# Evolution of the genus *Aristolochia*

# - Systematics, Molecular Evolution and Ecology -

Evolution der Gattung *Aristolochia*

- Systematik, Molekulare Evolution und Ökologie -

**Dissertation**

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1. Gutachter: Prof. Dr. Christoph Neinhuis, Dresden
2. Gutachter: PD Dr. Thomas Borsch, Bonn
3. Gutachter: Prof. Dr. Thomas Speck, Freiburg

to my parents

to my wife

[...] *the processes involved in ‚descent with modification', to use Darwin's classic phrase, can be shown clearly to apply to differentiation within species, as well as to the further divergence of species* [...] *once they have become separated from each other.*

G.L. Stebbins (1950)

Variation and evolution in plants. New York: Columbia University Press

# Table of Contents

# Acknowledgements

In earlier times Prof. Dr. Miguel Vences showed me unintended how big science is working, which is gratefully acknowledged.

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# Chapter 1    Introduction

Systematics and evolution are linked to each other and it is nearly impossible to investigate one of these topics without looking at the other. In addition the study of those two fields is not possible without understanding and studying related research fields, such as e.g. ecology. This is the reason, why the present thesis is not only consisting of one research topic, but also includes related fields like pollination biology or molecular evolution.

The family Aristolochiaceae, as currently circumscribed, consists of four genera, belonging to two subfamilies (Aristolochioideae and Asaroideae) each with two genera (Neinhuis et al. 2005, Wanke et al. 2006b, 2006c). *Saruma* (a monotypic genus) and the small genus *Asarum*, ~85 spp. are merged in Asaroideae. The systematics and phylogeny of *Asarum* has been comprehensively studied by (Kelly 1998). Systematic and evolutionary problems have been found only on the population and species level of closely related species within one section (Yamijdi et al., in press). Basically, this is due to hybridisation of this populations and species. The other subfamily Aristolochioideae consists of the genus *Thottea* (~30 spp., from southeast Asia), which is only poorly studied and the species rich genus *Aristolochia* (~500 spp., see Chapter 3). Besides the evolution of the genus *Thottea* more questions still need to be resolved.   These problems concern the monophyly of Aristolochiaceae, since Hydnoraceae, a parasitic family, and Lactoridaceae (a monotypic family from the Juan Fenadez Islands) cause the paraphyly of Aristolochiaceae (Nickrent et al. 2002, Wanke et al. 2006c). These questions are beyond the scope of the present thesis, but are currently under investigation or will be in due course.

Although the issue of big genera is a hot topic in systematics, attempts to study these groups comprehensively are rare, mainly because a detailed knowledge on the specific group is an essential precondition which is nearly impossible to achieve by only one scientist. It is generally recognized (e.g. Frodin 2004) that botanists should

not consider these big genera as "black holes" but should focus on their resolution. Large genera pose high taxonomic challenges as well as unparalleled opportunities to study phenomena such as character evolution, changes in evolutionary diversification rates, adaptive radiations, rapid speciation, key innovations and chromosomal rearrangement (Berry et al. 2005).

The present study deals with the species rich genus *Aristolochia* and tries to resolve the relationships within the genus (Chapter 3). Beside this, a kind of case study on molecular evolution, ecology, and biogeography has been performed on some specific topics raised during the investigation of the main clades (Chapter 3, 4, 5). These case studies will lead to further and more detailed investigations, and will be applicable to similar problems in other clades or geographical areas, as well as open the possibility to look at a specific topic from a different viewpoint.

The presented thesis starts with a detailed circumscription of Aristolochiaceae and its relatives of the order Piperales (Chapter 2) followed by a more intensive investigation of the subfamily Aristolochioideae (Chapter 3). Finally, a detailed study of the subgenus *Aristolochia* focusing especially on the Old World representatives is presented. Chapters 3 & 4 report on findings, which raised several new questions and ideas for further studies. Each subchapter has its own introduction and abstract resulting in a short generall introduction here, to avoid too much redundant information.

# Chapter 2  Evolution of Piperales – *matK* gene and *trnK* intron sequence data reveal lineage specific resolution contrast

**Abstract**

Piperales are one of the largest basal angiosperm orders with a nearly worldwide distribution. The order includes three species rich genera, *Piper* (ca. 1,000 species), *Peperomia* (ca. 1,500-1,700 species), and *Aristolochia* s. l. (ca. 500 species). Sequences of the *matK* gene and the non-coding *trnK* group II intron are analysed for a dense set of 105 taxa representing all families (except *Hydnoraceae*) and all generic segregates (except *Euglypha* within Aristolochiaceae) of Piperales. A large number of highly informative indels are found in the Piperales *trnK/matK* dataset. Within a narrow region approximately 500 nt downstream in the *matK* coding region (CDS), a length variable simple sequence repeat (SSR) expansion segment occurs, in which insertions and deletions have led to short frame-shifts. These are corrected shortly afterwards, resulting in a maximum of 6 amino acids being affected. Furthermore, additional non-functional *matK* copies were found in *Zippelia begoniifolia*, which can easily be discriminated from the functional open reading frame (ORF). The *trnK/matK* sequence data fully resolve relationships within *Peperomia*, whereas they are not effective within *Piper*. The resolution contrast is correlated with the rate heterogenity between those lineages. Parsimony, Bayesian and likelihood analyses result in virtually the same topology, and converge on the monophyly of Piperaceae and Saururaceae. *Lactoris* gains high support as sister to Aristolochiaceae subf. Aristolochioideae, but the different tree inference methods yield conflicting results with respect to the relationships of subfam. Asaroideae. In Piperaceae, a clade formed by the monotypic genus *Zippelia* and the small genus *Manekia* (=*Sarcorhachis*) is sister to the two large genera *Piper* and *Peperomia*.

**Introduction**

The order Piperales is one of the most species rich clades among basal angiosperms, comprising about 3,300 species, with three genera that include more than 500 species so-called "big genera" (Frodin, 2004,). Nearly all types of growth and life forms are represented, such as geophytes, herbs, succulents, lianas, shrubs, trees, parasites and epiphytes. Members of Piperales exhibit a diverse spectrum of specializations in floral morphology and pollination. On the one hand, Aristolochiaceae attract insects with their highly specialized flowers, on the other hand Piperaceae and Saururaceae possess perianthless reduced flowers that are pollinated by flies and bees (Semple, 1974; de Figuereido and Sazima, 2000; Marquis, 1988; Bornstein, 1989). Therefore, Piperales are an important lineage for understanding early angiosperm diversification. Piperales also comprise some economically important plants like *Piper nigrum* (black pepper) used as a spice and several *Peperomia* species that are widely used as ornamental plants. In addition, secondary metabolites of Aristolochiaceae such as aristolochic acids are important compounds in pharmacology (e.g. Nortier et al., 2000).

Molecular data provide evidence for a sister relationship between Piperales and Canellales (e.g. Qiu et al., 1999; Savolainen et al., 2000; Qiu et al., 2005; Zanis et al., 2002; Hilu et al., 2003; Borsch et al., 2003; Löhne and Borsch, 2005; Kim et al., 2004; Borsch et al., 2005). The close relationship of all four orders, Piperales, Canellales, Magnoliales and Laurales to form the magnoliid clade is meanwhile well supported by substitution based tree inferences (Borsch et al., 2005; Borsch et al., 2003; Qiu et al., 2005; Zanis et al., 2002) and chloroplast genome microstructural changes (Löhne and Borsch, 2005). Phylogenetic analyses with a dense taxon sampling in Magnoliales (Sauquet et al., 2003), Laurales (Renner, 2005; Renner and Chanderbali, 2000; Renner, 1999) and Canellales (Karol et al., 2000; Suh et al., 1993) have been published, whereas Piperales lack a thorough molecular analysis.

Characterisation and utility of the *trnK* intron including the *matK* gene

The *matK* coding region has a length of 1500-1600 bp in most angiosperms, and is located within domain V of the *trnK* $_{UUU}$ group II intron (Neuhaus and Link, 1987; Ems et al., 1995; Fig. 1). Based on structural similarities, this ORF has been suggested to code for a maturase (Neuhaus and Link, 1987; Mohr et al., 1993), and in fact *matK* is the only maturase of higher plant plastids (Vogel et al., 1997). Transcription experiments have shown that *trnK* including *matK* are co-transcribed (Chieba et al., 1996) and there is accumulating evidence for the expression of the gene (Du Jardin et al., 1994; Barthet and Hilu, pers. comm.). Sequence variation is considerable, however, the reading frame of *matK* has been found intact even in extremely fast evolving lineages, such as Lentibulariaceae (Müller and Borsch, 2005a). Frameshift mutations in *matK* have been reported from Poaceae (Hilu and Alice, 1999) and Lentibulariaceae (Müller and Borsch, 2005a), although only near the 3' end of the CDS where they apparently have minimal impact on function. Moreover, small inversions (2-4 codons) have been encountered in Amaranthaceae (Müller and Borsch, 2005b), again with minimal impact on the amino acid composition of the gene.

**Figure 1.** The *trnK*/*matK* region. Coding regions are represented by enlarged black boxes, highly length-variable regions by small black boxes (H1-H7). Location of the *trnK* intron domains (DI – DVI) as well as domain X (DX) in *matK* are indicated. For further reference of the internal primers see Table 2. Size and position of length variable regions change with study group. Length of the region is presented proportional based on the situation found in *Aristolochia reticulata*.
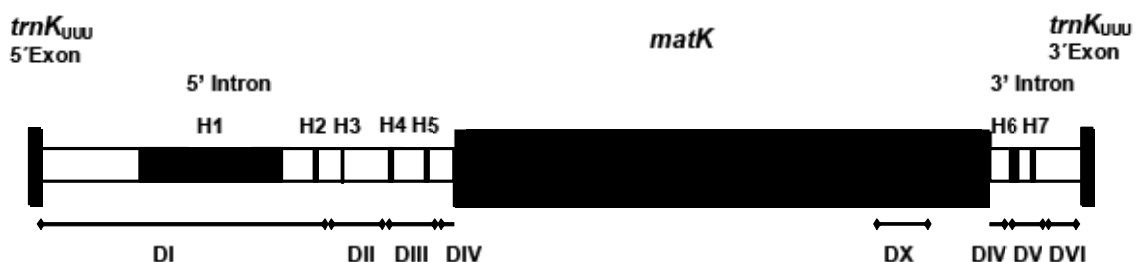


A peculiarity of *matK* are substitution rates in first and second codon positions approaching those in the third (Hilu and Liang, 1997), which contribute to the high overall evolutionary rate of *matK* in contrast to other chloroplast genes. *MatK* seems

to be single copy in the vast majority of plants, although some additional copies of pseudogenic nature have been discovered in Valerianaceae (Hidalgo et al., 2004; Bell et al., 2001), Nepenthaceae (Meimberg et al., 2006), as well as in some bryophyte lineages (Jankowiak et al., 2004). In all these cases, pseudogenic copies were easily identified and grouped together in phylogenetic reconstructions either based on distances or characters.

The *matK* gene has become one of the most frequently used chloroplast gene markers in angiosperm phylogenetic studies. Since *matK* can easily be co-amplified with the flanking non-coding intron parts, the complete *trnK* intron is increasingly used, expanding the dataset to 2400-2700 bp. As a consequence, the utility of this region could be extended to the inter- and intra-species level (e.g., Müller and Borsch, 2005a; Wanke et al., 2006b, Wanke et.al., 2006b).

Circumscription of Piperales

Piperales as considered here include the families Piperaceae, Aristolochiaceae, Saururaceae and Lactoridaceae as well as parasitic Hydnoraceae (Nickrent et al., 2002; APG, 2003). However, Aristolochiaceae and Lactoridaceae were sometimes placed in their respective own orders Aristolochiales and Lactoridales (Takhtajan, 1992, 1997). Hydnoraceae had been placed with other parasitic plants in Rafflesiales (Cronquist, 1988). Monophyly of Piperales is strongly supported by sequence (e.g. Chase et al., 1993; Graham and Olmstead, 2000; Mathews and Donoghue, 2000; Soltis et al., 2000; Borsch et al 2003; Hilu et al 2003; Jaramillo et al., 2004; Löhne and Borsch, 2005; Neinhuis et al., 2005; Borsch et al., 2005). A set of phenotypic characters has been suggested as synapomorphic for Piperales such as two-ranked leaves, sheathing leaf base, nuclear endosperm, a single adaxial prophyll, swollen nodes, distinct vascular bundles, wood with broad rays, vessel elements with simple perforation and secondary metabolism products like alkaloids from benzyl-isoquinolin and aporphine type (Doyle and Endress, 2000). All Piperales lineages show strong trends towards reduction and fusion of flower organs, with the genus *Peperomia* being considered to have the most reduced flowers in Piperales (Jaramillo et al., 2004).

Major lineages within Piperales

According to Jaramillo et al. (2004), Piperaceae comprise four genera, the large genera *Peperomia* Ruiz and Pavon (about 1700 species; Wanke et al., 2006d) and *Piper* L. (more than 1000 species; Jaramillo and Manos 2001) constituting the core of Piperaceae, and the small genera *Manekia* Trel. (considered as an earlier name of *Sarcorhachis*, Arias et al., in press), and *Zippelia* Blume. The distinctiveness of *Peperomia* has long been recognized, either as subfamily Peperomioideae (Thorne 1992), or, alternatively, as a separate family Peperomiaceae (e.g. Burger, 1977). The most detailed study on *Peperomia* was primarily based on fruit morphology by Dahlstedt (1900), dividing *Peperomia* into nine subgenera and seven sections. However *trnK*/*matK* data only support the monophyly: of subgenera *Micropiper*, *Sphaerocarpidium* (Wanke et al., 2006d). Monophyly of the genus has been substantiated by molecular approaches (Neinhuis et al., 2005, Jaramillo et al., 2004), flower morphology (Jaramillo et al., 2004) and pollen ultrastructure (Mathew and Mathew, 2001). *Piper* species are mostly shrubs, trees or lianas. Several generic names are included as synonyms within *Piper*, e.g. *Arctottonia* Trel., *Macropiper* Miq., *Pothomorphe* Miq., *Ottonia* Spreng. (Jaramillo and Manos 2001), and *Trianaeopiper* Trel. (Jaramillo and Callejas 2004). Analysis of ITS sequence data provides support for three major clades within *Piper*, which have diversified in Asia, the South Pacific and the Neotropics, respectively. *Zippelia* has been included in *Piper* or has been regarded as independent genus within Piperaceae or even Saururaceae. Recently, *Zippelia* together with *Manekia* has been proposed to be distantly related to *Piper* (Jaramillo and Manos, 2001), or to form a clade which is sister group to *Piper* and *Peperomia* (Jaramillo et al., 2004).

Saururaceae contain six species in four genera with an East Asian – North American disjunction: *Saururus*, *Gymnotheca*, *Anemopsis* and *Houttuynia* (Liang, 1995). Several studies have been conducted to reveal phylogenetic relationships in this small family. Most of these studies dealt with ontogenetic/morphological data as characters (e g. Tucker et al., 1993; Liang and Tucker, 1995; Liang and Tucker, 1990;

Tucker, 1975; Tucker, 1981; Meng et al., 2003). Two hypotheses on relationships were formulated: 1. *Anemopsis* + *Houttuynia* and *Saururus* + *Gymnotheca*; 2. *Saururus* branching first, followed by *Gymnotheca* and *Anemopsis* + *Houttuynia*. This is also substantiated by chloroplast sequence data (Meng et al., 2003; Jaramillo et al., 2004; Neinhuis et al., 2005), but contrasting with the signal obtained form the nuclear genome, which placed *Anemopsis* as sister to all other Saururaceae (Meng et al., 2003).

Aristolochiaceae are distributed worldwide. and are generally divided into two subfamilies, Asaroideae and Aristolochioideae (Huber, 1993). Asaroideae comprise about 85 species and occur mainly in northern temperate regions with a centre of diversity in Asia (Kelly, 1998), whereas Aristolochioideae have ca. 500 species and occur  predominantly in tropical-subtropical regions (Ma, 1990). The most recent classification for Asaroideae is based on Kelly (1997, 1998) who recognises two genera (*Asarum* and *Saruma*) with several segregates at the subgenus or section level in *Asarum*. González and Stevenson (2002) provide a detailed discussion of the various systematic treatments of Aristolochiaceae. They recognize five genera within Aristolochioideae: *Thottea*, *Isotrema*, *Endodeca*, *Pararistolochia* and *Aristolochia*. *Holostylis* (= *Aristolochia holostylis* (Duchartre) F.Gonzalez) and *Euglypha* have been included in a broadly circumscribed genus *Aristolochia*. Similarly, the most comprehensive analysis of morphological characters of Aristolochiaceae by Kelly and González (2003) recognizes seven genera: *Asarum* and *Saruma* in Asaroideae, in addition to the five in Aristolochioideae. Studies on Aristolochiaceae based on partial *matK* sequences (Murata et al., 2001) examined only four recognized genera. Other molecular inference (*trnL-F*) (Neinhuis et al. 2005) is largely congruent with the results of González and Stevenson (2002) and Kelly and González (2003). However, discrepancy exists among relationships between segregates within *Aristolochia.*
*Lactoris fernandeziana*, the only species in Lactoridaceae, is confined to the Juan Fernandez Islands, Chile. Its systematic position has been controversial among members of basal angiosperms (for a review see Stuessy et al., 1998; González and Rudall, 2001) partly caused by a difficult interpretation of convergent morphological

characters e.g. pollen with a saccus (apart from *Lactoris* only known from gymnosperms; Carlquist, 1964; Zavada and Taylor, 1986). Molecular data also support the position within Piperales (Qiu et al., 2005; Hilu et al., 2003; Borsch et al., 2003; Borsch et al., 2005), or even within Aristolochiaceae (Borsch et al., 2005; Neinhuis et al., 2005) making the family paraphyletic, or close to Aristolochiaceae and Hydnoraceae (Nickrent et al. 2002).

This study aims to: A) characterize patterns of molecular evolution for the *trnK*/*matK* region in Piperales, using a dense taxon sampling, B) investigate patterns of microstructural changes within the *trnK* intron and the *matK* gene, C) resolve major clades within Piperales, investigate the relationships between small and large genera (*Asarum*/*Saruma* versus Aristolochioideae, *Manekia*/*Zippelia* versus *Piper*/*Peperomia*).

**Material and methods**

Sampling strategy

A total of 105 accessions of Piperales are sampled, including a representative number of species/taxonomic groups for each genus in the order. Hydnoraceae (*Hydnora* and *Prosopanche*) have not been sampled as they lack the *matK* region in the cp genome (Nickrent, pers. comm.). In Piperaceae, 30 taxa are selected from the genus *Piper* (following the sampling of Jaramillo and Manos 2001)), 27 taxa of *Peperomia* (following the sampling of Wanke et al. (2006d), representing many subgenera recognised by Dahlstedt (1900)), as well as the monotypic genus *Zippelia* and two species of the genus *Manekia*. All four genera with in total five species of Saururaceae are sampled. Aristolochiaceae sampling includes 36 taxa from both subfamilies, representing all genera of Aristolochioideae accepted by González and Stevenson (2002) and Neinhuis et al. (2005) and a selection of Asaroideae. Lactoridaceae is represented by its only species *Lactoris fernandeziana*. Three genera of Canellales are used as outgroup.

Plant material has either been collected in the field or has derived from plants cultivated in botanical gardens (collections of the Botanical Gardens Bonn and Dresden, Germany) as well as herbarium specimens. A list of the sampled species, along with collection localities, vouchers and GenBank accession numbers is provided in Table 1. For generic segregates, of which the taxonomy is until know unclear, the most common name is used, e.g. *Macropiper* within *Piper* (Smith, 1975).

**Table 1.** Taxa used in the present study, including information about the correspondence to clades (for outgroup, family affiliation is given; for *Aristolochia, Piper, Peperomia* affiliation correspond to different taxonomic hierarchical ranks, depending on author), the origin of the studied material (field or collection), voucher information and the herbarium where the voucher is deposited, as well as GenBank accession numbers (incl. source), are given. GenBank accessions indicated with * are updated with the *trnK* intron from previously published data, based on *matK* gene only (*[1] Hilu et al. 2003, *[2] Müller et al. 2006).

| Taxon | Corresponding to clade | Origin / Garden accession | Voucher (Herbarium) | GenBank accession (incl. source) |
|---|---|---|---|---|
| **Aristolochiaceae Adans.** | | | | |
| *Aristolochia* L. | | | | |
| *A. acuminata* Lamk. | *Aristolochia s.str.* | BG Bonn, 17417 | Wanke & Neinhuis 146 (DR) | DQ 532063 |
| *A. albida* Duch. | *Aristolochia s.str.* | BG Bonn, 17419 | Neinhuis 92 (DR) | DQ 532064 |
| *A. arborea* Linden | *Isotrema* | BG Bonn, 02560 | Neinhuis 93(DR) | DQ 532044 |
| *A. baetica* L. | *Aristolochia s.str.* | BG Bonn, 14517 | Neinhuis 95 (DR) | DQ 882189 |
| *A. bracteolata* Lam. | *Aristolochia s.str.* | BG Bonn, 16714 | Neinhuis 94 (DR) | DQ 532059 |
| *A. californica* Torr. | *Isotrema* | BG Dresden, s.n. | Wanke & Neinhuis 143 (DR) | DQ 532039 |
| *A. chilensis* Bridges | *"Howardia"* | BG Bonn, 12878 | Neinhuis 97 (DR) | DQ 882185 |
| *A. cf. cordiflora* Mutis ex Kunth | *"Howardia"* | BG Lankester s.n. | Holst 8602 | DQ 532056 |
| *A. cruenta* Barringer | *"Howardia"* | Costa Rica: La Selva | Blanco 767 (USJ) | DQ 882186 |
| *A. erecta* L. | *Einomeia* | Travis, Texas, USA | privat coll. Westlund | DQ 882188 |
| *A. eriantha* Mart & Zucc. | *"Howardia"* | BG Bonn, 12952 | Neinhuis 99 (DR) | DQ 532054 |
| *A. gigantea* Mart. & Zucc. | *"Howardia"* | BG Bonn, 02099 | Neinhuis 101 (DR) | DQ 882187 |
| *A. gorgona* M.A.Blanco | *"Howardia"* | Heredia: Puerto Viejo de Sarapiquí, Costa Rica | Blanco 1752 (USJ) | DQ 532051 |
| *A. holostylis* (Duchartre) F. Gonzalez | *Holostylis* | BG Bonn, 02193 | Neinhuis 116 (DR) | DQ 532057 |
| *A. labiata* Wild. | *"Howardia"* | BG Bonn, 09867 | Neinhuis 96 (DR) | DQ 532055 |
| *A. macrophylla* Lam. | *Isotrema* | BG Dresden, s.n. | Neinhuis s.n. (DR) | DQ 882193 |
| *A. manshuriensis* Komarov | *Isotrema* | BG Bonn 13085 | Neinhuis 104 (DR) | DQ 532040 |
| *A. micrantha* Duch. | *Einomeia* | priv. coll. B.Westlund Texas, USA | Neinhuis 103 (DR) | DQ 532046 |
| *A. parvifolia* Sibth. & Sm. | *Aristolochia s.str.* | BG Bonn, 15274 | Neinhuis 105 (DR) | DQ 882190 |
| *A. pentandra* Jacq. | *Einomeia* | Cola de Caballo, Mexico | privat coll. B.Westlund | DQ 532045 |
| *A. pichinchensis* Pfeifer | *"Howardia"* | Rio Paleque, Prov. Los Rios, Ecuador | Moran 6928 (QCA, NY) | DQ 532050 |

| Taxon | Genus/Subgenus | Locality | Voucher | Accession |
|---|---|---|---|---|
| A. pistolochia L. | Aristolochia s.str. | France, Cassis, Calenque d'En Veau | Wanke 037 (DR 025372) | DQ 296652 |
| A. promissa MAST. | Pararistolochia | BG Bonn, 13014 | Neinhuis 118 (DR) | DQ 532065 |
| A. reticulata NUTT. | Endodeca | priv. coll. B.Westlund Texas, USA | Neinhuis 108 (DR) | DQ 532037 |
| A. salvadorensis LINDEN | Isotrema | BG Bonn, 10720 | Neinhuis 109 (DR) | DQ 882191 |
| A. serpentaria L. | Endodeca | priv. coll. B.Westlund Texas, USA | Neinhuis 112 (DR) | DQ 532038 |
| A. tomentosa SIMS. | Isotrema | BG Bonn, 02682 | Neinhuis 113 (DR) | DQ 532041 |
| A. westlandii HEMSL. | Isotrema | BG Bonn, 14211 | Neinhuis 115 (DR) | DQ 882192 |
| A. triactina HOOK. f. | Pararistolochia | BG Bonn, 12767 | Neinhuis 119 (DR) | DQ 532066 |
| **Thottea ROTTB.** | | | | |
| T. corymbosa (GRIFF.) HOU | Thottea | Malaysia | Weber & Anthonysamy 870519-1/1 (WU) | DQ 532036 |
| T. siliquosa (LAM.) HOU | Thottea | BG Bonn, 09037;India, Kerala (Bogner 86-3421) | Neinhuis 121 (DR) | DQ 532035 |
| T. dependens (PLANCH) KLOTZSCH | Thottea | field origin | A. Weber (WU) | DQ 882194 |
| **Asarum L.** | | | | |
| A. caudatum LINDL. | Asarum | Oregon, Mt Hood | Neinhuis 88 (DR) | DQ 532034 |
| A. chingchengense CHENG &YANG | Asarum | BG Bonn, 02680 | Neinhuis 90 (DR) | DQ 882196 |
| A. yakusimense MASAM. | Asarum | BG Bonn, 14276 | Neinhuis 91 (DR) | DQ 882197 *1 |
| **Saruma OLIVER** | | | | |
| S. henryi OLIV. | Saruma | BG Bonn, 02618 | Borsch 3456 (BONN) | DQ 532033 |
| **Lactoridaceae ENGLER** | | | | |
| L. fernandeziana PHIL. | Lactoris | Juan Fernandez Island, Chile | Crawford & Stuessy 11950 | DQ 882195 |
| **Saururaceae RICH.** | | | | |
| A. californica (NUTT.) HOOK & ARN. | Anemopsis | BG Bonn, 06422 | Wanke 002 (DR) | DQ 882198 |

| | | | | |
|---|---|---|---|---|
| G. chinensis DECNE. | Gymnotheca | BG Bonn, 17072 | Wanke 004 (DR) | DQ 882199 |
| H. cordata THUNB. | Houttuynia | BG Bonn, 08120 | Borsch 3481 (BONN) | DQ 212712, Wanke et al. (2006d) |
| S. cernuus L. | Saururus | Florida, USA | Borsch & Wilde 3108 (VPI, FR) | DQ 882200 *2 |
| S. chinensis (LOUR.) BAILL. | Saururus | BG Bonn, 00312 | Wanke 001 (DR) | DQ 212713, Wanke et al. (2006d) |
| **Piperaceae C. AGARDH** | | | | |
| **Peperomia RUIZ & PAVON** | | | | |
| P. argyreia (MIQ.) MORR. | Tildenia | BG Berlin, 062-56-74-83 | GH 13462 (B) | DQ 212734, Wanke et al. (2006d) |
| P. bicolor SODIRO | Sphaerocarpidium | BG Berlin, 107-84-74-83 | GH 13436 (B) | DQ 212761, Wanke et al. (2006d) |
| P. blanda (JACQ.) KUNTH | Sphaerocarpidium | BG Berlin, 054-24-74-73 | GH 13453 (B) | DQ 212763, Wanke et al. (2006d) |
| P. clusiifolia (Jacq.) Hook. | Rhynchophorum | BG Berlin, 062-58-74-83 | Schwerdtfeger GH13433 (B) | DQ 212753, Wanke et al. (2006d) |
| P. cuspidilimba C.DC. | Micropiper | BG Berlin, 054-48-74-83 | GH 23129 (B) | DQ 212733, Wanke et al. (2006d) |
| P. fagerlindii YUNCK. | Rhynchophorum | BG Berlin, 107-73-74-83 | GH 13431 (B) | DQ 212742, Wanke et al. (2006d) |
| P. fraseri C.DC. | Panicularia | BG Berlin, 285-64-89-80 | GH 27028 (B) | DQ 212719, Wanke et al. (2006d) |
| P. glabella (SW.) A. DIETR. | Sphaerocarpidium | BG Bonn, 18749 | Wanke 061 (DR) | DQ 212757, Wanke et al. (2006d) |
| P. gracillima S. WATSON | Geophila | BG Bonn, 06005 | Wanke 060 (DR) | DQ 212716, Wanke et al. (2006d) |
| P. graveolens RAUH & BARTHLOTT | Panicularia | El Oro, Ecuador | Rauh & Barthlott 35122 (HEID) | DQ 212722, Wanke et al. (2006d) |
| P. hylophila C.DC. | Sphaerocarpidium | BG Berlin, 173-23-95-33 | Horich (B) | DQ 212758, Wanke et al. (2006d) |
| P. inaequalifolia RUIZ & PAV. | Sphaerocarpidium | BG Berlin, 213-35-00-80 | GH 39585 (B) | DQ 212749, Wanke et al. (2006d) |
| P. macrostachya (VAHL) A. DIETR. | Rhynchophorum | BG Berlin, 039-82-89-23 | Leuenberger GH 26073 (B) | DQ 212744, Wanke et al. (2006d) |
| P. magnoliifolia (JACQ.) A. | Rhynchophorum | BG Berlin, 039-34-89- | Leuenberger & Hagemann GH | DQ 212752, Wanke et al. (2006d) |

| Species | Section | Locality | Voucher | Accession / Reference |
|---|---|---|---|---|
| DIETR. | | 20 | 26231 (B) | (2006d) |
| P. marmorata HOOK. f. | Tildenia | BG Bonn, 17527 | Wanke 064 (DR) | DQ 212725, Wanke et al. (2006d) |
| P. maypurensis KUNTH | Tildenia | BG Bonn, 11132 | Wanke 006 (DR) | DQ 212735, Wanke et al. (2006d) |
| P. metallica LINDEN & RODIGAS. | Tildenia | BG Bonn, 16189 | Wanke 066 (DR) | DQ 212740, Wanke et al. (2006d) |
| P. pereskiifolia (JACQ.) KUNTH | Micropiper | BG Berlin, 140-28-74-83 | Schwertfeger GH 13455 (B) | DQ 212726, Wanke et al. (2006d) |
| P. pernambucensis MIQ. | Rhynchophorum | BG Berlin, 062-62-74-83 | Schwertfeger GH 13432 (B) | DQ 212751, Wanke et al. (2006d) |
| P. pitcairnensis C.DC. | Sphaerocarpidium | BG Bonn, 17744 | Wanke 007 (DR) | DQ 212762, Wanke et al. (2006d) |
| P. ppucuppuccu TREL. | Micropiper | BG Berlin | Wanke 043 (DR) | DQ 212728, Wanke et al. (2006d) |
| P. rhombea RUIZ & PAV. | Micropiper | BG Berlin, 224-08-95-80 | GH 39582 (B) | DQ 212731, Wanke et al. (2006d) |
| P. rotundifolia (L.) KUNTH | Sphaerocarpidium | BG Berlin, 166-08-83-20 | Leuenberger GH 23064 (B) | DQ 212754, Wanke et al. (2006d) |
| P. spec. | Sphaerocarpidium | Zaire, Irangi | Fischer s.n. | DQ 212760, Wanke et al. (2006d) |
| P. trifolia (L.) DIETR. | Micropiper | BG Berlin, 078-06-97-80 | GH 37007 (B) | DQ 212727, Wanke et al. (2006d) |
| P. tuisana C.DC. | Sphaerocarpidium | BG Berlin, 173-24-95-33 | Horich GH 35526 (B) | DQ 212756, Wanke et al. (2006d) |
| P. vinasiana C.DC. | Rhynchophorum | BG Berlin, 173-25-95-33 | Horich & San Jose GH 35591 (B) | DQ 212743, Wanke et al. (2006d) |
| **Piper s.l.** | | | | |
| P. aduncum L. | Radula | Valle, Kolumbien | MAJ076 (DUKE) | DQ 882201 |
| P. aduncum L. | Radula | Samar, Philippinen | MAJ200 (DUKE) | DQ 882202 |
| P. arieianum C.DC. | Schilleria / Steffensia | Choco, Kolumbien | MAJ069 (DUKE) | DQ 882204 |
| P. augustum RUDGE | Schilleria / Steffensia | Choco, Kolumbien | MAJ122 (DUKE) | DQ 882203 |
| P. auritum KUNTH | Steffensia | Choco, Kolumbien | MAJ063 (DUKE) | DQ 882205 |
| P. bavinum C.DC. | Piper s.str. | Prov. Ha Tinh, Vietnam | MAJ392 (DUKE) | DQ 882210 |

