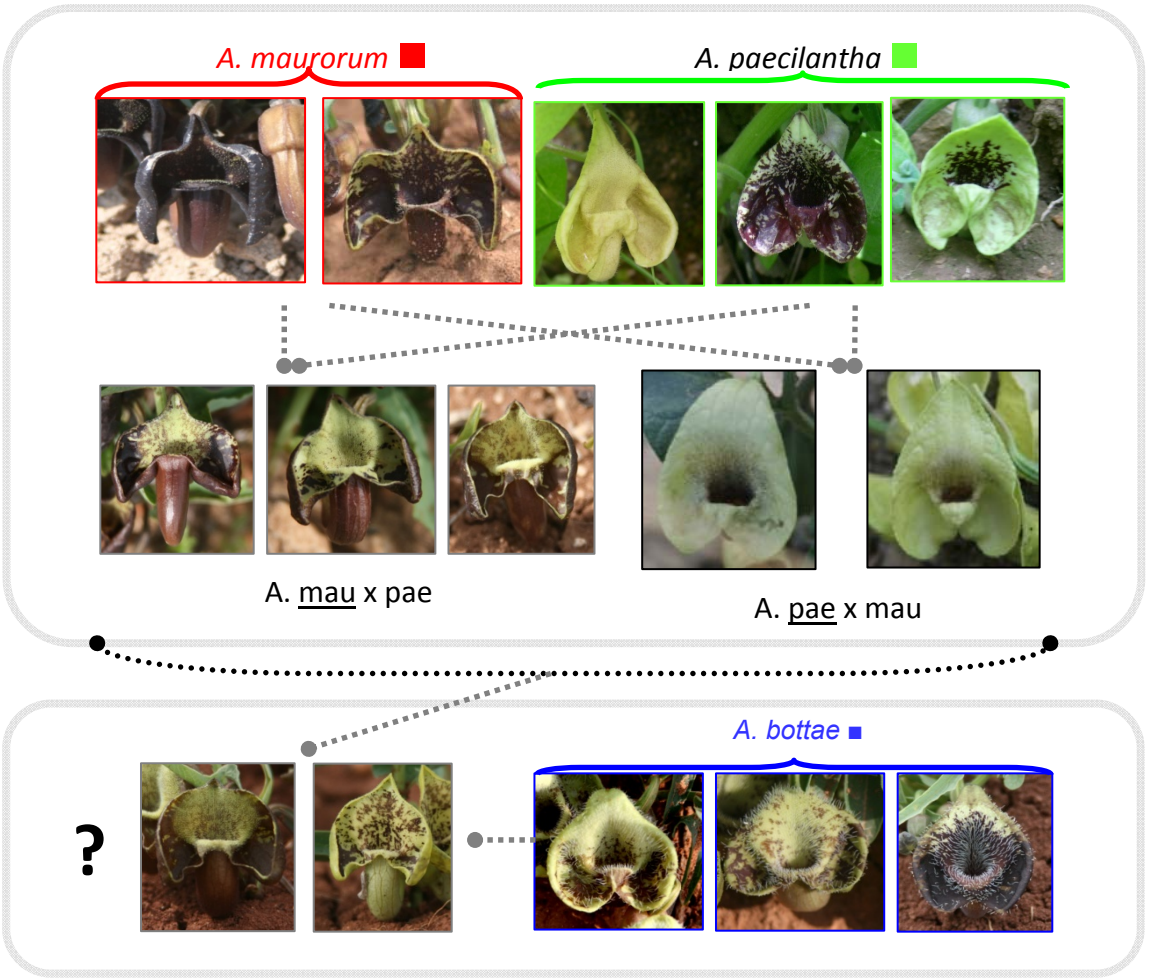
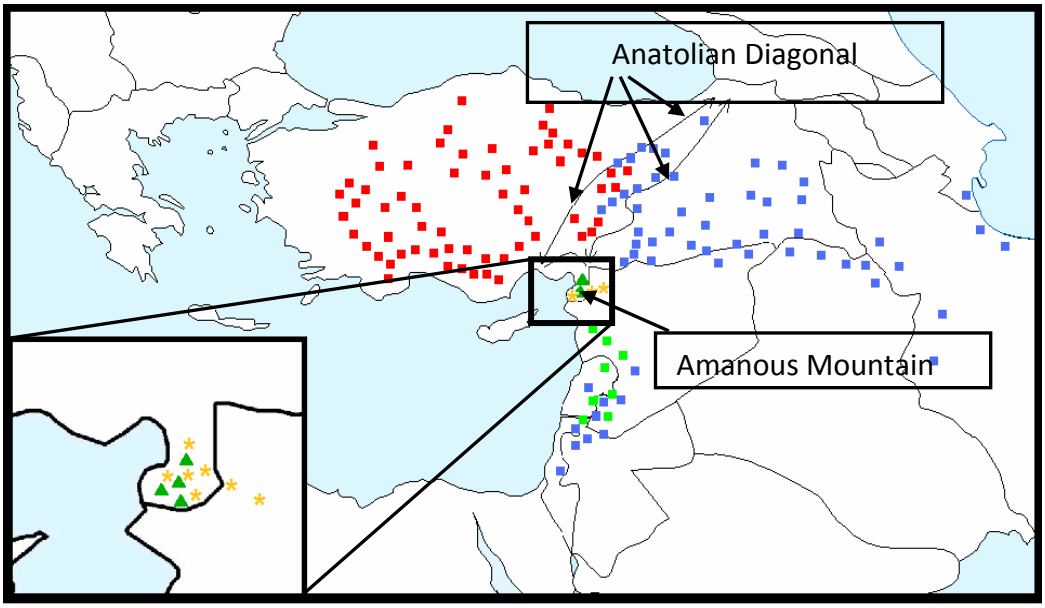


Figure 6 Pictures of the characteristic flowers of *A. maurorum* and *A. paecilantha* and the respective hybrid populations including the phenotypic variability of each. The map shows the distribution of the respective populations as well as the two important mountain regions which might function as incomplete geographic barriers between *A. maurorum* and *A. bottae*, which is known as the Anatolian Diagonal, as well as the hybrid zone between *A. maurorum* and *A. paecilantha* in the Amanous Mountain at the border of Syria and Turkey. Potential hybrids involving *A. bottae* have been recovered based on morphology but are not studied in detail in the present study.

See next page.





- *A. maurorum*
- *A. bottae* (group A, B, C)
- *A. paecilantha*
- *A. mau x pae*
- *A. pae x mau*

Discussion

Employing nuclear single copy genes in pilot studies

Recent publications showed that fast evolving markers (e.g. non-coding cpDNA) failed to provide resolved phylogenies for many plant lineages especially on the species level. Species and population level is becoming more and more important for conservation biology and is an invaluable tool in developmental biology and comparative genomics. Beside this, signal from independent inherited genomes is inevitable for the detection of hybridization (e.g. Sang et al. 1997), polyploidization (e.g. Doyle et al. 2004a), biogeography (Lavin et al. 2004), origin of domestication (e.g. Nesbitt & Tanksley 2002), or speciation in general (e.g. Barraclough & Nee 2001). Most of these topics can not be approached by only using cpDNA loci and the most frequently used nuclear loci (ITS) is limited in utility and increasingly subject to dispute (e.g. Doyle et al 2004a). To overcome the lack of knowledge from the bi-parentally inherited nuclear genome it is generally accepted that looking for nuclear single copy markers, implemented in pilot studies, is the most promising way (Whittall et al. 2006, Sang 2002, Hughes et al. 2006). In addition, greater resolution (high number of parsimony informative characters) is required by the combination and simultaneous analysis to either maximize the congruent signal or to maximize independent signals to detect e.g. reticulate evolution (e.g. Nixon & Carpenter 1996). Most of the nuclear gene regions (e.g. nuclear ribosomal DNA e.g. the ITS region, or gene families) occur in a few to thousands of copies, causing problems of orthology. Despite proposed mechanisms like concerted evolution those sequence differences complicate the phylogenetic reconstruction and require additional lab work and costs (e.g. cloning steps). Sequences of the nSCG region in this study were obtained without the need of cloning (no overlapping peaks), which clearly indicates the single copy nature of this region for the studied organisms. This region was already supposed to be present as only one single copy in asterids (Wu et al. 2006). The results presented here therewith support not only the evolutionary maintenance of the single copy nature but also the orthology in a fairly distantly related angiosperm lineage (magnoliids) compared to the study of Wu et al. (2006). This is also in congruence with Duarte et al. (in press). They also detected this region as a single copy gene comparing the fully sequenced genomes of *Arabidopsis*, *Populus*, *Vitis* and *Oryza* using bioinformatic approaches on which we built this study. Duarte et al. (in press) concluded that this region is likely to be single copy among all angiosperms and might only be present as low copy gene in recent polyploids. Although,

polyploidization has not been studied intensively in *Aristolochia* and to our knowledge never for any East Mediterranean *Aristolochia* species only chromosome counts for some species may provide evidence (all 2n=10). However, a recent study using FISH and probes of 5S and 45S rDNA indicate that both ancient and recent polyploidization has taken place in the genus *Aristolochia* (Berjano et al. 2009a), The closest relatives of the study of Berjano et al. (2009) to the species studied here, are from the sister group of the East Mediterranean. One species, *A. paucinervis*, was proven to be of recent hexaploid origin but this was already indicated by the chromosome counts for this species. Neither chromosome counts nor the sequences produced in this study reveals any evidence for recent polyploidization in the East Mediterranean *Aristolochia* species and reciprocal provide no evidence that the nuclear gene is not of single copy nature.

Consequences of comparing chloroplast loci versus nSCG regions

The nuclear genome is generally inherited bi-parentally in angiosperms and genetic information responsible for the phenotype of an organism is encoded here. Instead, the chloroplast genome mostly encodes proteins responsible for biochemical pathways and is inherited maternally (Hare 2001). Traditional systematists, who use phenotypic evidence i.e. leaf shape or flower shape and colour are thus somewhat relying on the information encoded in the nucleus. Consequently, the results from a nuclear encoded single copy gene should be closer to what tradition systematists would postulate in e.g. floristic treatments. However, by comparing the evidence from the maternally inherited cpDNA, hybridization should become clear through reticulate branching patterns and possible parents could be revealed (e.g. Sang & Zhang 1999, Sang & Zhang 2000; Linder & Rieseberg 2004). In addition, hybridization can only act when pollen can be transferred from one parent to the other and cross pollination is not inhibited trough genetic barriers (Thompson 2005). The transfer of pollen is simpler when populations of different species are occurring syntopic especially when pollen is transferred by small flies that might not be able to conquer large distances as is the case in Mediterranean *Aristolochia* (Rulik et al. 2007, Berjano et al. 2009b, Oelschlägel et al. in press). Possible patterns of biogeographical shared areas by populations of different species recovered in the cpDNA tree might indicate that hybridization is acting if the nuclear gene tree is recovering different groupings.

Our phylogenetic results, although the statistical support is not always to our satisfaction, clearly demonstrate reticulation through signals pointing into different

directions and support biogeographical orientation of potential parents when hybridization was supposed traditionally (see *A. maurorum* case below) but also reveal many more cases where hybridization might have influenced evolution in the East Mediterranean *Aristolochia* species.

Hybrid speciation in the *A. maurorum* species complex

Artificial hybridization in the genus *Aristolochia* is known from cultivations e.g. between the two Neotropical species *A. grandiflora* and *A. gorgona* (Blanco 2005). Only recently, natural hybridization was confirmed by molecules for species occurring in Asia belonging to *Aristolochia* subgenus *Isotrema* (Watanabe et al. 2008) and was assumed based on morphological intermediate phenotypes in the here studied *A. maurorum* complex (Davis & Khan 1961, 1982). Other cases for natural hybridization in the genus *Aristolochia* have not been reported, yet.

The phylogenetic tree based on chloroplast evidence clearly supports two independent lineages of the traditionally encountered *A. maurorum* species complex, *A. maurorum* (clade I, group A), and a clade representing all *A. paecilantha* populations together with populations which have been supposed to be of hybrid origin (clade II, group B). The fact that all potential hybrids are recovered together with *A. paecilantha* reveals that only *A. paecilantha* can be the pollen acceptor (maternal inherited) (Figure 5), independently if the hybrid phenotype resembles more the *A. maurorum* (mau x pae) or the *A. paecilantha* (pae x mau). Consequently, *A. maurorum* populations from clade I, group A have to be the pollen donor when the maternal evidence is compared with the results from the bi-parentally inherited nuclear evidence. Otherwise, the interspecific hybrids need to be found in both clades of the cpDNA tree.

As the offsprings display intermediate traits of both parents but can be grouped into two morpho types (mau x pae and pae x mau) the hybrid phenotype is not related to one parent (pollen donor vs. pollen acceptor). Therefore, it could be assumed that the displayed phenotype is of random origin. This would be supported by the fact that out of 23 populations sampled the number of each morpho type is nearly equally distributed (10 times pae x mau, 13 times mau x pae). However, the nuclear gene tree group the two types each together with the respective species (mau x pae together with *A. maurorum* group A and pae x mau together with *A. paecilantha*). Backcrossing, of the hybrids with either *A. maurorum* (clade I, group A) as the pollen donor might be the reason for the observation that in the nuclear gene tree (Figure 4)

the mau x pae type is clustering together with *A. maurorum* (clade I, group A of Figure 2), whereas the pae x mau type is clustering with the traditional *A. paecilantha* populations. Although, this observation would not be in accordance with the finding of no double peaks in the sequences or shifts due to indels in the two alleles of the single copy gene. Taking the genetic distance of the hybrid parents into account, one would expect at least double peaks due to allelic variation.

Hybridization also highlights other factors which then need to be involved in the building of a hybrid swarm, such as both species need to have the same fly pollinator which would be in contrast to Rulik et al. (2007) who postulated species specific pollinators. Another possibility for interspecific pollen transfer would be that both species share individual pollinators from individual pollinator guilds of each species. This would be in accordance with Berjano et al (2009b) who showed that two not closely related *Aristolochia* species (*A. baetica* & *A. paucinervis*) share a pollinator guild. Berjano et al (2009b) also demonstrated, that *A. baetica* is superior in pollinator attraction when both species are co-occuring.

Based on own field observations, the hybrid individuals produce fruits and seeds, and thus are not sterile, although, it is unknown if seeds are viable. As the hybrid swarm is found in a relatively restricted area in between the distribution area of *A. maurorum* (group A) and *A. paecilantha* (Figure 6) it is likely that the combination of traits does not provide evidence for heterosis. The local distribution of the hybrids, not conquering new habitats, might also indicate that hybrids are all of F_1 origin and consequently would indicate that the seeds are not viable.

Traditional view of the East Mediterranean *Aristolochia* species compared to findings

Aristolochia maurorum complex

Davis and Khan (1961) and Davis (1982) mentioned based on morphology a natural hybrid between the two species *A. paecilantha* and *A. maurorum* in Antakya (Amanous Mountain, Turkey, border to Syria). Davis and Khan (1961) described both perianth and flower characters as intermediate between *A. maurorum* and *A. paecilantha*. Although, in Davis (1982) the same specimen was described as possessing perianth characters more similar to *A. maurorum* whereas the leaf characters were more similar to *A. paecilantha*. Intensive field work which was the foundation for the present study, revealed many more potential hybrid populations (Table 2). However, most potential hybrid populations where found in the Antakya

region, but also hybrids have been found on the adjacent Syrian region even reaching the Aleppo area. The Aleppo region is the region of which the type of *A. maurorum* has been described from. According to Jarvis (2007) Linne based his description of *A. maurorum* on two drawings in Morison (1699) and Rauwolf (1583). To our current knowledge, this material is most likely belonging to the hybrid swarm as can be seen from Figure 6 and shows more phenotypic affinities to *A. maurorum* then to *A. paecilantha* (mau x pae).

The affinities of *A. cilicica* to other species of *Aristolochia* are unclear. In the first description of Davis & Khan (1961) a close relationship to *A. maurorum* was mentioned and they suggested peripheral isolation from *A. maurorum* in the Cilician Taurus in middle-south Turkey (Figure 2). As morphological differences, Davis and Khan mentioned the leaf shape and the flower shape ("blade being broader with much shorter, scarely divergent auricles and a perianth with the lower rim being broad and exauriculate"). However, in 1982, Davis did not mention these affinities anymore, but postulated a relationship to the Irano-Turanian element *A. bottae* and the newly described SW-Turkish *A. poluninii* (Davis & Khan 1977). Results presented here do support Davis & Khan (1961) view based on the chloroplast gene tree, but the nuclear gene tree support a relationship to *A. steupii*. Both from a morphological point of view and a biogeographic point of view this finding is currently not explainable.

In the light of the results of the *A. maurorum* complex and the inconsistency between the gene trees, it is likely that hybridization is playing an important role in the East Mediterranean *Aristolochia* species in general. Whenever possible we highlight the inconsistency of the different gene trees, biogeography and morphology to raise hypothesis to be tested in forthcoming studies.

Aristolochia bottae complex (*A. bottae, A. olivieri, A. hyrcana*)

The traditional view of this species complex (Davis & Khan 1961) can be substantiated by the findings in both gene trees (Figure 2 & Figure 4). *Aristolochia bottae* (group C), *A. hyrcana* and *A. olivieri* are closely related from a biogeographic point of view and the results presented here. As *A. bottae* itself is not monophyletic and falling apart in both gene trees it is likely that hybridization is also involved in this group of species as well. Davis & Khan (1961) mentioned that some of the species variability of *A. paecilantha* might be due to hybridization with *A. bottae*. This statement is not occurring in Davis (1982) anymore and our results provide no

evidence for this. However, we observed populations of *Aristolochia* which are closely allied to *A. bottae* (group B, Figure 2) where we are currently not able to assign a proper species affiliation (named as “potential hybrid of unknown origin”). These populations are occurring on the Anatolian diagonal (Figure 2, Figure 6) which splits *A. maurorum* from *A. bottae* and these plants show intermediate morphological characters of both species. Unfortunately, only two accessions are currently available and none of these amplified for the nuclear gene region. Additional lab work and field studies along the Anatolian diagonal will be required to unravel potential hybridization involving *A. bottae*. Davis and Khan (1961) noted some morphological differences between the geographical isolated populations of *A. bottae* such as the Irano-Turonian population’s show broader leafs but do not depict differences in flower morphology or colouring from the other *A. bottae* populations (group A, B). Especially the populations of *A. bottae* group A show strong relationships with *A. maurorum* and the mau x pae hybrid populations in the nuclear gene tree.

Aristolochia pontica complex (*A. pontica, A. iberica, A.steupii, A. bodamae*)

This complex includes the Hyrcano-Colchic species (*A. pontica*, *A. steupii* and *A. iberica*) as well as *A. bodamae* from NE-Turkey and adjacent part of Greece (Suppl. Fig. 4) (Davis & Khan 1961, 1982). Although, this complex is not recovered as monophyletic (unresolved), the chloroplast tree at least recovers two monophyletic clades containing *A. steupii* + *A. iberica* populations and *A. bodamae* + *A. pontica* populations. The nuclear gene tree does not show any of the chloroplast findings because it is largely unresolved with respect to those species. *Aristolochia bodamae* has also been assigned to the *Aristolochia hirta* complex by several authors according to morphological characters (Davis & Khan 1961, 1982; Nardi 1991). This assumption can not be validated by any of our findings.

Aristolochia billardieri complex (*A. billardieri*, *A. krausei*, *A. brevilabris*)

Morphological similarity of the species forming this complex is indicated by the not distinguish ability through only vegetative characters. Davis (1980) distinguished *A. krausei* from the other two through its biauriculate limb and separated *A. brevilabris* from *A. billardieri* through its reduced perianth limb. Although, the three species are phenotypically extremely similar, this is a rare case where small floral variations seem to be congruent with the monophyly of each species recovered by the

chloroplast gene tree. The nuclear gene tree does not allow conclusions with respect to this species complex.