| Species | Subgenus / group | Locality | Voucher | GenBank |
|---|---|---|---|---|
| *P. caninum* BLUME | *Piper s.str.* | Prov. Surigao, Philippinen | MAJ218 (DUKE) | DQ 882213 |
| *P. cf. longum* L. | *Macrostachys* | BG Köln, s.n. | Neinhuis s.n. (DR) | DQ 882218 |
| *P. cf. magnificum* Hort. ex TREL. | *Schilleria / Steffensia* | BG Bonn, 05020 | Wanke 069 (DR) | DQ 882209 |
| *P. cinereum* C.DC. | *Steffensia* | Choco, Kolumbien | MAJ066 (DUKE) | DQ 882216 |
| *P. confertinodum* (TREL. & YUNCK.) M.A. JARAM. & CALLEJAS | *Confertinodum group* | Choco, Kolumbien | MAJ054 (DUKE) | DQ 882227 |
| *P. crocatum* RUIZ & PAV. | *Piper s.str.* | BG Bonn, 18143 | Wanke 070 (DR) | DQ 212714, Wanke et al. (2006d) |
| *P. decumanum* L. | *Piper s.str.* | Prov. Leyte, Philippinen | MAJ210 (DUKE) | DQ 882212 |
| *P. flagellicuspe* TREL. & YUCK. | *Radula* | Choco, Kolumbien | MAJ065 (DUKE) | DQ 882206 |
| *P. hispidum* SWARTZ | *Radula* | Choco, Kolumbien | MAJ053 (DUKE) | DQ 882219 |
| *P. michelianum* C.DC. | *Arctottonia* | Edo. Jalisco, Mexico | MAJ537 (DUKE) | DQ 882217 |
| *P. munchanum* C.DC. | *Schilleria / Steffensia* | Choco, Kolumbien | MAJ120 (DUKE) | DQ 882207 |
| *P. nigrum* L. | *Piper s.str.* | Prov. Lagunas, Philippinen | MAJ181 (DUKE) | DQ 882215 |
| *P. ornatum* N.E.BR. | *Piper s.str.* | BG Bonn, 18144 | Wanke 005 (DR) | DQ 882211 |
| *P. parvulum* M. A. Jaram. & Callejas | *Trianae group* | Choco, Kolumbien | MAJ055 (DUKE) | DQ 882226 |
| *P. peltatum* L. | *Pothomorphe* | Choco, Kolumbien | MAJ045 (DUKE) | DQ 882208 |
| *P. penninerve* C.DC. | *Piper s.str.* | Prov. Surigao del Norte, Philippiene | MAJ213 (DUKE) | DQ 882214 |
| *P. pulchrum* C.DC. | *Macrostachys* | Antioquia, Kolumbien | MAJ100 (DUKE) | DQ 882222 |
| *P. reticulatum* L. | *Enckea* | Choco, Kolumbien | MAJ128 (DUKE) | DQ 882220 |
| *P. reticulatum* L. | *Enckea* | Antioquia, Kolumbien | MAJ062 (DUKE) | DQ 882221 |
| *P. spec* | | BG Bonn, 00854 | Borsch 3475 (BONN) | DQ 882225 |
| *P. spoliatum* TREL. & YUNCK. | *Macrostachys* | Choco, Kolumbien | MAJ060 (DUKE) | DQ 882223 |
| *P. subpedale* TREL. & YUNCK. | *Schilleria / Steffensia* | Choco, Kolumbien | MAJ057 (DUKE) | DQ 882224 |
| | | | | |
| **Macropiper MIQ.** | | | | |
| *M. excelsum* (FORST. f.) MIQ. | *Macropiper* | BG Bonn, 17450 | Wanke 071 (DR) | DQ 882229 |
| *M. hooglandii* HUTTON & GREEN | *Macropiper* | Cult. Auckland Museum, Neuseeland | ROG 8496 (AK) | DQ 882228 |

| | | | | |
|---|---|---|---|---|
| **Manekia** TREL. | | | | |
| M. naranjoana (C.DC.) CALLEJAS | Manekia | Costa Rica | O. Vargas s.n. (DUKE) | DQ 882239 |
| M sydowii (TREL.) ARIAS, CALLEJAS & BORNSTEIN | Manekia | Antioquia, Kolumbien | MAJ038 (DUKE) | DQ 882238 |
| | | | | |
| **Zippelia** BLUME | | | | |
| Z. begoniifolia BLUME | Zippelia | BG Kunming, s.n. | Wanke & Neinhuis s.n. (DR) | DQ 882230 |
| Z. begoniifolia D12 clone 2 | | | | DQ 882231 |
| Z. begoniifolia E12 clone 3 | | | | DQ 882232 |
| Z. begoniifolia F12 clone 4 | | | | DQ 882233 |
| Z. begoniifolia C12 clone 1 | | | | DQ 882234 |
| Z. begoniifolia F11 clone 5 | | | | DQ 882235 |
| Z. begoniifolia G12 clone 5 | | | | DQ 882236 |
| Z. begoniifolia D11 clone 4 | | | | DQ 882237 |
| | | | | |
| **Outgroup** | | | | |
| Canella winterana Gaertn. | Canellaceae | BG Bonn, 15293 | Borsch 3466 (BONN) | DQ 882240 *2 |
| Drimys lanceolata (POIR.) BAILL. | Winteraceae | BG Bonn, 00769 | Borsch 3484 (BONN) | DQ 882241 *2 |
| Pseudowintera colorata (RAOUL) DANDY | Winteraceae | BG Bonn, 00770 | Borsch 3490 (BONN) | DQ 882242 *1 |

**Methods**

<u>DNA-isolation, amplification and sequencing</u>

Total genomic DNA was isolated from fresh material, silica gel dried leaves or herbarium specimens. A modified CTAB procedure (with a triple-extraction was conducted as described in Borsch et al. (2003).

The *trnK*/*matK* region was generally amplified in two parts with an overlap of 250 to 400 bp, using the primers listed in Table 2. In some species, the *trnK*/*matK* region was amplified in three parts due to long insertions of AT rich microsatellites. Amplification profiles differed only with respect to annealing temperatures for the specific primer combination used, and were otherwise: 3 min at 96°C, 3 min at 50°C (48°C), 3 min at 72°C, 34 cycles (39 cycles) of 1 min at 94°C, 1.50 min at 48/50/52°C, 3 min at 72°C and a final extension 20 min at 72°C. Reactions of 25 µl containing 15 µl DNA template (2 ng/µl), 3.3 µl dNTP mix (1.25 mM each), 0.5 µl of each primer (20 pmol/µl) and 1 U Taq Polymerase (Promega) were conducted. After gel electrophoresis the PCR products were purified using a QiaQuick gel extraction kit (QIAGEN). Direct sequencing used the ABI Prism$^{TM}$ BigDye 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 CEQ 8000 sequencer, following standard protocols for each kit. In some cases, the PCR products were cloned using the pGEMT-easy vector kit (Promega) and sequenced with the amplification primers after plasmid isolation and purification through GFX microplasmid kit (AmerSham). Cloning followed standard procedures with 1 µl vector, 1 µl ligase, 5 µl Buffer (all provided with each kit) and 3 µl PCR product.

**Table 2.** Amplification and sequencing primers used in the present study.

| Primer name | Direction | Sequence (5'-3') | Taxonomic Group used for | Design |
|---|---|---|---|---|
| MG1 | rev. | AAC TAG TCG GAT GGA GTA GAT | Piperales[1] (excl. Zippelia + Sarcorhachis) | Liang & Hilu (1996) |
| MG15 | for. | ATC TGG GTT GCT AAC TCA ATG | Piperales[1] | Liang & Hilu (1996) |
| NYmatK 480F | for. | CAT CTG GAA ATC TTG STT C | outgroup + Asaroideae + Aristolochia | Borsch (2000) |
| trnK 3914Fdi | for. | GGG GTT GCT AAC TCA ACG G | Piperales[1] | Johnson & Soltis (1995) |
| psbA-R | for. | CGC GTC TCT CTA AAA TTG CAG TCA T | Piperales[1] | Steele (1994) |
| AR-matK-660R | rev. | A(CT)G GAT TCG CAT TCA TA | Aristolochiaceae + Saururaceae | this study |
| Ca-matK-1690R | rev. | AGA GGA TTG TTT ATG GAG | outgroup | this study |
| Pi-matK-470F | for. | TTC AAA CCC TTC G(CT)T (AG)CT GG | Piper + Saururaceae | Wanke et al. (2006a) |
| Pi-matK-730R | rev. | ATA GAA ATG GA(CT) TCG TTC AAG | Saururaceae + Peperomia | Wanke et al. (2006a) |
| Pi-matK- 500F | for. | TTT GCA TTT ATT GCG AAT C | Piperaceae + Saururaceae | this study |
| AS-matK-670R | rev. | GA(AG) AGG ATT GTT TAC G(AG)A G | Thottea + Asaroideae + Saururaceae + outgroup | Wanke et al. (2006b) |
| Pi-matK-700R | rev. | AT(AG) AGA AGA TTG TTT ACG G | Piper + Zippelia + Sarcorhachis | this study |
| AS-matK-460F | for. | TAC TTC CCT TTT T(ACT)G AGG | Asaroideae + Thottea + Saururaceae | Wanke et al. (2006a) |
| Pi-matK-560F | for. | TGG ATA CAA GAT GTT CCC | Peperomia + Saururaceae | this study |
| AR-matK-080R | rev. | ACT CCT GAA A(AG)A GAA GTG G | Aristolochiaceae + Saururaceae | Wanke et al. (2006b) |
| Pi-matK-1060F | for. | ACT T(AG)T GGT CTC AAC (CT)G | Piperales | Wanke et al. (2006a) |
| Pi-matK-370R | rev. | TTT (CT)CC TAT AAT TGG AGC | Piperaceae | Wanke et al. (2006a) |
| Pi-matK-1480F | for. | TCG TAA ACA (CT)AA AAG TAC | Piperaceae + Saururaceae + Asaroideae + Aristolochia | Wanke et al. (2006a) |
| TH-matK-420F | for. | AAC TGA ATA AAT GGA TAG AGC | Thottea | Wanke et al. (2006b) |
| AR-matK-420F | for. | AAG TGA ATA AAT GGA TAG AGC | Aristolochia | Wanke et al. (2006b) |
| AR-matK-1850R | rev. | CCA GGC AAG ATA CTA AT | Aristolochia | this study |
| ZiSa-matK-480F | for. | AGT TCA AAA CAT TCG CTA CTG G | Zippelia + Sarcorhachis | this study |
| ZiSa-matK-1820R | rev. | ACA CTA ATT GGA AGG AGA ATG G | Piper + Peperomia | this study |
| ZiSa-trnK-F | rev. | AAC CGT GCT TGC ATT TTT CAT TG | Zippelia + Sarcorhachis | Wanke et al. (2006a) |
| Pi-matK-950R | rev. | CCT ATC GCT CTT TTG ATT TTG GAA | Piper + Peperomia | Wanke et al. (2006a) |
| AR-matK-1200F | for. | TTC CAA AGT CAA AAG AGC G | Lactoris + Aristolochiaceae + outgroup | this study |
| Pi-matK-2800F | for. | AAT CTT TCT CAT TAT TAC AGT GG | Piper + Saururaceae + Aristolochioideae | this study |
| Pi-matK-2030F | for. | CCT CTT TGC ATT TAT TGC G | Piperaceae + Saururaceae + | Wanke et al. (2006a) |

| | | | Asaroideae | |
|---|---|---|---|---|
| AR-matK-1510R | rev. | TAG ACT CCT GAA A(AG)A GAA GTG G | Aristolochia | this study |
| AR-matK-960R | rev. | AAC CTT TTC CCG CAT CAG G | Aristolochia | this study |
| TH-matK-960R | rev. | AAC CTT TTC CCG CAT TAG A | Thottea | Wanke et al. (2006b) |
| TH-matK-930F | for. | TAA TGC GGG AAA AGG TTC | Thottea | Wanke et al. (2006b) |
| AR-matK-930F | for. | TAT TAG TAC CTG ATG CGG G | Aristolochia | this study |
| ZiSa-trnK-R | for. | AAT CCG TAT TCC TTT TTC TCC G | Zippelia + Sarcorhachis | this study |
| AR-matK-780R | rev. | GGT CTT CTG AAA ATG ATT AC | Aristolochia | this study |
| AR-matK-680R | rev. | CCG AGA AAA ACG AAT ATG GAT T | Aristolochia | this study |
| AR-matK-1400F | for. | CTC TTT CAG GAG TCT ATC TAT G | Aristolochia | this study |
| AR-matK-1450R | rev. | CGT TAG AGT TGC ACG TTA | Aristolochia | this study |
| AR-matK-1510R | rev. | TAG ACT CCT GAA ARA GAA GTG G | Aristolochia | this study |
| AR-matK-2100R | rev. | TGA AAA TGA TTA CAA AGC ACT AC | Aristolochia | this study |
| AR-matK-2400R | rev. | ATT TTC TAG CAT TTG ACT CC | Aristolochia | this study |
| AR-matK-2510R | rev. | AAA AAT CTC AAT AAA TGY AA | Aristolochia | this study |
| AR-matK-3500R | rev. | ATC CAA ATA CCA AAT ASA TTC C | Aristolochia | this study |
| AR-trnK-1320R | rev. | ATC GCT CTT TTG ACT TTG G | Aristolochiaceae + Lactoris | Wanke et al. (2006b) |
| Pe-matK-2000F | for. | TTC CTT ACG AAT CCA TAG A | Piper + Peperomia | Wanke et al. (2006a) |
| Pe-matK-2500R | rev. | TTC GCA ATA AAT GCA AAG AGG | Piperaceae + Saururaceae | Wanke et al. (2006a) |
| Pe-matK-2700F | for. | AAA CAA TCT TTT CAT TTA CG | Peperomia | Wanke et al. (2006a) |
| Pi-matK-1820R | rev. | ACA CTA ATT GGA AGG AGA ATG G | Piper + Peperomia | Wanke et al. (2006a) |
| trnK-med-150F | for. | AGA GAA TAC TTC CAT CCT TAC CG | Aristolochia | this study |
| trnK-med-440R | rev. | ATT CGT CTT TAC TCA CTC CGT A | Aristolochia | this study |

[1] Also used for outgroup amplification and sequencing

Sequence alignment and treatment of microstructural changes

Sequences were manually aligned using PhyDE[®] (Müller et al., 2005) following alignment rules proposed by Borsch et al. (2003) and Löhne and Borsch (2005) and guided by secondary structures of DNA especially for length mutations and inversions. Secondary structures and the resulting free energy ($\Delta G$) of hairpins was calculated using RNAstructure 4.2 (Mathews et al., 2005). The aforementioned alignment rules have been compiled to account for mutational events other than nucleotide substitutions. The observed motifs are largely the result of simple sequence repeats (one or several copies), deletions, and inversions (see also Benson et al., 1997; Graham et al., 2000; Kelchner, 2000; Löhne and Borsch, 2005; Müller and Borsch, 2005a). Seven mutational hotspots were excluded from the final matrix (Table 3), especially microsatellites, because of uncertain primary homology. An indel matrix was calculated using the "simple indel coding" approach (SIC, Simmons and Ochoterena, 2000) as implemented in SeqState (Müller 2005a). The alignment and the indel matrix are available from TreeBASE (www.treebase.org).

Phylogenetic analyses

Phylogenetic reconstructions using heuristic searches under maximum parsimony (MP) were performed using PAUP* 4.0b10 (Swofford 2002). The strict consensus tree was inferred with command files for PAUP* 4.0b10generated by PRAP (Müller, 2004), implementing the Parsimony Ratchet (Nixon, 1999). The following ratchet settings were employed: 10 random addition cycles of 500 iterations each with a 25% of upweighting of the characters in the iterations. In addition, indels were analyzed employing SIC (Simmons and Ochoterena, 2000) as implemented in SeqState (Müller 2005a). SeqState generates a ready-to-use Nexus file containing the sequence alignment with an automatically generated indel matrix appended. 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. Decay values as further measurement of support for the individual clades were

obtained using PRAP in combination with PAUP* and the same options in effect as in the ratchet.

Maximum likelihood analyses were executed assuming a general time reversible model (GTR), and a rate variation among sites following a gamma distribution (four categories represented by mean). GTR+G+I was chosen as the model that best fits the data by Modeltest v3.6 (Posada & Crandall 1998) employing the interface MTgui (Nuin, 2005). The settings proposed by Modeltest v3.6 [BaseFreq=(0.3384 0.1510 0.1455), Nst=6, Rmatrix=(1.0672 1.7089 0.2936 0.6604 1.7089), Shape=1.2190, Pinvar=0.0621] were executed in PAUP.

For Bayesian inference (BS) the program MrBayes v3.1 (Ronquist and Huelsenbeck, 2003) was used. To acknowledge possible deviating substitution models for the coding and non-coding regions the data set was divided into two partitions. For both partitions, the GTR model of nucleotide substitution was assigned, assuming site-specific rate categories following a gamma distribution. Two runs ($10^6$ generations each) with four chains each were run simultaneously, starting from random trees. Chains were sampled every 10 generations and the respective trees were written to a tree file. Calculation of the consensus tree and the posterior probability (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).

Relative rate test

Relative rate tests according to Sarich and Wilson (1967) were used to quantify the degree of rate divergence between taxon sets (e.g. clades).. Relative rate differences were calculated between the main Piperales groups (Asaroideae, Aristolochioideae (excl. *Thottea*), *Thottea*, Saururaceae, Lactoridaceae, *Manekia/Zippelia*, *Piper* s.l., *Peperomia*). As in the phylogenetic analyses *Canella*, *Drimys*, and *Pseudowintera* were chosen as reference taxa. Calculations of differences in substitutional rates between groups were based on ML estimates of distances (GTR +G +I model). Calculations were performed with help of GRate (Müller, 2002; see Müller et al., 2004) that allows to compare average rates of previously defined taxon sets.

**Table 3.** Hotspots excluded, due to ambiguous homology assessments. The location of the hotspots, length and their composition is shown.

| hotspot | position in the alignment | length distribution bp | | | | composition %[*] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Aristolochiaceae | Lactoridaceae | Piperaceae | Saururaceae | A | T | C | G |
| H1 | 472-916 | 14-437 | 5 | 4-5 | 5 | 34.8 | 56.0 | 4.2 | 5.0 |
| H2 | 1049-1068 | 8-17 | 9 | 9-11 | 9-10 | 0.9 | 68.8 | 29.7 | 0.6 |
| H3 | 1249-1260 | 2-5 | 3 | 2-12 | 3-9 | 93.9 | 2.3 | 2.1 | 1.7 |
| H4 | 1409-1426 | 8-12 | 8 | 6-8 | 7 | 64.8 | 3.2 | 11.4 | 20.6 |
| H5 | 1525-1541 | 0-17 | 0 | 0-1 | 1 | 2.1 | 97.9 | 0 | 0 |
| H6 | 3821-3847 | 0-27 | 5 | 4-9 | 6-8 | 72.0 | 14.6 | 7.0 | 6.4 |
| H7 | 3872-3899 | 5-8 | 7 | 1-18 | 7 | 12.7 | 57.8 | 16.9 | 12.7 |

[*] calculated for each hotspot from the whole dataset

**Table 4.** Sequence statistics for the coding and noncoding regions calculated with SeqState (Müller 2004). % divergence = overall sequence dissimilarity (uncorrected $p$-distance * 100).

| region | characters (bp)[1] | length range (bp)[1] | characters (bp)[*] | % divergence | variable[*] | parsimony informative[*] | % GC contents[1] |
|---|---|---|---|---|---|---|---|
| *trnK* intron incl. *matK* | 4256 | 2413-3278 | 3683 | 13.8 | 43.8 % | 32.1 % | 33:8 |
| *trnK* intron excl. | 2575 | 886-1754 | 2002 | 14.8 | 33.2 % | 22.1 % | 33.5 |
| *trnK* 5' intron | 1874 | 677-1538 | 1355 | 14.4 | 34.8 % | 24.4 % | 33.9 |
| *trnK* 3' intron | 701 | 179-701 | 647 | 16.8 | 29.8 % | 17.3 % | 32.3 |
| *matK* coding | 1681 | 1509-1557 | 1681 | 13,3 | 56.6 % | 44.0 % | 33.7 |

[1] calculated with hotspot regions included
[*] calculated with hotspot regions excluded

## Results

<u>Variability in the *trnK* intron and the *matK* gene</u>

In Piperales, the *trnK* intron (including the *matK* ORF) is 2533 bp on average, ranging from 2412-3258 bp. The *matK* gene itself has a length of 1509-1557 bp (mean 1534 bp). Frequent microstructural changes lead to a considerably longer aligned dataset of 4256 characters, including hotspots (Fig. 1, Table 4). All hotspots are located in the *trnK* intron and their position and extension are given in Table 3. Sequences in hotspots are microsatellites consisting of stretches of poly-As or Ts except H1 that comprises long and highly-variable AT-rich sequence parts inserted in Aristolochioideae (excl. *Thottea*) sequences (cryptic simple microsatellite, Wanke et al, 2006a). Table 5 summarises lineage specific characteristics for partitioned datasets (*Piper*, *Peperomia*, *Aristolochia* s.l.). It is clearly shown that the number of parsimony informative characters, compared to outgroup, is similar between the three large genera. But parsimony informative characters within the clades are considerably different. *Piper* displays only ~1/3 to ~1/2 the amount of informative sites compared to *Aristolochia* or *Peperomia*, respectively. Generally, the number of variable characters is twice as high within *Peperomia* and *Aristolochia* compared to *Piper*.

**Table 5.** Characterisation of the maximum parsimony trees obtained for the complete alignment (hotspots excluded) and partitioned sets representing the "giant genera", to evaluate the lineage specific resolution contrast based on alignment characteristics.

| | without indels* | with indels* | Aristolochia | Peperomia | Piper |
|---|---|---|---|---|---|
| trees found[#] | 440 | 3434 | 1 | 6 | 609 |
| steps[#] | 4303 | 5065 | 1371 | 1522 | 1029 |
| CI[#] | 0.559 | 0.555 | 0.761 | 0.787 | 0.835 |
| RI[#] | 0.913 | 0.906 | 0.842 | 0.810 | 0.863 |
| RC[#] | 0.511 | 0.503 | 0.641 | 0.637 | 0.720 |
| HI[#] | 0.441 | 0.445 | 0.239 | 0.213 | 0.165 |
| total char.[#] | 3684 | 4089 | 3684 | 3684 | 3684 |
| constant char.[#] | 2066 | 2066 | 2822 | 2695 | 2925 |
| uninformative char.[#] | 432 | 605 | 313 | 370 | 255 |
| pars. informative char.[#] | 1186 | 1418 | 549 | 619 | 504 |
| pars. informative char. %[$] | - | - | 10.365 | 6.743 | 3.740 |
| variable char. %[$] | - | - | 18.227 | 18.051 | 9.393 |

\* based on the complete alignment incl. outgroup
[#] based on charactersets for the mentioned members plus outgroup, based on substitutions only, if not different indicated
[$] based on charactersets for the mentioned members without outgroup, based on substitutions only

Microstructural variation in the *trnK* intron

A large number of length mutations has been identified and coded in a separate matrix (395 indels in total). Among these, 217 indels are located within the *trnK* 5' intron, 59 in the *matK* gene and 119 in the *trnK* 3' intron. Most of these indel events represent simple sequence repeats (SSR) of the flanking region (up to 25 bp in the intron and 15 bp, 5 codons, in the gene). An AT-rich microsatellite-like stretch is found in the domain I of the *trnK* intron (Fig 1, hotspot I). In Aristolochioideae (not present in *Thottea*), this cryptic simple microsatellite ranges from 29 bp in *Isotrema* to 443 bp in *Endodeca*. The internal structure of this repeat can be characterised as $(A_nT_m)_k$. This microsatellite region is absent in all other Piperales. A second poly-A/T microsatellite is observed in *Thottea* (Fig. 2). Examination of the flanking regions and subsequent structural analysis reveals a hairpin with the microsatellite forming a terminal loop, which appears to have been inverted in the common ancestor of *Thottea* (Fig. 2).

Compared to expected triplet insertions and in frame deletions, "self repairing" out-of-frame indels around 600 nt upstream of the *matK* ORF start (Fig. 3, Tab. 6), apparently associated with a microsatellite, have been identified. Based on primary homology assessment, length mutations involving one or two nucleotides must be assumed, which are followed downstream by an additional length mutational event involving one or two nucleotides respectively. Therefore, the frameshift affects only five or six amino acids, the restoration of the original reading frame is observed in all cases. However, an out-of-frame deletion of two codons is found in the two species of *Pararistolochia* (around alignment position 2050). The *matK* gene of the Piperaceae is highly variable in length on its 3' end, this variation is probably due to point mutations that result in early stop codons in several taxa (Fig. 4).

**Figure 2.** Position 356-397 of the alignment (*trnK* 5' intron), showing a selection of taxa and the potential inverted sequences (boldface) in *Thottea*. Below the potential part as "normal" and as reverse complemented and aligned is given. Lowest shows the reverse complemented part aligned into the original selection. The absolute number of inverted nucleotides could not be detected due to the insertion of poly T's. The secondary structure (ΔG = -3.5) for this region is given as example from *Thottea corymbosa*, demonstrating the perfect stem-loop region of the potential inverted region.

```
A. salvadorensis      TTTCTTTGAACGGGACTCAAA----------------AAAT-TAACCC----TTGGGTC
A. tomentosa          TTTCTTGGAACGGGACTAAAA----------------AAAT-TCACCC----TTGGGTC
A. manshurensis       TTTCTTGGAACGGGACTAAAA----------------AAAT-TCACCC----TTGGGTC
A. serpentaria        TTTCTTGGAACGGGACTAAAA----------------AAAT-GCACCC----TTGGGTC
Thottea dependens     ATTCTTGGAACGGGATGCATTTTTTTTTTTTTTTTTT-----TCATCT----TTAGGTC
Thottea corymbosa     ATTCTTAGAACGGGATGAATTTTTTTTTTTT------------TCATCT----TTGGGTC
Thottea siliquosa     ATTCTTGGAACGGGGTGCATTTTTTTTTTT--------------TCATCT----TTGGGTC
Asarum caudatum       ATTCTTGGAACGGGACCAAAT----------------CAATATCACCATTGATTGGGTC
Saruma henryi         ATTCTTGGAACGGGACCAAAT----------------CAATATCACCATTGATTGGGTC
Anemopsis californica ATTCTTGGAATGGGACCAAAT----------------CAAT-TCATCC----TTGGGTC
```

```
Thottea dependens          GATGCATTTTTTTTTTTTTTTTTTT-----TCATC
Thottea corymbosa          GATGAATTTTTTTTTTTT-----------TCATC
Thottea siliquosa          GGTGCATTTTTTTTTTT-------------TCATC
Thottea dependens          GATGAAAAAAAAAAAAAAAAA----AAAT-GCATC
Thottea corymbosa          GATGAAAAAAAAAA-----------AAAT-TCATC
Thottea siliquosa          GATGAAAAAAAA-------------AAAT-GCACC
```

```
A. salvadorensis      TTTCTTTGAACGGGACTCAAA----------------AAAT-TAACCC----TTGGGTC
A. tomentosa          TTTCTTGGAACGGGACTAAAA----------------AAAT-TCACCC----TTGGGTC
A. manshurensis       TTTCTTGGAACGGGACTAAAA----------------AAAT-TCACCC----TTGGGTC
A. serpentaria        TTTCTTGGAACGGGACTAAAA----------------AAAT-GCACCC----TTGGGTC
Thottea dependens     TTTCTTGGAACGGGATGAAAAAAAAAAAAAAAAA----AAAT-GCATCC----TTGGGTC
Thottea corymbosa     TTTCTTGGAACGGGATGAAAAAAAAAA-----------AAAT-TCATCC----TTGGGTC
Thottea siliquosa     TTTCTTGGAACGGGATGAAAAAAAA-------------AAAT-GCACCC----TTGGGTC
Asarum caudatum       ATTCTTGGAACGGGACCAAAT----------------CAATATCACCATTGATTGGGTC
Saruma henryi         ATTCTTGGAACGGGACCAAAT----------------CAATATCACCATTGATTGGGTC
Anemopsis californica ATTCTTGGAATGGGACCAAAT----------------CAAT-TCATCC----TTGGGTC
```
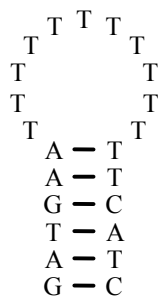


*Thottea corymbosa* ΔG = -3.5

**Figure 3.** Position 2020-2110 of the alignment. An example for the high amount of length mutational events within the *matK* gene. Frame shift mutations with the respective "self repairing part" are indicated in bold. Only parts of the complete matrix are shown. All effected AS are marked in bold and arranged how they should be aligned on nucleotide level to demonstrate frame shift events.

```
Canella          ATTACTCCAAAGAAA---------TCCATTTCCATT------TTT------TCA---------AAAGATAAT
Drimys           ATTACTCCAAAGAAA---------TCCATTTCCCTT------TTT------TCA---------AAAAGGAAT
Pseudowintera    ATTATTCCAAAGAAA---------TCCATTTCCATT------TTT------TCA---------AAAAGGAAT
A. pentandra     ATTAGTTCAAAGAAA---------TCCATTTTTTTT------TTC------TCA---------AAAGAGAAT
A. bracteolata   ATTAGTTCAAAGAAA---------TCCATTTCTTTT------TTC------TCA---------AAAGAGAAT
A. albida        ATTAGTTCAAAGAAA---------TCCATTTATTTT------TTC------TCATTCTCA---AAAGAGAAT
A. pistolochia   ATTAGTTCAAAGAAA---------TCCTTTTTTTTTTT-----TTCTCAAA-TCA---------AAAGAGAAT
A. parvifolia    ATTAGTTCAAAGAAA---------TCCTTTTTT---------TTC------TCA---------AAGGGGAAT
A. pichinch.     ATTAATTCAAAGAAA---------TCCATTTTTATT------TTA------TCA---------AAAGGGAAT
P. triactina     ATTAGTTCAAAGAAA---------TCCATTTTTTTT------TTC------TCA---------AAAGAGAAT
A. salvadoren.   GTTAGTTCAAAGAAA---------TCCATTCTTTTTTTT---TTC------TCA---------AAAGAGAAT
A. californica   GTTAGTTCAAAGAAA---------TCCATTCCTTTTTTTTTTTTTC------TCA---------AAAGAGAAT
A. macrophylla   GTTAGTTCAAAGAAA---------TCCATTCCTTTTTTT---TTC------TCA---------AAAGAGAAT
A. serpentaria   GTTAGTTCAAAGAAA---------TCCATTCCTTTTTTT---TTC------TCA---------AAAGAAAAT
Th. siliquosa    ATTAGTCCAAAGAAA---------TTCATTTCTTTT------TTC------TCA---------AAGGAGAAT
Asa. caudatum    ATTAGTCTAAAGAAA---------TCTATCTCTTTCTTT---TTT------TCA---------AAAGGGAAT
Saruma henryi    ATTAGTCCAAAGAAA---------TCTATCTCTTTCTTT---TTT------TCA---------AAAGGGAAT
Lactoris         ATTAGTCCAAAGAAA---------TCGATTTCTTTT------TTT------TCA---------AATGGGAAT
Anemopsis        ATTAGCCAAAAAAA----------TCCATCTCT---------TTT------TCA------AAAGAAGAGAAT
Gymnotheca       ATTAGCAAAAAAAA----------TCCATCTCT---------TTT------TCA------AAAGAGAGAAT
M. naranjoana    ATTAGCCAAAAAAA----------TTCTCTTCT---------TTT------TCA------AAAAAAGAGAAT
P. decuma        ATTAGCAAAAAAAA----------TTCTTTTCT---------TTT------TCA------AAAAAAGAAAT
Pep. gracill.    TTTAGCAAAAGA----------------------------------------------------AAAAAAAAT
Pep. fraseri     TTTAGCAAAATA----------------------------------------------------AAAAAAAAT
Pep. trifolia    TTTAGCAAAATAAAAAAAAA----TTCTTTTAT---------TTT------TCA-------AAAAAAAAAT
Pep. argyreia    TTTAAAAAAAAAAAAAAAAAA----TTCTTTTCT---------TTT------TCT-------AAAAAAAAAT
Pep. metallica   TTTAGCAAAATAAAAAAAAA----TTCTTTTAT---------TTT------GAA--------AAATAAAAAT
Pep. marmorata   TTTAGCAAAAGAAAAAAAAG----TTCTTTTAT---------TTT------TCA--------AAAAAAAAAT
Pep. fagerlin.   TTTAGCAAAATAAAAAAAAAAA--TTCTTTTAT---------TTT------TCA-------AAAAAAAAAAAT
Pep. clusiifo.   TTTAGCAAAATAAAAAAAA----TTCTTTTCT---------TTT------TCA--------AAAAAAAAAT
Pep. pernambu.   TTTAGCAAAAGA----------------------------------------------------AAAAAAAAT
```

```
Canella          IleThrProLysLys---------SerIleSerIle------Phe------Ser---------LysAspAsn
Drimys           IleThrProLysLys---------SerIleSerIle------Phe------Ser---------LysArgAsn
Pseudowintera    IleIleProLysLys---------SerIleSerIle------Phe------Ser---------LysArgAsn
A. pentandra     IleSerSerLysLys---------SerIlePhePhe------Phe------Ser---------LysGluAsn
A. bracteolata   IleSerSerLysLys---------SerIleSerPhe------Phe------Ser---------LysGluAsn
A. albida        IleSerSerLysLys---------SerIleTyrPhe------Phe------SerPheSer---LysGluAsn
A. pistolochia   IleSerSerLysLys---------SerPhePhePheP-----heLeuLys-Ser---------LysGluAsn
A. parvifolia    IleSerSerLysLys---------SerPhePhe---------Phe------Ser---------LysGlyAsn
A. pichinch.     IleAsnSerLysLys---------SerIlePheIle------Leu------Ser---------LysGlyAsn
P. triactina     IleSerSerLysLys---------SerIlePhePhe------Phe------Ser---------LysGluAsn
A. salvadoren.   ValSerSerLysLys---------SerIleLeuPhePhe---Phe------Ser---------LysGluAsn
A. californica   ValSerSerLysLys---------SerIleProPhePhePhePhe------Ser---------LysGluAsn
A. macrophylla   ValSerSerLysLys---------SerIleProPhe------Phe------Ser---------LysGluAsn
A. serpentaria   ValSerSerLysLys---------SerIleProPhe------Phe------Ser---------LysGluAsn
Th. siliquosa    IleSerProLysLys---------PheIleSerPhe------Phe------Ser---------LysGluAsn
Asa. caudatum    IleSerLeuLysLys---------SerIleSerPhePhe---Phe------Ser---------LysGlyAsn
Saruma henryi    IleSerProLysLys---------SerIleSerPhe------Phe------Ser---------LysGlyAsn
Lactoris         IleSerProLysLys---------SerIleSerPhe------Phe------Ser---------AsnGlyAsn
Anemopsis        IleSerGlnLysLys---------SerIleSer---------Phe------Ser------LysGluGluAsn
Gymnotheca       IleSerLysLysLys---------SerIleSer---------Phe------Ser------LysGluGluAsn
M. naranjoana    IleSerGlnLysLys---------PheSerSer---------Phe------Ser------LysGluGluAsn
P. decuma        IleSerLysLysLys---------PhePheSer---------Phe------Ser------LysLysGluAsn
Pep. gracill.    PheSerLysArg----------------------------------------------------LysLysAsn
Pep. fraseri     PheSerLysIleLysLysAs----nSerPheIl---------ePh------eGl--------nLysLysAsn
Pep. trifolia    PheLysLysLysLysLysAs----nSerPheLe---------uPh------eLe--------uLysLysAsn
Pep. argyreia    PheSerLysIleLysLysAs----nSerPheIl---------eLe------uLy--------sAsnLysAsn
Pep. metallica   PheSerLysArgLysLysSe----rSerPheIl---------ePh------eGl--------nLysLysAsn
Pep. marmorata   PheSerLysIleLysLysLysI--leLeuLeuP---------heP------heL-------ysLysLysAsn
Pep. fagerlin.   PheSerLysIleLysLysAs----nSerPheLe---------uPh------eGl--------nLysLysAsn
Pep. clusiifo.   PheSerLysArg----------------------------------------------------LysLysAsn
Pep. pernambu.   PheSerLysLys----------------------------------------------------LysLysAsn
```

**Figure 4.** The 3' part of the *matK* gene (position 3517-3570), showing the different positions of stop codons based on homology of the nucleotides a) nucleotide sequence b) amino acid sequence. Stop codons are indicated in boldface or as star respectively. Only a representing sampling of the dataset is indicated.

a)

| | |
|---|---|
| *Piper cinereum* | CATACAAATGACTTGACCAATCAT**TAA**------**TGA**----TTGATCACAAG |
| *Macropiper excelsum* | CATACAAATGACTTGACCAATCATGAA------**TGA**----TTGATCATAAG |
| *Piper hispidum* | CATACAAATGACTTGACCAATCATGAA------**TGA**----TTGATCATAAG |
| *Piper reticulatum* | CATACAAATGACTTAACCAATCATGAA------**TGA**----TTGGTCATAAG |
| *Piper pulchrum* | CATACAAATGACTTGACCAATCATGAA------**TGA**----TTGGTCATAAG |
| *Piper spoliatum* | CATACAAATGACTTGACCAATCATGAA------**TGA**----TTGGTCATAAG |
| *Peperomia gracillima* | CATACAAATGACTTGACCAATCATGAA------**TGA**----TTGGTCATAAG |
| *Peperomia fraseri* | CATACAAATGACTTGACCAATCAAGAA------**TAA**----TTGGTCATAAG |
| *Peperomia ppucuppucu* | CATACAAATGACCTGACCAATCAA**TAATAATAATAA**----TTGGTCATAAA |
| *Peperomia trifolia* | CATACAAATGACCTGACCAATCAA**TAA**------**TAA**----TTGGTCATAAG |
| *Peperomia rhombea* | CGTACAAAT**TAA**CTCACCAATCAA**TAA**------**TAA**----TTAGTCATAAG |
| *Peperomia cuspidilimba* | CATACAAAT**TAA**CTGACCAATCAA**TAA**------**TAA**----TTAGTCATAAG |
| *Peperomia pereskiifolia* | CATACAAATGACCTGACCAATCAA**TAA**------**TAA**----TTGGTCATAAG |
| *Peperomia maypuensis* | CATACAAATGACTTGAACAATCAAGAA------**TAA**----TTGGTCATAAA |
| *Peperomia argyreia* | CATACAAATGACTTGACCAATCAAGAA------**TAA**----TTGGTCATAAG |
| *Peperomia marmorata* | CATACAAATGACTTGACCAATAAAGAA------**TAA**----TTAGTCATAAG |
| *Peperomia vinasiana* | CAAACAAATGACTTGACCAATCAAGAA------**TAA**----TTAGTCATAAG |

b)

| | |
|---|---|
| *Piper cinereum* | HisThrAsnAspLeuThrAsnHis**EOF**------**EOF**----LeuIleThrArg |
| *Macropiper excelsum* | HisThrAsnAspLeuThrAsnHisGlu------**EOF**----LeuIleIleArg |
| *Piper hispidum* | HisThrAsnAspLeuThrAsnHisGlu------**EOF**----LeuIleIleArg |
| *Piper reticulatum* | HisThrAsnAspLeuThrAsnHisGlu------**EOF**----LeuValIleArg |
| *Piper pulchrum* | HisThrAsnAspLeuThrAsnHisGlu------**EOF**----LeuValIleArg |
| *Piper spoliatum* | HisThrAsnAspLeuThrAsnHisGlu------**EOF**----LeuValIleArg |
| *Peperomia gracillima* | HisThrAsnAspLeuThrAsnHisGlu------**EOF**----LeuValIleArg |
| *Peperomia fraseri* | HisThrAsnAspLeuThrAsnGlnGlu------**EOF**----LeuValIleArg |
| *Peperomia ppucuppucu* | HisThrAsnAspLeuThrAsnGln**EOFEOFEOFEOF**----LeuValIleArg |
| *Peperomia trifolia* | HisThrAsnAspLeuThrAsnGln**EOF**------**EOF**----LeuValIleArg |
| *Peperomia rhombea* | HisThrAsn**EOF**LeuThrAsnGln**EOF**------**EOF**----LeuValIleArg |
| *Peperomia cuspidilimba* | HisThrAsn**EOF**LeuThrAsnGln**EOF**------**EOF**----LeuValIleArg |
| *Peperomia pereskiifolia* | HisThrAsnAspLeuThrAsnGln**EOF**------**EOF**----LeuValIleArg |
| *Peperomia maypuensis* | HisThrAsnAspLeuAsnAsnGlnGlu------**EOF**----LeuValIleLys |
| *Peperomia argyreia* | HisThrAsnAspLeuThrAsnGlnGlu------**EOF**----LeuValIleArg |
| *Peperomia marmorata* | HisThrAsnAspLeuThrAsnLysGlu------**EOF**----LeuValIleArg |
| *Peperomia vinasiana* | GlnThrAsnAspLeuThrAsnGlnGlu------**EOF**----LeuValIleArg |

## Additional, non-functional *matK* copies in *Zippelia*.

While editing electropherograms of *Zippelia*, conspicuous overlapping peaks were observed from positions 3264 to 3271 in the alignment on the forward strand or positions 4047 to 4084 on the reverse strand. Different PCR products obtained with different primer combinations and sequencing of both strands showed the same pattern, thus exluding contamination or Taq errors. Subsequent to cloning and generating independent sequences per colony, five different products, differing by a

large number of point mutations and 9 length mutations, were obtained. All sequences were highly similar to *matK* sequences in Piperaceae, but most sequences exhibited a large number of internal stop codons. The sequence obtained from clone 2 showed a 7 bp gap within domain X, that was not corrected, thus clearly pointing to a non-functional copy of *matK*. The conserved domain X (135 nt in length), associated with the maturase activity of the protein, can be found from pos. 3237 to 3371 in the Piperales *matK* alignment (Fig. 1). This variability pattern in *Zippelia* could only be explained by assuming the presence of at least five additional copies of *matK*. The only copy without internal stop codons and out-of-frame mutations, and thus unambiguously recognized as functional was used for the phylogenetic analyses. Evidence for polymorphic sites or additional *matK* copies was not found in any other investigated taxon or generated sequence for this study.

Rate heterogeneity between lineages

Between the seven main Piperales clades we observed highly significant (P<0.0001) rate heterogeneity, with the Piperaceae having pronounced rates compared to the remaining Piperales. Within Piperaceae, *Peperomia* displays the fastest evolutionary rate, followed by *Piper* and *Sarcorhachis*/*Manekia*. For the remaining clades, Asaroideae have the lowest and *Lactoris* the fastest rates.

Trees resulting from maximum parsimony, likelihood and Bayesian inference

The different methods employed in this study, maximum parsimony (Fig. 5), likelihood and Bayesian inference (Fig. 6) resulted in a nearly identical tree topology for the major groups. The present data set (2066 constant and 1186 parsimony informative (PI) characters) results in 440 most parsimonious trees (MPT) of 4303 steps; the strict consensus tree is depicted in Fig. 5 with the bootstrap and decay values depicted aling the branches. The coding of length mutational events has added 300 PI sites to the data matrix. The combined analysis has resulted in 3434 MPTs (5065 steps) (Table 5). The likelihood phylogram (-ln 30144.36066 ) is shown in Fig. 6 with the significantly supported branches (prosterior probabilities above 90) being emphasized by thick lines.

The relationship between Aristolochiaceae and *Lactoris* remains uncertain, as the analyses do not resolve the tree with high support. MP analyses show Asaroideae sister to a *Lactoris* + Aristolochioideae clade (no support), whereas the Bayesian and the likelihood analysis show Asaroideae sister to Piperaceae + Saururaceae (no support; PP 64). The Aristolochiaceae are paraphyletic with respect to *Lactoris* and split into two well- supported clades (Asaroideae and Aristolochioideae). The sister group relationship of Aristolochioideae and Lactoridaceae receive BS of 82% / 83% as well as PP of 100. Within subfamily Aristolochioideae, *Thottea* branches first followed by the *Endodeca* and *Isotrema* clade (maximal support) which is sister to the remaining Aristolochioideae. Among the remaining Aristolochioideae, *Pararistolochia* as well as segregates recognised by e.g. Huber (1985, 1993) like *Einomeia* are monophyletic. Hubers's segregate "*Howardia*" is found to be polyphyletic, as the *Aristolochia grandiflora* complex has been treated as part of "*Howardia*". Relationships among these segregates are generally not well supported in parsimony analyses.

Saururaceae are monophyletic and sister to Piperaceae. Within Saururaceae, results obtained with the different search methods, are congruent, resolving *Houttuynia* and *Anemopsis* together and this clade sister to the remaining genera *Saururus* and *Gymnotheca*. Piperaceae are subdivided into two well-supported clades, the first one consisting of the small genera *Manekia* and *Zippelia*, and the two large genera *Piper* s. l. and *Peperomia* forming the second clade. Several infrageneric clades, such as *Pothomorphe*, *Macrostachys* and *Macropiper*, can be recognised with moderate to high support. In contrast to this, species relationships in *Peperomia* are fully resolved with generally high support, although the infrageneric clades are often polyphyletic (e.g. *Tildenia, Rhynchophorum*).

**Figure 5**. Strict consensus tree of 440 most parsimonious trees (MPT's; length 4303, CI = 0.559, RI = 0.913, RC = 0.511). Bootstrap support values as well as decay indices are depicted along the branches; support values derived with an appended indel matrix are shown below the branches, whereas regular support values are depicted above. Indels were coded with SeqState (Müller, 2005a) using the SIC-approach (Simmons and Ochoterena, 2000). Members of "Howardia" are indicated with an asterisk (*).The Asian tropics and South Pacific clade is highlighted by a grey box. (see next page)
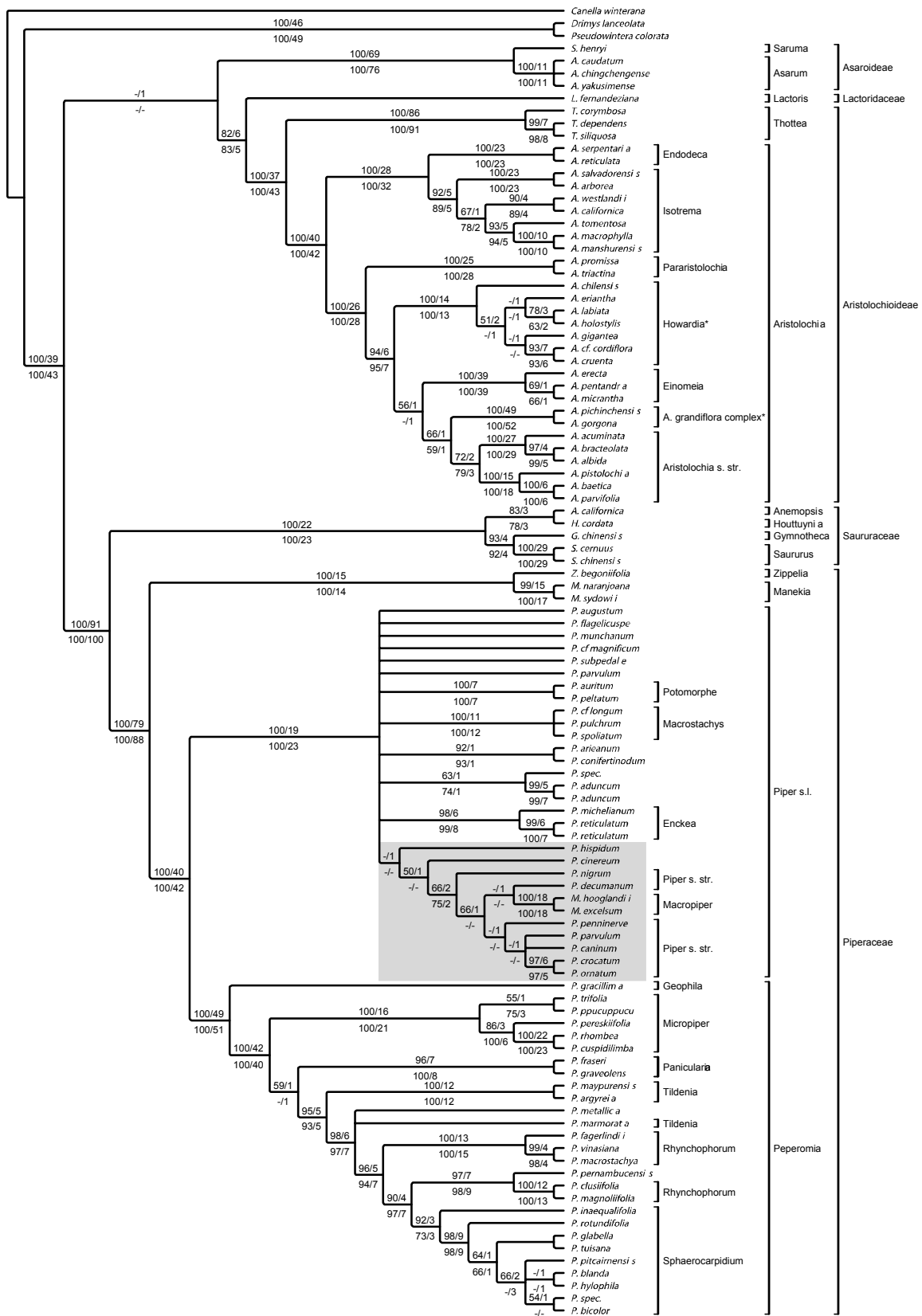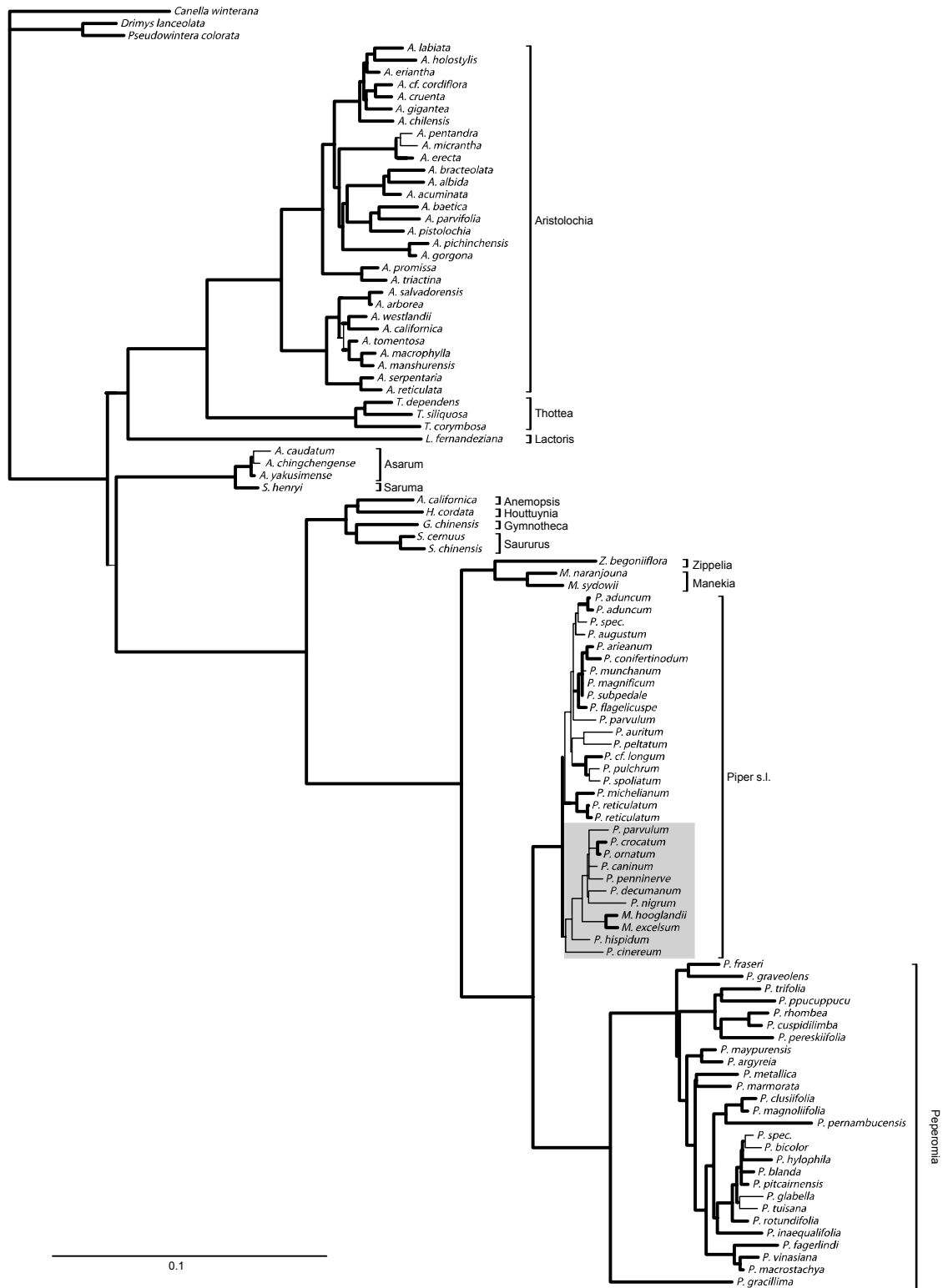
Canella winterana
Drimys lanceolata
Pseudowintera colorata
100/46
100/49

S. henryi — Saruma
A. caudatum — Asarum
A. chingchengense
A. yakusimense
100/69
100/76
100/11
100/11
Asaroideae

L. fernandeziana — Lactoris — Lactoridaceae

-/1
-/-

82/6
83/5

T. corymbosa
T. dependens — Thottea
T. siliquosa
100/86
100/91
99/7
98/8

100/37
100/43

A. serpentaria — Endodeca
A. reticulata
100/23
100/23

A. salvadorensis
A. arborea
A. westlandii — Isotrema
A. californica
A. tomentosa
A. macrophylla
A. manshurensis
100/28
100/32
100/23
100/23
90/4
89/4
100/10
100/10
92/5
89/5
67/1
78/2
93/5
94/5

100/40
100/42

A. promissa — Pararistolochia
A. triactina
100/25
100/28

A. chilensis
A. eriantha
A. labiata — Howardia*
A. holostylis
A. gigantea
A. cf. cordiflora
A. cruenta
100/14
100/13
-/1
78/3
-/1
63/2
-/1
93/7
93/6
51/2

100/26
100/28

A. erecta
A. pentandra — Einomeia
A. micrantha
100/39
100/39
69/1
66/1

A. pichinchensis — A. grandiflora complex*
A. gorgona
100/49
100/52

A. acuminata
A. bracteolata
A. albida — Aristolochia s. str.
A. pistolochia
A. baetica
A. parvifolia
100/27
100/29
97/4
99/5
100/15
100/18
100/6
100/6

94/6
95/7
56/1
-/1
66/1
59/1
72/2
79/3

Aristolochia

Aristolochioideae

A. californica — Anemopsis
H. cordata — Houttuynia
G. chinensis — Gymnotheca
S. cernuus — Saururus
S. chinensis
100/22
100/23
83/3
78/3
93/4
92/4
100/29
100/29

Saururaceae

Z. begoniifolia — Zippelia
M. naranjoana — Manekia
M. sydowii
100/15
100/14
99/15
100/17

100/91
100/100

P. augustum
P. flagelicuspe
P. munchanum
P. cf magnificum
P. subpedale
P. parvulum

P. auritum — Potomorphe
P. peltatum
100/7
100/7

P. cf longum
P. pulchrum — Macrostachys
P. spoliatum
100/11
100/12

P. arieanum
P. conifertinodum
P. spec.
P. aduncum
P. aduncum
92/1
93/1
63/1
74/1
99/5
99/7

P. michelianum
P. reticulatum — Enckea
P. reticulatum
98/6
99/6
99/8
100/7

P. hispidum
P. cinereum
P. nigrum — Piper s. str.
P. decumanum
M. hooglandii — Macropiper
M. excelsum
P. penninerve
P. parvulum — Piper s. str.
P. caninum
P. crocatum
P. ornatum
-/1
-/-
50/1
-/-
66/2
75/2
66/1
-/1
-/1
100/18
100/18
-/-
-/1
-/1
97/6
97/5

100/19
100/23

Piper s.l.

100/79
100/88

100/40
100/42

P. gracillima — Geophila

100/49
100/51

P. trifolia
P. ppucuppucu
P. pereskiifolia — Micropiper
P. rhombea
P. cuspidilimba
55/1
75/3
86/3
100/6
100/22
100/23
100/16
100/21

P. fraseri — Panicularia
P. graveolens
P. maypurensis — Tildenia
P. argyreia
96/7
100/8
100/12

100/42
100/40
59/1
-/1

P. metallica
P. marmorata — Tildenia
100/12

P. fagerlindii
P. vinasiana — Rhynchophorum
P. macrostachya
100/13
100/15
99/4
98/4

P. pernambucensis
P. clusiifolia — Rhynchophorum
P. magnoliifolia
97/7
98/9
100/12
100/13

P. inaequalifolia
P. rotundifolia
P. glabella
P. tuisana
P. pitcairnensis — Sphaerocarpidium
P. blanda
P. hylophila
P. spec.
P. bicolor
98/6
97/7
96/5
94/7
90/4
97/7
92/3
73/3
98/9
98/9
64/1
66/1
66/2
-/3
-/1
-/1
54/1
-/-
100/7

95/5
93/5

Peperomia

Piperaceae

**Figure 6**. Likelihood phylogram. Significantly supported branches are indicated by thick lines (Posterior Probability ≥ 90%). The Asian tropics and South Pacific clade is highlighted by a grey box.

**Discussion**

Microstructural changes in the *matK* CDS

The *matK* ORF is maintained in all taxa of Piperales analysed in this study. Length variability, resulting from stop codons, of the ORF is confined to the 3' end, where minimal impact on the protein structure is expected (Hilu and Liang, 1997). This parallels results in other groups of angiosperms with highly variable *matK* sequences such as Lentibulariaceae (Müller and Borsch, 2005a), and the general situation in angiosperms as derived from the molecular evolutionary analysis of a 550-taxon data set (Borsch et al., pers. comm.). Microstructural changes in Piperales mostly involve one to three codons, rarely up to five codons. Since non-trimeric structural mutations are generally suppressed in coding regions (Metzgar et al., 2000), the *matK* gene therefore appears as a functional gene. The only situation where microstructural changes are not in triplets is associated with an internal microsatellite (Fig. 2). SSR expansion and contraction does not seem to be a rare process in coding regions, but often may result in changes of gene function (Li et al., 2004).

Microstructural changes in the *trnK* group II intron

The perhaps most striking microstructural variation is the extensive insertion of an AT-rich, microsatellite-like sequence in the domain I of the *trnK* intron. For the overall Piperales analysis, this portion had to be excluded (hotspot I), but at the species and even population level it has been found to be highly informative (Wanke et al., 2006a). As in other non-coding chloroplast DNA, most microstructural changes are SSRs of four to nine nucleotides in length, whereas length mutations involving one to two nucleotides are particularly rare. An overall higher frequency of microstructural changes in the *trnK* intron as compared to the *matK* gene also conforms to a general trend and has been found in other studies with complete *trnK*/*matK* sequences in Amaranthaceae (Müller and Borsch, 2005b), Lamiales (Müller et al., 2004; Rahmanzadeh et al., 2005) and Magnoliales (Sauquet et al., 2003).

Presence of *matK* pseudogenes in Piperaceae

The highly deviant copies of *matK* found in *Zippelia* can only be explained by the occurrence of several additional non-functional copies (paralogous pseudogenes) of *matK*. The presence in some copies of non-triplet microstructural changes in domain X which are not corrected shortly downstream, as well as the frequent occurrence of stop codons, are evidence for a pseudogenic nature of these additional *matK* copies. Given that all *matK* sequences from *Zippelia* group together in a parsimony analysis (trees not shown), it can be hypothesized that either gene duplication events occurred rather recently in an ancestor of *Zippelia begoniifolia* or that plastids might harbour different copies of the genome. Additional pseudogenic copies of *matK* have already been reported for other land plant lineages such as Bryophyta and Marchantiophyta (Jankowiak, 2004), Antocerotophyta and Lycophyta (Quandt et al., unpublished) and were also found in Valerianaceae (Hidalgo et al., 2004; Bell et al., 2001), and Nepenthaceae (Meimberg et al., 2006). Similar to Piperaceae, pseudogenes in Valerianaceae could be clearly distinguished from the functional *matK* CDS, and excluded from the actual phylogenetic analysis. There has been some discussion on a possible pseudogenic nature of *matK* in Orchidaceae, based on low transition-transversion ratios and the presence of internal stop codons (Kores et al., 2000). However, this has not been confirmed by other recent studies (Kocyan et al., 2004; Van den Berg et al., 2005). Recently, the non-functional *trnK/matK* copies in Nepenthaceae were found to be translocated to the mitochondrial genome (Meimberg et al. 2006). Since multiple PCR amplification products of *matK* in *Zippelia* were obtained with primers annealing to internal parts of the ORF (at least one primer), there is currently no information on where these *matK* copies may be located.

Although duplication of chloroplast genes resulting in paralogous copies is rare, there are reports of extensive *trnF* duplications in Asteraceae (Vijverberg and Bachmann, 1999) and Brassicaceae (Koch et al., 2005). For *trnF*, Koch et al., (2005) clearly showed the tandem arrangement of copies in the plastome, whereas the situation is less clear for *rbcL* paralogues which occur in several angiosperm lineages. Cummings et al. (2003) provided evidence in Brassicaceae, Solanaceae and monocots for *rbcL* transfers into the mitochondrial genome, although there may also

be paralogous *rbcL* copies in the plastome of Orobanchaceae (Wolfe and Randle, 2004).

**Figure 7**. Relative substitutional rates in Piperales with reference to *Peperomia* (outgroup: *Canella*, *Drimys*, and *Pseudowintera*). X axis: taxa, y axis: *d = K(Peperomia, outgroup) – K(taxa, outgroup)*; with *K(i,j)* = maximum likelihood estimate (GTR +G +I model) of substitutions per site between taxa. Significant rate differences (<0.0001) compared to *Peperomia* are indicated with an asterisk.
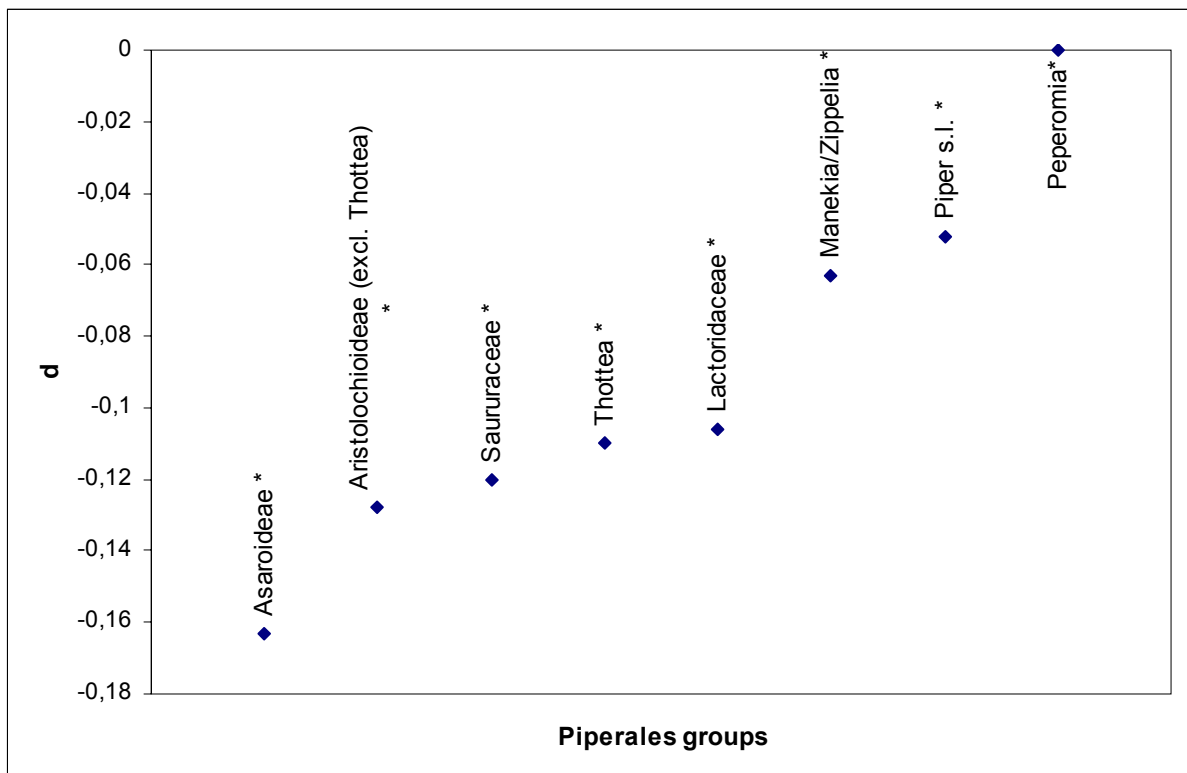


Rate heterogeneity and lineage specific resolution contrast

Relative rates of *Peperomia* and *Piper* are much higher as compared to Aristolochiaceae (Fig. 7) which is also paralleled by branchlength in Fig. 6. Nevertheless, internal resolution within the *Piper* clade is significantly less as compared to the *Peperomia* clade. Further comparison between those two lineages shows that internally the *Peperomia* clade exhibits about twice the amout of sequence variation in *trnK/matK* as compared to *Piper* (Table 5). The difference between genetic variation or parsimony informative sites within *Piper*, versus *Piper* compared to the outgroup, and the high relative rate indicates, that most of the parsimony

informative sites have been accumulated before the radiation of present *Piper* species but after the split of the *Piper-Peperomia* lineage. There are at least two possible explanations for the resolution contrast between *Piper* and *Peperomia*, two groups that show fairly similar global distribution patterns of extant species (although there are more species of *Piper* in tropical Asia). One is that rates continued to accelerate during the crown group diversification of *Peperomia*, thus leading to the accumulation of more variability among species. It seems that *Peperomia* species are more often narrow endemics, occupying specialized niches as epiphytes or succulents, what may lead to small effective population sizes. More work on these aspects is certainly needed. The other is that rates have slowed down in *Piper*, thereby hindering the accumulation of a fairly good amount of historic information. It may be assumed that the crowngroup diversification in both genera started at about the same time, considering their comparable global distribution. Unfortunately, in the absence of reliable fossil material, no molecular dating approach is available for Piperales. The situation described here parallels findings in Lentibulariaceae, where *trnK/matK* sequences with accelerated rates fully resolve the *Genlisea-Utricularia* clade (Müller and Borsch 2005) in contrast to its sister clade *Pinguicula* (Cieslack et al. 2005).