Aristolochia auricularia complex (*A. auricularia*, *A. geniculata*, *A. isaurica*, *A. rechingeriana*)

The auriculate limb is very rare trait in Mediterranean *Aristolochia*, and all species showing this character were traditionally grouped together in the *Aristolochia auricularia* complex (Davis & Khan 1961, 1982; Nardi 1983, Malyer & Erken 1996, Kit Tan & Sorger 1987). However, species boundaries were unclear and especially *A. rechingeriana* and *A. geniculata* were treated as synonym of *A. auricularia* (Malyer & Erken 1996). Nardi (1993) and Malyer & Erken (1996) agreed on accepting *A. isaurica*, but our own field and herbarium studies did not support morphological separation. Thus, *A. isaurica* is here included in *A. geniculata*. *Aristolochia rechingeriana* is only known from its type. This species was distinguished from other species of this complex by larger flowers, longer limb and longer auricules (Kit Tan & Sorger, 1987). The type originate from a locality were only *A. auricularia* could be found during own field work and it is doubtful if this species should be treated as independently or if it should be regarded as a phenotypic variation.

In contrast to morphological characters, our results do not support the *A. auricularia* complex as monophyletic. In the nuclear gene tree both *A. auricularia* and *A. geniculata* are found as monophyletic each, which is in accordance with traditional taxonomists, but in the chloroplast gene tree, *A. auricularia* is not. This might also indicate that hybridization has played a role in the evolution of this species complex and further studies are required.

Aristolochia hirta complex (*A. hirta, A. baseri*, *A. incisa, A. cretica*, *A. guichardii* and *A. poluninii*)

The distributions of the species belonging to this complex are limited to the south-western part of Turkey and adjacent Greek islands (Suppl. Fig. 5, 6). Most of the species (excluding *A. cretica*) are growing in close neighbourhood to *A. hirta* populations. Morphologically, they are clearly distinguishable from each other. Furthermore, floral features including perianth size, shape, and colour are the primary diagnostic characters distinguishing *A. hirta* (group C) from (group A and B). The results presented here indicate that all species belonging to this complex share maternally a common ancestor (together with remaining species of clade III) and that

hybridization is likely the reason for the splitting of e.g. *A. hirta* populations into different clades. Although, this hypothesis will need further confirmation.

Aristolochia baseri has been described by Malyer and Erken (1997) as a new species from South Anatolia and was assumed to be closely allied to *A. cilicica* from the Cilician part of Anatolia, which differs in its longer pedicles and in the colour and the shape of the perianth. The perianth shape of *A. baseri* is also similar to *A. cretica*, but differs in its oblong-lanceolate leaves, and in the revolute, flat and slightly recurved perianth limb which is considered as typical characters for this species. Except for the monophyly of this species in the chloroplast gene tree, the molecular results are to vague to support any morphological similarities or biogeographic patterns.

Duchartre (1864) described *A. incisa* from West Anatolia. Later Turrill (1960) described a new endemic species from Samos (Greece) and called it (*A. samia*). By comparison of the types, Davis & Khan (1961) considered *A. samia* as synonym of *A. incisa* based on a photo of the type and the morphological description of the type of *A. incisa.* However, all *A. incisa* populations split into two independant clades in the chloroplast tree, the first one includes *A. insica* from Samos together with *A. hirta* from the same locality, while the second one represents the Turkish populations of *A. incisa* together with *A. guichardii* populations from the Western part of Turkey. Morphologically, the Greece and Turkish populations of *A. insica* are almost identical and should be considered part of the same species, while they are divided into two separate populations depending on biogeography and chloroplast results. The nuclear gene tree supports different relationships for the included populations and thus hybridization might also be involved in these clades.

Aristolochia cretica is endemic to the southern Aegean region, from Crete to the western Dodecanese (Nardi 1991). Based on morphology, this species is closely allied to *A. pontica* but differs in its more rounded pubescent leaves which are smaller than the flowers and a perianth with a shortly biauriculate limb (Davis & Khan, 1961). The inclusion of two populations from two different islands clearly support the monophyly of the species based on the chloroplast gene tree. The species furthermore is likely to be a separate lineage in clade III, being not involved in hybridization, as both accessions are monophyletic in the chloroplast gene tree, although not included in the nuclear gene tree.

Aristolochia guichardii is endemic to SW- Anatolia (Mugla region) and Rhode (Davis & Khan, 1964). It has been described as closely related to *A. billardieri* and *A. brevilabris*. Resembling both in leaf shape and habit, but differs from both its allies in having an auriculate perianth limb that is broader and shorter than that of *A. billardieri*, hairy inside with different colours. The shape of the perianth resembles that of *A. hirta* which is quite common in West Anatolia and the neighbouring islands, but *A. guichardii* is showing much smaller flowers and differs also in having cordate to ovate leaves instead of narrowly deltoid leaves (Davis, 1982).

Davis & Khan (1977) described *A. poluninii* as a new species from the SW Anatolia (Suppl. Fig. 5) with affinities to *A. bottae* and *A. cilicica* but showing differences in perianth morphology (more hirsute and scattered with multicellular trichomes). Both morphology and chloroplast data support the monophyly of *A. poluninii* although without statistical support.

Aristolochia lycica and *Aristolochia stenosiphon*

Aristolochia lycica has been described by Davis & Khan (1961) from the southern part of Turkey as being morphologically intermediate between *A. stenosiphon* and *A. maurorum*. It differs from *A. stenosiphon* in its narrower leaves with divergent auricles and longer perianth limb (glabrous), with a very obtuse apex. From *A. maurorum* it can easily be distinguished by the shape of its flower, the slender tube, a smaller utricle and ovate to oblong very obtuse limb. The tube is minutely puberulent on the outside instead of glabrous. Although, *A. lycica* and *A. stenosiphon* are traditionally considered to be sister taxa, non of the molecular datasets provide evidence for this assumption. In addition biogeography would support a relationship to other taxa such as *A. hirta* (group C), which would also be in accordance with the chloroplast tree.

Conclusion

The results obtained from the chloroplast loci in combination with the nuclear marker clearly support the earlier hypothesis of hybridization involving the traditional *A. maurorum* species complex. Employing a nuclear single copy gene in a small scale pilot study meets the expectations of earlier authors to trace complex cases of evolutionary biology involving e.g. hybridisation. The results presented here support not only the evolutionary maintenance of the single copy nature but also the orthology in a fairly distantly related angiosperm lineage compared to published data. This first attempt to unravel the evolution of this species rich group of *Aristolochia* highlight the need for additional investigation on population level applying loci from differentially inherited genomes to test further hybridisation along distributional borders of separate clades and the relationships of taxa, prior to a complete taxonomic revision of this group. However, this study provides an updated status of several previously recognized taxa in the Near East.

Acknowledgment

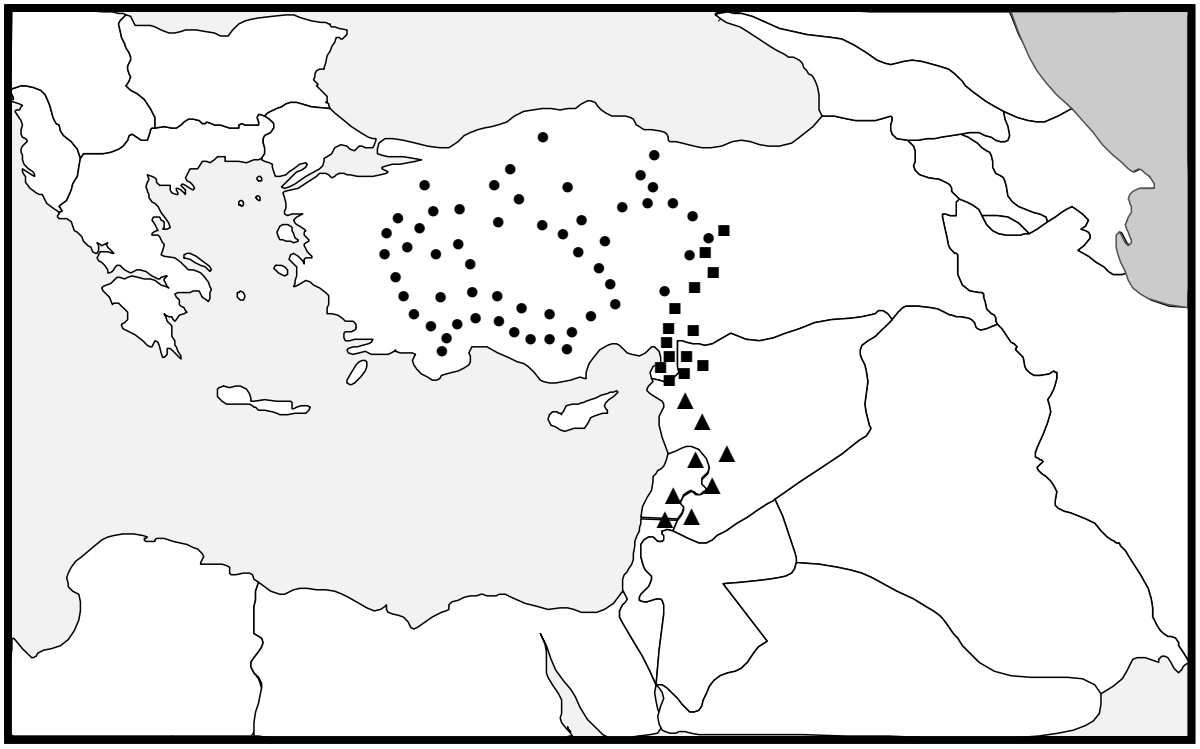
Financial sources: This work was supported by a TUBITAK research grant to H. Malyer, S. Wanke, C. Neinhuis and A. Tosunoglu (TBAG107T707). Additional funding to H. Mahfoud came from the German Academic Exchange Service (DAAD) and a grant from the Syrian Ministry of Education & Research. Initial work on the single copy gene region was supported by a postdoc grant to S. Wanke from the DAAD and is currently supported by a DAAD PPP project to S. Wanke and K. Müller together with C. dePamphilis. We thank M. Neumann, W. Lobin, O. Fragman Sapir for supplying plant material and the Botanical Garden Dresden (Barbara Ditsch) to take care of our *Aristolochia* collection. Several herbaria have provided herbarium specimens for morphological investigation and/or molecular phylogenetic studies which is gratefully acknowledged: (American University of Beirut, Lebanon (BEI), the Herbarium of the Friedrich Schiller University, Jena, Germany (JE), the Royal Botanic Garden Edinburgh, Great Britain (E), the Botanic Garden and Botanical Museum Berlin-Dahlem, Germany (B), the Hebrew University, Jerusalem, Israel (HUJ), the Uludag University (BULU), the Anadolu University (ESSE), the Ankara University (ANK), the Gazi University (GAZI), the Yuzuncu Yıl University (VANF), Turkey.

Supplementary Material

Suppl. Fig. 1 Distribution of the *Aristolochia cilicica* and *Aristolochia billardieri* group.

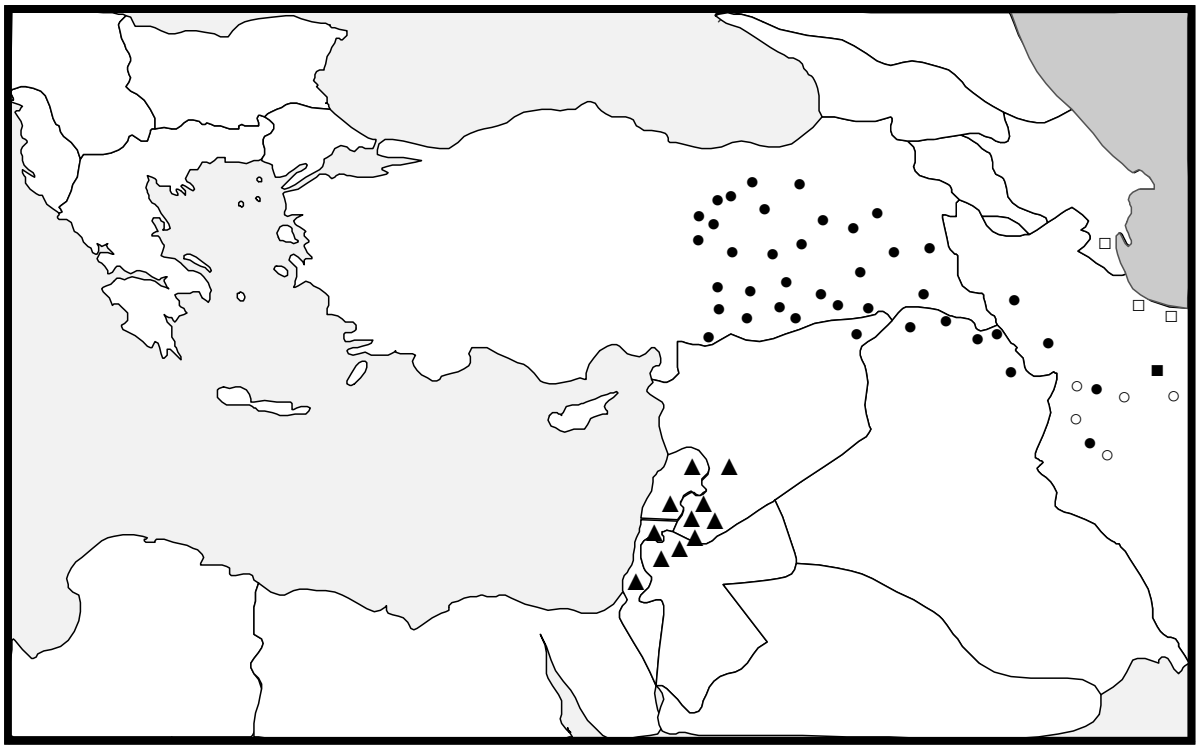
● *A. billardieri* □ *A. krausei* ▲ *A. brevilabris* ○ *A. cilicica*

Suppl. Fig. 2 Distribution of the *Aristolochia maurorum* group.



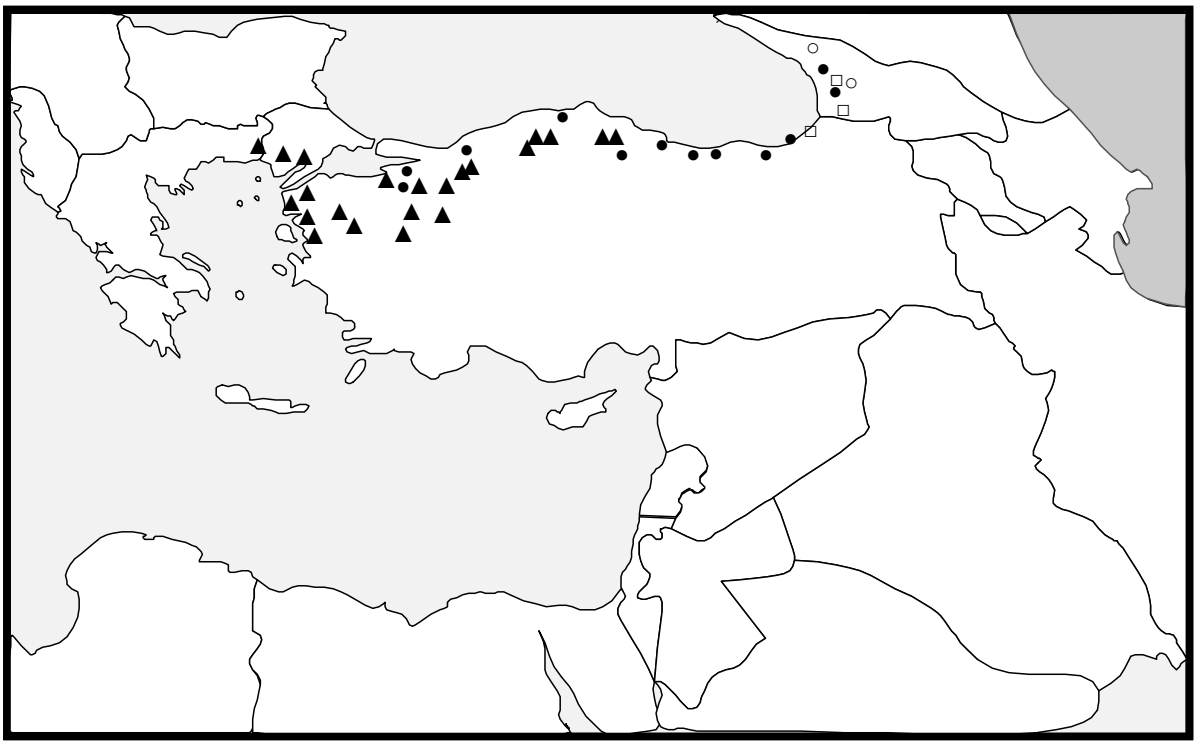
● *A. maurorum* group A ■ hybrid of *mau x pae* or *pae x mau* ▲ *A. paecilantha*

Suppl. Fig. 3 Distribution of the *Aristolochia bottae* group.



▲*A. bottae group A* ● *A. bottae group B* □ *A. bottae group C*
○ *A. oliveri* ■ *A. hyrcana*

Suppl. Fig. 4 Distribution of the *Aristolochia pontica* group.

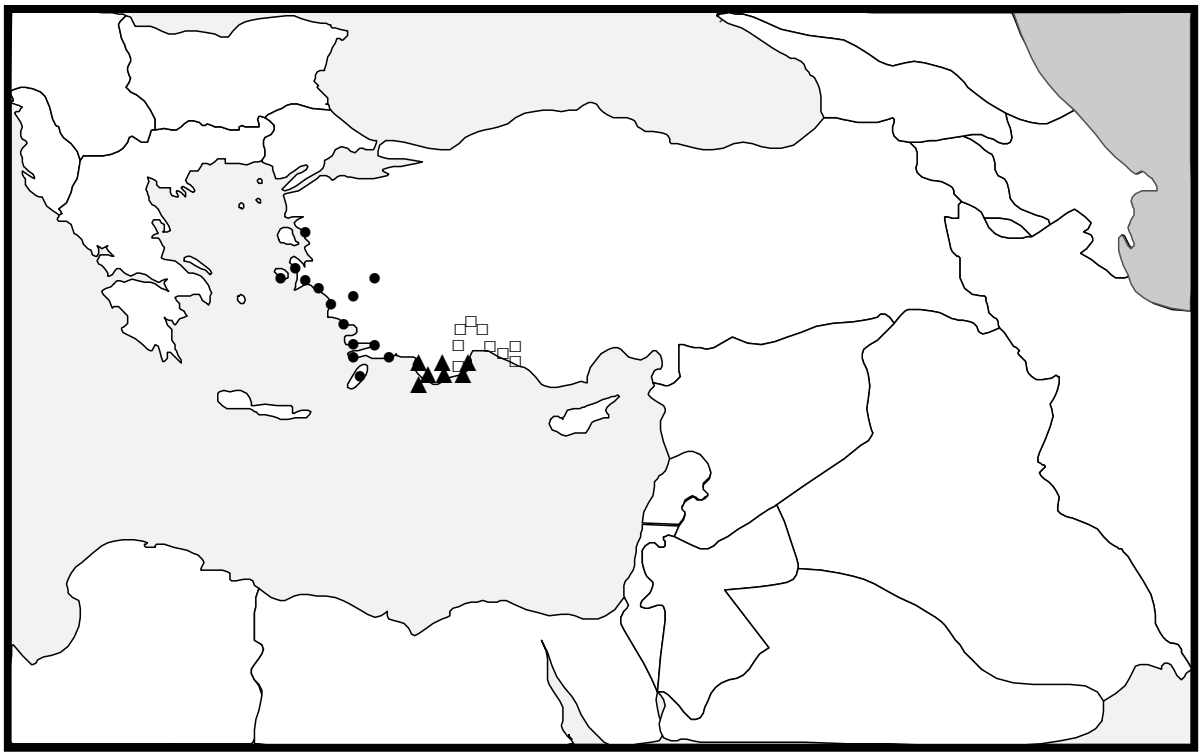


● *A. pontica* ▲ *A. bodamae* □ *A. iberica* ○ *A. steupii*

Suppl. Fig. 5 Distribution of *A. baseri*, *A. cretica*, *A. poluninii*, *A. incisa* and *A. guichardii*.