Relationships of *Lactoris*

This study provides strong evidence for the sistergroup relationship of *Lactoris* and Aristolochioideae and therewith the paraphyly of the family Aristolochiaceae as currently circumscribed. Depending on the combination of markers between different studies, or even within the same study, several molecular phylogenetic analyses have found Aristolochiaceae to be either para- or monophyletic (Duvall, 2000; Qiu et al., 2000; Doyle and Endress, 2000; Soltis et al., 2000; Savolainen et al., 2000; Hilu et al., 2003; Borsch et al., 2003; Borsch et al., 2005). However, several of these studies have not sampled both *Asarum* and *Saruma*, which could influence the branching pattern, and the supports for the relationships were often low. Based on morphological characters, the position of *Lactoris* was either hypothesized as close to Saururaceae (tenuinucellate ovules, development and morphology of stipules;

Igersheim and Endress, 1998; González and Rudall, 2001) or to Aristolochiaceae. The latter hypothesis was favoured by Doyle and Endress (2000) who listed several characters as potential synapomorphies for these taxa, e.g. presence of a perianth, presence of tepals, nearly sessile anthers that are strongly extrorse with a broad connective, and stamens basally fused with the gynoecium. But as already cited by González and Rudall (2001), most of these characters are symplesiomorphies of the whole order Piperales or even magnoliids. The basal fusion of stamens with the gynoecium as in *Lactoris* is present in *Aristolochia* but not in *Asarum*, *Saruma* or even *Thottea*. In addition, the only synapomorphy for Asaroideae and *Lactoris* cited by Doyle and Endress (2000) is the extended anther connective. From a molecular point of view, combining three fast evolving regions (Borsch et al., 2005) or a combination of nine genes from all three genomes (Qiu et al., 2005) increased the support for the most frequently found molecular tree (*Lactoris* sister to Aristolochioideae) to BS 89 (PP 100) in Borsch et al. (2005) or 78 (BS) for the protein coding genes in Qiu et al. (2005). In addition, the inclusion of Hydnoraceae could only enhance the poly-or paraphyly of Aristolochiaceae, as the datasets of Nickrent et al. (2002) already suggests.


Phylogeny of Aristolochiaceae

The two subfamilies of Aristolochiaceae, Aristolochioideae and Asaroideae, are each well supported in the present analysis. In most analyses, the monophyly of Aristolochiaceae was only well supported (molecular and morphology), if *Lactoris* was not included. Under inclusion of Lactoris the monophyly of Aristolochiaceae was often only poorly supported, but another position of Asaroideae within Piperales was not favored, thus the Asaroideae were unresolved and a branch of its own. The inclusion of this subfamily into Aristolochiaceae is a consequence of the historical treatment. Asaroideae and Aristolochioideae have never been seriously discussed as two independent lineages.

The subfamily Asaroideae consisting of the genera *Asarum* and *Saruma,* the sister relationship of these two genera, has been supported by other molecular (Qiu et al., 2000; Soltis et al., 2000; Neinhuis et al., 2005) and morphological analyses (Kelly and

González, 2003). The latter mentioned the PII sieve tube plastid inclusions, pluricellular stigmatic papillae and seeds with elaiosomes as synapomorphies. Aristolochioideae include the two major lineages *Thottea* and *Aristolochia* s.l.. Additionally, these two lineages are well circumscribed based on molecular (for an overview, see Neinhuis et al. 2005) and morphological data (for an overview, see Kelly and González (2003)). The *Aristolochia* s.l. clade comprises two lineages: the first containing *Isotrema* and *Endodeca*, the second containing *Pararistolochia* and *Aristolochia*. These results are congruent with former studies (González and Stevenson, 2002; Kelly and González, 2003; Neinhuis et al., 2005). Within *Aristolochia* the relationships of the informal group "*Howardia*" + segregates, *Einomeia* and *Aristolochia* s. str., all accepted by Huber (1985), remain unclear. These taxa have always been treated as *Aristolochia* except by Huber (1985), who further subdivided this clade into what he informally calls *"Howardia"* (hexandrous Central and South American species), *Einomeia* (pentandrous Central American species) and *Aristolochia* s.str. (Mediterranean and Paleotropical species). He also cited strong synapomorphies for the monophyly of these groups (e.g. the pentamerous organisation of the gynostemium in *Einomeia* (Huber, 1985; González and Stevenson, 2002, Kelly and González, 2003). The relationship of the segregates *Aristolochia grandiflora* complex, *Einomeia*, "*Howardia*" p.p. and *Aristolochia* s. str. are incongruent among the present and the analysis of Neinhuis et al. (2005), but the different branching patterns are in both poorly supported.


Phylogeny of Piperaceae

This study confirms the monophyly of the Piperaceae and its subdivision into two major clades: one including the large genera *Piper* and *Peperomia,* and another the small genera *Zippelia* and *Manekia*. The same topology was recovered by Jaramillo and collaborators (2004) based on the analysis of the slowly evolving genes 18S rDNA, *atpB*, and rbcL sequence data for a reduced sampling within *Piper* and *Peperomia*.

The first clade consists of the core Piperaceae with the large pantropical genera *Piper* and *Peperomia*. It is suggested that several outstanding characters in *Peperomia*

(e.g. paniculate inflorescences, peltate leaves) on which Dahlstedt's classification (Dahlstedt, 1900) has been based have evolved several times independently (Wanke et al., 2006d). Two subgenera from Dahlstedt (1900), *Micropiper* and *Sphaerocarpidium* (including *Erasmia*) and the three sections of subgenus *Rhynchophorum* could be regarded as monophyletic (Wanke et al., 2006d). This is also supported by morphological data. A very clear morphological synapomorphy for the subgenus *Micropiper* is the so-called pseudocupula at the base of the fruit (Dahlstedt, 1900). The fruits of all species of the subgenus *Sphaerocarpidium* are characterized by a large amount of sticky papillae distributed on the surface. The three sections of *Rhynchophorum* are each characterized by a typical fruit shape and fruit apex. The subgenus *Panicularia* was described on the basis of paniculate inflorescences but it is shown that this unusual character has evolved several times independently. The same accounts for the subgenus *Tildenia* in which the species with peltate leaves and shortened internodes are classified. The tuberous species belonging to the latter subgenus have been classified in a segregate section (Hill, 1907) which forms the basalmost clade in the genus *Peperomia*.

*Piper* (including *Macropiper*, *Pothomorphe*) is very diverse, varying in inflorescence position (terminal/axillary) and structure (solitary or clustered spikes or racemes), sexuality (bisexual or unisexual and then dioecious) and stamen number. The phylogeny presented here provides little resolution within *Piper* s.l. compared to earlier studies using the ITS region (Jaramillo and Manos, 2001). But the current analysis provides support for the monophyly of a Paleotropical clade, including taxa from both the Asian tropics and the South Pacific Islands. *Piper* species in the Paleotropics differ from their congeners in Tropical America in being dioecious plants with a climbing growing habit, while their Neotropical counterparts have bisexual flowers and several growing habits, i.e. shrubs, herbs treelets, but they are never real climbers. The monophyly of Paleotropical taxa had been suggested before (Callejas, 1986; Jaramillo and Manos, 2001) but it was never well supported. The segregates *Macrostachys*, *Enckea* and *Macropiper* are also highly supported (BS 100/100, 98/98 and 100/100), however, the present analysis does not provide support for many

segregates that had been supported in previous analyses using ITS sequence data (Jaramillo and Manos, 2001; Jaramillo et al., 2004; Jaramillo and Callejas, 2004).

The second clade consists of *Zippelia* (monotypic) and *Manekia* (5 to 6 species). Both taxa have been associated with *Piper* (de Candolle, 1866, 1923; Callejas, 1986). The herbaceous, Asiatic genus *Zippelia*, with a floral structure similar to *Saururus* (Omori, 1982), has been placed either in Saururaceae (Blume, 1830; Wu and Wang, 1957; Heywood, 1993) or in Piperaceae (Engler, 1893; Willis, 1957; Wu and Wang, 1958). In a cladistic analysis of taxa in Saururaceae and Piperaceae, mostly based on ontogenetic characters, the similarities between *Zippelia* and *Saururus* are identified as plesiomorphies and *Zippelia* appears as the basal taxon in Piperaceae (Tucker et al., 1993). *Manekia* was not included in this analysis. *Zippelia* shares several synapomorphies with Piperaceae, which indicate a close phylogenetic relationship with other taxa of Piperaceae, e.g. a double vascular cylinder in the stem, lack of discrete style, single ovule, basal placentation and fusion of two ventral bundles into one in each carpel (Liang and Tucker, 1995). *Zippelia* appears to represent a morphologically transitional genus between Saururaceae and Piperaceae, although indisputably belonging to the latter (Tucker et al., 1993). Several characters suggest that *Zippelia* is a more isolated evolutionary line in Piperaceae,  as expressed by floral development, which is different from the other piperaceous taxa (Liang and Tucker, 1995), as well as unique glochidiate fruits and a Drusa type of embryo sac (Lei et al., 2002).

The little studied genus *Manekia* from Central America, northern South America and the Atlantic Forest of Brazil is a liana with fleshy, axillary inflorescences, similar to those of *Peperomia*.


Phylogeny of Saururaceae

The close relationship of Saururaceae and Piperaceae and the monophyly of Saururaceae are unquestionable. Comprehensive studies support the relationship between the two families based on morphology (Doyle and Endress, 2000; Tucker et al., 1993) and the monophyly of Saururaceae based on molecular data (e.g. Neinhuis et al., 2005; Jaramillo et al., 2004; Meng et al., 2002, 2003). This is also supported by

our results. The relationships found based on *trnK*/*matK* support the relationships found from most other molecular studies ((*Saururus* + *Gymnotheca*) and (*Anemopsis* + *Houttuynia*)). Discrepancy occurs between morphological studies and molecular results within the family, and even among molecular results from different genomes. Most of the morphological and especially ontogenetic studies have considered *Saururus* to be the most basal branch, sister to all remaining Saururaceae (e.g. Tucker et al., 1993; Liang, 1995, Lei et al., 1991; Okada, 1986). The morphological characters, especially those of the flower within the perianthless Piperales have to be treated with caution. This was already mentioned by Jaramillo et al. (2004) and Neinhuis et al. (2005), as these plants show a high degree of reduction and fusion, which makes the detection of reversals or parallelisms more complicated.

Concluding the present study, the evolution of *trnK/matK* in Piperales presents a case of striking rate heterogeneity of this gene in flowering plants. Particularly high rates are present in *Peperomia*, leading to an internally well resolved gene tree for this clade. High rates are further reflected in the accumulation of numerous specific length mutations, including self repairing frame shifts. Nevertheless, a complete reading frame of the *matK* gene is maintained, with unrepaired frame shift mutations being restricted to the downstream end of the gene. Further work will be necessary to explore possible causes that lead to the observed rate heterogeneity, including sequence comparisons of other genomic regions, analysis of speciation patterns, population structures and effective population sizes.

**Acknowledgements**

# Chapter 3     Systematics of the genus *Aristolochia* (Aristolochiaceae)

## 3.1   Systematics of pipevines – Combining morphological and fast-evolving molecular characters to investigate the relationships within subfamily Aristolochioideae (Aristolochiaceae)

**Abstract**

A combined phylogenetic analysis of the Aristolochioideae was conducted based on 72 morphological characters and molecular datasets (*matK* gene, *trnK* intron, *trnL* intron, *trnL-trnF* spacer). The analysis sampled 33 species as the ingroup, including two species of *Thottea* and 30 species of *Aristolochia* and the monotypic genus *Euglypha*, which represent all the infrageneric taxa formally described; *Saruma henryi* and *Asarum caudatum* were used as the outgroup. The results corroborate a sister-group relationship between *Thottea* and *Aristolochia*, and the paraphyly of *Aristolochia* with respect to *Euglypha* that consequently should be included into *Aristolochia*. Two of the three subgenera within *Aristolochia* (*Isotrema* and *Pararistolochia*) are shown to be monophyletic, whereas the signal obtained from the different datasets about the relationships within subg. *Aristolochia* is low and conflicting, resulting in collapsed or unsupported branches. The relationship between the New World and the Old World species of subgenus *Aristolochia* is conflictive because morphological data support these two groups as monophyletic, whereas molecular data show the monophyletic Old World species of *Aristolochia* nested within the New World species. A sister group relationship is proposed between *A. lindneri* and pentandrous species, which suggests that a group of five species from central and southern South America (including *A. lindneri*) could be monophyletic and sister to *Aristolochia* subsection *Pentandrae*, a monophyletic taxon consisting of about 35 species from southern USA, Mesoamerica, and the West Indies.

**Introduction**

Aristolochiaceae, a member of the Piperales (Borsch et al. 2005, Qiu et al. 2005), consists of approximately 550 species, most of which are tropical, and subtropical. Although generic circumscription within the family has been in dispute for about two centuries (cf. González and Stevenson 2002, Neinhuis et al. 2005), recent authors recognize four genera in two subfamilies. The subfamily Asaroideae, characterized by an actinomorphic perianth, consists of two genera: the monotypic *Saruma,* endemic from central China, and *Asarum* with about 86 species from temperate areas of North America, Europe, and Asia.

The subfamily Aristolochioideae includes *Thottea*, with less than 30 species with an actinomorphic perianth, which are restricted to tropical Asia; and *Aristolochia* (including the monotypic South American generic segregates *Holostylis,* and *Euglypha*), which is by far the largest genus of the family. *Aristolochia* has a monosymmetric perianth and is primarily pantropical but with some offshoots in subtropical, and temperate areas. The most consistent synapomorphies that relate these genera are found on the seed coat (González and Stevenson 2002, González and Rudall 2003), which consists of a two cell-layered testa, and a three cell-layered tegmen. The cells of the inner layer of the testa have crystals and thickened inner walls; the three layers of the tegmen are tangentially elongated and fibrous; fibers of the outer, and inner layers are parallel to the longitudinal axis of the seed, whereas those of the middle layer are perpendicular to them.  In addition, the following unique combination of characters strongly suggests that these genera form a monophyletic family: alternate, distichous leaves with palmate, reticulate venation, adaxial prophylls, oil cells, trimerous perianth (double in *Saruma*), two (*Saruma*, *Asarum*, and some *Thottea* spp.) or one (*Aristolochia*, and the remaining *Thottea* spp.) whorl(s) of six stamens (five in *Aristolochia* subsection *Pentandrae* Duchartre, and more than six in most *Aristolochia* subgenus *Pararistolochia* Schmidt), six carpels, and pollen in monosulcate or inaperturate monads.

Throughout the taxonomic history of the family, every possible combination of generic relationships can be found (summarized in González and Stevenson 2002). However, the most accepted, but yet contradictory classifications at a subfamily level are those by Schmidt (1935), and Huber (1960, 1985, 1993). Schmidt (1935), following the classic treatment by Klotzsch (1859), proposed that Asaroideae consists of *Asarum* plus *Saruma* plus *Thottea*. In contrast, Huber (1985, 1993) transferred *Thottea* to the subfamily Aristolochioideae along with *Aristolochia.*

The genus *Aristolochia* has been treated in its broad sense by many authors (Duchartre 1854a, 1864, Hoehne 1942, Pfeifer 1966, 1970, Hou 1984, Nardi 1984, 1991, Ma 1989, among others). However, as many as 15 segregates have been proposed (for a detailed revision see González and Stevenson 2002), of which *Einomeia, Endodeca, "Howardia", Isotrema*, and *Pararistolochia* have recently been used at the generic level especially by Huber (1985, 1993). The splitting of *Aristolochia* is based primarily on floral, and fruit characters such as the morphology of the gynostemium, the gross shape of the perianth, the dehiscence of fruits, and the morphology of the seeds.

Furthermore, Huber (1985, 1993) recognized two tribes, Isotrematinae (with *Endodeca* Raf., and *Isotrema* Raf.), and Aristolochiinae (with *Aristolochia* s. str., *Einomeia, Euglypha* Chodat and Hassler*, Holostylis* Duchartre*, "Howardia"* Klotzsch, and *Pararistolochia* Hutch. and Dalziel). "*Howardia*" (an incorrect name; see discussion in Neinhuis et al. 2005) has been used for the hexandrous species of *Aristolochia* from the West Indies, and Central- and South America, and equals *Aristolochia* section *Gymnolobus* subsection *Hexandrae* Duchartre (1854a, 1864).

Recent phylogenetic analyses based on morphological (González and Stevenson 2002, Kelly and González 2003), and molecular data (Neinhuis et al. 2005) are consistent with Huber's inclusion of *Thottea* in Aristolochioideae. These analyses also support the monophyly of the generic segregates *Endodeca, Isotrema*, and *Pararistolochia*. Furthermore, they indicate that *Aristolochia* in its broad sense is

paraphyletic with respect to *Euglypha*, *Holostylis*, and *Einomeia* (= *Aristolochia* subsection *Pentandrae*)*.* Molecular data, in addition, separated *A. grandiflora*, and its allies from other "*Howardia*" (Neinhuis et al. 2005).

Murata et al. (2001) conducted a phylogenetic analysis of *Aristolochia* s. l. based on *matK* sequences. Although *Pararistolochia* was not sampled, their results supported the monophyly of subgenera *Isotrema*, and *Aristolochia*. Furthermore, they suggested that the New World species of *Aristolochia* could be paraphyletic with respect to the Old World species, and that the pentandrous *Aristolochia nelsonii* Eastwood, from Mexico, could form a clade with *A. burelae* Herz., from Bolivia and Argentina.

Because of the conflicts already mentioned, we conducted a simultaneous analysis of morphological, and fast evolving molecular data in order to test the monophyly, and the phylogenetic relationships within *Aristolochia s. l*., including all of the infrageneric taxa that have been proposed on the basis of morphological characters alone. In addition, we evaluated the congruence of morphological and molecular data, to propose or corroborate morphological synapomorphies of the major clades within the subfamily.

**Material and methods**

**Material**

DNA-Isolation, amplification and sequencing

Total genomic DNA was isolated from fresh, silica dried material or herbarium specimens. The voucher specimens are listed in Table 1. Details of protocols are given in Borsch et al. (2003) using a modified triple-extraction approach with CTAB following the miniprep procedure of Liang and Hilu (1996).

**Methods**

The *trnK*/*matK* region was generally amplified in two parts with an overlapping region of 250 to 400 bp, using the primers shown in Table 2. Amplification profiles generally followed an initial denaturation 1 min at 94°C, annealing 1.5 min at 48°C, elongation 3 min at 72°C, and a final extension 7 min at 72°C. Because of the high sequence variability within the *trnK* intron as well as the *matK* gene and because of periodically occurring poly-A or poly-T mononucleotide repeats, cryptic simple microsatellites, a large number of internal primers had to be designed specifically for the sequencing process. The Polymerase Chain Reaction (PCR) was carried out as a 25 µl reaction, containing 15 µl DNA template (1:100 delution genomic DNA), 3.3 µl dNTP mix (1.25 mM each), 0.5 µl of each primer (20 pmol/µl), and 1 u Taq polymerase with self adjusting $MgCl_2$ (Eppendorf).

The *trnL-F* region was amplified following the procedure of Neinhuis et al. (2005). Primer sequences are also given in Neinhuis et al. (2005).

Both PCR products, *trnK*/*matK*, and *trnL-F*, were purified by gel. The PCR products were then extracted using different commercial gel extraction kits (Macherey-Nagel NucleoSpin Extract II, and QIAGEN QiaQuick gel extraction) and directly sequenced with a CEQ DTCS Quick Start Kit (Beckman Coulter) on CEQ 8000 automated sequencer, following the standard protocol provided with each kit.

Sequence alignment and treatment of microstructural changes

Sequences were manually aligned using PhyDe[®] (Müller et al. 2005) following alignment rules proposed by Borsch et al. (2003), and Löhne & Borsch (2005). Microsatellite structures were excluded due to ambiguous homology assessment. Indels have been considered to be an additional source for phylogenetic information with a low level of homoplasy. The origin of indels was usually easy to recognize (simple sequence repeats). Thereafter, indels were automatically coded employing the simple indel coding algorithm (SIC) (Simmons and Ochoterena 2000) via SeqState (Müller 2004) using the PhyDe[®] plugin option. The alignment and the indel matrix can be obtained from www.treebase.org.

Selection of outgroup taxa

*Saruma henryi* and *Asarum caudatum* were used as outgroups. Choice of outgroup taxa is based on results from previous phylogenetic analyses of the family by González (1999a), González and Stevenson (2002), Kelly and González (2003), Murata et al. (2001), and Neinhuis et al. (2005). The family Lactoridaceae was excluded from our analysis (1) because of the conflictive relationships between Lactoris and Aristolochia (cf. González & Rudall 2001) and (2) because this study does not focus on the relationships within Aristolochiaceae but on the subfamily Aristolochioideae which is proven to be monophyletic. Therefore, *Lactoris* would be merely another outgroup member.

Phylogenetic analyses

Separate phylogenetic analyses were performed for each (morphological and molecular) dataset, and as combination of the molecular datasets only and as combination of molecular and morphological data. In both cases, the molecular datasets were analyzed either with substitutions only, or including coded length mutations. All trees calculated were based on the same maximum parsimony (MP) parameters, using the ratchet default settings of PRAP (Müller 2004) but with 10 random addition cycles. The generated PRAP file was executed in PAUP*4b10 (Swofford 2002). Evaluation of branch support was performed with 1000 bootstrap

replicates, and 10 random addition cycles on the MP results obtained from PRAP, and PAUP.

Table 1. Origin, accession number, and vouchers of the species included in the present analysis. Sequences which have been generated for a previous study are annotated with the source (# Wanke et al. 2006c; * Neinhuis et al. 2005).

| Species | Accession no. field origin / Botanical Garden | Voucher (Herbarium) | GenBank accession number | |
|---|---|---|---|---|
| | | | trnK/matK | trnL-F |
| A. acuminata Lamk. | BG Bonn, 17417 | Wanke & Neinhuis 146 (DR) | DQ532063# | AY689156* |
| A. acutifolia Duchartre | Colombia, Meta | González-4176 (COL) | DQ532048 | DQ532018 |
| A. albida Duchartre | BG Bonn, 17419 | Neinhuis 92 (DR) | DQ532064# | AY689153* |
| A. arborea Lindl. | BG Bonn, 02560 | Neinhuis 93 (DR) | DQ532044# | AY689175* |
| A. bracteolata Lamk. | BG Bonn, 16714 | Neinhuis 94 (DR) | DQ532059# | AY689159* |
| A. californica Torr. | BG Bonn, 15953 | Wanke & Neinhuis 143 (DR) | DQ532039# | AY689174* |
| A. clematitis L. | Croatia, Is Ilovik/Asinello | Starmühler (KL) | DQ532060 | DQ532019 |
| A. cordiflora Kunth | Colombia, Santander | González 3651 (COL) | - | DQ532020 |
| | BG Lankester | Holst 8602 | DQ532056# | - |
| A. eriantha Mart. & Zucc. | BG Bonn, 12952 | Neinhuis 99 (DR) | DQ532054# | AY689163* |
| A. gorgona Blanco | Costa Rica, Sarapiqui, Puerto Viejo | Blanco 1752 (USJ) | DQ532051# | DQ532022 |
| A. grandiflora Sw. | BG Bonn, 05629 | Neinhuis 122 (DR) | DQ532052 | DQ532029 |
| A. holostylis (Duchartre) F. González | BG Bonn, 02193 | Neinhuis 116 (DR) | DQ532057# | AY689162* |
| A. kaempferi Willd. | BG Dresden, 011482-16 | Wanke & Neinhuis s.n. (DR) | DQ532042 | DQ532023 |
| A. labiata Willd. | BG Bonn, 09867 | Neinhuis 96 (DR) | DQ532055# | AY689168* |
| A. leuconeura Linden | Panama, near STRI | González 4019 (COL) | DQ532058 | DQ532032 |
| A. lindneri Berg. | Bolivia, San Jose de Chiquitos | Ibisch s.n. (DR) | DQ532047 | DQ532031 |
| A. manshuriensis Komarov | BG Bonn, 13085 | Neinhuis s.n. (DR) | DQ532040# | - |
| | BG Würzburg s.n. | Neinhuis 104 (DR) | - | AY689184* |
| A. maxima Jacq. | Panama, Parnama | González 4018 (COL) | DQ532049 | DQ532030 |
| A. micrantha Duchartre | Westlund s.n., private coll. | Neinhuis 103 (DR) | DQ532046# | AY689170* |
| A. nummularifolia Kunth | Colombia, Meta | González 4175 (COL) | DQ532053 | DQ532025 |
| A. panamensis Standl. | Panama, Panama | González 4018B (COL) | DQ532043 | DQ532028 |
| A. pentandra Jacq. | Mexico, Cola de Caballo | Neinhuis 106 (DR) | DQ532045# | AY689169* |
| A. pichinchensis Pfeifer | Rio Paleque, Prov. Los Rios, Ecuador | Moran 6928 (QCA, NY) | DQ532050# | DQ532027 |
| A. pistolochia L. | France, Cassis, Calenque d'En Veau | leg. Kreft, Wanke 037 (DR) | DQ532062 | DQ532024 |
| A. promissa Mast. | BG Bonn, 13014 | Neinhuis 118 (DR) | DQ532065# | AY689173* |
| A. reticulata Nutt. | Westlund s.n., private coll. | leg. Westlund ,Neinhuis 108 (DR) | DQ532037# | AY689179* |
| A. rotunda L. | France, Corsica, Figaretto plage | Wanke 015 (DR) | DQ532061 | DQ532026 |
| A. serpentaria L. | USA, Texas, Travis | Westlund s.n. | DQ532038# | DQ532021 |
| A. tomentosa Sims. | BG Bonn, 02682 | Neinhuis 113 (DR) | DQ532041# | AY689178* |
| A. triactina Hook. f. | BG Bonn, 12767 | Neinhuis 119 (DR) | DQ532066# | AY689171* |

| | | | | |
|---|---|---|---|---|
| *Euglypha rojasiana* Chodat & Hassl. | BG München s.n., Brasil, Mato Grosso | Wanke s.n. (DR) | DQ861635 | DQ861636 |
| *Thottea corymbosa* (Griff.) Hou | Malaysia | Weber & Anthonysamy 870519-1/1 (WU) | DQ532036# | AY689152* |
| *Thottea siliquosa* (Lamk.) Hou | India, Kerala (BG Bonn, 09037) | Neinhuis 121 (DR) | DQ532035# | AY689151* |
| *Asarum caudatum* Lindl. | USA, Oregon, Mt. Hood | Neinhuis 88 (DR) | DQ532034# | AY689149* |
| *Saruma henryi* Oliv. | BG Bonn, 02618 | Neinhuis 120 (DR) | DQ532033# | AY145340* |

Table 2. Primers used in the present study (*trnK/matK*).

| Primer name | direction | Sequence (5'-3') | Design |
|---|---|---|---|
| MG1 | reverse | AAC TAG TCG GAT GGA GTA GAT | Liang & Hilu (1996) |
| MG15 | forward | ATC TGG GTT GCT AAC TCA ATG | Liang & Hilu (1996) |
| NYmatK 480F | forward | CAT CTG GAA ATC TTG STT C | Borsch (2000) |
| trnK 3914Fdi | forward | GGG GTT GCT AAC TCA ACG G | Johnson & Soltis (1995) |
| psbA-R | forward | CGC GTC TCT CTA AAA TTG CAG TCA T | Steele & Vilgalys (1994) |
| AR-matK-660R | reverse | A(CT)G GAT TCG CAT TCA TA | Scheplitz (unpublished) |
| AS-matK-670R | reverse | GA(AG) AGG ATT GTT TAC G(AG)A G | this study |
| AS-matK-460F | forward | TAC TTC CCT TTT T(ACT)G AGG | Wanke et al. 2006d |
| AR-matK-080R | reverse | ACT CCT GAA A(AG)A GAA GTG G | this study |
| TH-matK-420F | forward | AAC TGA ATA AAT GGA TAG AGC | this study |
| AR-matK-420F | forward | AAG TGA ATA AAT GGA TAG AGC | Wanke et al., 2006a |
| AR-matK-1850R | reverse | CCA GGC AAG ATA CTA AT | this study |
| AR-matK-1200F | forward | TTC CAA AGT CAA AAG AGC G | this study |
| AR-matK-1510R | reverse | TAG ACT CCT GAA A(AG)A GAA GTG G | this study |
| AR-matK-960R | reverse | AAC CTT TTC CCG CAT CAG G | this study |
| TH-matK-960R | reverse | AAC CTT TTC CCG CAT TAG A | this study |
| TH-matK-930F | forward | TAA TGC GGG AAA AGG TTC | this study |
| AR-matK-930F | forward | TAT TAG TAC CTG ATG CGG G | this study |
| AR-matK-780R | reverse | GGT CTT CTG AAA ATG ATT AC | this study |
| AR-matK-680R | reverse | CCG AGA AAA ACG AAT ATG GAT T | this study |
| AR-matK-1400F | forward | CTC TTT CAG GAG TCT ATC TAT G | this study |
| AR-matK-1450R | reverse | CGT TAG AGT TGC ACG TTA | this study |
| AR-matK-1510R | reverse | TAG ACT CCT GAA ARA GAA GTG G | this study |
| AR-matK-2100R | reverse | TGA AAA TGA TTA CAA AGC ACT AC | this study |
| AR-matK-2400R | reverse | ATT TTC TAG CAT TTG ACT CC | this study |
| AR-matK-2510R | reverse | AAA AAT CTC AAT AAA TGY AA | this study |
| AR-matK-3500R | reverse | ATC CAA ATA CCA AAT ASA TTC C | this study |
| AR-trnK-1320R | reverse | ATC GCT CTT TTG ACT TTG G | Wanke et al. 2006a |
| trnK-med-150F | forward | AGA GAA TAC TTC CAT CCT TAC CG | this study |
| trnK-med-440R | reverse | ATT CGT CTT TAC TCA CTC CGT A | this study |

The morphological data set is based on data published in González (1999a), and González and Stevenson (2002), which contained 66 species plus two composite terminals (subsection *Pentandrae* Duchartre, and the informal group *Dipharus* Klotzsch) in the ingroup.

This data set was reduced to match the sampling of the molecular data (33 species) for the present analysis; the two composite terminals were reduced to *A. micrantha,* and *A. pentandra* (representing subsection *Pentandrae*), and *A. labiata* (representing group *Dipharus*). On the other hand, the present analysis includes *A. albida*, *A. eriantha*, *A. gorgona*, and *A. pichinchensis*, which were not included by González (1999a) or González and Stevenson (2002). The morphological dataset (Table 3, Appendix 1) includes 72 characters, 11 of which are from vegetative structures, 10 from inflorescence architecture, 38 from flowers, and pollen, 6 from fruits, and 7 from seeds. All the multi-state characters (18) were treated as unordered.

**Results**

Four shortest trees were obtained in the simultaneous analysis of all available information (molecular, coded length mutations, and morphology; Figs 1, 2C). The trees have a length of 2461 steps (L), CI = 0.72, RI = 0.79, and RC = 0.57 (Tab. 4). About 22 % of the total number of characters (4162) are parsimony-informative (86% of the characters from the morphological dataset are parsimony-informative; Table 4). All main clades receive maximal or nearly maximal bootstrap support values. Only three (exclusively concerning the relationships of crown groups) clades have no bootstrap values. The analysis supports that *Thottea* and *Aristolochia s. l.* are sister taxa. Furthermore, it recovers at least nine taxa existing in the traditional classification, including *Thottea, Endodeca, Isotrema*, *Einomeia,* and *Pararistolochia*. Within subgenus *Aristolochia*, the Old World species appear as monophyletic; the monophyly of the New World species is still uncertain (Figs. 1, 2), due to the conflicts shown in the strict consensus tree (Fig. 2), which is affecting the whole subgenus *Aristolochia*. In addition, a sister group relationship between the two sampled species of *Aristolochia* subsection *Pentandrae* (= *Einomeia)* and *A. lindneri* is shown in all the analyses, with 100% bootstrap; however, the relationship of this subclade to the *A. grandiflora* complex, and to *Aristolochia* series *Thyrsicae* F. González (represented by *A. acutifolia*, and *A. maxima*) is also unsupported in the analysis based on morphology plus molecular plus coded length mutations.

The combined molecular analysis (included coded length mutations) comprises a sum of 4090 characters (843 of which are parsimony-informative; Table 4); only two trees are obtained (Fig. 2B), with L = 2281, CI = 0.74, RI = 0.79, and RC = 0.58. Bootstrap values are also higher than 90% in most of the clades; only the relationships of crown subclades of subgenus *Aristolochia* have bootstraps lower than 50% (Fig. 2B). *Euglypha rojasiana* gains maximum support as sister to "*Howardia*" pro parte (p.p.) in most analyses or as part of a polytomy with "*Howardia*" p.p..

Table 3. Morphological matrix.; characters and character states are detailed in Appendix 1 (A = 1, 2; - = inapplicable; ? = unknown).

| | 0 0 - | 1 0 - | 2 0 - | 3 0 - | 4 0 - | 5 0 - | 6 0 - | 7 0 - |
|---|---|---|---|---|---|---|---|---|
| Saruma henryi | 0000-00000 | 0000000000 | 0000000000 | 000000000- | ---0000000 | 0000000000 | 0000000000 | 00 |
| Asarum caudatum | 0000-00000 | 0000000000 | 0110000000 | 000000000- | ---0000000 | 0001000110 | 0110000000 | 02 |
| Thottea corymbosa | 1?01001000 | 12?1?????? | 1110000?0- | ---0000000 | 2100100320 | 0111?2?1?0 | 0012002101 | 10 |
| T. siliquosa | 1101001000 | 111000010- | 1110000?0- | ---0000000 | 2101100320 | 0111?2?1?0 | 0012002101 | 10 |
| Euglypha rojasiana | 1101001000 | 10001---0- | 0121012101 | 0111011000 | 3101111000 | 0111011000 | 003-010001 | 00 |
| Aristolochia acutifolia | 1101011101 | 1010000100 | 1120012102 | 0111011000 | 3101110420 | 0111011000 | 0001001011 | 10 |
| A. acuminata | 1101101000 | 1010010101 | 0121012110 | 0111011000 | 3101110420 | 2111012010 | 0001001021 | 10 |
| A. arborea | 1101101001 | 1010000100 | 1120011100 | 0020010001 | 3111111220 | 2111012010 | 0010000000 | 00 |
| A. bracteolata | 1101001000 | 100001010- | 0121012110 | 0111011000 | 3101110420 | 0111010010 | 0001000001 | 00 |
| A. californica | 1101001001 | 000001010- | 0120011000 | 0020000000 | 3111111220 | 0111012010 | 0010000001 | 00 |
| A. clematitis | 1101101000 | 100000010- | 0120012110 | 0111011000 | 3101110420 | 0111010010 | 0000001001 | 00 |
| A. cordiflora | 1101001000 | 11011---1- | 0120012101 | 1111011000 | 3101111100 | 0111010000 | 0001001001 | 00 |
| A. gorgona | 1101001000 | 100001110- | 1120011102 | 0001111100 | 3101111100 | 0111010000 | 0010001001 | 30 |
| A. grandiflora | 1101001000 | 100001110- | 1120011102 | 0001111100 | 3101111100 | 0111010000 | 0001001001 | 30 |
| A. holostylis | 1101001000 | 11011-0-01 | 1001210000- | ---0011000 | 3101111220 | 0111010000 | 0010000001 | 00 |
| A. kaempferii | 1101001001 | 000001010- | 0120011100 | 0020100000 | 3111111220 | 0111012010 | 0010000001 | 00 |
| A. labiata | 1111001010 | 10001---0- | 0120012101 | 1111012000 | 3101110000 | 0111010000 | 0001001021 | 10 |
| A. leuconeura | 1101001000 | 11011---1- | 0120012102 | 0111011010 | 3101111010 | 0111010000 | 0001001001 | 01 |
| A. lindneri | 1101001000 | 100001110- | 1120012102 | 0020010000 | 3111111140 | 0111010000 | 0001001001 | 30 |
| A. manshuriensis | 1101001001 | 000001010- | 0120011100 | 0020100000 | 3111111220 | 0111012010 | 0010001000 | 00 |
| A. maxima | 1101001101 | 100000010- | 1120012102 | 0111011000 | 3101111220 | 0111010000 | 0001101011 | 10 |
| A. micrantha | 1101001000 | 100001010- | 0120012101 | 0111011000 | 4101111220 | 1111110000 | 0010001001 | 30 |
| A. nummularifolia | 1101001000 | 10001---0- | 0120012101 | 0111011000 | 3101111220 | 0111012010 | 0010000001 | 00 |
| A. panamensis | 1101001001 | 1000000100 | 1120011101 | 0020010000 | 3111111220 | 0111012010 | 0010000000 | 00 |
| A. pentandra | 1101001000 | 100001010- | 0120012102 | 0111011000 | 4101111420 | 1111111000 | 0010001001 | 30 |
| A. pichinchensis | 1101001000 | 100001110- | 1120011102 | 0001111100 | 3101110420 | 0111010000 | 0001001001 | 30 |
| A. pistolochia | 1101101000 | 100000010- | 0120012110 | 0111011000 | 3101110420 | 0111010010 | 0001001001 | 00 |
| A. promissa | 1101011000 | 1010000101 | 0120011102 | 0111110100 | 2101111222 | 0111013011 | 113-000001 | 00 |
| A. reticulata | 0101001000 | 1011010110 | 0120011100 | 0010100000 | 3111111220 | 01101A010 | 0010000000 | 00 |
| A. eriantha | 1101001010 | 10001---0- | 0120012100 | 1111013010 | 3101113010 | 0111010000 | 0001001001 | 30 |
| A. rotunda | 1100001000 | 10001---0- | 0120012101 | 0111011000 | 3101110420 | 0111010010 | 0000000000 | 00 |
| A. serpentaria | 0101001000 | 100101010- | 0120011100 | 0010100000 | 3111111220 | 01101A010 | 0010001001 | 00 |
| A. tomentosa | 1101011001 | 0010000000 | 0120011100 | 0020100000 | 3111111220 | 0111012010 | 0010001001 | 00 |
| A. triactina | 1101011000 | 1010000101 | 0120011102 | 0111011000 | 2101111222 | 0111013011 | 113-000001 | 00 |
| A. trilobata | 1111001010 | 10001---0- | 0120112101 | 1111011100 | 3101110420 | 0111010000 | 0001001021 | 10 |

Table 4. Statistics comparing the results from different datasets and the analyses conducted.

| | Morphology | Indels* | trnK/matK | trnK/matK + Indels | trnL-F | trnL-F + Indels | comb. molecular | comb. molecular + Indels* | morphology + molecular | morphology + molecular + Indels* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Figure** | **n. s.** | **n. s.** | **n.s.** | **n.s.** | **n.s.** | **n. s.** | **2A** | **2B** | **2C#** | **1, 2C** |
| total number of characters. | 72 | 234 | 2690 | 2817 | 1166 | 1273 | 3856 | 4090 | 3928 | 4162 |
| constant characters | 1 | - | 1771 | 1771 | 903 | 903 | 2674 | 2674 | 2674 | 2674 |
| uninformative characters | 11 | 121 | 331 | 402 | 121 | 171 | 452 | 573 | 463 | 584 |
| number and percentage of parsimony-informative characters | 61 (85.9) | 113 (48.3) | 588 (21.9) | 644 (22.9) | 142 (12.2) | 199 (15.6) | 730 (18.9) | 843 (20.6) | 791 (20.1) | 904 (21.7) |
| number of most parsimonious trees | 43 | 730 | 68 | 2 | 6 | 229 | 8 | 2 | 6 | 4 |
| length | 164 | 334 | 1555 | 1732 | 382 | 552 | 1942 | 2281 | 2122 | 2461 |
| CI | 0.60 | 0.70 | 0.73 | 0.73 | 0.80 | 0.75 | 0.74 | 0.74 | 0.73 | 0.72 |
| RI | 0.80 | 0.70 | 0.80 | 0.80 | 0.82 | 0.76 | 0.80 | 0.79 | 0.80 | 0.79 |
| RC | 0.48 | 0.49 | 0.59 | 0.59 | 0.70 | 0.60 | 0.60 | 0.58 | 0.58 | 0.57 |
| clades resolved | 18 | 9 | 25 | 31 | 25 | 18 | 28 | 31 | 26 | 28 |
| polytomies in the strict consensus tree | 4 | 4 | 3 | 1 | 2 | 3 | 2 | 1 | 3 | 2 |

* calculated from the combination of all molecular datasets (coded length mutation)

n. s. trees not shown

# topology as figure

Fig. 1 Strict consensus tree based on the combined molecular and morphological datasets (substitutions and coded length mutations). Supports above each branch are bootstrap values. Segregated genera or infrageneric taxa previously proposed, and New World/Old World distribution are shown to the right. Asterisks indicate taxa originally treated as part of "*Howardia*". Pentandrous species are indicated with [#]. 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 ring-like 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 *A. serpentaria*): herbaceous shoots, with scale-like, not clasping 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.
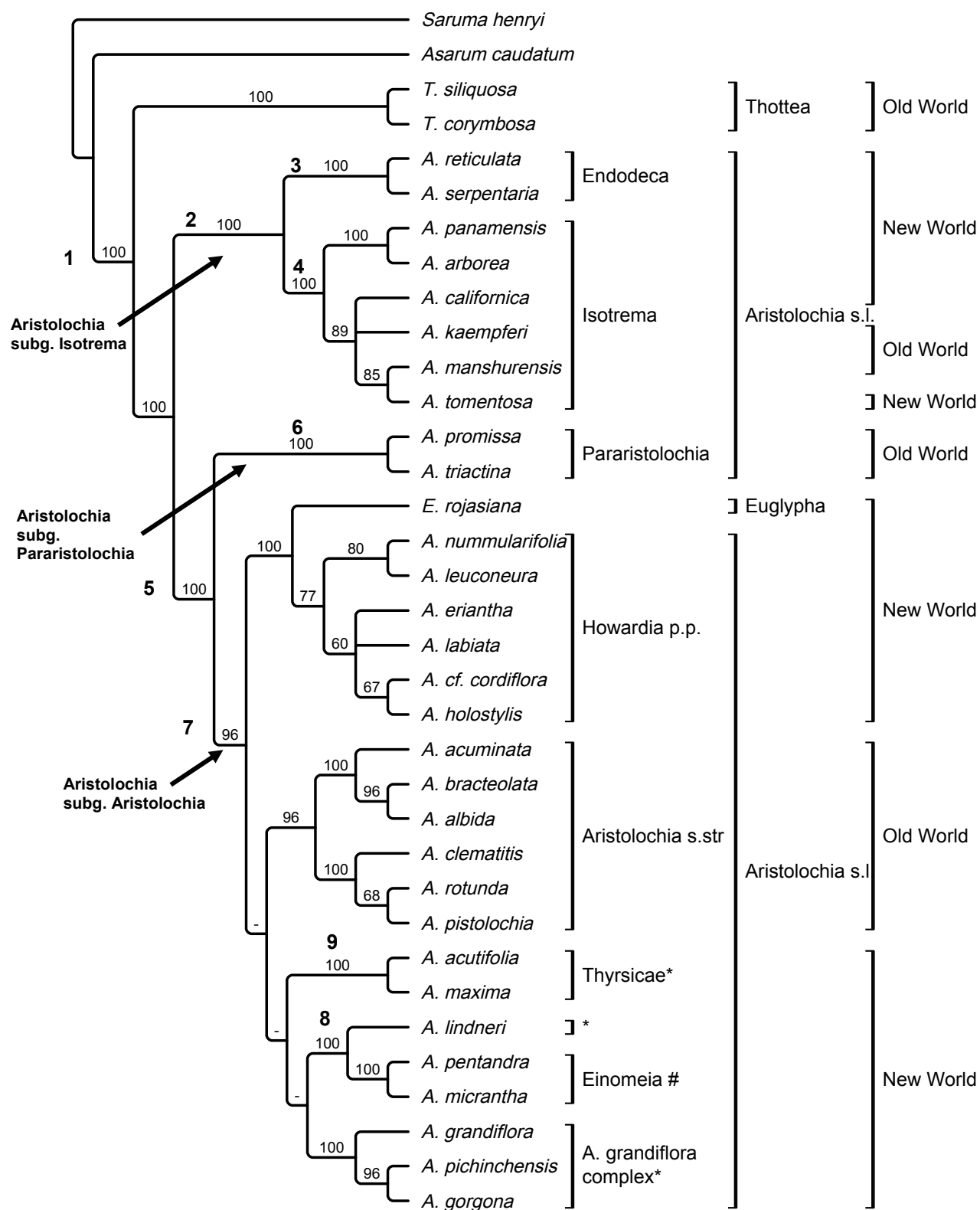
See next page.

Fig. 2 Simplified summary of trees (strict consensus trees, with bootstrap values) resulting from combination of different datasets, showing the incongruence between them. The taxa *Isotrema* and *Endodeca* are not shown, as relationships remain constant throughout all analysis. A) combined molecular datasets (based on substitutions only), B) combined molecular datasets (incl. coded length mutations), C) combined molecular datasets (based on substitutions + coded length mutations) + morphology.
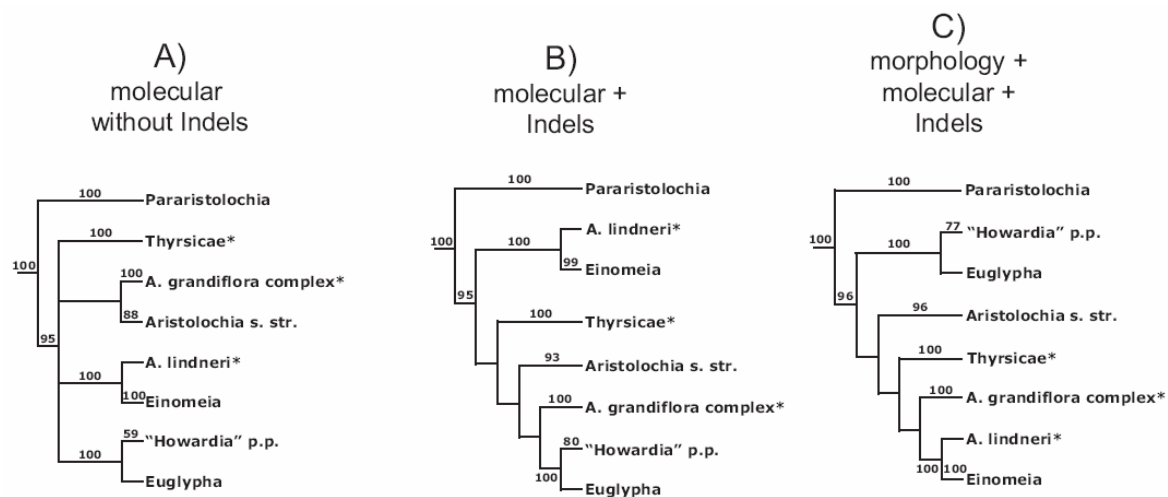
**Discussion**

The results of the simultaneous analyses based on molecular data alone, and on molecular plus morphological data are consistent with Huber's (1985, 1993) inclusion of *Thottea* in Aristolochioideae. The inclusion of *Thottea* in Aristolochioideae is supported by the following morphological synapomorphies: primarily woody plants, base of the petiole `U´ or `V´ shaped, hooked trichomes, partial inflorescence consisting of more than one flower (González 1999b), bracts distinct in shape from the normal leaves, abscission zone between the inferior ovary, and the rest of the flower, and stamens primarily arranged in one whorl.

Our analysis also supports a sister-group relationship between the *Aristolochia* segregates *Isotrema* and *Endodeca* and confirms the sister-group relationship between subgenera *Aristolochia* and *Pararistolochia*. Two morphological synapomorphies (a three-lobed gynostemium, and an annulus, a ring-like structure around the mouth of the perianth that is variously modified in some Central American species; González and Stevenson 2000b) support the sister-group relationship within the two main clades of subgenus *Isotrema* (that is, the segregates *Isotrema*, and *Endodeca*). On the other hand, the sister group relationship between subgenera *Pararistolochia*, and *Aristolochia* is supported by the presence of conical trichomes, especially throughout the inner surface of the perianth tube which are partially responsible for the trap mechanisms of the flowers. Subgenus *Pararistolochia* is represented by more than twenty species from Africa, and Australasia; morphological support for the monophyly of this subgenus comes from the gynostemium primarily with more than six (and up to 24) stigmatic lobes, and from the pollen grains, which develop a long, and massive exine ridge (González 1999a; González and Stevenson 2002).

Conflictive topologies are found within subgenus *Aristolochia* (Figs 1, 2A-C), where nearly all possible sistergroup relationships could be observed based on the combination of different data sets. The main incongruence involves the relationships of the Old World clade, and the New World species. The strict consensus trees resulting from all combined datasets (Figs. 2A, 2B, and 2C, respectively) are in favor of the hypothesis that the New World species are paraphyletic, although the support values are low. In contrast, the analysis based on the morphological data set alone

(not shown; see González 1999a, González and Stevenson 2002), supports the monophyly of the New World species of subgenus *Aristolochia*, which in turn are sister to the Old World species (i.e. *Aristolochia* s. str.). The tree obtained from the combined molecular data set (incl. indels) (Fig. 2B) differs from that obtained in the analysis based on morphological plus molecular data (incl. indels) (Fig. 1, 2C) in the placement of the Old World species of subgenus *Aristolochia* (=*Aristolochia* s. str.) which appear as sister only to the *Aristolochia grandiflora* complex + "Howardia" p.p. (incl. *Euglypha*) in the former (Fig. 2B), but to the New World subclade (*Aristolochia* ser. *Thyrsicae* (*Aristolochia grandiflora* complex (*Aristolochia lindneri* plus *Einomeia*))) in the latter (Fig. 2C).

Most of the infrageneric taxa traditionally described within subgenus *Aristolochia* (cf. Duchartre 1854a, 1864, Masters 1875, Schmidt 1935, Hoehne 1927, 1942) were established primarily on the gross shape of the mature perianth, which is extremely variable and supplies some of the most important diagnostic characters at the species level. However, its phylogenetic information is limited because it is highly homoplastic (González and Stevenson 2000b, 2002). Our results identify only a few characters related to the perianth that are synapomorphic at different levels (see Conclusions). These are: a ring-like structure around the perianth fauces in segregates *Isotrema* plus *Endodeca*; the floral tube evenly inflated and almost as wide as the utricle in subgenus *Isotrema*; conical hairs inside the perianth, and a floral tube slightly curved in subgenera *Pararistolochia* plus *Aristolochia*; a perianth abaxially concave and completely monosymmetric (formed by the complete fusion of the three sepals) in subgenus *Aristolochia*.

González (1990, 1991, 1997) proposed an alternative classification for the New World species based on characters from leaves, inflorescences, fruits, and seeds. So far, this classification is in general consistent with the phylogenies derived from molecular and combined datasets, especially because of the recognition of subseries *Thyrsicae*, a taxon of about 30 species supported as monophyletic by the following characters: presence of a zone of abscission in the base of the petiole, the base of the partial inflorescences, and the base of the peduncle; capsules with latticelike septa; and seeds broadly oblong with two wings.

The sister-group relationship between *A. lindneri*, from Bolivia and Paraguay, and the pentandrous species (*A. micrantha,* and *A. pentandra*) is supported by 100% bootstrap in all the analyses substantiating earlier results from Murata et al. (2001) based on only one pentandrous species sister to the hexandrous *A. burelae*, from Bolivia and Argentina. These two independent pieces of evidence are also supported by the following morphological characters in common between all the pentandrous species and a group of five species from central, and southern South America (*A. burelae*, *A. lindneri, A. lozaniana, A. stukhertii,* and *A. urbaniana*): Partial inflorescences cymose and reduced to one or two bracteate flowers, the bracts peltate or at least wide, and strongly clasping, the pollen grains reticulate with large supratectal warts, the capsules opening from the tip, and the seeds smooth, triangular, thick, non-winged, with the funicle linear, and completely fused to the seed proper (detailed descriptions of these characters are fully explained in González 1999a, 1999b, 2001, González and Stevenson 2002, and González and Rudall 2003). The disjunct distribution of these two monophyletic taxa (subsection *Pentandrae*, on one hand, and *A. burelae, A. lindneri, A. lozaniana, A. stukhertii,* and *A. urbaniana* on the other) is similar to that found in closely related taxa in the Apiaceae, and Hydrophyllaceae (Constance 1963; Heckard 1963; see also the review by Raven 1963), which suggests the existence of a biogeographic pattern between sister taxa primarily found in the subtropical belt of North America, and Mesoamerica, on one hand, and the subtropical belt of South America, on the other.

Finally, both the simultaneous, and the independent analyses corroborates that *Aristolochia holostylis* (formerly *Holostylis reniformis*) is nested within a group of South American species of *Aristolochia*, near *Aristolochia cordiflora*, as was previously suggested (González 1997, 1999a, González and Stevenson 2002). In fact, the new combination *Aristolochia holostylis* (Duchartre) F. González has already been made (González 1999c). Nesting of *Euglypha rojasiana* Chodat & Hassl. within the main New World clade of *Aristolochia* has also been proposed based on morphological data (González 1997, 1999a, González and Stevenson 2002), and is substantiated by our molecular data. *Euglypha,* a small climber of central South America, had been separated due to the constriction at the base of the utricle (resembling the subsection Podanthemum in *Aristolochia* s.str.) and the unusual fruit containing only one or two seeds per locule.

## Conclusions

The simultaneous molecular, and morphological analysis supports the recognition of two main pairs of lineages within *Aristolochia* s. l.: the segregates *Endodeca* plus *Isotrema*, and the subgenera *Pararistolochia* plus *Aristolochia* (incl. *Euglypha*). All main clades within Aristolochioideae reconstructed in the analysis are easily recognized by at least one morphological synapomorphy. In fact, names as old as *Endodeca* (Rafinesque 1828), *Isotrema* (Rafinesque 1819), and *Pararistolochia* (Hutchinson and Dalziel 1927), described on the bases of morphological grounds, could easily be validated as monophyletic taxa, which demonstrate the congruence between molecular data, and morphological traits. *Aristolochia holostylis* (=*Holostylis reniformis*) is nested within the bulk of South American species of *Aristolochia*, perhaps as sister to the *Aristolochia cordiflora* group; therefore *Holostylis reniformis* should be definitely abandoned. The genus *Euglypha* should also be abandoned as proposed by González (1997) and included into *Aristolochia*, and consequently named *Aristolochia rojasiana* (Chodat & Hassl.) F. González.

## Acknowledgments

**Appendix 1.** Morphological characters used in the present analysis. Most of the morphological characters included in this analysis were taken from living material, herbarium and spirit specimens, and field observations. Literature has been employed only when data were insufficient or unclear. All the multistate characters were treated as unordered.

0. Habit. (0) herbaceous, (1) woody at least in the roots and/or rhizomes.

1. Growth units. (0) sympodial (1) monopodial (indeterminate). Whereas growth units of *Saruma* and *Asarum* are sympodial, those of *Aristolochia*, *Euglypha*, *Holostylis*, and *Thottea* are monopodial; some species of subgenus *Pararistolochia* from Australia and New Guinea have sympodial growth units (González 1999b).

2. Elongating shoots. (0) nearly straight, (1) strongly sinuous. The elongating shoots of most of the Aristolochiaceae are nearly straight, but in some neotropical members of *Aristolochia*, they are strongly sinuous.

3. Number of axillary buds. (0) one, (1) two or more. In *Asarum* and *Saruma* there is one axillary bud per node whereas in *Aristolochia*, *Euglypha*, *Holostylis* and *Thottea* there are at least two axillary buds per node (González 1999b).

4. Arrangement of axillary buds. (0) uniseriate, (1) biseriate.

5. Mature stems. (0) circular, (1) medially constricted. Stems in some species of *Aristolochia* become medially constricted thus producing a "figure 8" form in transverse section.

6. Hooked trichomes. (0) absent, (1) present. Hooked trichomes are present in both vegetative and reproductive organs of *Aristolochia*, *Euglypha*, *Holostylis* and *Thottea*. Although the number of cells on each trichome can vary from 3 to 8 in these taxa, the apical cell is always hook-shaped.

7. Leaf expansion. (0) normal, (1) delayed. In most of the species, the leaf primordium begins differentiation into petiole and blade in plastochrone 3-4 and the blade expands relatively rapidly; in other species the differentiation occurs at a late stage, and blade expansion is delayed (González and Stevenson 2002).

8. Vegetative prophylls. (0) non-pseudostipular, (1) pseudostipular. In a group of neotropical species, the prophyll of each renewal shoot develops into a sessile, round, clasping leaf called a pseudostipule (Duchartre 1854b, González 1990).

9. Petiole abscission zone. (0) absent, (1) present (González 1990, 1991, 1997, 1999b).

10. Petiole base. (0) U-shaped, (1) semicircular (González and Stevenson 2002).

11. Position of the partial florescences. (0) along leafy, elongated, main branches, (1) lateral racemes (Fig. 11D), (2) anthoblasts (González 1999b).

12. Partial florescence. (0) uniflowered, (1) bi/multiflowered (González 1999b). Inflorescence development of *Thottea corymbosa* and *T. grandiflora* is not known, preventing the coding of this and characters 13-19 in these species.

13. Pherophylls (0) leafy, (1) reduced (González 1999b).

14. Flower. (0) bracteate, (1) non-bracteate (González 1999b).

15. Bract expansion. (0) non-clasping, (1) clasping.

16. Bract base. (0) non-peltate, (1) peltate.

17. Bract shape. (0) similar in shape and size to leaves, (1) reduced (González and Stevenson 2002).

18. Inflorescence internodes. (0) elongated, (1) shortened (González 1999b).

19. Inflorescence phyllotaxis. (0) distichous, (1) helicoid (González and Stevenson 2002).

20. Peduncle abscission zone. (0) absent, (1) present (González 1990, 1991, 1997, 1999b).

21. Perianth series. (0) two, (1) one.

22. Perianth shape. (0) rotate, (1) bell-shaped, (2) tubular.

23. Perianth. (0) non-stipitate, (1) stipitate. This character is not applicable to *Asarum* and *Saruma*, because the perianth in these genera is continuous with the peduncle. In the other genera, there is a deep constriction between the perianth and the peduncle, above which the perianth may have a stipe or not.

24. Perianth base. (0) symmetrical, (1) strongly asymmetrical.

25. Perianth. (0) not differentiated, (1) differentiated into utricle, tube and limb. In *Aristolochia s.l.* (that is, including, *Euglypha* and *Aristolochia holostylis*) the perianth has three parts, an inflated portion at its base called utricle, which extends into a more or less narrowed portion, the tube; the distal expanded part of the perianth, above the tube, is called the limb. The tube in *Aristolochia holostylis* is shortened, but can be detected as an area between the utricle and the limb that lacks trichomes on the inside. The perianth in the remaining genera is not differentiated into distinct parts (González and Stevenson 2000b).

26. Perianth concavity. (0) absent, (1) adaxial, (2) abaxial (González and Stevenson 2000b).

27.  Perianth abscission zone.  (0) absent, (1) present.  In all species of *Aristolochia* and *Thottea*, a constriction is formed above the ovary that functions as an abscission zone by means of which the perianth falls off along with the gynostemium at late anthesis.

28.  Second order perianth veins.  (0) present, (1) absent.  Flowers of some species of *Aristolochia* lack the second order veins that run longitudinally along  the base of the perianth.  Thus, the perianth is supplied only by the six veins that enter the perianth from the ovary (González and Stevenson 2000b).

29.  Syrinx.  (0) absent, (1) incomplete, (2) complete.  The syrinx is an inner flange formed between the utricle and the tube.  This character is not applicable to *Asarum*, *Saruma* and *Thottea*, because the flowers in these genera are not differentiated into utricle and tube.

30.  Tube position.  (0) longitudinal, (1) oblique.  At anthesis, the tube extends straight out from the utricle or is oblique to it, thus forming a sharp angle.

31.  Tube curvature.  (0) strong (U-shaped), (1) slight (González and Stevenson 2000b).

32.  Tube shape.  (0) distally inflated, (1) not inflated, (2) evenly inflated and almost as wide as the utricle.

33.  Conical perianth trichomes. (0) absent, (1) present (Huber 1985, González and Stevenson 2000b).

34.  Annulus.  (0) absent, (1) present.  The annulus is a circular flange at the juncture between the tube and the limb.

35.  Limb symmetry.  (0) regular, (1) monosymmetric (González and Stevenson 2000b).

36.  Limb lobes at anthesis.  (0)  three, (1) one, (2) two, one upper and one lower, (3)  two, lateral.

37.  Tail-like appendages on perianth.  (0) absent, (1) present.

38.  Limb.  (0) non fimbriate, (1) fimbriate.

39.  Limb protrusion.  (0) absent, (1) present.

40.  Stamen number.  (0) 12, (1) 24, (2) 8-10, (3) 6, (4) 5, (5) >25.

41.  Stamen series.  (0) two, (1) one.

42.  Stamens.  (0) equidistant, (1) grouped (González and Stevenson 2000a).

43.  Stamens.  (0) free, (1) fused forming a gynostemium (González and Stevenson 2000a).

44.  Stamen dehiscence.  (0) functionally introrse, (1) extrorse.  Anthers are functionally introrse in *Saruma* (Oliver 1889, Dickison 1992, Endress 1995, González and Stevenson 2000a).

45.  Anthers.  (0) with filament, (1) sessile.

46.  Anther length.  (0) short, (1) long.  In mature flowers of some species of *Aristolochia*, the length of the anthers is less than half the length of the gynostemium.  In others, the anthers are considerably longer (González and Stevenson 2002).

47.  Pollen sculpturing. (0) reticulate, (1) microreticulate, (2) fossulate, (3)  areolate, (4) psilate (González 1999a).

48.  Pollen aperture.  (0) sulcate, (1) porate, (2) inaperturate (González 1999a).

49.  Pollen ridge.  (0)  absent, (1) poorly differentiated, (2) markedly differentiated (González 1999a; González et al. 2001).