▲ *A. cretica* ● *A. incisa group A* ◙ *A. incisa group B* □ *A. baseri* + *A. poluninii*
■ *A. guichardii group A* ⁎ *A. guichardii group B* ○ *A. guichardii group C*

Suppl. Fig. 6 Distribution of the *Aristolochia hirta* group.



● *A. hirta* group A ▲ *A. hirta* group B □ *A. hirta* group C

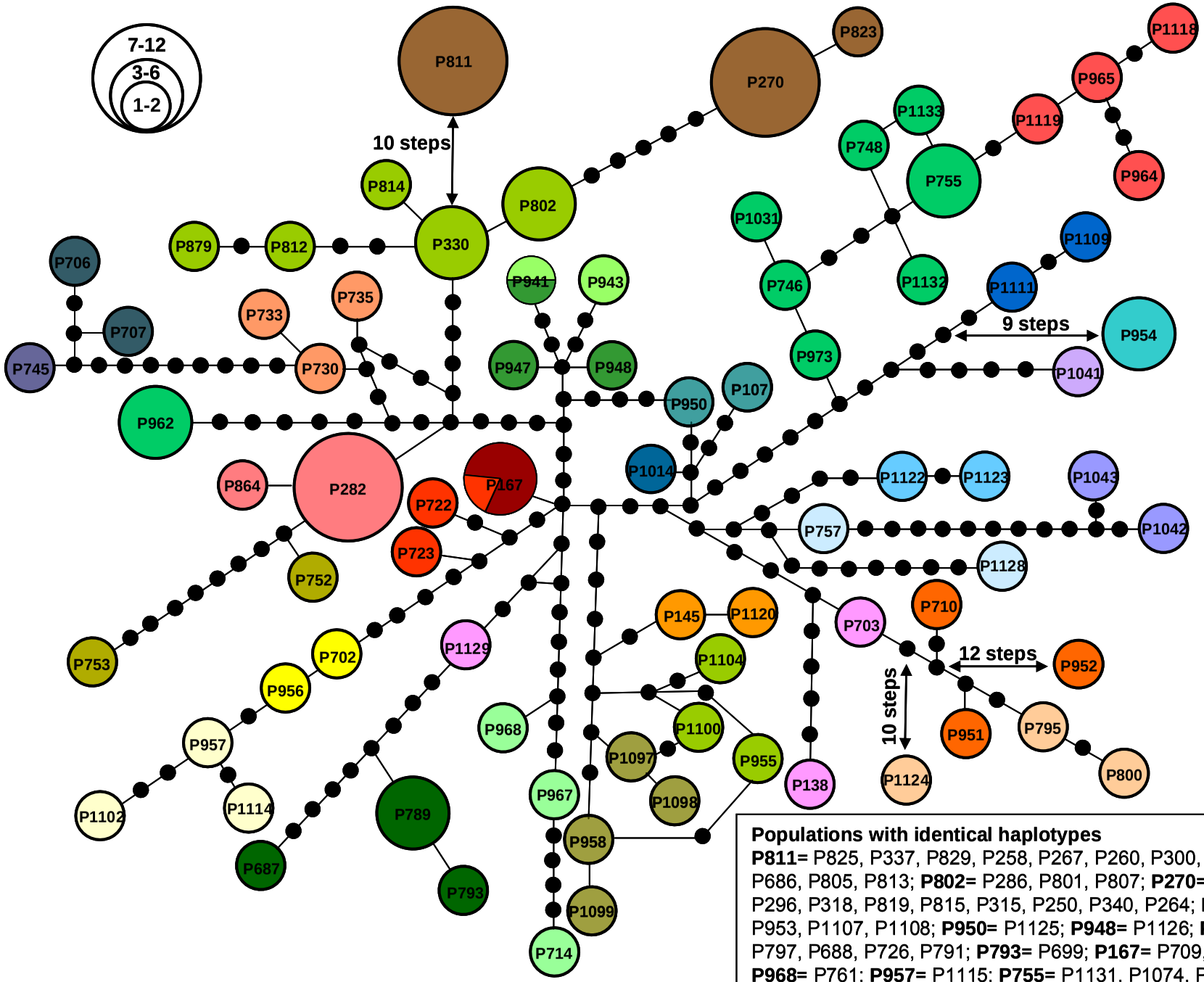
Suppl. Fig. 7 Distribution of *A. lycica*, *A. stenosiphon* and the *A. auricularia* group.

● *A. auricularia* □ *A. rechingeriana* + *A. lycica*

▲ *A. geniculata* < *A. stenosiphon* ○ *A. isaurica*

Suppl. Fig. 8 Haplotype network based on the cpDNA dataset. This reconstruction was used to plot the haplotype network together with the distribution maps in Figure 3 (A, B, C). Number of the respective population in the figure has been chosen randomly. A full list of populations sharing the same haplotype is given in the box. The size of a circle represents the number of populations sharing the same haplotype.

See next page.



Populations with identical haplotypes

P811= P825, P337, P829, P258, P267, P260, P300, P344, P305; **P330=** P686, P805, P813; **P802=** P286, P801, P807; **P270=** P256, P380, P342, P296, P318, P819, P815, P315, P250, P340, P264; **P954=** P787, P785, P953, P1107, P1108; **P950=** P1125; **P948=** P1126; **P947=** P944; **P789=** P797, P688, P726, P791; **P793=** P699; **P167=** P709, P164, P165, P163; **P968=** P761; **P957=** P1115; **P755=** P1131, P1074, P970; **P748=** P758; **P1111=** P860; **P1109=** P1110; **P1041=** P716; **P962=** P961, P963; **P282=** P257, P277, P836, P273, P274, P329, P331, P880, P734, P732

Chapter 2

Geographical structure, haplotype variation and evolution of *Aristolochia sempervirens L*. and *Aristolochia baetica L*. (*Aristolochiaceae*) in the Mediterranean.

Abstract

The present study documents DNA variation in the two closely related species*Aristolochiasempervirens* and *A. baetica*, also called the *A. sempervirens* species complex. This species complex is distributed throughout the Mediterranean from Morocco to Syria and Turkey, with (nearly) no area overlap of both species. This study uses multiple fast evolving cpDNA loci (*trnK* intron, *matK* gene, *trnK-psbA* spacer, ~3500 bp) for some 70 populations covering the whole distribution area. We demonstrate the overall good performance of this region on population level, showing a fully resolved phylogeny with high statistical support, as well as a high degree of haplotype variation between populations. We use a modified indel coding method, which takes the evolution of a microsatellite like structure into account. In general indel coding methods have not been able to increase support significantly. In our case we show, that our approach can increase support between 10-20% compared to traditional indel coding methods or to a substitution based approach only. The results are compared with a nuclear single copy region (~1200bp) for a representative selection of populations from the larger dataset. Beside the unusual high degree of resolution and support obtained from cpDNA, it is demonstrated that the nuclear region provides the same resolution with an overall identical topology and support. Given that only $^1/_3$ of the sequencing effort is required, it is concluded that nuclear single copy regions and especially their introns are of high applicability for species and population level evolutionary biology and may outperform fast evolving cpDNA regions. The two morphologically distinct species split in all analyses in three highly supported clades, representing geographically isolated areas. Hybridization as a source for the split into three instead of two clades is rejected as both nuclear and chloroplast DNA provide an identical signal. In addition the phylogenetic reconstructions give credit to a complex colonization scenario of the Mediterranean area by this species complex, detect relict populations, multiple independent colonization events and incomplete speciation after geographic isolation. All populations of *A. baetica* show a similar if not identical morphology, which is likely explained by cryptic speciation.

Introduction

The Mediterranean region comprises only 1.6 % of the earth dry land, but exhibits more than 25.000 known vascular plant species (~10% of the world total). Current characteristics of this area have been shaped by its paleogeological/-climatic history, its ecogeographical heterogeneity and more recently by human influence, resulting in a complex biogeography and a high degree of endemism (>50%) (e.g. Comes & Kadereit 1998, Thompson 1999, Comes 2004, Thompson 2005). During the last decades studies investigating a particular species complex (e.g. *Primula*, Guggisberg et al. 2006), a genus (e.g. *Cyclamen*, Gielly et al. 2001, Debussche & Thompson 2003) or multiple populations and individuals of only one species (*Campanuladichotoma*, Nyman 1991) to infer the underlying pattern which have shaped the current distribution of particular groups have been carried out. Many studies had the character of model studies or case studies. Unfortunately, the complex history of the Mediterranean and adjacent regions faces the nearly unlimited possible combinations of how a taxon might have evolved.

Today, sequence data of more than 50 different coding and non-coding DNA regions of all three plant genomes are used in order to address phylogenetic, phylogeographic but also general evolutionary biological questions on different taxonomic levels. The current focus is clearly on the chloroplast genome for ongoing phylogenetic research in angiosperms to infer relationships among and within the different lineages. One of the most frequently used so called “fast evolving” locus is the *matK* gene, which can easily be co-amplified with the flanking non-coding intron parts (*trnK* intron) (Müller et al. 2006). This region has been shown to have a broad application range, from representative angiosperm phylogenies (e.g. Hilu et al. 2003) to species level systematics (e.g Wanke et al. 2006a). Studies below the species level applying this region are still rare (e.g. Ohsako & Ohnishi 2001; Fujii et al. 2002; Watanabe et al. 2006) but show promising results leading to an extended application even down to population level. The *trnK-psbA* region has not been used extensively but provides at least the variability of the *trnK* intron (Shaw et al. 2005, 2007) although smaller in length. Moreover, with the growing number of studies that use length variable regions, it has been shown that microstructural length-mutations (indel characters), which are frequent in noncoding chloroplast DNA provide valuable additional information for phylogenetic inference (Simmons & Ochoterena 2000, Simmons et al. 2001, Löhne & Borsch 2005, Müller 2006, Wanke et al. 2007). The *trnK* intron including the *matK* gene in Piperales comprises about 2533 bp, ranging

from 2412 to 3258bp in Piperales (Wanke et al. 2007) while the length of the *matK* coding region in most angiosperms is about 1500-1600 bp. The *trnK* 5' intron includes a highly variable microsatellite region, which is located in the domain I of the *trn*K intron (Wanke et al 2006a). In *Aristolochioideae* this cryptic simple microsatellite ranges from 29 bp to 443 bp and is absent in all other Piperales lineages with known sequences (Wanke et al. 2007).

Molecular data has become one of the most powerful sources for revealing the evolutionary history among organisms (Baldauf 1999, Mathews & Donoghue 1999, Soltis et al.1999, Graham and Olmstead 2000, Brown 2001, Nozaki et al. 2003, Hassanin 2006).In most cases, however, only a few molecular markers are employed for reconstructing evolutionary history. Chloroplast gene and multicopy nuclear ribosomal DNA regions are prevalent. Unfortunately the most frequently used nuclear region on species and population level, the ITS-region (internal transcript spacer), has been recognized as inadequate for addressing a variety of phylogenetic questions (Baldwin et al 1995). Reasons for this include: a) multi-copy sequences buffered by complete concerted evolution, and consequently loss of testimony for bi-parental inheritance (reticulation) (Baldwin et al 1995, Wendel et al 1995), b) the untraceable extension and direction of concerted evolution, resulting in varying nrDNA repeats even among the same species (Doyle et al 2004) and c) the lack of concerted evolution causing paralogy (e.g. Doyle et al 2004b). Although analysis of single or low copy nuclear genes is a proposed solution (Sang 2002, Alvarez and Wandel 2003, Zhang and Hewitt 2003), they have rarely been used as e.g. a characterization was lacking until recently (pre-full genome and transcriptome period). A number of low or single copy nuclear genes have been previously identified including LEAFY, ACCase, PGK, petD, GBSSI, GPAT, ncpGS, GIGANTEA, PHYA, PHYB and PISTILLATA – primarily for their use as phylogenetic markers for evolutionary studies (Small et al. 2004). To cope with this Duarte et al. (in press, BMC Evol. Biol) identified approximately 700 genes that are assumed to exist only as one copy in the nucleus (comparing fully sequenced genomes of *Arabidopsis*, *Oryza*, *Vitis*, and *Populus*. Starting from several hundred potential nSCGs discovered by Duarte et al. (in press at BMC Evol. Biol.) ESTs (Expressed Sequence Tags) were screened for homologs among basal angiosperm lineages, the number and size of introns and an appropriate overall length for amplification and sequencing. Comparison of EST data and further characterization was done using PlantTribes (http://fgp.bio.psu.edu/tribedb/index.pl, Wall et al. 2008) and TIGR Plant Transcript

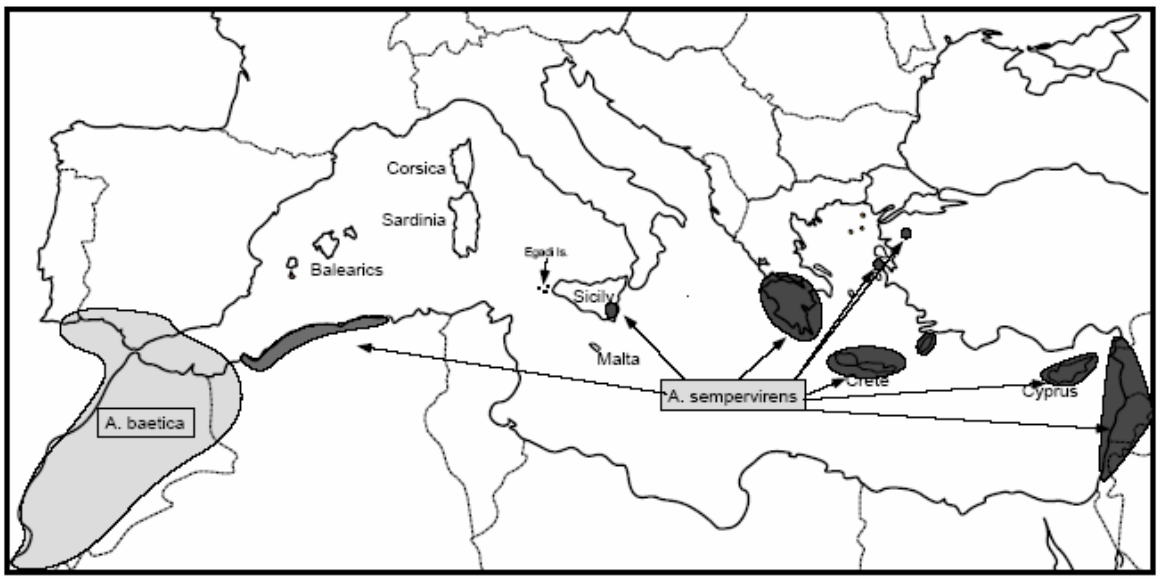
Assemblies (http://plantta.jcvi.org, Childs et al. 2007) datasets and further processed through bioinformatic pipelines to automatically obtain the required information and a ranking for useful loci concerning a specific question and the therewith required variability (unpublished data). One of these regions is evaluated on species and population level in a case study using two closely related species of the Mediterranean region as a model group.

The *Aristolochia sempervirens* complex is part of the Mediterranean representatives comprising about 50 species in a clade called *Aristolochia* s. str. (subgenus *Aristolochia*) (Wanke et al 2006a). This species complex is characterized by its climbing habit, a feature unique among Mediterranean *Aristolochia* and only found in Asian and African tropical species of *Aristolochia* s. str. and other clades in the tropics of the New World (Neinhuis et al 2005, Wanke et al. 2007). A climbing growth form is prevalent in *Aristolochia* species of forest habitats, but this vegetation type has largely disappeared due to large scale forest clearings by human activity in the past 5000 or even 10.000 years. Three species have been described within *Aristolochia sempervirens* complex (*A. baetica* L., *A. sempervirens* L., *A. altissima* Desf.), from which the latter two have been subject of controversial discussions on their synonymization by various authors (e.g. Davis and Kahn 1961) and sometimes one author changed the view of synonymization and non-synonymisation among different publication (Nardi and Nardi 1987, Nardi 1991). *Aristolochia sempervirens* was said to occur in Crete only (Linnaeus 1753, Davis and Kahn 1961), while *A. alttissima* was thought to occupy a larger range from Algeria to the Near East (Ducharter 1864, Boissier 1879, Halacsy 1904, Hayek 1924, Mouterde 1966). Currently only *A. sempervirens* is accepted occurring from the Near East (Turkey, Syria, Lebanon, Palestine, Israel) via islands of the Mediterranean Sea (Cyprus, Crete, Peloponnesus, Sicily) to coastal Algeria (see Fig. 1). *Aristolochia baetica* L. instead is found in Algeria, Morocco, Portugal and Spain, with no overlapping areas to *A. sempervirens* (Fig. 1) (de Groot et al 2006, Wanke 2007).

We here present a study carried out in the “circum Mediterranean” species complex *Aristolochia sempervirens* using both phylogenetic and phylogeographic methods based on both fast evolving chloroplast (*trnK* intron, *matK* gene, *trnk-psbA* spacer) and a single copy nuclear gene region, to test if the current distribution pattern has been subject of anthropogenic influence or is the result of natural colonization. Furthermore, we test applicability and phylogenetic power of a single copy nuclear

gene (nSCG) region to reconstruct well resolved and highly supported gene genealogies as a prerequisite to study evolutionary historyon population level.

Figure 1 Distribution of the *Aristolochia sempervirens* complex. Distribution of *A. baetica* is shown in pale gray, where as *A. sempervirens* is highlighted in dark grey. It is important to notice, that *A. baetica* show a superficially enclosed area, whereas *A. sempervirens* show a scattered distribution involving many Mediterranean islands.



Material & Methods

Material from 67 populations has been investigated representing the whole distribution area of both *A. sempervirens* and *A. baetica*. All populations have been sequenced for the cpDNA datasets and a representative set of these, based on the results from the cpDNA phylogeny, has been chosen to be sequenced for the nSCG region. *Aristolochiaclematitis*,*Aristolochia pistolochia* or both were used as outgroup for the gene genealogy reconstruction based on previous phylogenetic results of Wanke (2007). Most material was taken from living plants, either collected in the field or collected in the field and cultivated in the Botanical Garden Dresden. Few others were taken from herbarium specimens. A list of investigated species along with their collection localities, voucher information, and GPS data are given in Table 1.

DNA isolation was performed with either fresh or silica gel dried material. In five cases DNA was extracted from herbarium specimens collected between eight and 161 years ago. Genomic DNA was isolated using a modified triple-extraction approach with CTAB as described in Borsch et al. (2003) following the miniprep procedure of Liang and Hilu (1996). Genomic DNA was usually clean enough for direct PCR amplification although in some cases a column cleaning process was

performed using the Macherey-Nagel NucleoSpin Extract II columns and the protocol provided by the manufacturer for DNA extraction from agarose gels. Amplification of the *trnK-matK-psbA* region was performed in 25 µL reactions containing (1 µL genomic DNA, varying concentration depending on quality of isolation and used material), 1 µL of 25 mM $MgCl_2$, 4 µL of dNTP mix (each 1,25mM), 0.5 µL of each primer(20pmol/µL), and 0.2 µL self-made Taq polymerase (~5 units/µL). The single copy gene was amplified in 50 µL reactions containing (1 µL genomic DNA, varying concentration depending on quality of isolation and used material), 1.5 µL of 25 mM $MgCl_2$, 8 µL of dNTP mix (each 1,25mM), 1 µL of each primer (50pmol/µL), and 0.5 µL Taq polymerase (5 units / µL, PeqLab).

Because of the overall size of the cpDNA region it was amplified in two parts with an overlap of several 100 bp. In few cases especially when degraded herbarium specimens have been used, the two PCR parts were further subdivided. The following primer combinations have been used for the respective parts: trnKFbryo1 + AR-matK-1510R for the first part and AR-matK-1200F + psbAR for the downstream part. Primer sequences are listed in Table 2 (chloroplast marker) and Table 3 (nuclear marker). For herbarium specimens the primer given for sequencing have also been used for the amplification to obtain shorter products.