50.  Supratectal warts.  (0) none, (1) small, (2) large (González 1999a).

51.  Ovary position.  (0) half-inferior, (1) inferior.

52.  Ovary shape.  (0) globose, (1) linear (elongated and narrow).

53.  Carpels.  (0) partially apocarpous, (1) syncarpous.

54.  Mature carpels.  (0) 6, (1) 5, (2) 4.

55.  Stigmas.  (0) free, (1) connate.

56.  Gynostemium lobes.  (0) 6, (1) 5, (2) 3, (4) 8-10, (5) 12 (González and Stevenson 2000a, 2002).

57.  Stigmatic papillae.  (0) present, (1) absent.

58.  Position of the stigmatic papillae.  (0) terminal, (1) lateral/basal (González and Stevenson 2000a, 2002).

59.  Fruit surface.  (0) smooth, (1) verrucate.

60.  Pericarp.  (0) membranous to chartaceous, (1) strongly lignified.

61.  Mesocarp.  (0) dry, (1) fleshy.

62.  Fruit.  (0) ventricidal, (1) septifragal, (2) irregularly dehiscent, (3) indehiscent.

63.  Fruit dehiscence.  (0) basipetal, (1) acropetal.

64.  Fruit septae.  (0) entire, (1) lattice-like.

65.  Seeds per carpel.  (0) >5, (1) 1-2.

66.  Seed contour.  (0) concave-convex, (1) flattened, (2) trigonous.  In transverse section, the contour of the seed proper appear concave-convex, flattened, or extremely curved and with the margins touching each other (Hou 1981, Huber 1985, González and Stevenson 2002).

67.  Shape of the seed proper.  (0) ovoid, (1) ellipsoid (González and Stevenson 2002).

68. Seed wings. (0) absent or vestigial, (1) two, rectangular, (2) one, triangular-rhomboidal (González and Stevenson 2002).

69. Funicle. (0) free from the seed, (1) fused to the seed (Huber 1985, González 1999a, González and Stevenson 2002, González and Rudall 2003).

70. Funicle. (0) massive, (1) filiform, (2) papery, incomplete, (3) papery, complete. (González and Stevenson 2002, González and Rudall 2003).

71. Sticky aril. (0) absent, (1) chalazal-funicular, (2) *Asarum* type, (3) funicular. (González 1990, González and Stevenson 2002, González and Rudall 2003).

## 3.2 Colonisation, phylogeography and evolution of endemism in Mediterranean *Aristolochia* (Aristolochiaceae)

**Abstract**

This study provides evidence for a multiple colonisation of the western Old World from Asian ancestors within *Aristolochia* section *Diplolobus* (subsection *Aristolochia* and *Podanthemum*). Within subsection *Podanthemum* it is assumed, that the colonisation of the African continent happened at least two times independently. In contrast, for subsection *Aristolochia*, a rapid morphological radiation in the Near East (or close to this area) with subsequent star like colonisation of the different current distribution areas, which is not paralleled on the molecular level, appears to be more likely. Phylogenetic tree reconstruction is unsupported for these clades, but most clades are highly supported as monophyletic. Interestingly the Mediterranean and temperate Eurasian species, which are morphologically distinct (*A. pistolochia*, *A. clematitis*) are not clustering within the main clades, but are independent lineages. Analogue, *A. rigida* a species from Somalia is well-supported sister to the subsection *Aristolochia*. Within subsection *Podanthemum* the colonisation event from an Asian ancestor is clearly traceable, whereas in subsection *Aristolochia* the path is not traceable, since the ancestors are extinct or not present in the connecting areas. Within the Mediterranean, Near East and Caucasian species of subsection *Aristolochia* two morphologically and biogeographically well supported groups can be identified: the Near East/Caucasian species and the West Mediterranean species. The previous groupings for the latter, based on morphological characters, could be substantiated only partly by our results. This study provides the first phylogeny of all West Mediterranean species. In addition an independent complex is established including some micro endemic species. The phylogenetic results are discussed with respect to biogeography, and morphology, to give a first insight into the radiation and colonisation of the genus *Aristolochia* in the Mediterranean region.

**Introduction**

*Aristolochia* in the extended Mediterranean region, including Turkey, Caucasus and the Near East, comprises up to 60 species and subspecies and thus represents one of most diverse lineages within the genus, even if the exact species number for Turkey, Caucasus and the Near East needs revision. Beyond this, it is one of the most northern occurrences of *Aristolochia*.

A recent molecular phylogeny recovered the Mediterranean *Aristolochia* species as monophyletic (de Groot et al 2006). However, only very few members were included, as the aim of the study were to revise the African representatives of the genus. The Mediterranean species of the genus *Aristolochia* are part of *Aristolochia* s.str. (Neinhuis et al. 2005, Wanke et al. 2006b), an Old World clade probably nested within or sister to Neotropical clades (Wanke et al. 2006b). *Aristolochia* s. str. has been subdivided into two lineages based on morphological characters, subsection *Podanthemum* (unilabiate flowers with a stiped utricle) and subsection *Aristolochia* (unilabiate or bilabiate flowers with a sessile utricle), each recovered as monophyletic by molecular based phylogenies (Ohi-Thoma 2006) and in addition, supposed to be sister to each other (de Groot et al. 2006). The Old World distribution of the two subsections is similar to each other, as both occur in Asia and Africa. In contrast to the syntopic distribution in Asia, the African species of the two clades show distinct distribution areas. Subsection *Podanthemum* occurs south of the Sahara, whereas subsection *Aristolochia* is confined to North Africa, namely Tunisia, Algeria and Morocco, including the Canary Islands and Madeira (de Groot et al. 2006). In the Mediterranean and the adjacent Near East/Caucasia only members of subsection *Aristolochia* are found. For subsection *Podanthemum* a colonisation out of Asia has been postulated, based on the distribution of *A. bracteolata* from India, Sri Lanka, and Pakistan via the southern part of the Arabian Peninsula to East Africa and Central Africa (Sahel zone) and a subsequent radiation (de Groot et al. 2006). For subsection *Aristolochia* a similar colonisation route, starting from Asia towards Europe could be assumed, but was never studied in detail. *Aristolochia rigida* which occurs only in Somalia and Yemen, displays morphological affinities to the East Mediterranean/Caucasian species (bilabiate, curved perianth, sessile utricle), but from a biogeographic point of view a close relationship to other East African species could be assumed. Molecular results were unable to assign this species to any of the taxonomic groups mentioned above (de Groot et al. 2006).

Relationships within the west Mediterranean *Aristolochia* species

Several floras dealing with the Mediterranean region have been published in the last decades treating also the genus *Aristolochia* e.g., Les Quatre Flores de France (Fournier 1990), Flora Iberica (Castroviejo 1986) or Flora Europaea (Nardi & Akeroyd 1993). The assignment of a name to a taxon has been used inconsistently throughout these publications, as different names are used for the same species and vice versa. One of the most prominent examples, posing a floristic, taxonomic and nomenclatural problem, is *Aristolochia longa* = *A. fontanesii* (for details see Nardi 1983, 1988). Actually, some of the species were unknown until recently, others simply mistreated.

The west Mediterranean species (circumscribed here as plants with unilabiate, not curved perianth) have been placed in groups (complexes) indicating their relationships (e.g. Nardi 1984, 1991). These complexes are predominantly based on morphological similarities. Four species complexes have been proposed: the *A. rotunda* complex, the *A. pallida* complex, the *A. fontanesii* complex, and the *A. sempervirens* complex (Table 1). In contrast, *A. sicula* a microendemic and ecologically poorly studied species from the mountains of Sicily is not placed within any of the complexes. Further on, *A. clematitis* and *A. pistolochia* are well known and widespread in the Mediterranean area, but were not placed in any of the groups, as they are morphologically distinct.

Table 1. Traditional affiliation of the West-Mediterranean species to species complexes (according to different authors). Chromosome numbers are provided (incl. source) as well as current distribution of the species in Mediterranean region.

| Species complex (according to) | Species included traditionally | Chromosome numbers (according to) | Current Distribution |
|---|---|---|---|
| *A. rotunda* (Nardi 1984) | *A. rotunda* | 2n=12 (Fabbri & Fagioli 1971, Nardi 1984) | Spain (incl. Majorca), S-France (incl. Corse), Italy (incl. Sicily, Sardinia), W-Croatia, Slovenia, Greece |
| | *A. bianorii* | 2n=12 (Castroviejo 1986) | Majorca, W-Minorca |
| *A. sempervirens* (Nardi 1984) | *A. sempervirens* | 2n=14 (Montmollin 1986) | N-Algeria, E-Sicily, Peloponnesus, Crete, Rhode, Cyprus, Syria |
| | *A. baetica* | Unknown | S-Spain, Morocco, NW-Algeria |
| *A. fontanesii* (Nardi 1984, 1991) | *A. fontanesii* | Unknown | N-Algeria |
| | *A. paucinervis* | 2n=36 (Nardi 1984) | S-France, Spain, Morocco |
| | *A. navicularis* | 2n=24 (Fabbri and Fagioli 1971, Nardi 1984) | S-Sardinia, Egadi Islands (west of Sicily), N-Tunisia |
| | *A. parvifolia* | 2n=12 (Nardi & Nardi 1987) | Peloponnesus, Crete, Kos, Rhode, S-Turkey, Syria, Lebanon, Israel |
| | *A. clusii* | 2n=12 (Nardi 1984) | S-Italy, Sicily |
| *A. pallida* (Nardi 1984, 1989, 1991) | *A. pallida* | 2n=10 Nardi 1984 | France, Italy, Croatia, Greece (?) |
| | *A. lutea* | 2n=8 (Fabbri & Fagioli 1971, Nardi 1984, Fiorini 1988 | Italy, Slovenia, Balkan Peninsula |
| | *A. elongata* | 2n=10 (Nardi 1989) | Greece |
| | *A. merxmuellerii*[#] | unknown | Kosovo (former Yugoslavia) |
| | *A. microstoma* | 2n=10 (Constantinidis et al. 1997) | Greece |
| | *A. tyrrhena* | 2n=26 (Nardi 1984) | W-Corse, Sardinia |
| | *A. sicula** | 2n=16 (Nardi 1984) | Sicily |

[*] according to Ball (1964)
[#] according to Mayer & Greuter (1985)

## Morphological characters and chromosome numbers

As already mentioned above, the methods used to delimitate the complexes within the Mediterranean *Aristolochia* species are purely descriptive, more based on superficial morphological similarities than on cladistic concepts to test character evolution, and are probably posthoc assumptions for assumed probabilities of relationships. Morphological characters, which in the past were assumed to have great systematic value, are the subterranean parts of the plants, especially the rootstocks of the west Mediterranean species, the shape and size of the leaves, or petiole length alone and in comparison with the length of the peduncle (some of those have only been used within one species complex). Some perianth characters have been accepted as most reliable as well: the colour, the hairs inside the tube as well as the tube and limb shape (e.g. Nardi 1984).

Generally, *Aristolochia* species in the Mediterranean are calcicole plants (at least tolerate limestone grounds) and the most common growth form is geophytic, with summer dormancy and growth and flowering in spring. The two lianescent species instead are perennial, evergreen woody climbers with accelerated growth and flowering in spring.

Chromosome numbers have been shown to be a valuable character to circumscribe clades in the genus *Aristolochia.* (Sugawara et al. 2001, Ohi-Thoma et al. 2006) and have been approved to fit largely to the evolutionary history inferred from molecular based phylogenies (Ohi-Thoma et al. 2006). The *Aristolochia* subg. *Isotrema* (tropical and temperate species) exhibit 2n = 32 chromosomes, 2n = 12 have been reported for *Aristolochia* subg. *Pararistolochia* and more diverse chromosome numbers in *Aristolochia* subg. *Aristolochia* with 2n = 14 present in *Howardia* p.p., the *Aristolochia grandiflora* complex, and *Aristolochia* subsect. *Pentandrae*. In series *Thyrsicae* chromosome counts of 2n = 16 and in *Aristolochia burelae* the lowest chromosome numbers in *Aristolochia*, 2n = 6, have been reported (Ohi-Thoma et al. 2006). In contrast to the numbers given above one of the crown groups, *Aristolochia* s.str., namely the Mediterranean species, exhibit numerous different chromosome numbers. The Asian and African species of subsection *Podanthemum* display a constant number of 2n = 12 whereas the subsection *Aristolochia* possesses mainly 2n = 14 with the exception of the Mediterranean species, where 2n = 8, 10, 12, 14, 16, 24, 32, 36 have been observed. The number of chromosomes had been used to divide the west Mediterranean species into complexes, if they fit to the a priori assumptions of the authors. If not, the numbers were neglected.

This study aims to provide: A) the first phylogeny of west Mediterranean *Aristolochia* species to elucidate species relationships and to evaluate the former proposed groupings; B) an assessment of the evolution of major morphological characters, traditionally used to group and circumscribe species complexes, as well as to investigate chromosome numbers in the light of phylogenetic tree reconstruction, if available; and C) provide a first insight into the radiation and colonisation of *Aristolochia* in the Mediterranean region inferred from phylogenetic reconstruction, as well as to get access to the number of colonisation events of the Western Old World.

**Material and Methods**

<u>Sampling strategy – outgroup choice</u>

All West Mediterranean *Aristolochia* species presently accepted (following Flora Europaea, Nardi & Akeroyd 1993) are included in the analysis plus a selection of Near East, Caucasian and Asian species, all from subsection *Aristolochia*. In addition subsection *Podanthemum* was sampled by Asian and African representatives, covering the whole distribution area, to re-evaluate possible independent colonization events.

For species, from which subspecies have been described, the first described subspecies is included, as unpublished preliminary results indicate that later published subspecies do not necessarily belong to the species to which they have been assigned as subspecies. Detailed studies with a broad sampling within these species complexes are beyond the scope of the present study, but will be the topic of upcoming studies on populations. Attention was especially paid to micro endemics, which are often morphologically distinct (e.g. *A. microstoma*, *A. sicula*). All West Mediterranean, East Mediterranean and Caucasian species were collected in the field. In the case of *A. merxmuelleri*, material was taken from the paratype (herbarium specimen) since the area of occurrence belongs to the most heavily mined areas in Kosovo. Most species from Asia, were also taken from herbarium specimens. A list of investigated species along with their collection localities, vouchers and GenBank accessions are given in Table 2 (for *trnK* intron and *matK* gene together). As outgroup neotropical *Aristolochia* species were used (*A. salvadorensis, A. westlandii, A. macrophylla A. eriantha*), who belong to different taxonomic groups (the first three belong to subgenus *Isotrema*, the last to "*Howardia*").

Table 2. List of investigated species, the origin of the studied material (field or collection; collector only indicated when different from voucher), voucher information and the herbarium where the voucher has been deposited, GenBank accession numbers for the *trnK*/*matK* region (all sequences have been generated for this study, if not otherwise indicated, only some GenBank Accession have been received, sequences are available for reviewers, by Reviewer's PIN Number: 11026 at TreeBase.org). The affiliation to one of the two Old World subsections of genus *Aristolochia* subgenus *Aristolochia* is provided, if possible according to morphological characters.

| *Aristolochia species* | affiliation | Origin | Voucher (Herbarium) | GenBank accession |
|---|---|---|---|---|
| *A. acuminata* Lam. | Pod. | BG Dresden, s.n. | Wanke & Neinhuis 146 (DR) | DQ 296646 |
| *A. albida* Duch. | Pod. | BG Bonn, 17419 | Neinhuis 092 (DR) | DQ 296648 |
| *A. baetica* L. | Aristo.[#] | BG Bonn, 14517 (Spain) | Neinhuis 095 (DR) | DQ 296653 |
| | | Morocco, Tiznit, Assara | Wanke s.n. (DR) | - |
| *A. bianorii* Sennen & Pau | Aristo.[+] | Spain, Majorca, Betlem | Wanke 034 (DR) | DQ 296664 |
| | | Spain, Majorca, Soller | Wanke 036 (DR) | - |
| *A. bottae* Jaub. & Spach | Aristo.[#] | Turkey, BG Bonn 02790, leg. Koenen | Wanke 042 (DR) | DQ 296659 |
| *A. bracteolata* Lam. | Pod. | BG Bonn, 16714 | Neinhuis 094 (DR) | DQ 296647 |
| *A. clematitis* L. | Aristo.[*] | Croatia, Ilovik/Asinello Island | Meister et al. s.n. (DR) | DQ 296651 |
| *A. clusii* Lojacono | Aristo.[+] | Italy, Sicily, Villa di Marchese | Wanke & Neinhuis 104 (DR) | DQ 296666 |
| | | Italy, S-Italy, Bernalda | Wanke 192 (DR) | - |
| *A. debilis* Siebold & Zucc. | Aristo. | BG Dresden | Wanke 118 (DR) | - |
| *A. gaudichaudii* Duch. | Pod. | ? | Murata et al. SETS8 (TI) | - |
| *A. guichardii* P.H. Davis & Kahn | Arist. | Greece, Rhode, Asklepio | Wanke 186 (DR) | - |
| *A. pontica* Lam. | Aristo.[#] | BG Bonn, Turkey | Neinhuis s.n. (DR) | - |
| | | Georgien, Guria, Quabga, Karzchlis tawi, leg. Gröger & Lobin 304-2 | Wanke 210 (DR) | DQ 296656 |
| *A. elongata* (Duchartre) Nardi | Aristo.[+] | Greece, Peloponnesus, Mt. Kyparissias | Wanke 169 (DR) | DQ 296671 |
| | | Greece, Is. Euboea | Wanke 155 (DR) | - |
| *A. eriantha* Mart. & Zucc. | outg | BG Bonn, 12952 | Neinhuis 99 (DR) | DQ 532054[a] |
| *A. fontanesii* Boiss. & Reut. | Aristo.[+] | Algeria, Algier, leg. Abdelkrim | Wanke & Neinhuis 123 (DR) | DQ 296663 |
| *A. foveolata* Merr. | Aristo. | ? | Murata et al. SETS4 (TI) | - |
| *A. hirta* L. | Aristo.[#] | Greece, Samos, Pirgos-Platanos | Neinhuis 134 (DR) | DQ 296657 |
| *A. iberica* Fisch. & C.A. Mey. ex Boiss. | Aristo.[#] | Georgia, Lagodechi National Park, leg. Gröger & Lobin 319-3 | Wanke 210 (DR) | DQ 296655 |
| *A. incisa* Duch. | Aristo.# | Greece, Samos, Pirgos-Platanos | Neinhuis 127 (DR) | DQ 296658 |
| *A. jackii* Steud. | Pod. | ? | SETS56 (TI) | - |
| *A. kankauensis* Sasaki | Pod. | ? | SETS35 (TI) | - |
| *A. lutea* Desf. | Aristo.[+] | N-Italy, Passo di S. Boldo | Wanke 100 (DR) | - |
| | | S-Italy, Monticchio | Wanke 190 (DR) | - |

| | | | | |
|---|---|---|---|---|
| *A. macrophylla* Lam. | outg. | BG Dresden, s.n. | Neinhuis s.n. (DR) | DQ 882193[a] |
| *A. merxmuelleri* Mayer & Greuter | Aristo.[+] | Kosovo, Mirusa, Konznik | Mayer 10.4.1968 (LJU,M) 63193 | DQ 296673 |
| *A. microstoma* Boiss. & Spruner | Aristo.[+] | Greece, Peloponnesus, Palea Epidaurus | Neumann 008 (DR) | DQ 296672 |
| | | Greece, Attica, Mt. Parnitha | Wanke 183 (DR) | - |
| *A. navicularis* Nardi | Aristo.[+] | Italy, Sardinia, Donori | Wanke 019 (DR) | - |
| | | Italy, Sardinian Nora | Wanke 021 (DR) | - |
| *A. pallida* Willd. | Aristo.[+] | N-Italy, Valdobiadene, Guitta-Santo Steffano | Wanke 101 (DR) | DQ 296669 |
| | | S-Italy. Mt. Li Foi | Wanke 204 (DR) | - |
| *A. parvifolia* Sm. | Aristo.[+] | Greece, Rhode, Haraki | Wanke 177 (DR) | - |
| | | Syria, Sallah Alden | Mafoud 033/2 (DR) | - |
| *A. paucinervis* Pomel | Aristo.[+] | BG Coimbra 135 | Wanke & Neinhuis 148 (DR) | DQ 296662 |
| | | Spain, Alto del Mirlo | Costa PA5 (MA) | - |
| *A. pierrei* Lecomte | Pod. | Thailand s. loc. | s.coll, s.n. (DR) | DQ 296649 |
| *A. pistolochia* L. | Aristo.[*] | France, Cassis, Calenque d'En Veau, leg. Kreft | Wanke 037 (DR 025372) | DQ 296652 |
| *A. rigida* Duch. | unknown | Somalia, Bulo Burti | Bally & Melville 15331 (K,MO) | - |
| *A. rotunda* L. | Aristo.[+] | France, Corse, Figaretto Plage | Wanke 015 (DR) | DQ 296665 |
| | | Greece, Loutroupigi | Wanke 161 (DR) | - |
| *A. salvadorensis* Standl. | outg. | BG Bonn, 10720 | Neinhuis 109 (DR) | DQ 882191[a] |
| *A. sempervirens* L. | Aristo.[*] | Italy, Sicily, Avola | Wanke & Neinhuis 103 (DR) | DQ 296654 |
| | | Syria, Umaltueur | Mafoud 025/2 (DR) | - |
| *A. sicula* Tineo in Guss. | Aristo.[+] | Italy, Sicily, Piano Zucchi | Wanke 209 (DR) | DQ 296668 |
| | | Italy, Sicily, Piano Zucchi II | Wanke 191 (DR) | - |
| *A. tyrrhena* Nardi & Arrigoni | Aristo.[+] | Italy, Sardinia, San Nicolao | Wanke 024 (DR) | DQ 296667 |
| | | France, Col de la Croix | Wanke 009 (DR) | - |
| *A. westlandii* Hemsl. | outg. | BG Bonn, 14211 | Neinhuis 115 (DR) | DQ 532041[a] |

[+] grouped into W-Mediterranean species, based on morphology
[#] grouped into E-Mediterranean species, based on morphology
[*] grouping into west or east Mediterranean species not possible due to morphological transitional character states
[a] Wanke et al 2006b

Table 3 Primers used in the present study.

| Primer | Sequence 5'-3' | Design |
|---|---|---|
| MG1 | AAC TAG TCG GAT GGA GTA GAT | Liang & Hilu (1996) |
| MG15 | ATC TGG GTT GCT AAC TCA ATG | Liang & Hilu (1996) |
| NY-matK-480F | CAT CTG GAA ATC TTG STT C | Borsch (2000) |
| AS-matK-460F | TAT TTC CCT TTT HGA GG | Wanke et al. 2006d |
| Pi-matK-1060F | ACT TRT GGT CTC AAC YG | Wanke et al. 2006d |
| Pi-matK-1480F | TCG TAA ACA YAA AAG TAC | Wanke et al. 2006d |
| AR-trnK-420F | AAG TGA ATA AAT GGA TAG AGC | Wanke et al. 2006a |
| AR-trnK-1320R | ATC GCT CTT TTG ACT TTG G | Wanke et al. 2006a |
| trnK-med-150F | AGA GAA TAC TTC CAT CCT TAC CG | Wanke et al. 2006b |
| AR-matK-960R | AAC CTT TTC CCG CAT CAG G | Wanke et al. 2006b |
| AR-matK-930F | TAT TAG TAC CTG ATG CGG G | Wanke et al. 2006b |
| trnK-med-440R | ATT CGT CTT TAC TCA CTC CGT A | Wanke et al. 2006b |
| AR-matK-1200F | TTC CAA AGT CAA AAG AGC G | Wanke et al. 2006b |
| AR-matK-1450R | CGT TAG AGT TGC ACG TTA | Wanke et al. 2006b |
| AR-matK-1510R | TAG ACT CCT GAA ARA GAA GTG G | Wanke et al. 2006b |
| AR-matK-1400F | CTC TTT CAG GAG TCT ATC TAT G | Wanke et al. 2006b |
| AR-matK-1850R | CCA GGC AAG ATA CTA ATT | Wanke et al. 2006b |
| AR-matK-2510R | AAA AAT CTC AAT AAA TGY AA | Wanke et al. 2006b |
| AR-matK-680R | CCG AGA AAA ACG AAT ATG GAT T | Wanke et al. 2006b |
| AR-matK-660R | AYG GAA TCG CAT TCA TA | Wanke et al. 2006b |
| AR-matK-2100R | TGA AAA TGA TTA CAA AGC ACT AC | Wanke et al. 2006b |
| AR-matK-780R | GGT CTT CTG AAA ATG ATT AC | Wanke et al. 2006b |
| AR-matK-2400R | ATT TTC TAG CAT TTG ACT CC | Wanke et al. 2006b |
| AR-matK-3500R | ATC CAA ATA CCA AAT ASA TTC C | Wanke et al. 2006b |


<u>Molecular methods, tree reconstruction, and evaluation of nodes</u>

The phylogeny is based on chloroplast sequence data of the *trnK* intron and the *matK* gene. This region has been shown to be a powerful marker to resolve species relationships (e.g. Müller & Borsch 2005). Amplification and sequencing follows methods described in Wanke et al. (2006c, 2006b). The primers used for amplification and sequencing are given in Table 3. 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* 4.0b10 (Swofford 2002) with 500 ratchet replicates, 10 random addition cycles and 1000 bootstrap replicates. In contrast to the manual indel coding method used in Neinhuis et al. (2005) the automated simple indel coding (SIC) (following Simmons & Ochoterena 2000) and the modified complex indel coding (MCIC) method described by Müller (2006) were used, both implemented in SeqState (Müller 2005). The alignment is available from TreeBase.org.

Morphological characters, chromosome numbers and ecological framework

Morphological characters were recorded in the field, from species collected in the field and grown in the Botanical Garden of the Technical University Dresden, as well as from herbarium specimens. In total nine characters were studied, covering the vegetative part of the plants, flower, and fruit morphology. Characters were chosen according to former publications where they have been proposed to be of "great systematic value", to test their real inheritance, as well as characters, which are assumed to be less biased by parallel evolution (Tab. 4, Appendix 1). Chromosome numbers were taken from previously published papers (see Table. 3 and Ohi-Thoma et al. 2006, Verlaque & Filosa 1993, Fiorini & Nardi 1993, Murin 1976).

As the ecological framework is a highly important factor for speciation, especially for closely related species, with a high degree of endemism, general factors were acquainted here. Correlations between morphological traits and ecological factors could either detect possible parallel evolution, as adaptation to abiotic factors or could identify key characters circumscribing niches for wide distributed or endemic plants, as reported by Rowe and Speck (2005). All general ecological factors were recorded in the field during extensive field work.

**Results**

All results obtained for different datasets, which produced identical topologies, are compiled into one tree using TreeGraph (Müller & Müller 2004) (Fig. 1). Evaluation of nodes indicated on the branches, are bootstrap values (parsimony), (i) based on substitutions only (gaps treated as missing data), (ii) indels coded with SIC and, (iii) coded with MCIC.

<u>Characterisation and structure of the molecular dataset</u>

The total length of the alignment of the *trnK*/*matK* region comprises 3502 characters. The *trnK* 5' intron represents 1673 characters, the *matK* gene 1562 and the *trnK* 3' intron 265 characters. The absolute sequence length varies within the *trnK* 5' intron from 710 to 903 bp, within the *matK* gene from 1509 to 1527 bp (503-509 AS) and within the *trnK* 3' intron from 204 to 229 bp. The great length variation in the *trnK* 5' intron is mainly based on several microsatellite structures, which were excluded due to uncertain homology assessment (Table 5). Among the West-Mediterranean species, homology assessment was possible for hotspot H1, due to secondary structure calculation, by applying a minimum free energy model and tracing simple sequence repeats (data not shown). Therefore, this hotspot was divided into two parts, from which the part for the West-Mediterranean species was used for tree calculations. For the East Mediterranean / Caucasian species a similar approach will be applicable, but is beyond the scope of the present study. The most frequent microsatellite structure (found 3 times) is a mononucleotide repeat or a combination of two mononucleotide repeats. Structures like that have been proposed to be highly homoplastic (e.g. Provan et al. 2001, Hale et al. 2004). A cryptic simple microsatellite region, which has been found only in *Aristolochia* (Wanke et al. 2006a) with the motive $(A_n T_m)_k$ was also found in the *trnK* 3' intron for the individuals sampled. A second cryptic simple microsatellite region was found only in the Mediterranean species, non Mediterranean species have a poly T mononucleotide repeat. A characterisation of these hotspots is shown as example for the West-Mediterranean species in Table 5. Tree statistics for the three different datasets are shown in Table 6. In the aligned sequence parts, 128 indels were found and coded (each indel coded as one character for SIC). For MCIC this resulted in 19 complex coded characters and 31 simple coded characters. Most of the complex coded characters were found

in the cryptic simple microsatellite region. Most indel events appear as direct simple sequence repeats (SSR).

Table. 5 Hotspots excluded due to ambiguous homology assessments. The location of the hotspots, characterisation and their composition is shown. I = *A. sempervirens* complex, II = *A. clusii* complex, III = *A. pallida* complex, IV = *A. rotunda* complex, V = *A. fontanesii* complex.

| Hotspot | position in the alignment | microsatellite type | length distribution for the species complexes (bp) | | | | | Composition %* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | V | A | C | G | T |
| H1 | 373-1743 | cryptic simple | 69-113 | 91-146 | 87-181 | 105-130 | 90-179 | 43.88 | 0.94 | 1.96 | 53.22 |
| H2 | 1850-1865 | mononucleotid | 12 | 10-14 | 11-12 | 12 | 12 | 0.00 | 27.46 | 0.00 | 72.54 |
| H3 | 2136-2281 | cryptic simple | 23 | 22-25 | 22-23 | 23-31 | 22-28 | 16.37 | 1.81 | 0.30 | 81.52 |
| H4 | 3060-3073 | mononucleotid | 8 | 9-11 | 8 | 5 | 5 | 0.45 | 2.24 | 0.00 | 97.31 |
| H5 | 4118-4141 | mononucleotid | 14-19 | 13-19 | 14-24 | 8-9 | 11-14 | 93.25 | 0.16 | 5.03 | 1.57 |

* calculated for each hotspot from the whole dataset

Table. 6 Tree statistics of the parsimony analysis (SIC=simple indel coding, MCIC=modified complex indel coding). Number of trees retained by filter (= only trees with different topology)

| matrix | number of characters | number of variable uninformative characters | number of parsimony informative characters | steps | number of trees retained by filter | CI | RI | RC |
|---|---|---|---|---|---|---|---|---|
| original matrix | 3503 | 249 | 285 | 797 | 35 | 0.76 | 0.90 | 0.68 |
| with SIC | 3695 | 344 | 380 | 1028 | 44 | 0.77 | 0.89 | 0.69 |
| with MCIC | 3553 | 268 | 314 | 991 | 30 | 0.78 | 0.90 | 0.70 |

CI = consistency index; RI = retention index; RC = rescale consistency index

Phylogenetic Relationships

The Old World species of *Aristolochia* subgenus Aristolochia split into two clades, first the subsection *Podanthemum* and second the subsection *Aristolochia* (Fig. 1). The Asian and African representatives of *Podanthemum* appear mixed in a highly supported monophyletic clade, including the widely distributed *A. bracteolata* (India to Africa). *Aristolochia rigida* is branching first within subsection *Aristolochia*, with high to moderate support depending of the analysis, followed by temperate Asian and temperate Eurasian representatives of subsection *Aristolochia* and *A. pistolochia*, which is endemic to Southern France and Spain.

Within the remaining species of subsection *Aristolochia,* two major clades could be found: 1. the east Mediterranean species together with the Caucasian species and 2. the west Mediterranean species together with the circum Mediterranean lianas (*A. sempervirens* complex) (Fig. 1). The relationships among the East Mediterranean and Caucasian species remain unclear, due to very low variability in the *trnK/matK* region, except the microsatellites that were excluded. Within the west Mediterranean species the former proposed groupings are only partly supported. The *A. sempervirens* complex remains unchanged. The *A. pallida* complex could be confirmed, but *A. tyrrhena* that was formerly thought to belong to the complex needs to be excluded. The following groupings were found in contrast to former proposed affinities: *A. fontanesii*, *A. parvifolia*, *A. paucinervis* and *A. navicularis* are included in the *A. fontanesii* complex, *A. clusii* should be excluded. *A. rotunda* and *A. bianorii* could be confirmed as the *A. rotunda* complex but *A. tyrrhena* should be excluded. In contrast to earlier concepts we found different relationships for *A. clusii* (formerly *Aristolochia fontanesii* complex), *A. tyrrhena* and *A. sicula* (formerly *Aristolochia pallida* complex). These species form a distinct clade with maximum support (Fig 1.).