Amplification profiles of the cpDNA PCR followed an initial denaturation of 1, 5 min at 96°C, elongation for 1min at 50°C, and extinction for 3 Min at 68-74°C. Whereas the PCR amplification profiles of the nuclear single copy gene was set as followed: 2min denaturation at 94°C, elongation for 1min at 55°C, and extinction for 2min at 72°C. The PCR product was purified by Qia Quick gel extraction kit (QIAGEN). Direct sequencing used the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) with subsequent electrophoresis on ABI 310 or 377 automated sequencers, or the CEQ DTCS Quick Start Kit (Beckman Coulter) with the SEQ 800 sequencer, following standard protocols for each kit. Sequences were aligned using PhyDe (Müller et al 2005), following alignment rules proposed by Borsch et al (2003), Löhne and Borsch (2005). The phylogenetic trees were calculated using methods described by Neinhuis et al. (2005) and Wanke et al. (2006b, 2006c), as implemented in PRAP (Müller 2004) and PAUP*4b10 (Swofford 2002) with 500 ratchet replicates, 10 random addition cycles and 1000 bootstrap replicates. The haplotype network was calculated using TCS 1.21 (Clement et al. 2000) (see also chapter 1 for more detailed information on the methods used).

Table 1 Accessions used in the present study (only for the molecular study), including information about the origin of material (field orcollection), voucher information and the herbarium where the voucher is deposited (incl. lab number referring to a specific specimen of one population on which the voucher and the DNA sequence is based on)

Aristolochia* species	**origin**	**voucher (**herbarium**) and coll. date**	**GPS and altitude (if available)**
A. baetica P414	Morocco, Cap Mazari	Wanke (DR) 09.04.2006	N35°32'11'', E05°12'40'', 12m
A. baetica P419	Morocco, Chefchaouen	Wanke (DR) 09.04.2006	N35°07'41'', E05°17'19'', 415m
A. baetica P422	Morocco, Mokrisset	Wanke (DR) 09.04.2006	N35°00'51'', E05°23'28'', 415m
A. baetica P424	Morocco, Zinat	Wanke (DR) 09.04.2006	N35°29'10'', E05°25'13'', 145m
A. baetica P434	Morocco, Tetouan	Wanke (DR) 09.04.2006	N35°34'48'', E05°17'18'', 14m
A. baetica P444	Morocco, Berkane	Wanke (DR) 10.04.2006	N34°52'29'', E02°21'33'', 250m
A. baetica P454	Morocco, Berkane	Wanke (DR) 11.04.2006	N34°52'50'', E02°22'13'', 290m
A. baetica P496	Morocco, Qued Massa	Wanke (DR) 15.04.2006	N29°44'15'', E09°14'36'', 830m
A. baetica P498	Morocco, Essaouira	Wanke (DR) 18.04.2006	N31°03'07'', E09°38'29'', 161m
A. baetica P502	Morocco, Asif Tamrakhit	Wanke (DR) 17.04.2006	N30°34'27'', E09°32'16'', 180m
A. baetica P503	Morocco, Qued Massa	Wanke (DR) 15.04.2006	N29°44'15'', E09°14'36'', 830m
A. baetica P512	Morocco, Tiznit, Assaka	Wanke (DR) 15.04.2006	N29°41'22'', E09°31'55'', 192m
A. baetica P517	Morocco, Tamzargout	Wanke (DR) 17.04.2006	N30°33'03'', E09°33'45'', 178m
A. baetica P671	Morocco, Bij Agadir	Hose (GENT) 27.12.1971	
A. baetica P656	Algeria, Oran	Santa Cruz 26.01.1931	
A. baetica P47	Portugal, Algarve	Koch, W127 (DR)	30 km west of Faro. 15m
A. baetica P573	Portugal, Algarve	Charpin 21.04.2001	
A. baetica P655	Portugal, Algarve	Siegel s.n. (DR)	
A. baetica P524	Spain, Mt. Esparteros	Wanke 03 (DR) 08.10.2005	
A. baetica P529	Spain, Petrera, Gileana	Wanke 08 (DR) 08.10.2005	
A. baetica P533	Spain, Coin	Wanke 12 (DR) 09.10.2005	
A. baetica P538	Spain, Tolox	Wanke 17 (DR) 09.10.2005	
A. baetica P540	Spain, Sotogrande	Wanke 19 (DR) 09.10.2005	
A. baetica P544	Spain, Manilva	Wanke 24 (DR) 09.10.2005	
A. baetica P549	Spain, Torrox	Wanke 29 (DR) 10.10.2005	
A. baetica P554	Spain, Olivar	Wanke 34 (DR) 10.10.2005	
A. baetica P559	Spain, Orginiva	Wanke 39 (DR) 10.10.2005	
A.clematitis P142	Croatia, Liovik	W. Starmühler (DR)	San Pietro die Nembi

A. pistolochia P136	France, Cassis	Wanke (DR, 025372)	Calenque d'En Veau
A. sempervirens P75	Greece, Cyprus, Pedoulas	Albach 137 (BG Bonn) 13.04.1999	N34°97' , E32°82'
A. sempervirens P720	Greece, Cyprus	Edmonson (E 2984) 28.04.1979	Akapnou to Vikla.
A. sempervirens P578	Greece, Peloponnese	Wanke 152 (DR) 28.03.2005	N37°01'16'', E21°41'23'', 122m
A. sempervirens P580	Greece, Peloponnese	Wanke 154 (DR) 26.03.2005	N36°44'07'', E22°30'45'', 22m
A. sempervirens P586	Peloponnese, Platanos	Wanke 149 (DR) 25.03.2005	N37°19'48'', E22°39'20'', 473m
A. sempervirens P585	Peloponnese, Kosmas	Wanke 151(DR) 26.03.2005	N37°06'40'', E22°45'46'', 1011m
A. sempervirens P651	Peloponnese, Kastania	Wanke 153 (DR) 26.03.2005	N36°51'19'', E22°18'22'', 502m
A. sempervirens P653	Greece, Samos- Mesagio	Neinhuis 145 (DR) 21.05.2005	
A. sempervirens P654	Samos- Mesagio	Neinhuis 144 (DR) 21.05.2005	
A. sempervirens DB512	Greece, Crete	Risse 1529 (B) 07.12.2006	N35°03', E25°29'30'', 800m
A. sempervirens P666	Greece, central Crete	Kalpotzakis s.n. 05.01.2007	N35°06', E24°55', 350m
A. sempervirens P52	Italy, Avola	Wanke & Neinhuis W103 (DR)	N36°55'03'', E15°09'25'', 5m
A. sempervirens P54	Italy, Sicily	Wanke & Neinhuis W106 (DR)	N36°58'46'', E15°10'17'', 190m
A. sempervirens P652	Italy, Sicily	Wanke & Neinhuis (DR)	N36°57'29'', E15°12'23'', 20m
A. sempervirens P278	Syria, Um Altueur	Mahfoud 25/2 (DR) 08.04.2006	N35°45'36'', E35°52'74'', 102m
A. sempervirens P309	Syria, Lattakia-Ariha	Mahfoud & Wanke (DR).01.04.2006	N35°39'73'', E36°00'48'', 87m
A. sempervirens P323	Syria, Slunfa	Mahfoud & Wanke (DR).31.03.2006	N35°35'92'', E36°10'95'', 1142m
A. sempervirens P325	Syria, Safeta	Mahfoud & Wanke (DR). 30.03.2006	N34°49'56'', E36°03'75'', 755m
A. sempervirens P335	Syria, Balloran	Mahfoud & Wanke (DR). 03.04.2006	N35°48'72'', E35°56'50'', 288m
A. sempervirens P402	Syria, Albdruseah	Mahfoud 054/3 (DR) 04.05.2006	N35°52'22'', E35°54'66'', 62m
A. sempervirens P738	Syria, Kassab	Mahfoud 005/1 (DR) 30.04.2007	N35°53'22'', E35°55'66'', 653m
A. sempervirens P739	Syria, Kassab, Alsamra	Mahfoud 003/1 (DR) 29.03.2007	N35°55'08'', E35°58'19'', 755m
A. sempervirens P740	Syria, Lattakia, Zgren	Mahfoud 002/1 (DR) 22.03.2007	N35°41'94'', E35°51'28'', 142m
A. sempervirens P741	Syria, Alhafah	Mahfoud 011/ (DR) 06.04.2007	N35°35'39'', E36°03'44'', 892m
A. sempervirens P742	Syria, Tartus, Yahmur	Mahfoud 006/1 (DR) 02.04.2007	N34°49'40'', E36°04'19'', 831m
A. sempervirens P743	Syria, Lattakia, Alesaweah	Mahfoud 011/1 (DR) 29.03.2007	N35°46'41'', E35°53'62'', 157m
A. sempervirens P744	Syria, Banyas, Alkadmus	Mahfoud 017/1 (DR) 06.04.2007	N35°10'46'', E36°01'36'', 468m
A. sempervirens P784	Syria, Lattakia, Maschketa	Mahfoud 043/1 (DR) 01.05.2007	N34°54'06'', E35°39'42'', 222m
A. sempervirens P670	Israel, Lower Galilee	Joel (BG Dresden) 11.08.2006	
A. sempervirens P1040	Israel	BG Dresden	
A. sempervirens P684	Algeria, Alger	Salle(JE) 06.01.1848	
A. sempervirens P276	Turkey, Belen	Mahfoud 038/2 (DR) 28.04.2006	N36°24'25'', E36°02'64'', 208m
A. sempervirens P294	Turkey, Yayladagi	Mahfoud 053/1 (DR) 02.05.2006	N35°54'60'', E36°06'18'', 725m
A. sempervirens P343	Turkey, Kirikhan	Mahfoud 037/1 (DR) 27.04.2006	N35°44'12'', E36°12'15'', 855m

A. sempervirens P689	Turkey, Bursa	Malyer & Bikaku (BULU) 15.03.1993	
A. sempervirens P690	Turkey, Edremit	Malyer & Baser (BULU) 26.06 1981	
A. sempervirens P736	Turkey, Samandag	Mahfoud 035/1 (DR) 27.04.2007	N36°04’19’’, E36°01’04’’, 155m
A. sempervirens P737	Turkey, Eskandarun,	Mahfoud 004/1 (DR) 30.03.2007	N35°55’32’’, E35°55’11’’, 755m
A. sempervirens P974	Turkey, Amasya	Malyer (ESSE 9916) 25.05.1992	
A. sempervirens P975	Turkey, Bursa	Bilisik & Malyer (BULU) 19.06.2007	N40°01’16’’, E29°07’15’’, 225m

Table 2 *trnK-matK* Primers used in the present study.

Primer name	Sequence 5'-3'	Design
AR-matK-2400R	ATT TTC TAG CAT TTG ACT CC	Wanke et al. 2006b
Pi-matK-1060F	ACT TRT GGT CTC AAC YG	Wanke et al. 2006d
AR-matK-1200F	TTC CAA AGT CAA AAG AGC G	Wanke et al. 2006b
AR-matK-1510R	TAG ACT CCT GAA ARA GAA GTG G	Wanke et al. 2006b
AR-matK-2510R	AAA AAT CTC AAT AAA TGY AA	Wanke et al. 2006b
trnK-Fbryo1	GGGTTGCTAACTCAATGGTAGAG	Worberg et al. 2007
psbA-R	CGCGTCTCTCTAAAATTGCAGTCAT	Steele (1995)
AR-matk- 2100R	TGAAAATGATTACAAAGCACTAC	Wanke et al. 2006b

Table 3 nSCG Primers used in the present study

Primer name	Sequence 5'-3'	Design
AR-5443-1010R	GCATTTATACARCCATCATTCTCTGG	This study
AR-5443-600F	GCGGGTAAATGGGAGGTTCC	This study
AR-5443-980R	GAACATCTCATCTTCAGCAACAGGTCTC	This study
AR-5443-1230F	CAAGCARTGGAAAAGGATGGTGACC	This study
AR-5443-980F	GAGACCTGTWGCTGAAGATGARATGTT	This study
AR-5443-1230R	GGTYACCATCCTTTTCCAYTGCTT	This study

Results

Phylogenetic findings

The total length of the alignment of the multiple coding and non-coding chloroplast regions (*trnK* intron, *matK* gene, *trnK-psbA* spacer) comprises 3384 characters (Table 4). Four highly variable regions have been detected.Three of them showed typical A or T mononucleotide repeats (ranging from 7 to 15pb).The poly A and T microsatellites region, located within the *trnK*5' intron, and was described as cryptic simple in nature (Wanke et al. 2006a), had a variable length between 33 - 122pb (Table 4).All variable regions described above have been included in our analysis by using an unpublished modified indel coding method (Müller unpublished).

The maximum parsimony (MP) strict consensus tree (Figure 2) based on the combined chloroplast loci displayed the following phylogenetic relationships and is largely congruent with the phylogenetic hypothesis reconstructed with the nuclear gene region alone (Figure 3) or the combined tree, which is based on both the combined chloroplast and the nSCG region (Figure 4).

Three independent main lineages among the two species have been observed.The first lineage represents all *A. sempervirens* populations (from Algeria to the Near East) as monophyletic with a bootstrap value (BS) of 100. This clade is further subdivided into two sub-clades:

A) the populations from Syria, Turkey, Israel, Cyprus, Samos (Greece) and Algeria together in a largely unresolved and unsupported clade, and

B) populations from Crete and Peloponnesus (Greece) together with populations form Sicily (Italy).

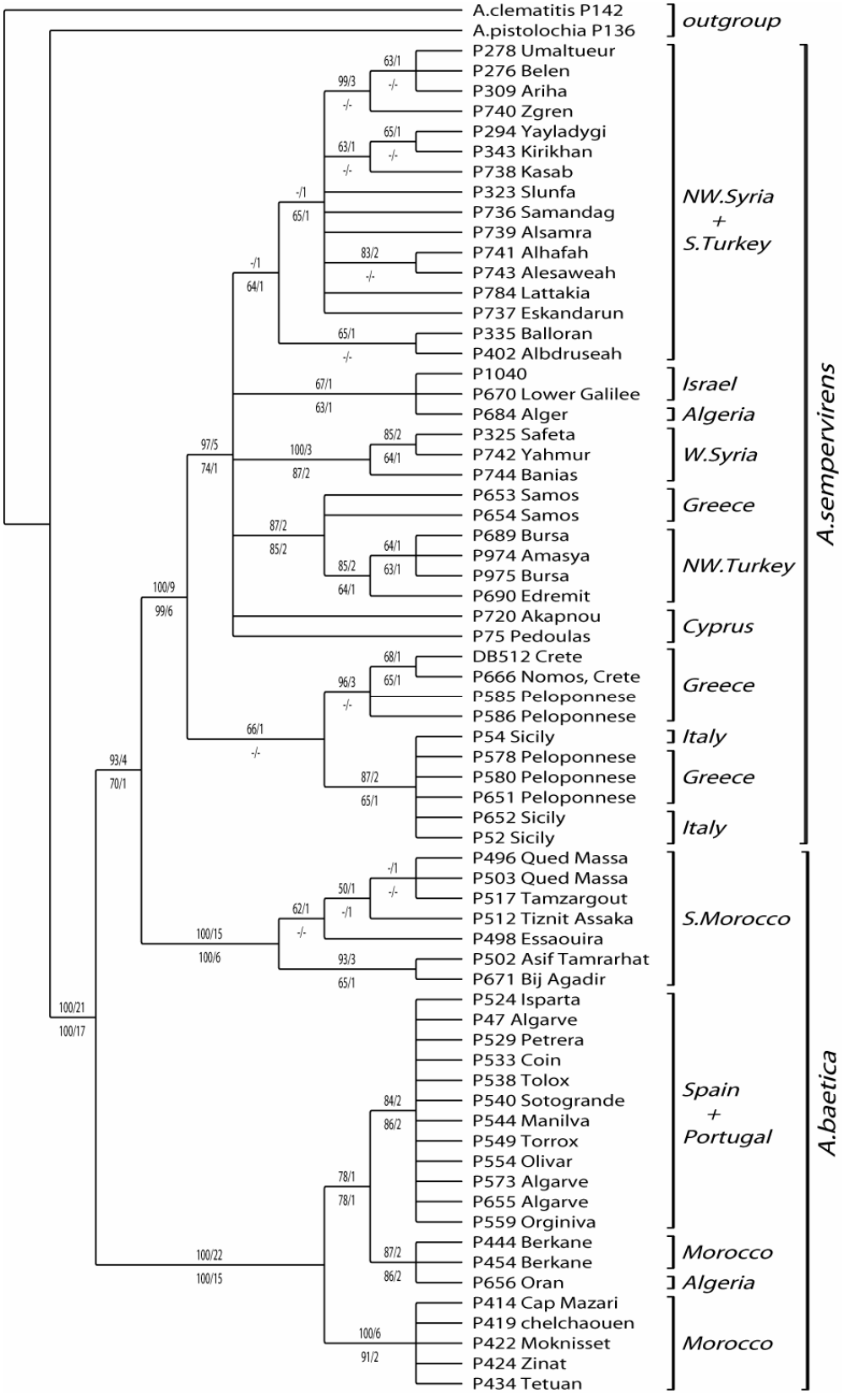
For *A. baetica* two major clades are recovered which are successive sister to the *A. sempervirens* clade and are therewith not monophyletic. The first *A. baetica* subclade contains all Portuguese, all Spanish, all Northern Moroccan and Algerian populations with a BS of 100. The second clade represents *A. baetica* populations from southern Morocco (south from the Anti Atlas and the adjacent coastal area) and is also supported with 100 BS.

Applying a new indel coding method for the chloroplast region the statistical support was generally increased by 10 to 25 BS and in few cases from no support to 99 BS. Similar, the Decay value increased by 1 to 8 steps. In total 21 nodes received higher support whereas only two nodes receive lower support and for the remaining 9 nodes no significant changes were observed (Fig. 2).

Table 4 Alignment characteristics of the chloroplast regions on which the phylogenetic tree reconstruction are based on (original dataset). Annotations of intron, exon and spacer boundaries are based on the full chloroplast genome of Piper cenocladum (DQ887677). Dataset length used for final calculations as well as the type of microsatellite due to which a region was excluded and the spans in bp is provided in a separate column. PIC are the parsimony informative characters found in the respective loci.

loci	position	characters	length range	type of satellite	PIC
trnK 5' intron	1- 1126	1126	815-877	AT rich repeat (33-122), T mononucleotide repeat (7-8)	20
matK gene	1227-2759	1533	1516	T mononucleotide repeat (12-13)	35
trnK 3' intron	2760-3032	273	261-266	A mononucleotide repeat (10-15)	5
trnK 3' exon	3033-3067	35	35	-	0
trnK-psbA spacer	3068-3384	317	280-296	-	11

Figure 2 Phylogenetic tree reconstruction based on the complete sampling for the *trnK-matK-trnK-psbA* chloroplast region. Topology of the strict consensus maximum parsimony tree is shown as cladogram (for topological differences between indel coding and non indel coding tree, see results). Values above branches are bootstrap supports first and decay values second inferred from the matrix with indel coding and below branch without indel coding.



The nSCG region with a reduced but representative sampling (26 populations) is 1163 bp in size, which is about ⅓ of the combined chloroplast datasets. It contains multiple introns and exons (Figure 5). This region was alignable without ambiguous areas thus no highly variable regions had to be excluded. The full region contains nine exons and eight introns in *Arabidopsis thaliana* (Figure 5). Flanking primers were placed in exon V and exon IX, amplifying three full exons and two exons in part as well as four highly variable introns for the respective *A. sempervirens* and *A. baetica* populations (Figure 5).

The topology of the phylogenetic tree based on the nSCG region for the reduced sampling represents all clades recovered by the chloroplast phylogeny. Also the geographic range of the two species was almost the same as that of the chloroplast sequence data. The differences among those two trees are basically the statistical support for individual nodes. The most striking case is the sister group relationship of the south Moroccan populations of *A. baetica* to all the populations of *A. sempervirens*, which is only 88 (BS) compared to 93 (BS) in the chloroplast gene tree.

Because no topological differences could be observed regarding the main branches of the tree between the two gene genealogies, a combined tree was calculated, which is presented in Fig. 4. This tree is largely resolved and supported with respect to the main branches and provides additional support for the findings from the individual gene trees alone.