Figure 1. Strict consensus tree (parsimony) based on original matrix with coded indels included (SIC) (topology identical among all datasets). Support values from original matrix without coded length mutations above branches, below branches with simple indel coding (SIC) (first) and with modified complex indel coding (MCIC) (second). Chromosome counts taken from Table 1 according to authors listed therein or according to Ohi-Thoma et al. (2006), Verlaque & Filosa (1993), Fiorini & Nardi (1993), Murin (1976) if not listed in Table 3.
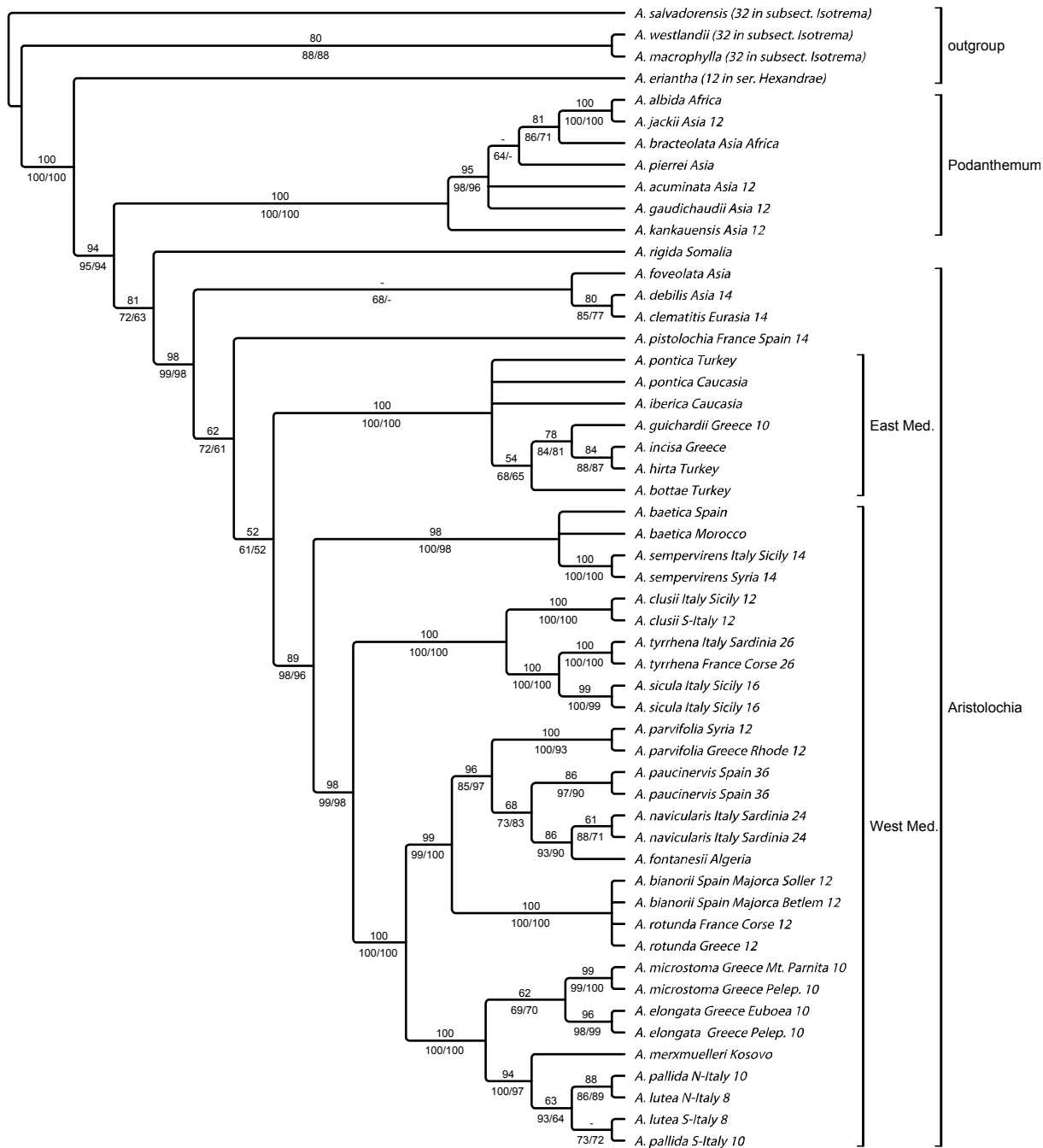
Table 4. Morphological characters and important ecological factors coded according to Appendix 1

| species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A. salvadorensis | 3 | 1 | 4 | 1 | 2 | 0 | 2 | 1 | 0 | 0 | 2 | 0 |
| A. macrophylla | 3 | 1 | 4 | 1 | 3 | 0 | 2 | 1 | 0 | 1 | 1 | 0 |
| A. westlandii | 3 | 1 | 4 | 1 | 1 | 0 | 2 | 1 | 0 | 2 | 2 | 1 |
| A. eriantha | 3 | 1 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | ? |
| A. gaudichaudii | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 2 | 1 |
| A. kankauensis | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 2 | 1 |
| A. jackii | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 2 | 1 |
| A. acuminata | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 2 | 1 |
| A. bracteolata | 3 | 1 | 4 | 1 | 2 | 1 | 0 | 0 | 1 | 3 | 1 | 2 |
| A. albida | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 1 |
| A. pierrei | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 2 | 1 |
| A. rigida | 3 | 1 | 4 | 1 | 2 | 0 | 1 | 1 | ? | 3 | 3 | 2 |
| A. foveolata | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 1 | 1 | 2 | ? | ? |
| A. debilis | 0 | 1 | 4 | 1 | 1 | 1 | 0 | 1 | 1 | 2 | ? | ? |
| A. clematitis | 2 | 1 | 4 | 0 | 0 | 1 | 0 | 1 | 0 | 2 | 2 | 1 |
| A. pistolochia | 1 | 1 | 4 | 1 | 0 | 1 | 0 | 1 | 1 | 2 | 3 | 3 |
| A. baetica | 0 | 0 | 3 | 1 | 1 | 0 | 0 | 1 | 1 | 2 | 1 | 1 |
| A. sempervirens | 0 | 0 | 3 | 1 | 1 | 0 | 0 | 1 | 1 | 2 | 1 | 1 |
| A. iberica | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| A. pontica | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 0 |
| A. hirta | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| A. cretica | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| A. guichardii | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| A. incisa | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| A. bottae | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| A. parvifolia | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 3 | 3 | 4 |
| A. navicularis | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| A. paucinervis | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 2 | 1 |
| A. fontanesii | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 2 | 1 |
| A. bianorii | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 3 | 3 |
| A. rotunda | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| A. clusii | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 2 | 1 |
| A. tyrrhena | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 3 | 4 |
| A. sicula | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. pallida | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| A. lutea | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| A. elongata | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| A. microstoma | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 2 | 1 |
| A. merxmuelleri | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 2 | 5 |

The underground parts as adaptation to ecological factors

Within subsection *Aristolochia* different types of rootstocks have been evolved. This differentiation has to be interpreted without doubt as an adaptation to ecological conditions. Especially in all East Mediterranean and Caucasian species the very long rootstock is correlated with the limited availability of humidity, since the soil is deeply drying up during summer. In contrast, in the Western Mediterranean the interpretation of the underground parts is more complicated. The western Mediterranean *Aristolochia* are not occurring in similar habitats as Eastern Mediterranean species, or they are growing along streams, rivers, or year round wet places, which makes the development of deep penetrating rootstocks unnecessary. Those species never develop a rootstock, which is more than 5 times longer than broad. Instead most of the species exhibit a globose rootstock, as sufficient water is always availible. West Mediterranean species, which show an elongated rootstock (*A. parvifolia, A. thyrrena, A. clusii*) are growing in calcareous gravel or rock crevices, were a globose rootstock does not make sense as water permeability is high and water retention is low.

**Discussion**

Relationships of subsect. *Aristolochia* versus subsect. *Podanthemum* and its consequences for colonisation routes

The two Old World subsections of subgenus *Aristolochia* are revealed as monophyletic, which is in accordance to previously published results (Ohi-Thoma et al. 2006). The morphological synapomorphy for those two clades are the stiped utricle in subsection *Podanthemum*, which is not present in subsection *Aristolochia* (Ohi-Thoma et al. 2006, de Groot et al. 2005). In contrast to the results obtained by de Groot et al. (2005), who postulated a colonisation of Africa out of Asia via Pakistan and the Arabian peninsula (based on data from *A. bracteolata*) and a subsequent radiation in Africa, the nesting of *A. jackii* (Asia) sister to *A. albida,* is contradicting this assumption or would suggest a second independent colonisation of Africa. Such a second colonisation scenario is substantiated by the frequent occurrence of *A. albida* in Madagascar, adjacent islands and the East African coast, whereas the rest of Central Africa is only poorly colonised, as inferred from investigated herbarium specimens (de Groot et al. 2005). The two species (*A. jackii and A. albida*) are morphologically very similar.

The radiation of subsection *Aristolochia* is even more complicated. *Aristolochia rigida* (endemic to Somalia) gains reasonable support as the first branch of the subsection. De Groot et al. (2005) did not place this species in one of the two subsections of the Old World *Aristolochia*, due to the lack of statistical support. However, a close relationship to the Near East-Caucasian species has been postulated, because of morphological characters (trumpet-like flower tube and bilabiate flower limb) somehow similar to East Mediterranean and Caucasian species. Since the next branches represent temperate Asian (A. *foveolata*, A. *debilis*), temperate Eurasian (*A. clematitis*) and Mediterranean (*A. pistolochia*) species, a similar colonisation scenario as postulated for *Podanthemum* from an Asian ancestor, to the western Old World is not likely. Instead a rapid morphological radiation in the Near East (or close to this area) with subsequent star like colonisation of the different current distribution areas, which is not paralleled on the molecular level, appears to be more likely. Unfortunately, the common ancestor, who would support this assumption is not identified yet or does not exist any more. This assumption would also explain the low support for the nodes that lead to the different highly supported geographical clades.

Evolution among west Mediterranean species – synopsis of chromosome numbers and phylogenetic tree reconstruction

Newly found relationships – the *Aristolochia clusii* complex

In contrast to earlier systematic treatments new relationships are proposed based on molecular data for *A. clusii*, *A. tyrrhena* and *A. sicula*. These species form a distinct clade revealing maximum statistical support. *Aristolochia clusii* has been proposed to be part of the *A. fontanesii* complex due to the elongated rootstock, short petioles and peduncles and the chromosome number 2n=12. *Aristolochia clusii* has been considered as the basal most taxon in a ploidy series within the *A. fontanesii* complex (2n = 12, 2n = 24, 2n = 36, Nardi 1984) and due to its distribution close to members of *A. fontanesii* complex (Tunisia, Sicily, and Sardinia) (Eriksson et al. 1974, Nardi 1983, 1984). Morphological "affinities" of *A. microstoma* to *A. clusii*, as proposed by Nardi (1991), without specifying the characters, are in contrast to the obtained molecular results. The second species of this group, *A. tyrrhena,* has been proposed to belong to the *A. pallida* complex, displaying "characteristic" flower and leaf structures such as the elongated utricle (Nardi 1984, Nardi & Arrigoni 1983, Nardi & Ricceri 1987). Former authors did not use the chromosome counts in this particular case to explain their proposed grouping, since the *A. pallida* complex displays 2n = 8, 10 and *A. tyrrhena* 2n=26. Therefore this character did not help to substantiate the formerly proposed grouping. The rootstock, a character that was generally used to explain relationships in Mediterranean *Aristolochia*, also differs from *A. pallida/A. lutea* (globose) and *A. tyrrhena* (cylindrical, thin and elongated). The last species of this group, *A. sicula*, has been proposed to be distantly related to the *A. pallida* complex (Nardi 1984) or even as subspecies of *A. pallida* (Ball 1964), without mentioning any synapomorphy for this assumption. This species complex, recovered here for the first time, consists exclusively of micro-endemic species (paleo-endemics), and show a great variability in chromosome numbers, compared to other species complexes. These numbers are not explainable by simple polyploidisation. The most reasonble explanation for this variability is chromosome duplication followed by a subsequent loss of individual chromosomes or the addition of chromosomes to the diploid set (aneuploidy). *Aristolochia clusii* exhibits 2n=12 chromosomes, whereas *A. sicula* and *A. tyrrhena* show 2n=16 and 2n=26, respectively.

The *Aristolochia fontanesii* complex

All species traditionally treated here are characterised by their elongated rootstock. This character has been used to separate this complex from other Mediterranean *Aristolochia* species. Since we are able to show that some species with an elongated rootstock, e.g. *A. clusii* (formerly proposed to be part of this complex, Nardi 1984), belong to another complex, this character needs to be treated with caution (Fig. 1, Tab. 6). The same applies to other characters, which are cited for the *A. fontanesii* complex such as the "rather short" petioles and peduncles (e.g. Nardi 1984).

Polyploidy within a single species (autopolyploidy) is postulated here to be the driving force behind speciation within this complex because hybridisation can be neglected (see below). Chromosome doubling is known to open possibilities for new genetic combinations, likewise for the phenotypic plasticity and the adaptive capacity to the environment (e.g. Levin 1983). The resulting apo-endemic species would be *A. paucinervis*, *A. navicularis,* and *A. fontanesii*, whereas the patro-endemic species is branching from the first node of the phylogenetic tree (*A. parvifolia*, 2n=12, x=2). Morphologically all apo-endemic species are clearly distinguishable from each other, by the size of the flower and the number of coloured nerves on the inner limb wall (de Groot et al 2006). The patro-endemic *A. parvifolia* exhibits a totally different phenotype, showing much smaller vegetative parts and a twisting flower limb, coloured with reticulate brownish nerves. All apo-endemic species show a normal limb, which is greenish-yellow.


The *Aristolochia pallida* complex

This complex contains one of the most widespread Mediterranean species (*A. lutea*) as well as species with a very restricted distribution (*A. merxmuellerii*). The phylogenetic tree gains high support in most parts, except for the sistergroup relationship of *A. elongata* and *A. microstoma*. Morphologically, both species are easily distinguishable form each other and the rest of the species belonging to this complex. *Aristolochia microstoma* is easy to distinguish from all other *Aristolochia* species by the unique perianth morphology. The limb is reduced to a small beak or missing and the mouth of the tube is reduced to a pore (Nardi 1991). The function and pollination ecology of this unusual flower is unknown. Most likely the pollinators are small arthropods, which are living near the ground or even in the

forest litter, as the flower is often subterranean or only the brownish tip of the flower reaches the soil surface (Nardi 1991, Wanke personal observation). *Aristolochia elongata* shares an elongated rootstock with *A. microstoma*, which is globose in all other species of this complex. The remaining species are not easy to distinguish because differentiating characters were not used consistently and always in comparison to differing sets of species (e.g. Nardi 1984, 1991). For all species, except *A. lutea* (2n=8), the same chromosome number has been reported (2n=10), the main reason why *A. lutea* and *A. pallida* are still kept as distinct species. It should be mentioned here, that based on intensive fieldwork and cultivation of many accessions we tend to include at least *A. lutea* into *A. pallida* as the main morphological character distinguishing the two species (e.g. limb/tube length ratio ≥ 1 in *A. pallida*; < 1 in *A. lutea*) is recovered to be inconsistent within populations and under cultivation conditions. The here included accessions of *A. lutea* and *A. pallida* are not monophyletic in the phylogenetic analyses, but are clustering as geographical clades, which is further substantiated by unpublished data sampling more than 60 populations (Wanke, in prep).

Ball (1964) treated *A. sicula* as subspecies of *A. pallida*, which was never accepted. Another microendemic species, *A. merxmuelleri*, only known from serpentine rich areas in Kosovo was also considered to be part of the *A. pallida* complex (Mayer & Greuter 1985).


*Aristolochia sempervirens* complex

Both species are lianas and share that they are able to spread by the formation of stolons. In addition, they are evergreen and produce flowers during the whole year, with a main flowering period in spring. All other species are only growing during the winter rainy season and the flowering period is restricted to one or two months in spring. For *A. sempervirens* the normal chromosome number for subsection *Aristolochia* (2n=14) has been reported (Montmollin 1986), which substantiates the position recovered in the phylogenetic analyses.


*Aristolochia sempervirens* and *A. baetica* differ also in flower and leaf morphology and show similarities to the Near East and Caucasian species, as the limb is not elongated, the tube opens gradually and is slightly curved. *Aristolochia baetica* shows more cordate leaves, with a glaucous waxy surface, whereas the leaves of

*A. sempervirens* are more elongated, coriaceous and glabrous. These characters remain consistent throughout the distribution area. The flower form and colouration is also largely different. The results from the phylogenetic tree support the monophyly of of *A. sempervirens* but not of *A. baetica*. This is basically due to the fact, that unpublished results including more than 60 populations covering the whole distribution area show three independent lineages among the two species. One represents the core *A. sempervirens* and two clades among *A. baetica.* One *A. baetica* clade consists of South Moroccan populations (south from the High Atlas and the adjacent coastal area), which is sister to the *A. sempervirens* clade (Mahfoud et al. in prep). As the accession of *A. baetica* used here is belonging to the South-Moroccan clade, they appear as a polytomy.

*Aristolochia rotunda* complex

*Aristolochia rotunda*, the type species of the genus, is one of the most widespread *Aristolochia* species in the Mediterranean. The purple/greenish (*A. rotunda*) and purple/brownish (*A. bianorii*) inner part of the limb, as well as leaves without or with very short petioles, characterises the whole complex. Both species share the same chromosome number (2n=12) (Fabbri & Fagioli 1971, Nardi 1984, Castroviejo 1986). The biggest phenotypic differences between the two species is the size, *A. bianorii* is much smaller than *A. rotunda*. In addition they colonise two completely different ecological habitats. (dry places, often rock crevices vs. humous soil which is never completely dry) Lavergne et al. (2004) detected a clear trend for endemic species to be smaller in size, having also smaller flowers than their widespread congeners, which is also evident for *A. bianorii* and *A. rotunda*.

Highly specialized pollination systems as shown by Rulik et al. (submitted) for A. pallida, may contribute to reproductive isolation and could therefore be a driving force for the evolution of endemism in many West-Mediterranean *Aristolochia*. This syndrome is often associated with highly modified flowers such as in *Aristolochia*. This is well known in the Mediterranean also for *Ophrys* species (morphological and chemical attraction and pollination barrier) (Paulus & Gack 1990).

Pollination and other reasons for neglecting hybridisation

Hybridisation, a common factor for speciation in the Mediterranean (Thompson 2005), is only possible between sympatric, or closely neighbouring species, with pollinators able to cover the distance between the two species. This is only applicable for the minority of west Mediterranean *Aristolochia* species. If they have similar distribution patterns, they are normally separated, by altitude, or different ecological habitats. Occasionally we observed that some species occur syntopic, but never two species, that belong to the same species complex. In addition no morphologically "intermediate" individuals have been observed (excluding *A. pallida* / A. *lutea*). Furthermore results from *A. pallida* indicate that the Mediterranean species have a highly specialized interdependence with their pollinators (*Phoridae*) (Rulik et al. submitted.). Self-fertilisation is only poorly studied, but is likely to occure. Beside this, hybridisation is only known from very few neotropical *Aristolochia* species e.g. *A. grandiflora* and *A. gorgona* (Blanco 2005).


Biogeography, radiation and colonisation events of west Mediterranean endemics

Mediterranean climate is highly affected by strong seasonality, temperate to cold winter, with rainfall and often extremely dry summer, with high mean temperatures. Geophytes generally outlast the summer period by summer dormancy with abandoning the aerial parts of the plant after flowering and fruiting, at hand for *Aristolochia*, but also well known for *Cyclamen*, *Crocus*, *Iris*, or many Orchids. This outlasting could have influenced the radiation of Mediterranean species, as adaptation to a comparatively drier habitat will necessarily lead to an underground structure, which is able to penetrate deeper into the soil to acquire humidity (elongated rootstock). Seeds are only germinating under humid conditions, and need to be dispersed by ants into their den (Wanke & Neinhuis, unpublished). Seedling survival could also be an important factor for the colonisation and survival of the present plant group, as it is known, that plant mortality is high at seedling stage (e.g. Herrera 1991) and ant dispersal is very local dispersal agent (Gomez & Espadaler 1998) (and necessitates several other preconditions).

Micro-endemic species as ad hand (e.g. *A. sicula*, *A. bianorii*), should entirely be treated as endangered, as colonisation of new habitats from their current distribution area is not likely, as they now either occur on islands (dispersal by ants

from one island to the next or the continent is improbable) or island like habitats, with surrounding areas of unsuitable ecological framework. Habitats of such micro-endemics are generally unique (Kruckeberg and Rabinowitz 1985, Debusche and Thompson 2003), and adaptation to surrounding and changing habitats is less likely in endemics, because of their morphological uniformity, which is often due a higher amount of inbreeding (Lavergne et al 2004).

All West-Mediterranean species are endemic to the Mediterranean region except *A. lutea*, which also occurs on the Balkan Peninsula. A high degree of endemism is a widespread phenomenon in the Mediterranean area (Greuter 1991). Climatic changes have caused two common distribution patterns. First, schizo-endemic species evolved via fragmentation and isolation under climatic pressure and second, east west vicariance (Thompson 2005), substatiated by several studies in the Mediteranean such as the genus *Senecio* (Comes and Abott 1999a, 2001).

Fig. 2 Distribution of the *Aristolochia pallida* complex.
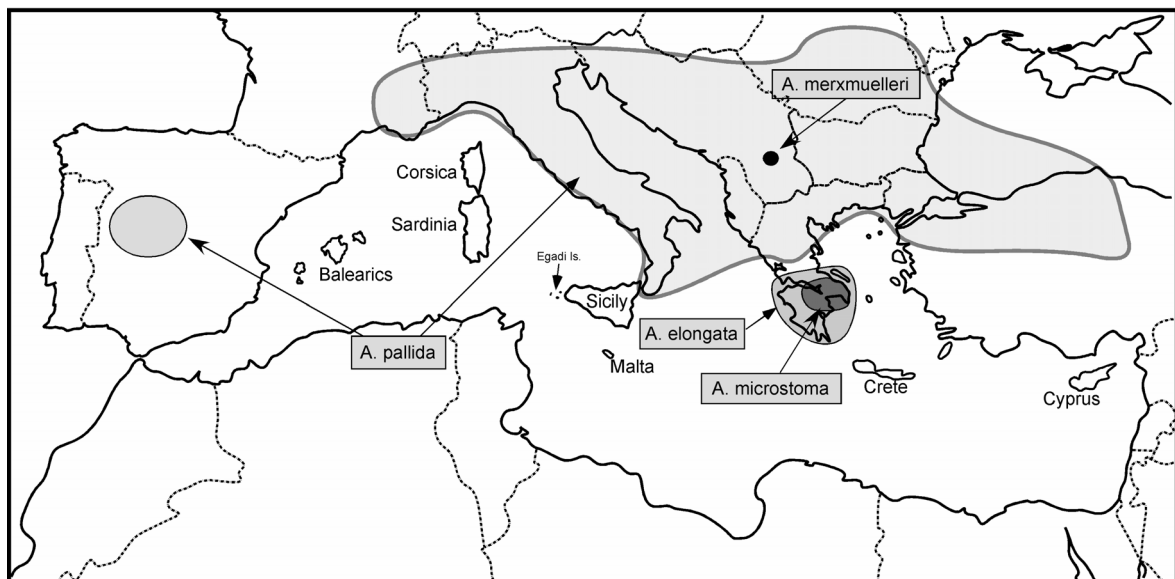


The *Aristolochia pallida* complex could belong to the first group of endemics, since they show the same number of chromosomes and their present day distribution is either widespread (*A. lutea*) or narrow (*A. merxmuelleri*). *Aristolochia pallida* is restricted to S-France and the adjacent part of N-Italy, besides some disjunct populations in NW Italy, S-Italy, N-Croatia and N-Greece (Nardi & Nardi 1987, Nardi 1989, Trinajstic 1990, Nardi 1991, Franjic & Trinajstic 1999). Interestingly, the isolated populations of *A. pallida* are surrounded by populations of *A. lutea* occurring from Italy to the Balkan Peninsula and Asia Minor and could even be

syntopic (Nardi 1984, 1991, Wanke unpublished data). In addition *A. elongata* and *A. microstoma* are adapted to temporarily dry habitats (elongated rootstock) in contrast to the species adapted to a year round humid soil (small, globose rootstock (*A. pallida*, *A. lutea*, *A. merxmuelleri*). The latter group primarily occurs in forests or similar habitats. These complex distribution patterns could be due to a re-colonisation from glacial refuges in southern Italy and Greece (*A. pallida* and *A. lutea*) or are the glacial refuges (*A. elongata*, *A. microstoma* and *A. merxmuelleri*), but these assumptions will need further investigation on the population level.

Fig. 3 Distribution of the *Aristolochia sempervirens* complex.
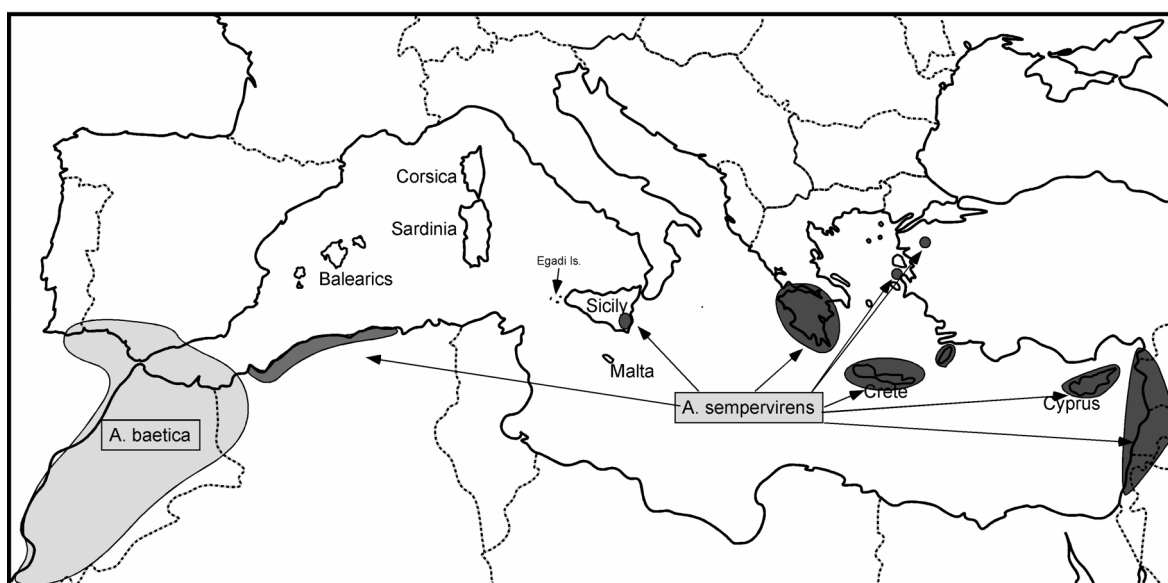


*Aristolochia sempervirens* and *A. baetica* should be regarded as east west vicariant endemics. *A. baetica* is restricted to Morocco and the southern Iberian Peninsula, a well known distribution pattern for many W-Mediterranean. In contrast, the distribution of *A. sempervirens* from Near East to Algeria including some islands of the Mediterranean (Cyprus, Rhode, Crete, the southern tip of the Peloponnesus, SE-Sicily) (Nardi 1984) may be the result of ancient anthropogenic introduction to the Islands and the coastline of N-Africa. This would explain why *A. sempervirens* is restricted to old settlements especially on Sicily, Rhodes and Cyprus. On the other hand the current distribution may reflect fragments of a larger contiguous area prior to the tectonic fragmentation. Both hypotheses would support also vicariance of both species, as they have no overlapping distribution area, as shown by de Groot et al. (2006). Interestingly, a similar distribution

pattern of two closely related species in NE-Morocco has also been found for *A. paucinervis* and *A. fontanesii* (de Groot et al. 2006).

A second species complex for which east west vicariance is likely is the *Aristolochia fontanesii* complex. This group has a connection to the Near East via Cyprus and Greece, due to the occurrence of the first branching species *A. parvifolia*. All other species belonging to this complex are distributed in the west Mediterranean (Iberian Peninsula, S-France, N-Africa, S-Sardinia and the Egadi Islands) (Figure 3) (Nardi 1984, Wanke personal observations). The radiation of this species complex could be close to the present day distribution of *A. parvifolia* as inferred from phylogenetic tree reconstruction. However, it should be mentioned that a similar colonisation pathway could have been used by the *A. sempervirens* complex, which is also distributed in the Near East, Cyprus, the southern most Greek islands, Sicily and N-Africa (see above).

Fig. 4 Distribution of the *Aristolochia fontanesii* complex.
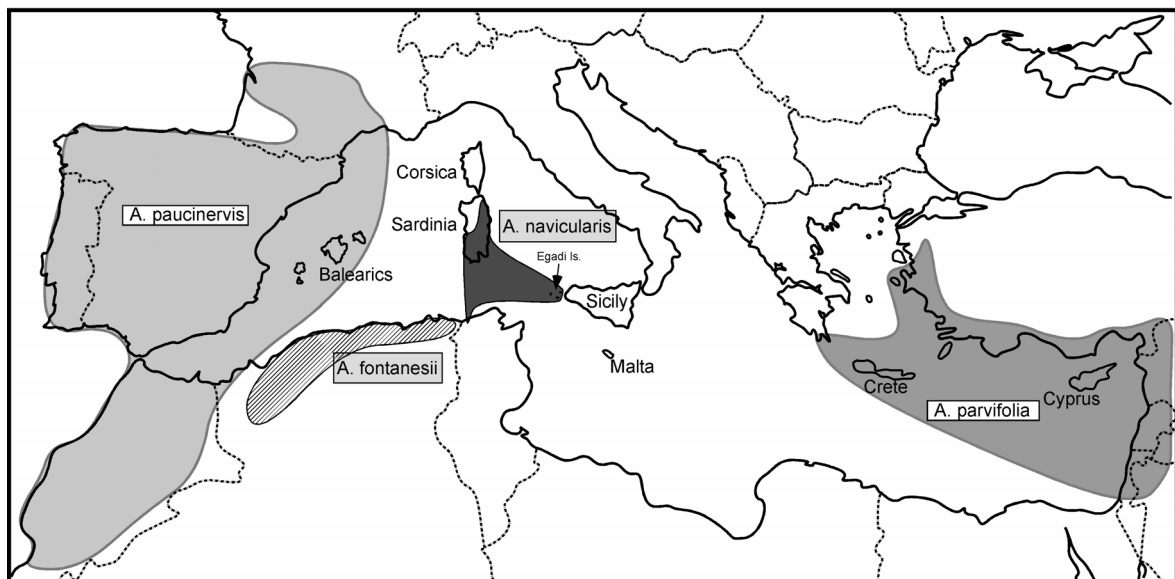


As observed by Nardi (1984) and de Groot et al. (2006) the species of the *A. fontanesii* complex are geographically isolated. *A. paucinervis* is found in S-France and the Iberian Peninsula and in N-Morocco. *A. fontanesii*, characterised by the largest flower of the west Mediterranean species, is only found in coastal Algeria. Following the coastline to the east, *A. navicularis* is found in Tunisia, with a disjunction in S-Sardinia and the Egadi Islands (west of the coast of Sicily), but not on Sicily itself (Nardi 1984, de Groot 2006). The similar distribution pattern of the two species complexes could be an indication for a similar colonisation event, at

the same time period and therewith under similar ecological condition and would in the reverse sense be in favour of a natural colonisation event for *A. sempervirens* instead of introduction.

Many of the areas, where *Aristolochia* species occur such as Sicily, Crete, Corsica, Sardinia and the Balearic islands are among the regions of highest species diversity (Lobo et al. 2001) and show the highest rate of endemism in the Mediterranean (Medail and Quezel 1997). Beside this, these regions are zones of high tectonic activity, microplate fragmentation and isolation (e.g. Rosenbaum et al. 2002) and are at least partly among the areas of the Mediterranean, which have been less affected by human activities (Thompson 2005). Unfortunately dating approaches are not suitable due to lack of reliable fossils within the genus *Aristolochia* or other calibration possibilities, to test the congruence of present day distribution and tectonic activity. The evolution of the newly found species complexes is likely to have evolved via influence of such activity.