Figure 3 Phylogenetic tree reconstruction based on the reduced sampling for the nuclear single copy gene (nSCG) without indel coding. Values above branches are bootstrap values.

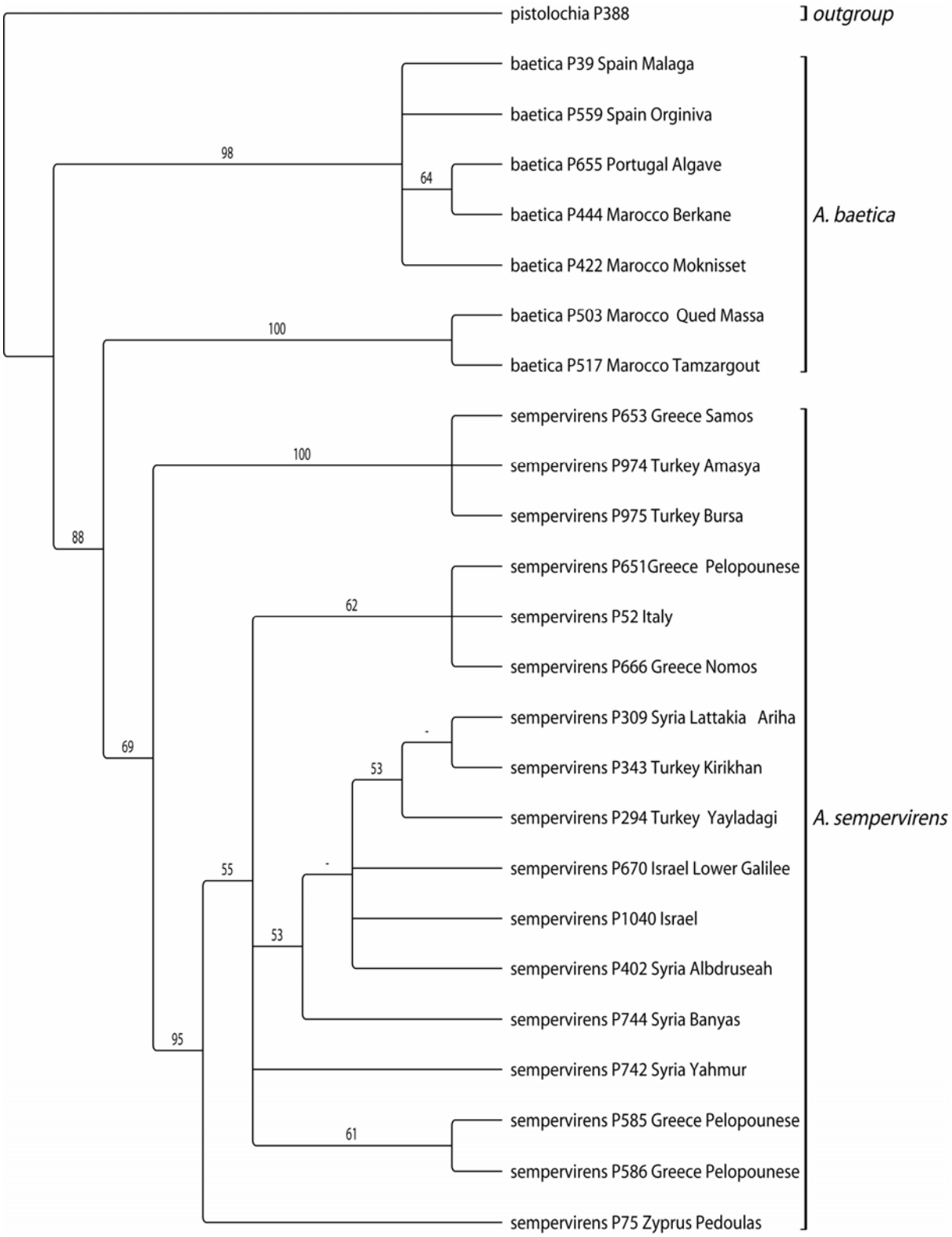


Figure4 Phylogenetic tree reconstruction based on the reduced sampling for the *trnK-matK-trnK-psbA* (chloroplast) included coded indels, combined with the nuclear single copy gene without indel coding. Values above branches are bootstrap values and decay indicesare shown below branches.

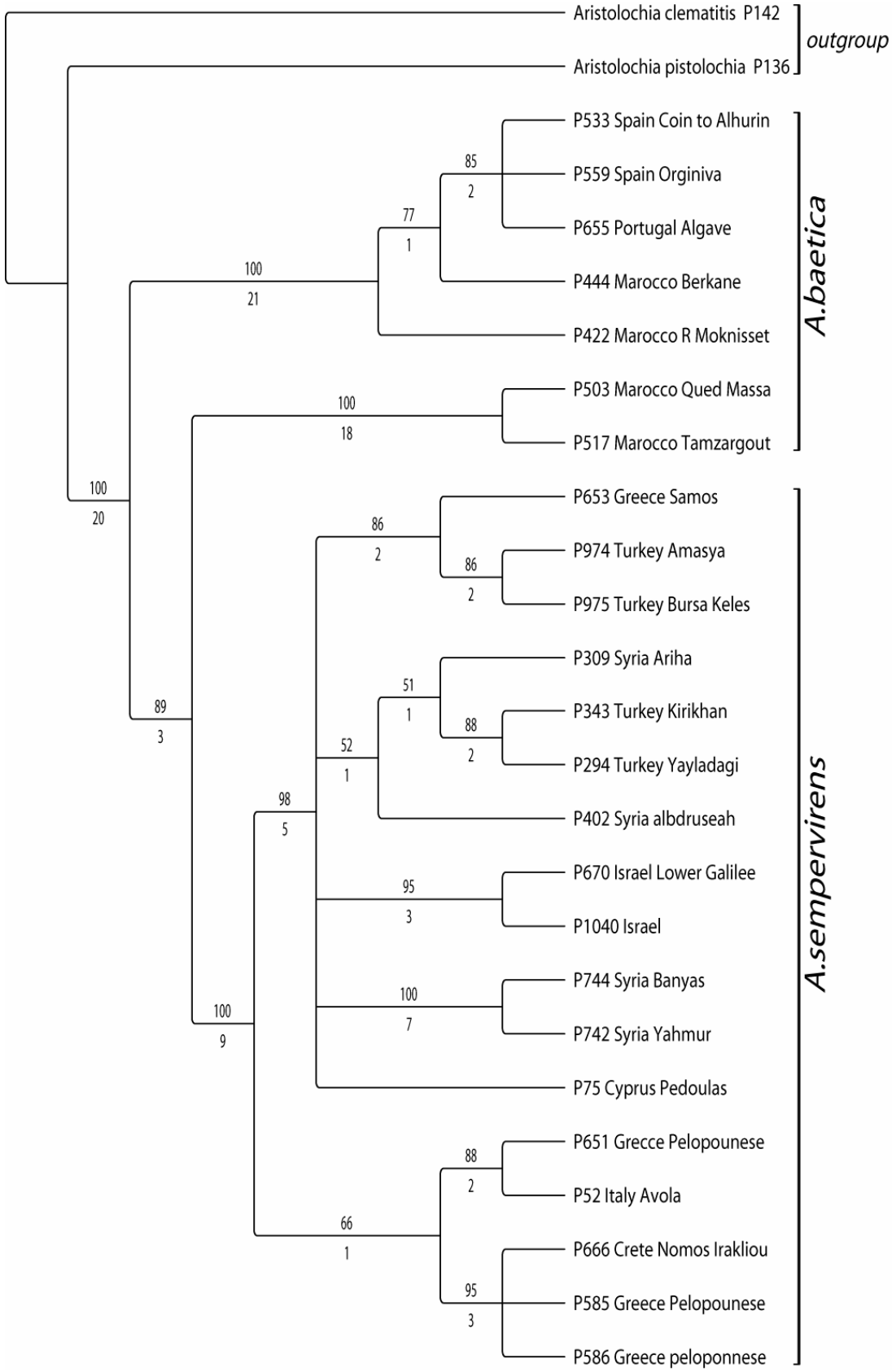
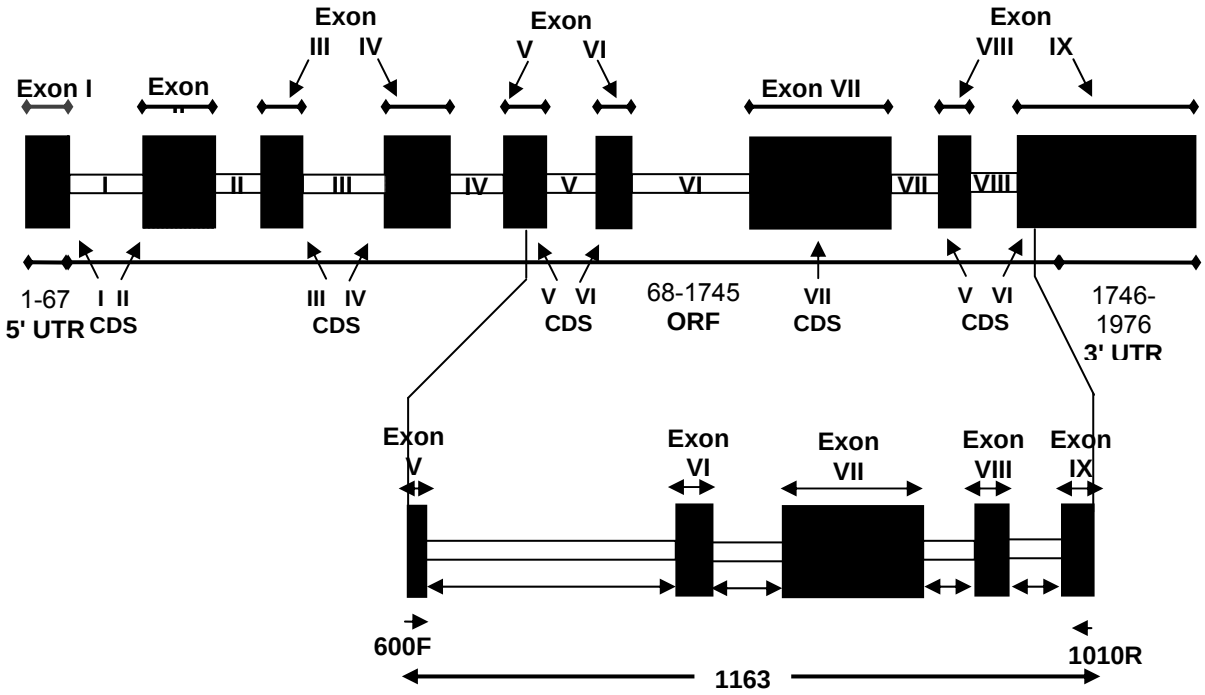


Figure5 Gene model for the tribe 5443 in *Arabidopsis thaliana* (upper model) and *Aristolochia baetica* P39 (lower model). Length of the region is presented proportional based on the situation found in the respective accession.

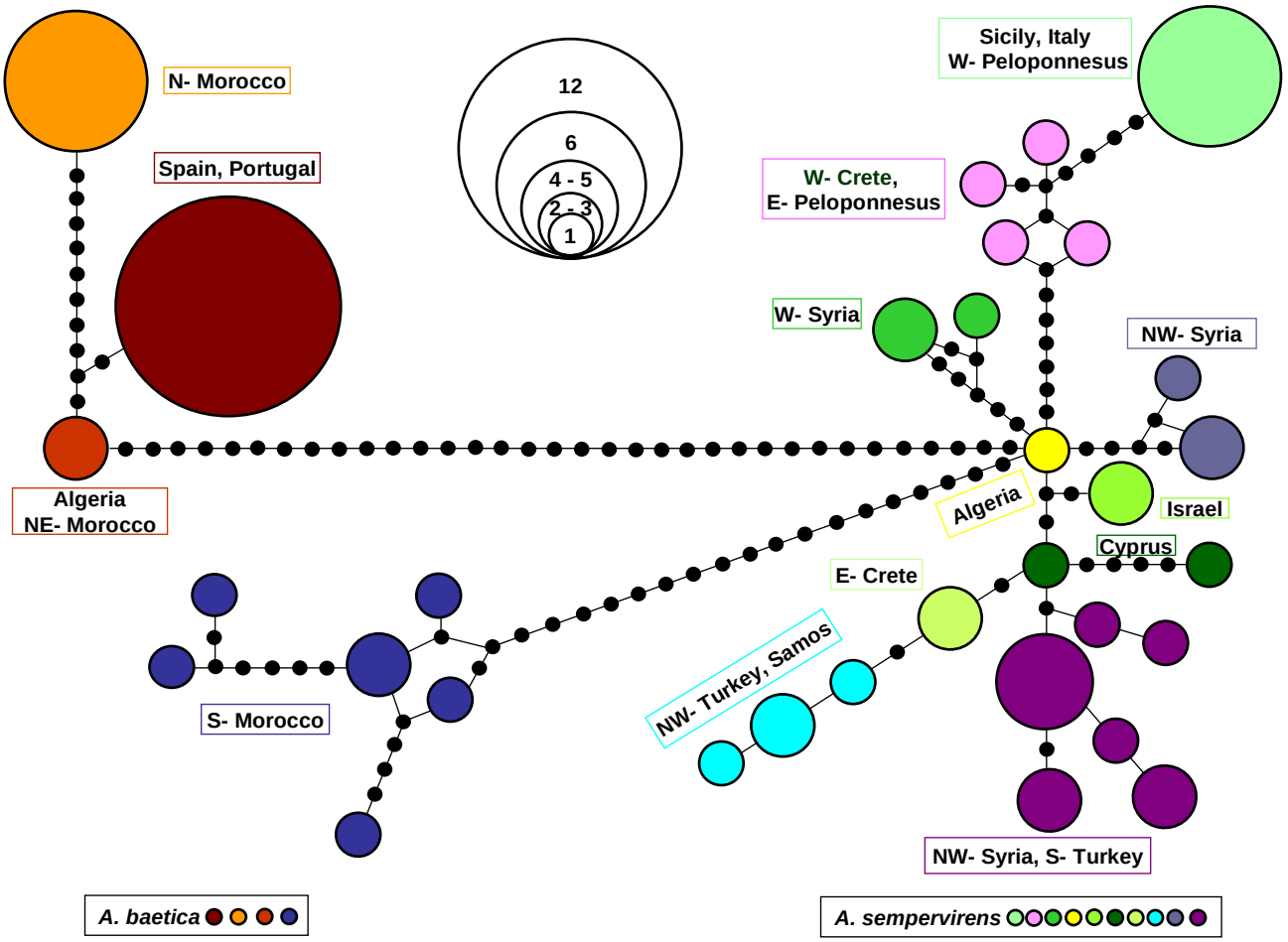


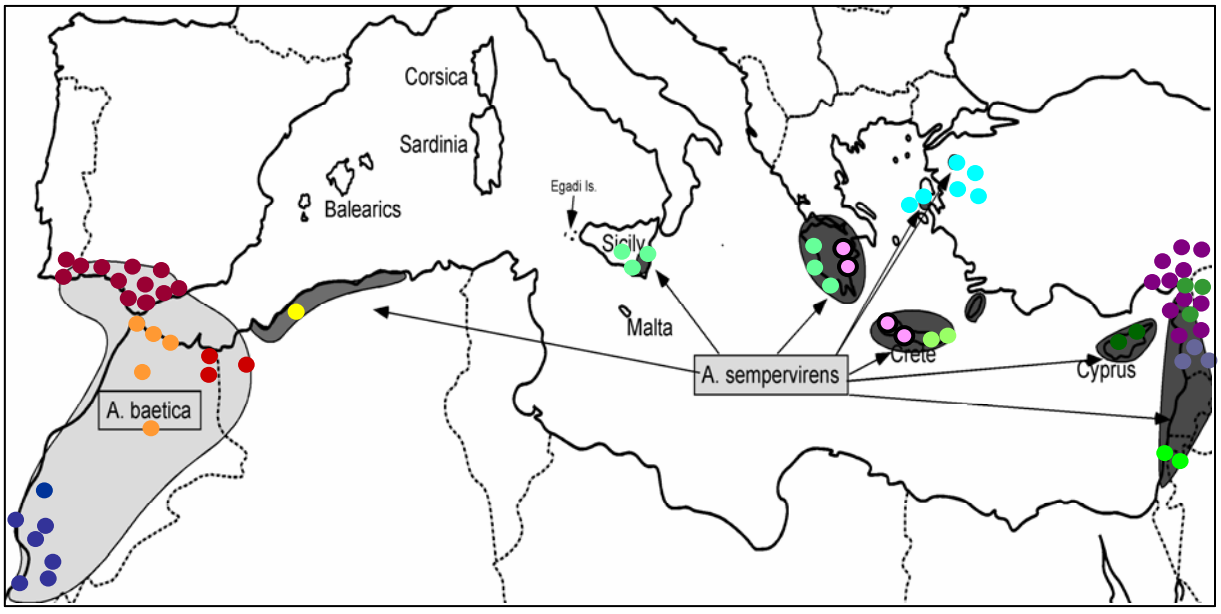
Phylogeographic findings

The haplotype network of the two studied species (40 populations of *A.sempervirens* and 27 populations of *A. baetica*) based on mutational steps of the chloroplast regions (*trnK* intron, *matK* gene, *trnK-psbA* spacer) show clearly different patterns of chloroplast variability in the two species.Overall, three main haplotypeshave been observed, one represents all *A. sempervirens* populations, while *A. baetica* populations were divided into the two remaining groups.The geneticdistances of populations are shown in Figure6.

As a general observation the haplotype diversity among *A. sempervirens* populations is extremely high. However, all *A. sempervirens* populations are found inone haplotype cluster, although several independent lineages can be observed. These lineages are each characteristic for different island population as wellas the remaining continental populations representing most likely independent waves of colonization of the same area. Furthermore, the different haplotypes found in *A. sempervirens* on different islands (e.g. Crete, Sicily, Cyprus) indicate, that colonization of the Mediterranean islands took place long time ago. All *A. sempervirens* populations from different areasin the Mediterranean converge at the central haplotype from Algeria. Furthermore, the *A. sempervirens* populations from Creteshow two differenthaplotypes which are shared with populations from neighboring islands as follows: A) western Crete populations share the same haplotype with Italian, Sicilian and Peloponnesus populations and B) eastern Crete populations share the same haplotypewith populations from Cyprus, Turkey and South-western Syria.Syria and adjacent areas have been colonized at least three times independently from an unknown ancestor being linked to the Algerian haplotype (see discussion).

Figure 6 Distribution and haplotype network (cpDNA & nSCG) of the *Aristolochia sempervirens* L. and *Aristolochia baetica* L. species complex. Sizes of circles are equivalent to the number of populations showing the same haplotype. Small black circles are proportional to the number of mutations between haplotypes.





Only three haplotypes are found among all Spanish, Portuguese, N–Moroccan and Algerian populations of *A. baetica* (Figure 6). These populations,found together in one haplotype cluster, show low haplotype diversity for the range of distribution compared to *A. sempervirens* and especially to the S-Moroccan populations of *A. baetica*. Only three different haplotypes, which are geographically separated by either the isthmus between Morocco and the Iberian Peninsula (Street of Gibraltar) or the different parts of the Atlas Mountains (Figure 7), indicate a more recent evolution. Populations belonging to this cluster are additionally characterized by an inversion within the *trnK* group II intron (not shown).Furthermore, the colonization of the Iberian Peninsula took place in one single colonization event, as only one haplotype could be observedfor populations of the Iberian Peninsula. All populations from the Iberian Peninsula are likely originating from populations found in North-East Morocco, close to the border of Algeria (Berkane). The genetic distance between these populations is the smallest observed (3 steps), whereas 11 steps are needed from the remaining N-Moroccan populations to the Iberian populations.

The second haplotype cluster of *A. baetica* is only found in southern Morocco, south from the Anti Atlas and the adjacent coastal area.Although this area is comparatively small, two times as many haplotypes are observed compared to the first haplotype cluster north of the Atlas Mountains.

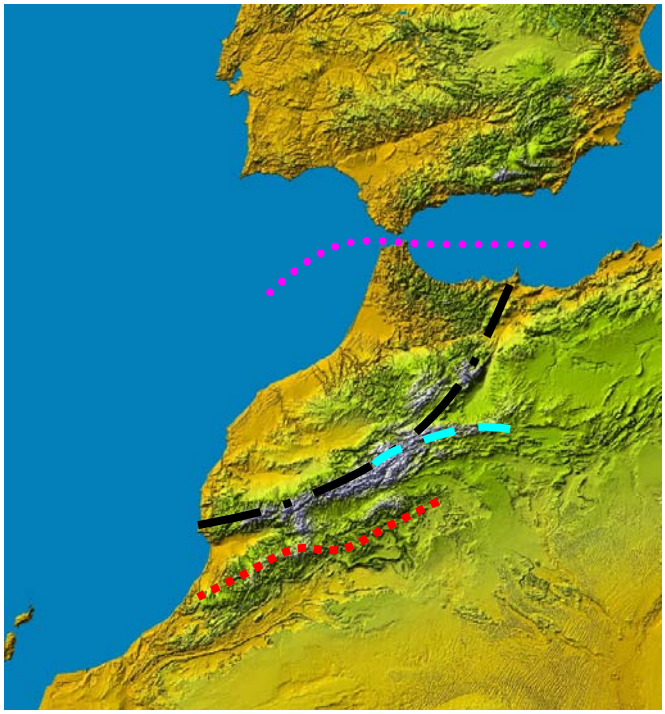
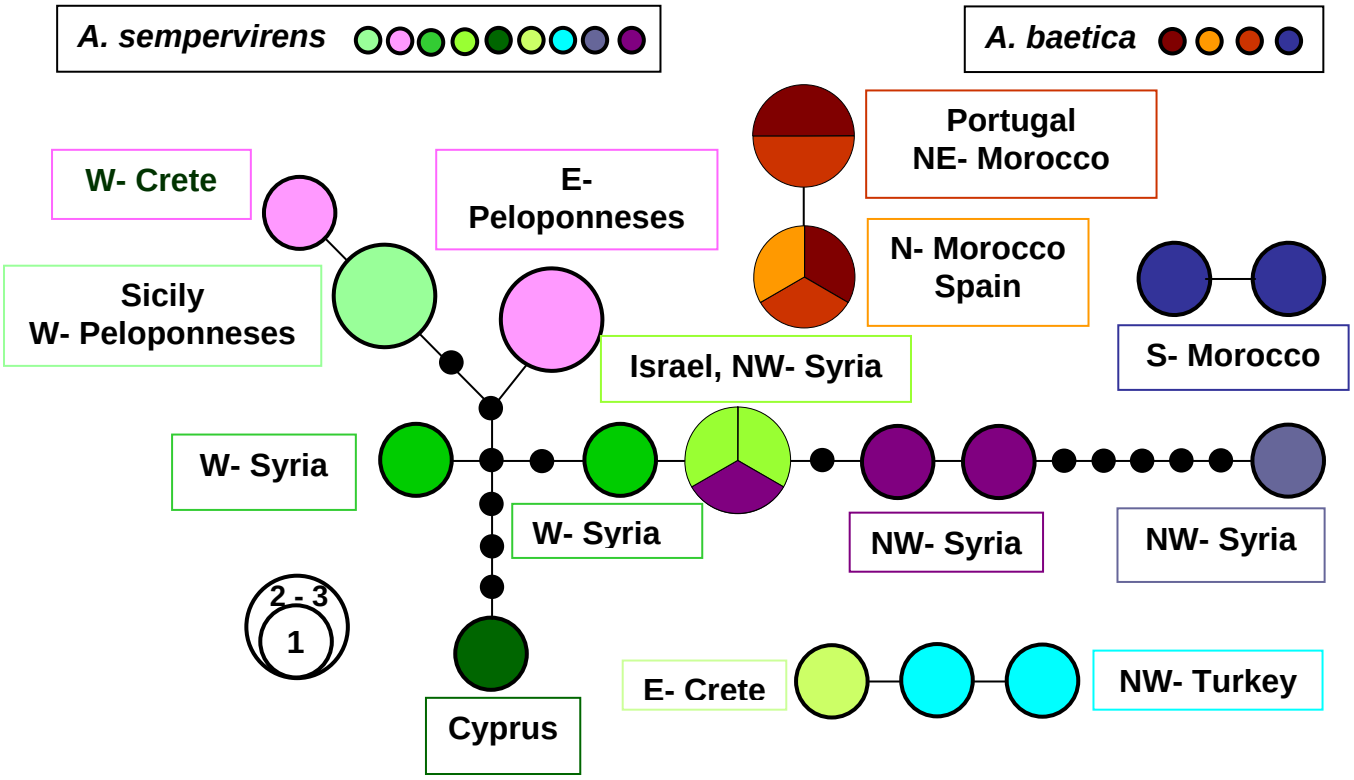


Figure 7 Physical map of the distribution area of *A. baetica* in Morocco with lines indicating geographical barriers formed by the High, Middle and Anti Atlas. In Morocco, north of the black line, all population show the same haplotype, being different from the populations from the triangle between the black and the turquoise line. The southern Moroccan populations are geographically separated from the other *A. baetica* populations by two barriers (black and red) as they occur south of the red line. The pink line (Atlantic Ocean, Strait of Gibraltar and Western Mediterranean Basin) separates the African from the Iberian population, showing two different haplotypes. (www.mygeo.info)

For the nuclear gene region an additional haplotype network has been reconstructed (Figure 8) which is different from the network calculated from the chloroplast one. The populations from the Iberian Peninsula, N-Morocco and S-Morocco could not be linked to each other and not to the *A. sempervirens* populations. This is most likely due to the large amount of mutations between the different clusters of haplotypes already observed in the chloroplast network. Another explanation for this result may be the incomplete sampling, since the Algerian population of *A. sempervirens*, being the connection between the three main clusters of haplotypes in the chloroplast network could not been amplified and sequenced from the 100+ year old herbarium specimens. Populations representing *A. sempervirens* also fall into two clusters which could not be linked to each other. The populations from NW-Turkey, Samos and E-Crete are one cluster and the remaining populations of *A. sempervirens* are the second cluster. The genetic distance and the connections between all the populations of *A. sempervirens* are overall highly similar to the one recovered based on the chloroplast network. Differences are found for shared haplotypes between N-Syrian, S-Turkish and populations from Israel, which are linked to the populations of NW-Syria and W-Syria.

Figure 8 Haplotype network based on the nSCG region. Four independent clusters are found which could not be linked to each other. Colors are identical to Figure 6.



Within *Aristolochia baetica* populations the results obtained from the nSCG haplotype network are also highly similar to the results obtained from the chloroplast network. The two main clusters are characterizing the S-Moroccan populations (two haplotypes) and populations from Algeria, N-Morocco, Spain and Portugal.The latter are represented by only two haplotypes, which are shared between the different occurrences of individual of populations. All NE-Moroccan populations including the Algerian populations and the populations from Spain and Portugal share one type. The other type is shared between all three areas, which indicates gene flow by pollen, but not the dispersal of seeds.

Discussion

Congruence of chloroplast and nuclear loci

The chloroplast loci (*trnK* intron, *matK* gene, *trnK-psbA* spacer) and the nuclear single copy gene, are virtually identical with respect to their phylogenetic signal. The chloroplast tree is better supported in lower nodes whereas the nSCG dataset provides better signal for upper branches. Overall, the combination of both datasets reveals the best resolution and support for major nodes, which supports the congruence of the datasets also without performing e.g. Shimodaira-Hasegawa tests (Shimodaira Hasegawa 1999). The fact that the combined tree does not receive higher support and is better resolved than the single gene trees, is likely a result of signal incongruence among the datasets. This can be expected if multiple colonization events are assumed and subsequent gene flow is not prevented by e.g. geographic isolation (see later). Beside the recommendation of using single or low copy genes alone, for the reconstruction of species and population level systematics, the combination and simultaneous analysis of independent datasets, as suggested by e.g. Nixon & Carpenter (1996) is shown in the present study.

Phylogeographicimplications

Colonization and isolation are critical events in the evolutionary dynamics of plant populations. The review of Thompson (1999) illustrates that the Mediterranean flora is full of examples that provide key insights into such evolutionary issues. The *A. sempervirens* complex represents one of these examples, which elucidate or illustrate the colonization and subsequent isolation of populationson the Mediterranean islands.

With respect to the present day distribution of *A. sempervirens*, three different scenarios of colonization and/or introduction could be assumed:

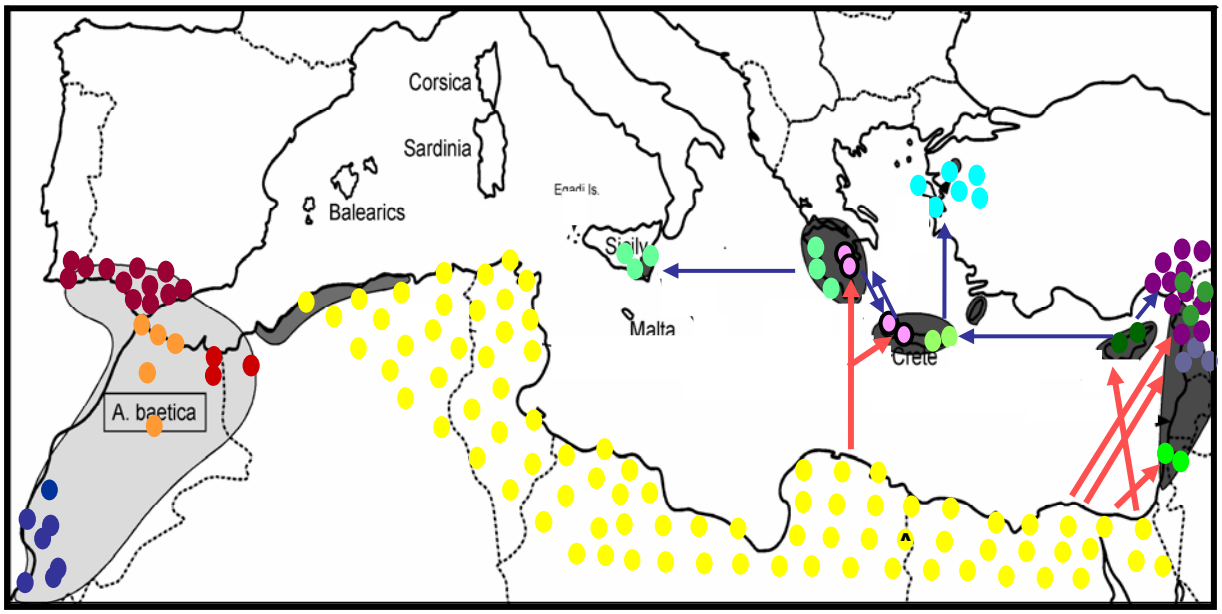
I) Many of the localities and islands inhabited today by *A. sempervirens* are places of intense human settlements in at least the last 5000 years. In addition, *A. sempervirens* has been cultivated as both ornamental plant but more importantly for medical use (Heinrich et al. 2009). As a consequence, the current distribution could be a result of human mediated introduction to the Mediterranean islands such as Cyprus, Crete, Sicily and along the continental shoreline. The origin of this species would therefore likely be the area of greatest genetic diversity, whereas introduction would be postulated for genetic homogeneity.

II) The present day distribution is a result of either East-West colonization or West-East colonization. Both scenarios would implicate that older populations show greater genetic diversity than the more recent populations. Although, many islands of the Mediterranean were connected to each other or to the mainland, in former times, such as Sicily to North Africa (until ~6 MYA) (Rosenbaum et al. 2002) or Peleponesus, Crete and SW-Turkey through a land bridge. None of the routes would be easily explainable by only plate tectonic drifts or sea level fluctuation building at least temporary bridges without taking long distance dispersal into account.

III) Current distribution patterns are relict populations of a widespread *A. sempervirens*in former times. Especially the climatic history, in combination with geological events, has shaped the current distribution. In contrast to other species of *Aristolochia* in the Mediterranean region, both *A. baetica* and *A. sempervirens* show an atypical growth form. They do not show prominent underground structures which enable to survive unfavorable climatic changes or seasonality. In addition, both species are evergreen climbers. Judging from the overall habit, both species are more likely an (understory)-forest plant than adapted to the present day Mediterranean climate. Taking into account, that the Mediterranean region including N-Africa showed predominantly forest vegetation until 10.000 years ago (Templeton 2005), the relict scenario is also likely.

The multiple waves of colonization as can be inferred from the chloroplast haplotype network (Figure 6) and are reconstructed in Figure 9. Based on the maternal inherited lineage, the colonization only happens once and further colonization of neighboring islands took place later on. The different haplotypes found in *A. sempervirens* on different islands (e.g. Crete, Sicily, Cyprus) indicates, that colonization of the Mediterranean islands took place long time ago and possibly in chronologically discrete waves. For some populations, e.g. Sicily and W-Peloponnesus gene flow is still occurring, as the nuclear gene haplotype does not recover the separation. Similarly, the populations on the Peloponnesus themselves exchange genetic information. In contrast, on Crete the eastern and western populations do not show gene flow anymore, as the populations could even not be linked to each other in the nuclear gene network. A high degree of isolation was also observed in the genus *Cyclamen* on western and eastern mountain chains on Crete (Gilly et al. 2001)being historically a mountain arc connected to the Peloponnesus.The populations from NW-Turkey are also separated from the remaining populations and show independent accumulation of genetic diversity in the nuclear gene network, which indicated reproductive isolation and initiated “speciation” through geographic isolation. Although the Near East has been colonized multiple times independently, the nuclear gene network indicates no reproductive isolation as one haplotype is shared among populations initially originating from independent colonization events of Syria and adjacent areas. The finding that all *A. sempervirens* populations are linked to a central Algerian population strongly indicates that this population is a relict population (Castelloe and Templeton 1994) of a N-African widespread haplotype in former times, from which all current areas have been colonized. Support for such a scenario is provided by the overall habit of this species, which is much better adapted to forest vegetation then to a seasonal climate (see above). Climatic changes coming along with vegetation type changes are likely the reason for population fragmentation and geographical disjunction (Comes and Kadereit 1998, Hewitt 2000, Petit et al. 2002) currently observed in A. sempervirens.

Figure 9 Reconstruction (based on cpDNA) of primary (red arrows) and secondary (blue arrows) colonization routes by now extinct populations of*A. sempervirens* from N-Africa. Crete was colonized once from N-Africa and a second time from Cyprus. The shared haplotypes between W-Crete and E-Peloponnesus indicate a simultaneous colonization of both areas. Peloponnesus populations subsequently subdivided to form a second haplotype in the Western part, which later colonized Sicily. E-Crete populations have colonized NW-Turkey. These populations are likely a relict population of a more widespread E-Crete haplotype on the Greek Islands and adjacent W-Turkey. The Near East (coastal area of the Mediterranean) was colonized at least three times independently from a N-African ancestral haplotype and once from Cyprus, which was colonized earlier also by an N-African haplotype. The nuclear gene network supports this scenario and further indicates that gene flow is occurring between e.g. Syrian populations originating from different colonization events(see also main text).



Only three haplotypes are found among all Spanish, Portuguese, N–Moroccan and Algerian populations of *A. baetica* based on the cpDNA network (Figure 6). In the nuclear network the observed genetic variability is even lower. Only two types could be found although the whole distribution area has been densely sampled. Both results together indicate a comparatively young colonization event. Both networks converge also on a single colonization event from N-Africa to the Iberian Peninsula from Berkane (border Morocco-Algeria) or an area close to this (shared haplotypes in the nDNA and fewest steps in the cpDNA). Human mediated introduction can however be excluded as otherwise the genetic distance between Iberian and the N-African population would be smaller. Taking only the network deriving from the nuclear gene into account, gene flow is the likely they reason for the observed

homogeneity among this haplotype cluster. Otherwise all NE-Moroccan populations including the Algerian populations and the populations from Spain and Portugal would not share one type and the other type would even not be shared between all three areas.

Southern Moroccan populations of *A. baetica*, however, morphologically identical to the populations north of the Atlas, share only a common ancestor with all *A. sempervirens* populations and the northern populations and are therewith not closely related. Because of the morphological similarity, cryptic speciation is postulated for the findings as hybridization could be excluded as a possible explanation. It is also likely that the southern Moroccan populations started to diversifyearlier, as the number of nucleotide changes is much higher. Although both share a common ancestor with *A. sempervirens,*most likely from northern Africa, the split between both clusters happened in a timely parallel manner.

Systematics of the *A. sempervirens* complex

The phylogenetic trees support the monophyly of *A. sempervirens* but not of *A. baetica*. This is basically due to the fact that the two morphologically distinct species split in all analyses in three highly supported clades, one represent all *A. sempervirens* populations, while *A. baetica* is divided in two supported clades, representing geographically isolated areas. This finding is especially unexpected with respect to *A. sempervirens*, because this species was traditionally split in two but the monophyly of *A. baetica* was never questioned. *Aristolochia sempervirens L*. and *A. altissima Desf*. were a subject of intensive discussions in earlier studies, *A. altissima* was described from Algeria (Desfontaines 1799) and said to occupy a larger range, from Algeria to the Near East (Lebanon and Palestine) through Sicily, Greece and Cyprus (Ducharter 1864, Boisser 1879, Halacsy 1904, Hayek 1924, Mouterde 1966), while *A. sempervirens L.* was said to occur in Crete only (Linnaeus 1753). They were kept separate as distinct species, because of some morphological differences (flower color, stem and leaf size) (e.g. Boissier 1879, Hayek 1924, Mouterde 1966).Detailed studies on this two taxa conducted by Davis & Khan (1961, 1982) on few Cretan and Cyprus populations found no morphological difference between them and they considered *A. altissima* as a synonym of *A. sempervirens*. Similarly the study by Nardi & Nardi (1987) proved no remarkable differences between populations from Crete and Arcadian populations as well as between the Greek and the Italian

populations.They suggested instead that differences in morphological characters (e.g. stem and leaf size)fall into the variabilityof the plants according to the age,as well as influenced by environmental differences (soil). For this reason they believe that all Mediterranean populations occurring from Oran to the Near East belong to the same taxonomic unit and must be combined into one species (*A. sempervirens*). This conclusion was again supported later by Browicz (1990) and now for the first time by molecules in the present study.

Judging from our results and morphology, *A. baetica* and *A. sempervirens* are two separated species, although closely related (Wanke 2007).They are the only evergreen and climbing species in the Mediterranean, and are easily distinguishable from each other. *Aristolochia baetica* has glaucous leaves and a glabrous ovary and pedicels, while *A. sempervirens* has dark green leaves and a pubescent ovaryand pedicels (Ball 1964, de Groot et al. 2006, Wanke 2007).

With respect to *A. baetica*, morphological characters do not yet support separation of a third species in the *A. sempervirens* complex but more detailed investigations of especially the south Moroccan species are needed and might reveal e.g. micromorphological characters supporting the molecular findings.

Conclusion

- This study presents high overall variability and good performance of the fast evolving coding and non-coding cpDNA loci (*trnK-psbA* region). Even better on species and population level is the performance of the single copy nuclear gene region, taking into account that only one third of the sequencing effort had to be applied (more cost and time efficient).
- No hybridization could be detected as a reason for the groupings found.
- Haplotype reconstructions suggest a complex colonization scenario of the Mediterranean area by this species complex, detecting young and old relict populations (Algeria, NW-Turkey, respectively) as well as multiple colonization’s out of N-Africa (now extinct populations).
- Great haplotype diversity between populations strongly indicates that colonization took place long time ago and that human mediated introduction did not play an observable role by shaping the current distribution..

Acknowledgment

This work was supported by the German Academic Exchange Service (DAAD) and a grant from the Syrian Ministry of higher Education & Research. Initial work on the single copy gene region was supported by a postdoc grant to S. Wanke from the DAAD and is currently supported by a DAAD PPP project to S. Wanke and K. Müller together with C. dePamphilis. We thank A. Charpin, E. Kalpoutzakis, H. Malyer, K. Koch and the Botanical Garden Dresden for supplying plant material. Several herbaria have provided specimens for molecular phylogenetic studies (The Herbarium of the Friedrich Schiller University, Jena, Germany (JE), the Royal Botanic Garden Edinburgh, Great Britain (E), and herbaria from the Anadolu University (ESSE) and the Uludag University (BULU), Turkey. Specimens from Israel provided by Ori Fragman Sapir.

Chapter 3

Survey of leaf epicuticular waxes and trichomes in the genus *Aristolochia* (*Aristolochiaceae*) using scanning electron microscopy (SEM)

Abstract

A scanning electron microscope survey of leaf surface features in the genus *Aristolochia* was performed including samples from the New and Old World with a special emphasis on the Mediterranean species. The present SEM-study in the genus *Aristolochia* revealed that the investigated taxa can be divided into three groups according to the type of leaf epicuticular waxes. Furthermore, the Mediterranean *Aristolochia* species can be divided into two different groups: all west Mediterranean species are characterized by wax platelets of the "*Convallaria* type" whereas the Caucasian and Near East species together with *Aristolochia pistolochia* show wax rodlets known as the "*Aristolochia* type". Our results are in agreement with the phylogeny of the Mediterranean *Aristolochia* which separate the Mediterranean species into two clades described above. However, *A. pistolochia* occurs in the western Mediterranean this species has been found as sister to all Mediterranean species, which indicates that wax rodlets are a plesiomorphic character. Other investigated characters are the absence or presence and the type of trichomes on the adaxial leaf surface. In general a glabrous group (~ 19% of the investigated taxa) and the pubescent group (~ 81%) could be separated. The latter can further be subdivided into four basic types of trichomes: a) hook-shaped trichomes (two cells) which was present in most taxa, b) conical multi-cellular and erect to sub-erect trichomes being characteristic for some East Mediterranean species (*A. hirta* and *A. paecilantha*), c) multicellular-elongated trichomes with cylindrical base and an elongated acute apex , which were found among species belonging to the *Aristolochia grandiflora* complex and the *Aristolochia subgenus Isotrema*, and d) glandular trichomes with rounded tip found only in *A. triactina* which is the only representative of the subgenus *Pararistolochia* from Africa in this study.

Introduction

In the past, systematic studies also dealing with *Aristolochiaceae* was preliminary based on morphological and anatomical characters of seeds (e.g. Barthlott 1981, Huber 1985, Adams et al. 2004), flowers (e.g. Gonzales and Stevenson 2000) and leaf characteristics (Barthlott 1981)

In generel, characteristics of the leaf surface, especially the morphology and chemical composition of epicuticular waxes, has been proofed to be of high taxonomical value in both major plant lineages (Stace 1965, Henning et al. 1994, Barthlott et al. 1998, Barthlott et al. 2003) and among species (Hallam and Chambers 1970, Tomaszewski 2004, Yousuf 2008). In addition to the wide range of usability of epicuticular wax morphology, this research was enhanced by the ease to obtain data by scanning electron microscopy (SEM) from e.g. air-dried samples (Mankovska et al. 2004). More elaborate preparation methods like chemical fixation (Riccio et al. 2006) or glycerol substitution (Ensikat and Barthlott 1993) have been established allowing conservation of cellular structures of the leaf samples.

Micromorphology of epicuticular waxes in *Magnoliidae*, *Ranunculidae* and *Hamamelididae* have been studied by Hennig et al. (1994), also including few species of *Aristolochiaceae* (21 species). The results showed that more than two third of *Aristolochiaceae* species have a special type of wax rodlets known as "*Aristolochia* type", while six species showed wax platelets known as "*Convallaria* type". Later, Barthlott et al. (1998) screened more than 13000 species for epicuticular wax crystals, representing all major groups of seed plants. Barthlott et al. (1998) identified at least 23 wax types. For *Aristolochiaceae*, Barthlott et al. (1998) confirmed the results from Hennig et al. (1994) and characterised the "*Aristolochia* type" as transversally ridged rodlets being characteristic for "ancestral woody angiosperms", and the "*convallaria* type" as parallel oriented platelets, being characteristic for Liliiflorous families. The latter, being present in major land plant groups (angiosperms, gymnosperms and mosses).

Another type of characters that can serve for morphological systematics, are the form and density of trichomes. Trichomes can be found on the leaf epidermis as well as other plant organs. Trichome characteristics were used for the classification as well

as identification purposes by many systematists on different taxonomic levels (Theobald et al. 1979, Prabhakar et al. 1985, Dickison 2000, Batterman and Lammers 2004). Plant-hair types have been successfully used in the classification of genera and species and in the recognition of hybrids within certain groups (Rollins 1944, 1945). In *Cruciferae*, for example, Schultz (1936) used the type of hairs as a major criterion in the subdivision of the family into tribes and genera. In *Rhododendron* (*Ericaceae*), Cowan (1950) has shown that the trichomes afford useful characters for taxonomic separation on the infrageneric level as well as the species level. Ramayya (1962) provided a generic key for the Indian members of *Compositae* on the basis of trichomes. In recent times, Khalik (2005) studied the model family *Brassicaceae* and discovered a great diversity of trichome forms. Kalik (2005) concluded that this diversity provide by far the most important taxonomic characters of the epidermis and in many cases are as valuable as any of the other morphological character of the plants. In addition, most researchers agree that leaf hairs could differ among related species and were used as taxonomic characters among species (Davis and Heywood, 1973) or even among subspecies and varieties (Khalik, 2005), others found no difference in hair type within plant groups. They concluded that leaf trichomes could be useful for taxonomic purposes among some related species but might not be useful among others (Clark et al. 1980).

Aristolochia, a genus of *Piperales* (Borsch et al. 2005, Neinhuis et al. 2005, Qiu et al. 2005), has a nearly world wide distribution with the highest diversity in tropical and subtropical regions. The adaptation to different abiotic conditions is likely to be mirrored on leaf characteristics. Taxonomic treatments of *Aristolochia* have been discussed controversially.

Systematics of *Aristolchia* from a pre-molecular aera to the molecular phylogenetic aera has changed. A recent study (Wanke et al. 2006a) summarizing molecular results from published molecular phylogenetic hypothesis (Murata et al. 2001, Neinhuis et al. 2005, Ohi-Toma et al. 2006) as well as own data subdivided *Aristolochia* into three subgenera, which are in accordance t both the molecular based phylogenies as well as morphological data. However, flower and seed attributes were essential for the differentiation between infrageneric groups of the genus, molecular studies shaded light into natural relationships and largely confirmed

pre-molecular studies. In recent years the focus has shifted from generic und subgeneric level to species level systematics. The Mediterranean species represent one of these groups. Most important contributions to the knowledge of species from the Mediterranean were published by Nardi (1984, 1988, 1991, 1993), Nardi & Nardi (1987), Nardi & Ricceri (1987) and Davis & Khan (1961, 1964, 1982). Wanke (2007) provided the first phylogeny of all West Mediterranean *Aristolochia* species also including few members of Caucasia and the East Mediterranean region.

As detailed studies on epidermal trichomes and micromorphological studies on waxes are largely lacking and if available are only based on few representatives being not representative for all taxonomically recognized groups, the present study evaluates the presents of such structures and access possible phylogenetic importance for the systematics of the genus. Results are discussed in the light of traditional classifications as well as hypotheses derived from molecular data.

Material & Methods

This study on micromorphology of *Aristolochia* leaves comprises 54 species representing all three subgenera (Suppl. Tab. 1). Leaf material was taken from living plants cultivated in the Botanical Garden Dresden. Fresh and uncontaminated leaves were collected preferentially. Squares of leaves (approximately 3 mm x 3 mm) were excised using a razor blade. To obtain a relatively flat surface of the sample the midrib area has been avoided. Leaf samples were prepared for SEM by liquid substitution of glycerol according to Ensikat & Barthlott (1993). Samples were fixed to an aluminum sample holder (10mm in diameter, Plano GmbH, Wetzlar, Germany) using a carbon adhesive tape (Leit Tabs, Plano GmbH) and sputter-coated under argon atmosphere with an approximately 10 nm thin gold layer in EMITECH K550 (Emitech Ltd., Ashford Kent, UK). The adaxial leaf surface was observed using a Leo 420 (Leo Electron Microscopy Ltd., Cambridge, UK) and the software LeoUIF 420 and Diss5. Measurements on wax crystal dimensions were performed using Diss5. Description of wax crystal morphology the nomenclature introduced by Barthlott et al. (1998) was followed.

Results

Epicuticular waxes of the adaxial leaf surface

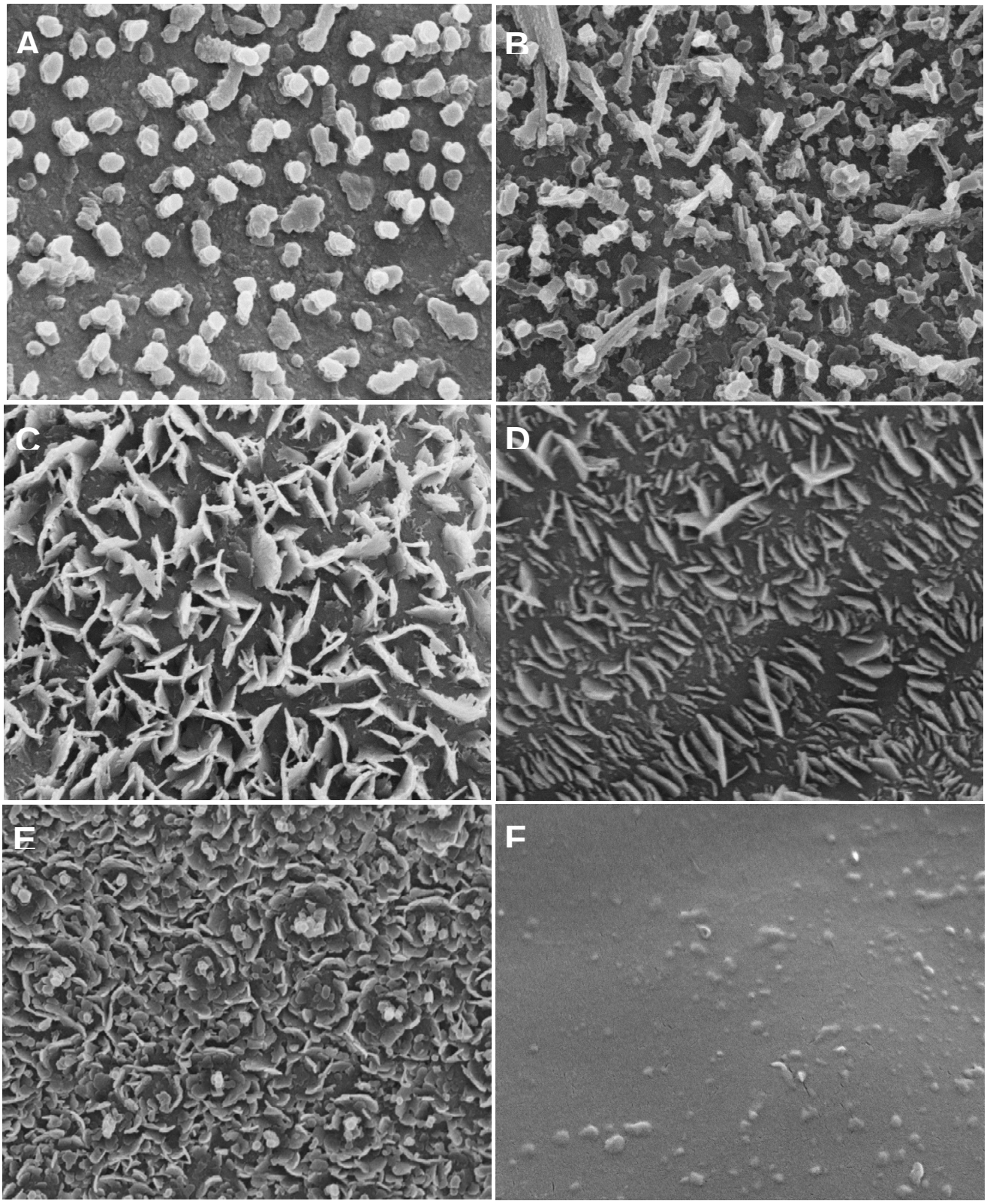
Two different types of wax crystals were found on the adaxial leaf surface of *Aristolochia*: transversely ridged rodlets and wax platelets. Different types are characteristic for the specific species and only two cases were observed where both types occur on one single species (Table 1). 70% of the investigated species possess transversely ridged rodlets (Figure 1-A, B) on the adaxial leaf surface. These waxes of the "*Aristolochia*" type occur likewise in Old World as well as in New World species. Surprisingly, the “Aristolochia” type is the unique wax found in East Mediterranean Aristolochia species. This is in contrast to most other groups which show usually both types on different species. According to the variability in length and density of the transversely ridged rodlets, the species showing this type of wax could be divided into two groups: A) short, homogenous and less dense rodlets (Figure 1-A) and B) short to long, heterogeneous and more dense rodlets (Figure 2-B). All included species from the East Mediterranean region represent the previous form (e.g. *A. guichardii*, *A. krausei*), while the second form is more common within *Howardia* s.str. (e.g. *A. brasiliensis*, *A. guentheri*).

In 26 % of all investigated species wax platelets with an irregular margin were found. The platelets protrude more or less perpendicular from the underlying surface and are usually connected to it by their narrow side. In some species (e.g. *A. lutea*, *A. parvifolia, A. elongata*) the wax platelets are arranged in parallel rows (Figure 1-D), but predominantly arrangement of wax crystals does not show any order (Figure 1-C). With the exception of *A. pistolochia*, which possess transversally ridged rodlets, irregular platelets were found in all investigated species belonging geographically to the West Mediterranean species. This type also occurs in few species distributed in Asia and America (*e.g. Howardia* s.str., subgenus *Isotrema*).

In two species *(A. cucurbitifolia* and *A. mollissima*) belonging to subgenus *Isotrema* both irregular wax platelets and transversally ridged rodlets were found. Loosely scattered rodlets are surrounded by platelets forming a bloom like pattern (Figure 1-E).

No waxes could be observed on the adaxial leaf surface of subg. *Pararistolochia* (*A. triactina* and *A. pravenosa*) (Figure 1-F).

Figure 1. A and B showing both transversally ridged rodlets; A *Aristolochia guichardii* with short, homogenous and less dense rodlets; B *Aristolochia guentheri* with short-long, heterogeneous and more dense rodlets; C and D showing both wax platelets typical for West Mediterranean species; C *Aristolochia clematitis* showing irregular wax platelets, and D *Aristolochia parvifolia* has parallel rows of wax platelets; E *Aristolochia cucurbitifolia* possessing transversally ridged rodlets and irregular platelets; F *Aristolochia triactina* without any waxes.



Trichomes on the adaxial surface of *Aristolochia* leaves

The majority of the investigated taxa are pubescent (Figure 2-A) but 20 % do not show any evidence for trichomes (Table 1). Four different types of trichomes could be observed: hook-shaped trichomes, gland-like trichomes, relatively thick and conical trichomes and long, thin trichomes often with longitudinally collapsed cells. Hook-shaped trichomes are at least two-celled, with a long, curved cell forming the hook and one to several cells forming a broad base (Figure 2-B). This type of trichome was found in all three subgenera and 70 % of the investigated species. Conical trichomes are inserted with their broad base on the underlying surface. More or less erect trichomes occur only in three species of subgenus *Aristolochia* also possessing hook-shaped trichomes (Figure 2-C,D). These more or less long and thin trichomes are cylindrical at their base and possess an elongated acute apex. Cells are always longer than broad and often longitudinally collapsed (multicellular-elongated trichome) (Figure 2-E). Both investigated species of the *Aristolochia grandiflora* complex as well as some species of subgenus *Isotrema* are characterized by this type of trichomes. (iv) Gland like trichomes (Figure 2-F) with a round tip are found in the only African species of subgenus *Pararistolochia (Aristolochia triactina)*. Other species are characterized by a combination of erect and hook-shaped trichomes types.

Figure 2. A *Aristolochia clematitis* with glabrous leaves; B *Aristolochia hirta* with hook-shaped trichomes; C conical trichomes and D conical together with hook-shaped trichomes both found in *Aristolochia paecilantha*; E *Aristolochia tomentosa* shows multicellular-elongated trichome together with hook-shaped trichomes; F glandular trichome observed in *Aristolochia triactina*.

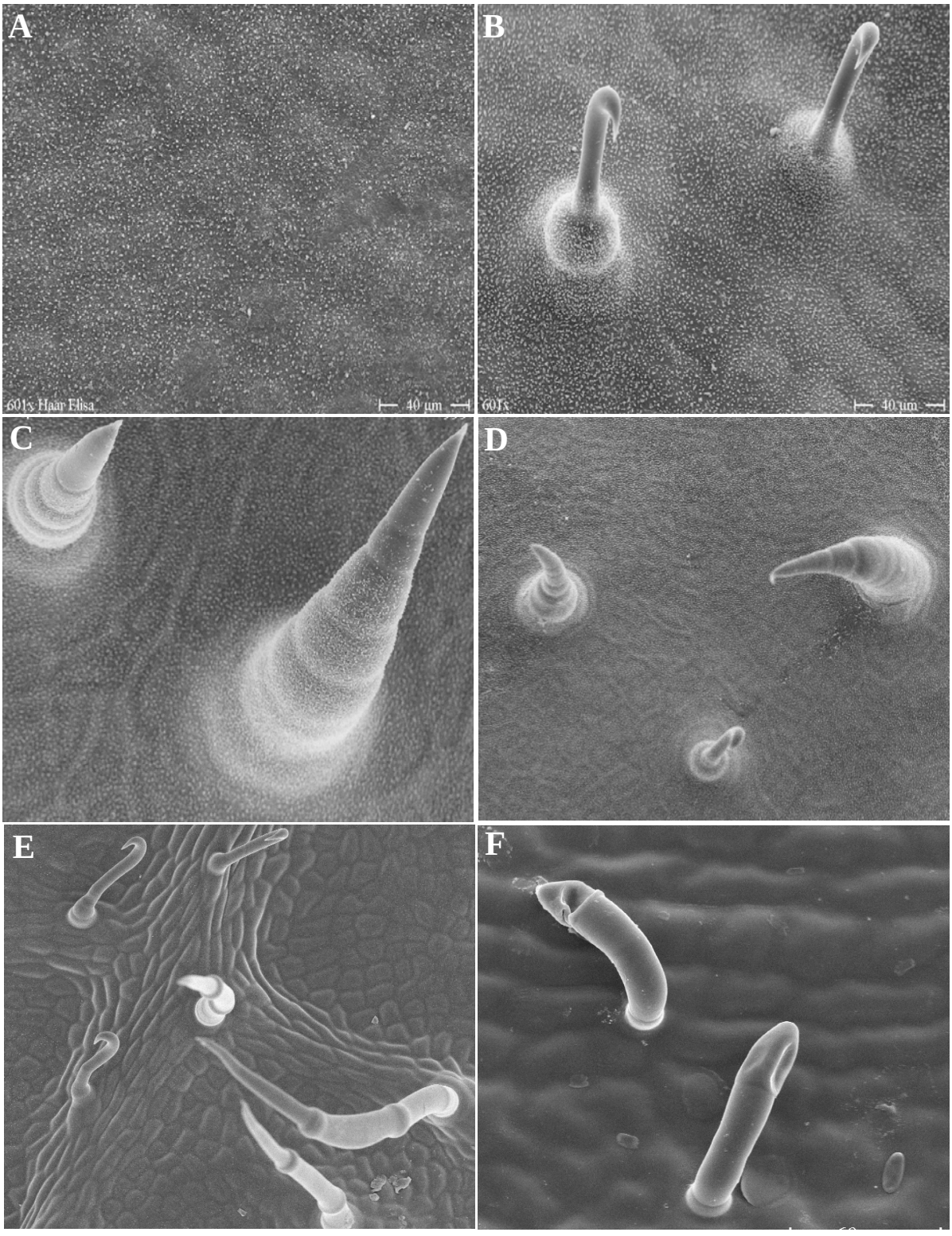


Table 1. Presence and morphology of trichomes and epicuticular wax crystals on the lower leaf surface of *Aristolochia* species.

Species	additional affiliation (e.g. section/subsections or equal)	wax crystals	Trichomes
subg. *Aristolochia*			
A. bottae	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. guichardii	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. hirta	*Aristolochia/Aristolochia*	rodlets	hook-shaped + conical
A. krausei	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. maurorum	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. paecilantha	*Aristolochia/Aristolochia*	rodlets	hook-shaped + conical
A. scabridula	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. baetica	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. clusii	*Aristolochia/Aristolochia*	platelets	hook-shaped
A. elongata	*Aristolochia/Aristolochia*	platelets	hook-shaped
A. lutea	*Aristolochia/Aristolochia*	platelets	absent
A. microstoma	*Aristolochia/Aristolochia*	platelets	hook-shaped
A. navicularis	*Aristolochia/Aristolochia*	platelets	absent
A. pallida	*Aristolochia/Aristolochia*	platelets	hook-shaped
A. parvifolia	*Aristolochia/Aristolochia*	platelets	hook-shaped
A. pistolochia	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. rotunda	*Aristolochia/Aristolochia*	platelets	absent
A. sempervirens	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. clematitis	*Aristolochia/Aristolochia*	platelets	absent
A. debilis	*Aristolochia/Aristolochia*	platelets	absent
A. rigida	*Aristolochia/Aristolochia*	rodlets	hook-shaped
A. acuminata	*Aristolochia/Podanthemum*	rodlets	hook-shaped
A. chlamydophylla	*Aristolochia/Podanthemum*	rodlets	hook-shaped
A. kankauensis	*Aristolochia/Podanthemum*	rodlets	hook-shaped
A. gorgona	*grandiflora complex*	rodlets	hook-shaped + long
A. grandiflora	*grandiflora complex*	rodlets	hook-shaped + long
A. anguicida	*Howardia s.str.*	rodlets	hook-shaped
A. brasiliensis	*Howardia s.str.*	rodlets	hook-shaped
A. cymbifera	*Howardia s.str.*	rodlets	hook-shaped
A. elegans	*Howardia s.str.*	rodlets	hook-shaped
A. eriantha	*Howardia s.str.*	rodlets	hook-shaped + conical
A. fimbriata	*Howardia s.str.*	platelets	hook-shaped
A. galeata	*Howardia s.str.*	rodlets	hook-shaped
A. gigantea	*Howardia s.str.*	rodlets	absent
A. guentheri	*Howardia s.str.*	rodlets	absent
A. leuconeura	*Howardia s.str.*	rodlets	absent
A. odoratissima	*Howardia s.str.*	platelets	hook-shaped
A. pohliana	*Howardia s.str.*	rodlets	absent
A. prostrata	*Howardia s.str.*	platelets	hook-shaped
A. ringens	*Howardia s.str.*	rodlets	hook-shaped
subg. *Isotrema*			
A. arborea	*Isotrema*	rodlets	absent
A. californica	*Isotrema*	rodlets	hook-shaped + long
A. austroyunnanesis	*Isotrema*	rodlets	long
A. cucurbitifolia	*Isotrema*	mixed	hook-shaped
A. manschuriensis	*Isotrema*	rodlets	hook-shaped
A. mollissima	*Isotrema*	mixed	hook-shaped + long
A. moupinensis	*Isotrema*	rodlets	long

A. nakaoi	*Isotrema*	rodlets	long
A. shimadai	*Isotrema*	rodlets	hook-shaped + long
A. tomentosa	*Isotrema*	rodlets	hook-shaped + long
A. westlandi	*Isotrema*	rodlets	long
subg. *Pararistolochia*			
A. triactina		without	glandular
A. praevenosa		without	hook-shaped

Discussion

Based on the classification and nomenclature of plant epicuticular waxes by Barthlott et al. (1998), and after examining nearly all Mediterranean species and a representative selection belonging to other clades, two completely different types of wax crystals occur within the genus. This is in accordance with previous studies (Hennig et al. 1994). Platelets of the “Convullaria” type are widely known from land plants (angiosperms, gymnosperms, mosses) (Theisen & Barthlott 1994, Meusel et al. 1994). This type of wax seems to be characteristic for the West Mediterranean species. However, few species of subg. I*sotrema* and *Howardia* show this features as well.

Rodlets have been found among all Caucasian and East Mediterranean species as well as for most of the Old and New World taxa, indicating that this is likely a plesiomorphic character in East Mediterranean species. Although, *A. pistolochia* is geographically occurring in the Western Mediterranean area (France, Spain), molecular phylogenetic studies showed this species as sister to all Mediterranean species more closely allied to *A. clematitis* (Wanke et al 2006a, Wanke 2007), which has a Eurasian distribution and is not part of the Mediterranean clade neither (Wanke et al 2006a, Wanke 2007). Also from a morphological point of view these species are sepereted from other Mediterranean Aristolochia species as thes do not posses a rootstock but have either a rhizome or show fleshy root (Wanke 2007). Former molecular hypothesis as well as macromorphological characters are therewith supported by the micro-morphological characters presented in this study. Among the included taxa, our results show that this type of wax is most common in the family and could be found among species belonging to all clades. Our results support the findings of Hennig et al. (1994) although the present study is based on more representative sampling and include many more species. The absence of both wax forms in all included species from subgenus *Pararistolochia* is likely a synapomophy

for this group, although more detailed investigations on closely related species need to be added from both Asia and Africa to further support this finding.

The presents or absence as well as the type and density of trichomes of the lower leaf surface seem not to correlate with the taxonomic affiliation of the species in most cases.

Plant hairs are among the most interesting features of plant surfaces. The woolly, velvety, or bristly appearance of many plants is mostly a result of trichome types and density. Also the systematic value in angiosperms is well documented in the botanical literature (Theobald et al. 1979, Batterman and Lammers 2004). In the present study, pubescences or glabrous appearance of the epidermal surfaces of the leaf were found to be less informative systematically for especially the main focal point, the Mediterranean *Aristolochia* species. Their value is greatly limited although revealing the presence in all Caucasian and East Mediterranean species, while for the West Mediterranean species both pubescent and glabrous leaves are observed. Although, the pubescence of leaves might be influenced by abiotic factors and adaptation to e.g. drier habitats co-occurring with higher sun radiation, this study does not support this assumption as for examples, species which are usually growing in extreme sun exposure, do not show any trichomes (*A. navicularis*) whereas species preferring shady places show trichomes (*A. pallida*). In the *Aristolochia grandiflora* complex, which was previously grouped in *Howardia* (Huber 1993) but based on molecular findings separated from it (Wanke et al. 2006a, Wanke et al. 2007) (Suppl. Fig. 1), a special type of trichomes was observed. This group can be characterised by hooked shaped trichomes, which are elongated compared to other hooked-shaped trichomes present in other clades.

The wax type (rodlets) and the presence of the hook-shaped trichomes in the *Aristolochia sempervirens* complex (*A. sempervirens* and *A. baetica*) indicates a closer relationship of this group to the East Mediterranean species, which might be a plesiomorphic trait and would therewith only partly contradict molecular findings as this group is recovered as sister to all remaining West Mediterranen species and show also many floral features of East Mediterranean species (limb is not elongated, tube opens gradually and it is slightly curved, fruits forming a “basket”). This group also shows a growth form more similar to tropical representatives of the genus and

also the biogeographic distribution (circum Mediterranean) highlight the exceptional combination of traits found in this species complex.

The wax type and trichome form observed in *Aristolochia rigida* which is occurring in Somalia and Yemen are similar to the West Mediterranean species and therewith are in contrast to the findings of deGroot et al (2006) and Wanke (2007) who described *Aristolochia rigida* as a species displays morphological affinities to the East Mediterranean and Caucasian species (bilabiate, curved perianth, sessile utricle). The intermediate traits between the West and the East Mediterranean *Aristolochia* species seem to be symptomatic for this species, which could also not be placed in molecular based phylogenetic trees (Wanke 2007). From a biogeographic point of view a close relationship to other East African species could be assumed but molecular results were unable to assign this species to any taxonomic group.

The glandular form of the trichomes observed only in *A. triactina* (subgenus *Pararistolochia*) could be characteristic for the African species belonging to this subgenus, but the inclusion of additional accessions and species from Africa are needed to confirm this hypothesis**.**

Conclusion

Although not all of the *Aristolochia* species have yet been examined, it is possible to draw some general conclusions. First, the study of the epicuticular wax of *Aristolochia* revealed a number of important micromorphological characters, and these characters exhibit interesting variations that are of significance for identification of some clades. Second, the variation in trichomes is an equally useful character for taxonomic purposes within some clades as well.

Supplementary Material

Suppl. Table 1 *Aristolochia* species investigated: origin of the voucher specimen and its accession number in the Botanical Garden Dresden; as well as the distribution areas of the species are given.

Species	Garden accession number	Origin of the investigated plant	Distribution area
A. acuminata	M. Chiang BG DD (BG Bonn 7417)		S. Asia
A. anguicida			S. America
A. arborea	BG DD1001680-15		C. America
A. austroyunnanesis	J. Murata. Japan		S. Asia
A. baetica	Wanke (034)	Spain, Majorca	W. Mediterranean
A. bottae	Mahfoud (18/2)	Syria, Alzebdane	E. Mediterranean
A. brasiliensis	09867	Brazil	S. America
A. californica	BG DD 013310-08		N. America
A. chlamydophylla	BG DD 01.10.2004		S. Asia
A. clematitis	BG DD 002034-09		Eurasia
A. clusii	Wanke (193)	Italy, Pomarico	W. Mediterranean
A. cucurbitifolia	BG DD 014030-08		S. Asia
A. cymbifera	BG Göttingen 2005		S. America
A. debilis	03.06.2002		S. Asia
A. elegans	BG DD 001306-10		America
A. elongata	Wanke (162)	Greece, Peloponnesus	W. Mediterranean
A. eriantha	BG DD 013305-12		S+C America
A. fimbriata	BG DD 0001870-16		S. America
A. galeata	L.Barabino (BG DD 014075-17) 2003		S. America
A. gigantea	BG DD (BG Bonn 14213)		S. America
A. gorgona	13.03.2006		C. America
A. grandiflora	L. Barabino (BG DD 0038-19-21)		S+C America
A. guentheri		Peru	S. America
A. guichardii	Wanke (186)	Grecce, Rhodes	E. Mediterranean
A. hirta	Mahfoud (29/1)	Turkey, Alanya	E. Mediterranean
A. kankauensis	BG DD (BG. Tokio 2262) Japan		S. Asia
A. krausei	Mahfoud (23/1)	Turkey, Erdemli	E. Mediterranean
A. leuconeura	L.Barabino (BG DD 0140332-12) 20.07.2004		C. America
A. lutea	Wanke (W135)	Croatia, Istria	W. Mediterranean
A. maurorum	Mahfoud (20/4)	Syria, Aleppo	E. Mediterranean
A. manschuriensis	BG DD 003860-17 China		S. Asia
A. microstoma	Wanke (171)	Greece, Peloponnesus	W. Mediterranean
A. mollissima	J. Murata, Japan		S. Asia

Species	Garden accession number	Origin of the investigated plant	Distribution area
A. moupinensis	Edinburgh , Japan 11.06.2004		S. Asia
A. nakaoi	BG. Tokio (22617) Japan, Tokio		S. Asia
A. navicularis	Wanke (21)	Italy, Sardinia	E. Mediterranean
A. odoratissima	J. Urban (BG DD)	Bolivia	S+C America
A. paecilantha	Mahfoud (24/1)	Syria, Alzebdane	E. Mediterranean
A. pallida	Wanke (129)	Italy, Alpicelli	W. Mediterranean
A. parvifolia	Wanke (163)	Greece, Rhodes	E. Mediterranean
A. pistolochia	Wanke (199)	France, Cassis	W. Mediterranean
A. pohliana	L. Barabino, Brazil 20.07.04	Brazil	S. America
A. pravenosa	BG DD 014799-30807		Australia
A. prostrata	M. Könen, Bolivia 16.02.2002	Bolivia	S. America
A. rigida	C. Neinhuis	Somalia	Somalia - Yemen
A. ringens	Gonzales (3575) BG.München	Bolivia	S. America
A. rotunda	Wanke (205)	Italy, Sant-Eufemia	W. Mediterranean
A. scabridula	Mahfoud (16/1)	Syria, Msyaf	E. Mediterranean
A. sempervirens	Wanke (106)	Italy, Sicily	Mediterranean
A. shimadai	J. Murata (22624) Japan, Tokio		S. Asia
A. tomentosa	BG DD 006146-17 (BG Bonn 0282) Malaysia		N. America
A. triactina	Neinhuis 119(DR) BG Bonn 12767		Africa
A. westlandi	BG DD 014736-21 (BG Bonn 14211) China		S. Asia

Suppl. Fig 1. Tree based on the combined molecular and morphological data sets (substitutions and coded length mutations). Supports above each branch are bootstrap values. Segregated genera or infrageneric taxa previously proposed. Asterisks indicate taxa originally treated as part of ‘‘*Howardia*.’’ Pentandrous species are indicated with pound signs. Large numbers along branches correspond to sets of characters: 1. *Thottea* plus *Aristolochia s.l.*: woody plants with hooked trichomes, a V- or U-shaped petiole base, a single whorl of stamens, and a completely inferior ovary that is separated from the rest of the flower by an abscission zone; 2. *Endodeca* plus *Isotrema*: a ringlike structure around the perianth fauces (variously modified in some Central American species), a 3-lobed gynostemium, and grouped stamens; 3. *Endodeca* (i.e., *Aristolochia reticulata* plus *Aristolochia serpentaria*): herbaceous shoots with scalelike, nonclasping, subtending leaves and shortened internodes on the partial inflorescences; 4. subgenus *Isotrema* (minus *Endodeca*): abscission zone on the base of the petiole, floral tube evenly inflated, and almost as wide as the utricle. 5. Subgenus *Pararistolochia* plus subgenus *Aristolochia*: Conical hairs inside the floral tube, and/or limb; and slightly (not U-shaped) curved tube. 6. Subgenus *Pararistolochia*: a broad exine ridge on pollen grains, indehiscent, and warty fruits that have a strongly lignified pericarp, and a fleshy mesocarp. 7. Subgenus *Aristolochia*: abaxially concave perianth, completely monosymmetric floral limb with a complete fusion of the three sepals into one or two lobes, and ventricidal, and acropetal capsules. 8. *Aristolochia* subsection *Pentandrae* plus (*A. burelae, A. lindneri, A. lozaniana, A. stuckertii,* and *A. urbaniana*): bracteate flowers, large supratectal warts on the pollen grains, and basipetal, and loculicidal capsules. 9. *Aristolochia* series *Thyrsicae*: presence of an abscission zone in the base of the petiole, the base of the partial inflorescences, and the base of the peduncle, lattice-like septa on the capsules, and broadly oblong seeds with two wings. (Wanke et al. 2006a)

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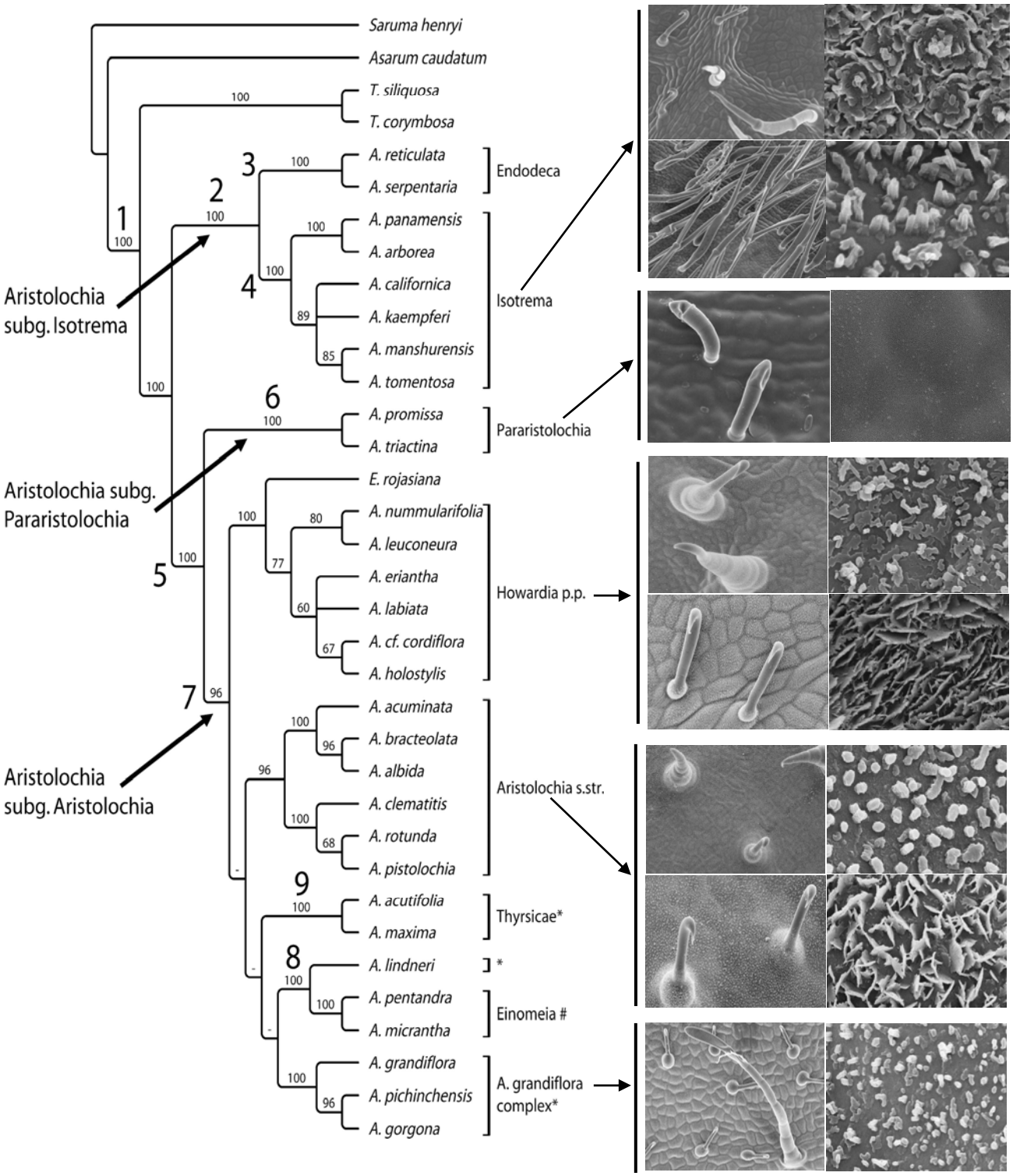
Saruma henryi
Asarum caudatum
T. siliquosa
T. corymbosa
A. reticulata
A. serpentaria
Endodeca
A. panamensis
A. arborea
A. californica
A. kaempferi
A. manshurensis
A. tomentosa
Isotrema
A. promissa
A. triactina
Pararistolochia
E. rojasiana
A. nummularifolia
A. leuconeura
A. eriantha
A. labiata
A. cf. cordiflora
A. holostylis
Howardia p.p.
A. acuminata
A. bracteolata
A. albida
A. clematitis
A. rotunda
A. pistolochia
Aristolochia s.str.
A. acutifolia
A. maxima
Thyrsicae*
A. lindneri
*
A. pentandra
A. micrantha
Einomeia #
A. grandiflora
A. pichinchensis
A. gorgona
A. grandiflora complex*
Aristolochia subg. Isotrema
Aristolochia subg. Pararistolochia
Aristolochia subg. Aristolochia
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Tosunoglu, A., Malyer, H., **Mahfoud, H.**, Neinhuis, C., Wanke, S. (2010). The Anatolian Diagonal – A hotspot for natural hybridisation for the genus Aristolochia? XIIITH OPTIMA MEETING (Organization for the Phyto-Taxonomic Investigation of the Mediterranean Area), Antalya, Turkey.

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