The affinities between *A. sicula*, *A. clusii* and *A. tyrrhena* (*Aristolochia clusii* complex) perfectly fit to their geographical distribution (Fig. 2). *A. clusii*, as the most widespread species, is found in the southern part of Italy including Sicily and Malta (e.g. Nardi 1984, Borg 1927, Haslam et al. 1977). *A. sicula*, the least known and surely most endangered *Aristolochia* species of the Mediterranean, is only occurring in the understory of mountain forests in Sicily and *A. tyrrhena* is endemic to Sardinia and Corsica (e.g. Nardi 1987, Nardi & Arrigoni 1983). The occurrence of *A. tyrrhena* on both islands, Sardinia and Corsica, indicates that *A. tyrrhena* was present before the breaking of the landbridge connection between Corsica and Sardinia, 11.5-6 MYA (Orszag-Sperber et al. 1993) and its present day distribution on Corsica could be a relict of more widespread occurrence on Corsica in former times. All these limitations lead to the assumption that the current distribution of *A. clusii* complex are relicts and that the current species or their ancestors were distributed in the whole area, otherwise the colonisation of Corsica and Sardinia seems problematic (ant dispersed).

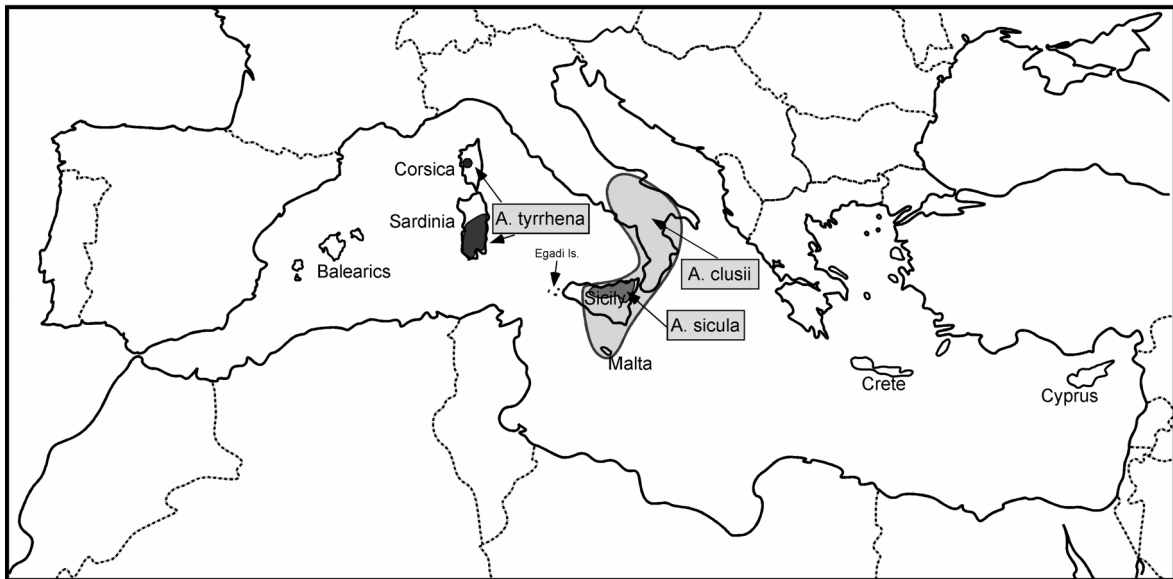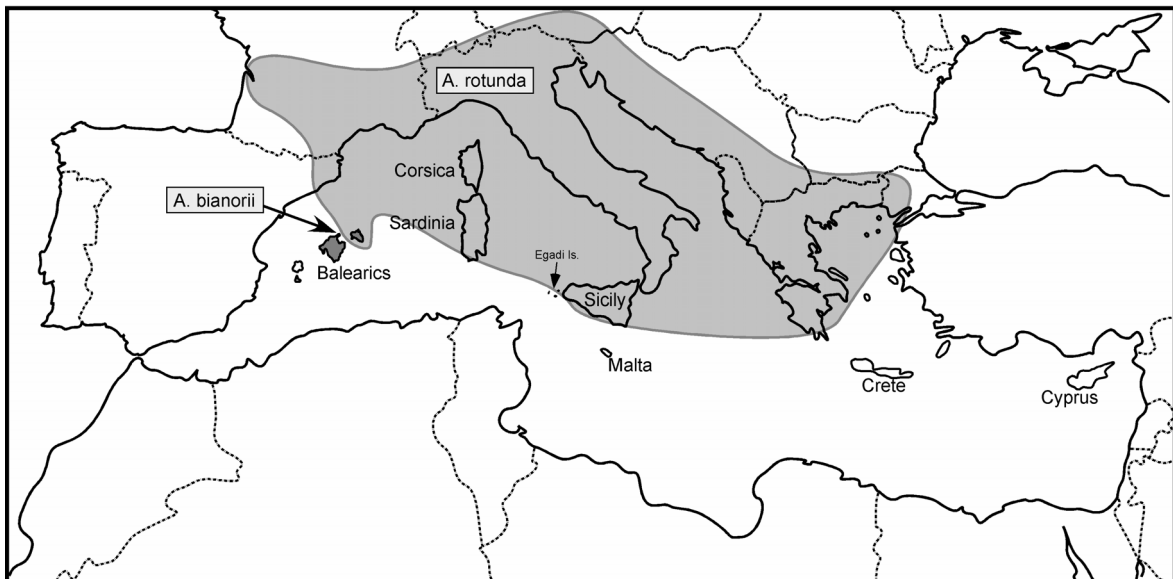Fig. 5 Distribution of the *Aristolochia clusii* complex.



Fig. 6 Distribution of the *Aristolochia rotunda* complex.



The distribution of *A. rotunda* covers NE-Spain, S-France, Italy to NW-Turkey (e.g. Nardi 1984, 1991). In contrast, *A. bianorii* is only found on the Balearic Islands Majorca and W-Minorca (Knoche 1921, Bonafe 1978, Bonner 1985, Castroviejo 1986, Bechett 1988, Romo 1994), whereas *A. rotunda* on the Balearic Islands is found only on the NE part of Minorca (Fig. 3). The populations of *A. bianorii* on Majorca are rather small and the number of individuals is very low. During own field and herbarium studies only six populations could be identified. This distribution pattern is interpreted here as neo-endemism, evolved via speciation at

the border of the distribution area (Minorca) of *A. rotunda*, by adaptation to different ecological conditions. *Aristolochia bianorii* occupies rock crevices by deep, thin rootstocks, tolerating low humidity, whereas *A. rotunda* generally grows at field margins, and abandoned fields with humus rich soil and higher water availability. In *Cyclamen balearicum*, a similar distribution is observed, likewise in accordance with ecological speciation (e.g. Debusche et al. 1997). In contrast to *C. balearicum* no occurrence of *A. bianorii* outside the Balearic Islands has been reported (e.g. S-France), negating a wider distribution in former times and the interpretation as relict refuge. Pollination of both species, which is not yet studied, could elucidate the speciation, as selfing could have played a major role for reproductive isolation of *A. bianorii* from the widespread *A. rotunda*.

A detailed population genetic study, sampling the whole distribution area of *A. rotunda* and all six populations on the Balearic Islands is currently in preparation (Wanke et al, unpublished data) and will elucidate the genetic diversity of both sister taxa with respect to the current distribution.


Evolution of growthform and underground parts

The two lianas represent the first branch of the west Mediterranean species. Lianas are predominant in Asian species of subsection *Aristolochia*, but also widely distributed among other clades of the genus *Aristolochia* indicating parallel evolution of this growth form in several closely related lineages. Parallel evolution of growth forms is hardly studied in most plant lineages, but has been reported for pendant life forms in Bryophyta (Quandt and Huttunen 2004) and Secamonoideae within Apocynaceae (Lahaye et al. 2005), besides few others. This implicates, that being a liana represents an underlying developmental constrain in the genus *Aristolochia* (Speck et al 2003), which is occurring in several monophyletic clades independently. It should be made clear that the two different growth forms (herb versus liana) are both arising from rootstocks. The East and the West Mediterranean species (excl. the lianas) have developed the same underground parts, which develop only from seeds, whereas the lianas are able to develop their rootstock at each node of branches or cuttings. Based on our data, globose (*A. pallida*, *A. lutea*, *A. sicula* and *A. rotunda*), elongated (*A. fontanesii* complex, *A. elongata*, *A. microstoma* and *A. bianorii*), and very thin, elongated (*A. tyrrhena*, *A. parvifolia*) rootstocks are most likely a parallel adaptation to the substrate and the

climatic condition. The globose rootstock is mostly found in forests with year-round humid soil. The elongated (sometimes up to 1m long) vertical rootstock is a character found usually in plants, which have to survive a very long and dry summer (e.g. N-Africa, Syria). In stony places, lacking almost any soil, the plants are sometimes becoming lithophytes like *A. bianorii.* This species develops a very thin elongated rootstock penetrating into cracks. Summarizing, our data clearly indicate, that the underground parts of *Aristolochia* are inappropriate to circumscribe clades. Only within a given species the rootstock is more or less homogeneous and might be used as a character, e.g. to estimate the age of the plant.

**Acknowledgements**

Appendix 1: Morphological characters and general ecological factors

1. underground parts:
(0) rootstock; (1) no rootstock, fleshy roots; (2) rhizome; (3) normal roots.

2. tiller offshoots:
(0) present; (1) absent.

3. rootstock shape:
(0) elongated, at least 5 times longer than broad; (1) elongated, less than 5 times longer than broad; (2) globose; (3) knobby; (4) no rootstock.

4. stem architecture:
(0) simple, not branching; (1) branching (above ground).

5. growthform:
(0) geophyte, dormant in summer; (1) evergreen liana; (2) perennial shrub; (3) deciduous liana.

6. perianth shape:
(0) curved; (1) not curved.

7. limb architecture:
(0) unilabiate; (1) bilabiate; (2) trilabialte.

8. utricle position:
(0) stiped utricle; (1) sessile utricle.

9. capsule dehiscent:
(0) apical; (1) basal.

10. sun exposure:
(0) complete shade; (1) medium shade; (2) sun; (3) full sun.

11. humidity of soil:
(0) high, along streams, rivers, wet places; (1) temporarily wet, never completely dry; (2) mixed, in forests or shrubbery, macchia, temporarely dry, very dry during summer; (3) very low, calcerous gravel or garigue, high permeability, low retaining.

12. soil:
(0) humose; (1) clay; (2) sand; (3) rock crevices; (4) gravel; (5) serpentin.

# Chapter 4     Molecular Evolution

## 4.1  Universal primers for a large cryptically simple cpDNA microsatellite region in *Aristolochia*.

**Abstract**

We provide a new and valuable marker to study species relationships and population genetics in order to trace evolutionary, ecological, and conservational aspects in the genus *Aristolochia*. Universal primers for amplification and subsequent sequencing of a chloroplast microsatellite locus inside the *trn*K intron are presented. Utility of the primers has been tested in 32 species representing all clades of *Aristolochia*, including population studies within the *A. pallida* complex, *A. clusii* and *A. rotunda*. The microsatellite region is characterized as a $(A_nT_m)_k$ repeat of 22–438 bp containing a combination of different repeats arranged as 'cryptically simple'.

Generally, microsatellites have been defined as short repeated sequences of not more than six nucleotides that are multiplied in arrays up to 100 nucleotides (cf. Goldstein & Schlötterer 1999 and references therein). During the last decade the definition is becoming vaguer and the term is now used for all kinds of short motif repeats including minisatellites and the derived cryptically simple regions. Nuclear microsatellites serve as the most common marker for all kinds of studies on the population level, whereas studies using cpDNA microsatellites are rather limited, as they show lower mutation rates than nuclear microsatellites (e.g. Provan et al. 1999). Furthermore, cpDNA microsatellites that originally have been defined as mononucleotide stretches (i.e. poly A or T stretches; Weising & Gartner 1999) have been reported to be highly homoplastic (e.g., Provan et al. 2001, Hale et al. 2004). More complex cpDNA microsatellites consisting of repetitive elements with varying size and repeat patterns as reported here seem to have more phylogenetic potential on the population level and within closely related species. These features as well as the non-recombinant, uniparental inheritance and the possible detection of reticulate evolution when compared with nuclear results might offer a new scope of cpDNA microsatellite studies on the population level.

The genus *Aristolochia* (Aristlochiaceae) differs from other angiosperms in several features e.g. expression patterns of the unique perianth (Jaramillo & Kramer 2004) but most importantly the utilization of plant material in herbal medicine makes studies necessary. Recent analyses indicate that some of the reported secondary components, such as "aristolochic acids" are nephrotoxic, carcinogenic and mutagenic. Therefore many countries prohibited the distribution of herbal medicines containing *Aristolochia* extracts or leaf material etc. As secondary metabolites are known to change conformation very fast, even within species, different effects on human health could be expected when different species or mixtures were used. Therefore, tools to distinguish between species or populations are urgently needed. The described primers represent a highly polymorphic tool for all kinds of studies in the increasingly important plant genus *Aristolochia*.

Table 1 List of the examined species, with the corresponding taxonomical affiliation. Species with analyses on population level are indicated with an asterisk. Cross species amplification using the primers AR-trnK-420F and AR-trnK-1320R was successful for all taxa.

| Taxonomic Group | Investigated species |
|---|---|
| *Isotrema* | A. reticulata, A. serpentaria |
| *Endodeca* | *A. manshuriensis, macrophylla, A. californica, A. tomentosa, A. westlandii, A. arborea, A. salvadorensis* |
| *Pararistolochia* | *A. promissa, A. triactina* |
| *Aristolochia s.l.* incl. groups like *Howardia*, *Einomeia* and *Holostylis* | *A. labiata, A. eriantha, A. cordiflora, A. cruenta, A. chilensis, A. gigantea, A. pentandra, A. micrantha, A. erecta, A. bracteolate, A. albida, A. acuminata, A. baetica, A. pistolochia, A. parvifolia, A. pichinchensis, A. gorgona, A. clusii*, A. rotunda*, A. pallida*, A. holostylis* |

The microsatellite region is located within the *trnK* intron and was found during a study on the evolution of Aristolochioideae (Wanke et al., 2006b). Interestingly, the genus *Aristolochia* is the only plant genus known to accumulate such microsatellites in the *trn*K intron (Wanke & Quandt unpublished data) that can be described as an $(A_nT_m)_k$ repetitive region ranging from 22 to 438 bp. AT repeats of plastid regions spanning more than 20 repeats have previously only been found in the liverwort genus *Fossombronia* ($AT_{75+n}$, Quandt & Stech 2005). The presence and utility of the region was tested among 32 species of *Aristolochia* (Wanke et al., 2006b) representing all clades (Neinhuis et al. 2005). A list of examined species with the indication of the corresponding grouping is presented in Table 1, cross-species amplification was successful for all taxa. In order to illustrate the intraspecific variability of the region in *Aristolochia* we sequenced and analysed 30 populations of the *Aristolochia pallida* complex. Alignment was carried out manually using PhyDE[®] (Müller et al. 2005). Thereafter, indels within the satellite region were automatically coded employing the simple indel coding algorithm (sic, Simmons and Ochoterena 2000) via Seqstate (Müller 2005) using the PhyDE[®] plugin option (Table 2). Alignment of the $(A_nT_m)_k$ repeats over the whole genus was impossible due to the "cryptically simple" nature of the repetitive elements (Tautz et al. 1986) that rendered the homology assessment ambiguous. Primers were designed manually and placed in more conserved areas of the group II intron, taking general primer rules into account: AR-trnK-420F AAG TGA ATA AAT GGA TAG AGC ($T_m$: 55,6°C); AR-trnK-1320R ATC GCT CTT TTG ACT TTG G ($T_m$: 56,2°C). PCR-reactions were carried out employing a T3 (Biometra) using the following parameters: an initial denaturation at 96°C for 1.5 min. followed by 34 cycles of denaturation at 95°C for 30 sec., annealing at 50°C for 1 min. and extension at 72°C for 1.5 min plus a final extension at 72°C for 20 min. The 25 µl

PCR reactions contained: 0.2 $\mu$l DNA template, 3.3 $\mu$l dNTP mix (1.25 mM each), 0.5 $\mu$l of each primer (20 pmol/$\mu$l), 1 U Taq Polymerase (PeqLab) and 1 x Taq Polymerase buffer S (PeqLab). After separation by gel electrophoresis (1,2% Agarose Gel for 2.5 hours) the PCR products were purified using the NucleoSpin II kit (Macherei Nagel) and directly sequenced using the CEQ DTCS Quick Start Kit (Beckman Coulter) on a CEQ 8000 automated sequencer, following the standard protocol provided by the manufacturer.

The universal primers described above will not only provide a useful marker to study populations, ecology and evolution in *Aristolochia*, but hold the potential to detect *Aristolochia* tissue in an unknown drug sample as well as to identify the species and its origin.

Table 2: Characterization and relative frequency of haplotypes within the *Aristolochia pallida* complex, including EMBL accession number. Among 30 tested individuals 21 haplotypes were observed, with a size ranging from 386 to 465 bp. The gene diversity was calculated as 0.9632 +/- 0.0213. In order to characterize the complex cryptically simple repeat motifs that correlate with alternations of an $(A_nT_m)_k$ motif, a simple indel coding (sic) approach as advocated by Simmons and Ochoterena (2000) was used.

| Haplotype | relative haplotype frequencies | size (bp) | indel-matrix (sic) | EMBL accession number |
|---|---|---|---|---|
| 01 | 0.0667 | 388.0 | 10??100?1??10?0????1????????111 | AM237617, AM237618 |
| 02 | 0.0667 | 393.0 | 10??100?1?10000??????1?????????111 | AM237604, AM237605 |
| 03 | 0.0667 | 397.0 | 1000000011?100000??????1?????????111 | AM237606, AM237607 |
| 04 | 0.1 | 442.0 | 10?10000110001??????1000?110111 | AM237629, AM237628, AM237630 |
| 05 | 0.167 | 399.0 | 10?10000171000000??????1?????????111 | AM237608, AM237609, AM237610, AM237613, AM237615 |
| 06 | 0.0333 | 387.0 | 11??100?1??10?0????1????????111 | AM237603 |
| 07 | 0.0333 | 404.0 | 10?10000171000000??????1?????????011 | AM237614 |
| 08 | 0.0333 | 402.0 | 11???10??70000011100000??????111 | AM237620 |
| 09 | 0.0333 | 442.0 | 11??10??1?10?0??10000001111111 | AM237621 |
| 10 | 0.0333 | 399.0 | 11??????1??70000000??????1?????????100 | AM237602 |
| 11 | 0.0333 | 392.0 | 10?10000171??1?????????1?????????111 | AM237612 |
| 12 | 0.0333 | 445.0 | 10?10000171?10?0???1000000101111 | AM237624 |
| 13 | 0.0333 | 401.0 | 1010000010000010???????1?????????111 | AM237619 |
| 14 | 0.0333 | 447.0 | 10?1000001000010???10000001111111 | AM237625 |
| 15 | 0.0333 | 420.0 | 00??100?0?100000000000001?????111 | AM237611 |
| 16 | 0.0333 | 386.0 | 11??????1????000???????1?????????111 | AM237601 |
| 17 | 0.0333 | 394.0 | 10?10000171?10?0??1?????????111 | AM237616 |
| 18 | 0.0333 | 441.0 | 10?10000171?10?0??10000011111111 | AM237626 |
| 19 | 0.0333 | 436.0 | 10?10000171?10?0??710000?1111111 | AM237622 |
| 20 | 0.0333 | 464.0 | 10?10000171?10?0??710000000011111 | AM237623 |
| 21 | 0.0333 | 465.0 | 10?10000110000??????1000?010111 | AM237627 |

# Chapter 5    Ecology and Biomechanics

## 5.1  Trapped! Pollination of Aristolochia pallida Willd. in the Mediterranean

**Abstract**

A first study of the pollination biology of a Mediterranean *Aristolochia* species in its natural habitat is presented. 183 flowers of *Aristolochia pallida* were investigated, which in total contained 73 arthropods, dominated by two groups of Diptera, Sciaridae (37%) and Phoridae (19%). However, only Phoridae are regarded as potential pollinators, since pollen has been found exclusively on the body surfaces of these insects. All Phoridae belong to the genus *Megaselia* and are recognised as four undescribed species. The measurements of flower and insect dimensions suggest that size is an important constrain for successful pollination: 1) the insects must have a definitive size for being able to enter the flower and 2) must be able to get in touch with the pollen. Only very few insect groups found in *Aristolochia pallida* fulfil these size requirements. However, size alone is not a sufficient constrain as too many fly species of the same size might be trapped but not function as pollinators. Instead, specific attraction is required as otherwise pollen is lost. Since all trapped Phoridae are males, a chemical attraction (pheromones) is proposed as an additional constrain. Since *A. pallida* flowers are protogynous, the record of *Megaselia* loaded with pollen found in a flower during its female stage proves that this insect must have been visited at least one different flower during its male stage before. Further on, this observation provides strong evidence that the flowers are cross-pollinated. All these factors indicate a highly specialised pollination of *Aristolochia pallida* by *Megaselia* species.

**Introduction**

The genus *Aristolochia* (Aristolochiaceae) consists of approximately 500 species, most of which are found in tropical, subtropical, and Mediterranean regions (Neinhuis et al. 2005; Wanke et al. 2006b, 2006c). *Aristolochia* flowers are highly derived, functioning as a trap for arthropods in order to ensure pollination (Knoll 1929). They are generally supposed to be pollinated by flies (Insecta: Diptera), attracting potential pollinators by a stinky odour (Sprengel 1793; Hildebrand 1867; Müller 1873; Correns 1891, 1892; Knuth 1899; Faegri and van der Pijl 1979; Proctor et al. 1996). However, detailed studies in different *Aristolochia* lineages are largely lacking.

It is not known whether the attracting mechanism is an odour deceiving decaying organic materials, animal excrements, carrion or fungi, a visual attraction, a chemical mimetic to fly pheromones or a combination of all of these features. Regarding the different flower types, its modifications, and different sizes, it is most probable that different mechanisms of pollinator attraction have been evolved in *Aristolochia.*

The flower morphology poses a tubular perianth that is monosymmetric, 1-3(-6)-lobed and extremely modified. The perianth is subdivided into 1) an utricle, a balloon like structure forming the trap for the pollinators, containing the gynostemium, 2) a syrinx, a narrow zone between the tube and the utricle, forming the sluice of the trap and 3) the tube/limb, which appearance is diverse in size, form and colour (Gonzalez and Stevenson 2000a) (Fig. 1). The inner part of the tube is densely covered with downward directed trichomes to prevent the escape of potential pollinators (Oelschlägel et al. in press). At the base of the utricle, around the gynostemium, a somewhat circular transparent area exists which deceives an exit for the trapped pollinators. The gynostemium itself is the product of the fusion of the styles, stigmas, and stamens. During the male stage the pollen sacs open and spread considerably resulting in an enlarged surface (Gonzalez and Stevenson 2000b), presenting the pollen to the insect, which subsequently transports the pollen to a different flower.

**Fig. 1** Drawing of an *Aristolochia* flower



Limb

Mouth
trapping hairs

Tube

Syrinx
Utricle
Gynostemium

Ovary
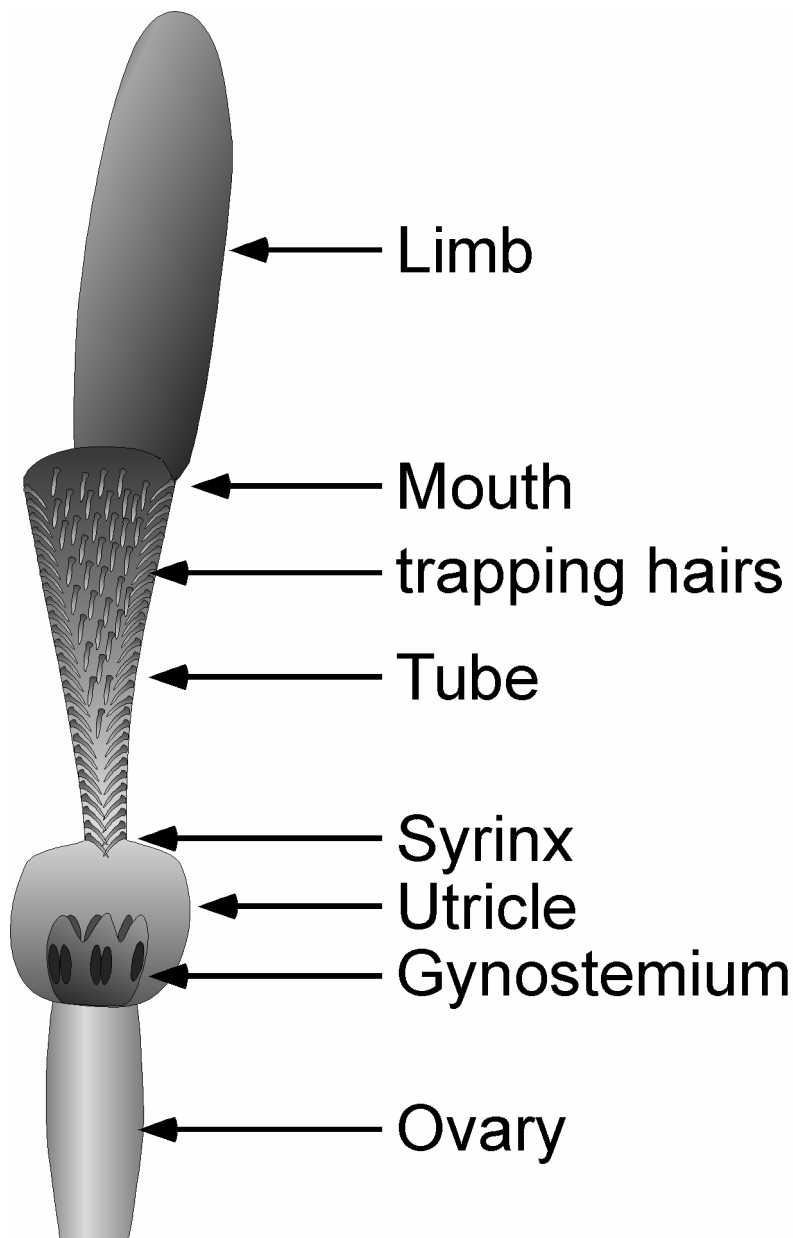
Attracted pollinators arriving on the flowers limb or the tube wall slide into the tube as the surface is covered with wax crystals. After being trapped, an escape is prevented by the downward directed hairs, so that only the direction deeper into the tube is possible. After passing the syrinx, the pollinator is attracted by the light, which shines through an annular, translucent window pane at the base of the utricle (Oelschlägel et al. in press). Due to the attraction by light, the pollinator eventually deposits the pollen on the stigmatic surface during the female stage while trying to escape through the 'window'. Since the flowers are protogynous, the flower enters the male period shortly after closing of the stigmatic lobes by opening the anthers and exposing the pollen. At this stage, the pollinator is loaded with pollen. The whole procedure of

being trapped may last several days. In order to keep the pollinators alive during this time, two nectaries secrete a sugar rich solution to feed the flies (Daumann 1959). In addition hairs cover most of the inner walls of the utricle in order to provide enough humidity for captives (Neinhuis, unpublished data). At the end of the male stage, the trichomes, which form the trap wilt, lose their stiffness, and the pollinator is released. The syrinx and tube hairs shrivel and the flower moves from vertical to horizontal orientation to ensure that imprisoned flies are able to escape and pollinate the next flowers (Oelschlägel et al. in press).

In the Mediterranean and adjacent Near East ~50 *Aristolochia* species occur, all of them are endemic to the area (Wanke et al., see chapter 3.2). The only exception is *A. clematitis* L., which is probably not native to this region (Wanke et al., see chapter 3.2.) and for which several observations on their pollination biology have been made clearly outside its natural range or in cultivation (Ule 1898; Daumann 1971; Havelka 1978). The most recent studies on the pollination biology of Mediterranean *Aristolochia* species date back to the 19th century (Delpino 1868, 1869), dealing with *A. pallida* Willd., *A. rotunda* L. and *A. sempervirens* L. (= *A. altissima* Desf.) from Italy. Unfortunately, these historic publications do not allow verification whether the studied plants are the same species that are currently accepted under these names. Reported flower visitors of *A. clematitis* are Phoridae: *Megaselia sordida* (Zetterstedt) (as *Phora carbonaria* Zetterstedt); *Megaselia pulicaria* (Fallen) (as *Phora pulicaria* Fallen) and Chironomidae: *Chironomus gracilis* Macquart (a nomen dubium, cf. Ashe and Cranston 1990: 353), but no information is given whether these insects carried pollen and thus the real pollinators remain unknown. Therefore, the pollination biology of the Mediterranean *A. pallida* is investigated in its natural habitat with special reference to the composition of the animal assemblage attracted by the flowers and the animals carrying pollen.

**Materials and methods**

Flowers were collected in the field in natural occurring populations (in April). Sample sites of *Aristolochia pallida* are as follows (including information on vouchers): Italy, Mt. li Foi II (40°40'03,6"N 015°43'35,3"E), 1179 m, 5/16/2005, Wanke 196 (DR); Mt li Foi I (40°40'06,04"N 015°43'45,4"E), 1144 m, 5/16/2005, Wanke 204 (DR); Sant Eufemia, (38°15'16,0"N 015°51'03,9"E), 658 m, 5/18/2005, Wanke 206 (DR); Mt. Vulture (40°56'56,6"N 015°38'42,7"E), 1067 m, 5/16/2005, Wanke 207 (DR); Monticchio (40°56'13,2"N 015°37'02,1"E), 747 m, 5/15/2005, Wanke 190 (DR). The flower and the pedicel were preserved in 70% ethanol. The different stages of anthesis ranged from recently opened flowers to nearly dropped flowers. Subsequent investigation was made using a stereomicroscope Olympus SZX12 with magnification from 7x to 144x times.

Measurements of functional perianth parts

In order to evaluate the minimum - maximum size of potential pollinators we measured: a) the most narrow part of the tube, the syrinx; b) the distance between utricle wall and gynostemium, and c) the height of trapped arthropod (between underside of the coxae and highest point of the thorax). Measurements were carried out using an integrated ocular micrometer calibrated with stage micrometer. The stage of the stigma was recorded as pale or dark, as indication of the female or male phase of the particular flower.

Taxonomy of possible pollinators

Trapped arthropods were determined and counted. Identification and nomenclature of Diptera follows Papp and Darvas (2000), for scuttle flies (Phoridae) Disney (1989, 1994) and for black fungus gnats (Sciaridae) Menzel and Mohrig (2000).

Scanning Electron Microscopy (SEM) is used to search for pollen on the body surfaces of the insects as well as to distinguish phorid species.

## Results

Altogether, 183 flowers were investigated, 49 of which (27%) contained in total 73 arthropod specimens. The majority of trapped species are Diptera (87.7%), beside other arthropods. Within the Diptera, scuttle flies (Phoridae) and black fungus gnats (Sciaridae) are dominating in numbers with 19.2 % and 37 % respectively (Table 1). 30 (61.2%) of the 49 flowers containing arthropods showed a pale stigma indicating the female stage and 19 (38.8%) a dark stigma (male stage, open anthers).

**Table 1** Sum of arthropod specimens trapped in the flowers of *A. pallida*. The number of trapped individuals is given per order (and family), their dominance (portion on the entire catch) and the portion found in pale (female stage) and dark (male stage) flowers. *One specimen outside the flowers, floating in ethanol

| Order | Family | Number of individuals | Dominance (%) | Flower stage: pale/dark |
|---|---|---|---|---|
| Acari | | 6 | 8,22 | 2/4 |
| Coleoptera | | 3 | 4,11 | 1/2 |
| Collembola | | 1 | 1,37 | 1/0 |
| Diptera | Cecidomyiidae | 2 | 2,74 | 2/0 |
| Diptera | Chironomidae | 1 | 1,37 | 0/1 |
| Diptera | Empididae | 2 | 2,74 | 2/0 |
| Diptera | Phoridae | 14 | 19,18 | 10/3* |
| Diptera | Sciaridae | 27 | 36,99 | 14/13 |
| Diptera | Sphaeroceridae | 1 | 1,37 | 1/0 |
| Heteroptera | indet. (larvae) | 7 | 9,59 | 4/3 |
| Homoptera | Aphidae (larvae) | 4 | 5,48 | 3/1 |
| Hymenoptera | | 2 | 2,74 | 2/0 |
| Lepidoptera | (larva) | 1 | 1,37 | 1/0 |
| Thysanoptera | | 2 | 2,74 | 1/1 |

<u>Phoridae (Diptera)</u>

All captured Phoridae are males (Table 2) and could be separated into four morphospecies A, B, C and D of the genus *Megaselia.* The species are recognised by differences of the bristle patterns of mesopleuron, epandrium, hypandrium, penis and anal tube as well as the colour of the halteres (Table 3). Phoridae are the only insects that carried pollen, which was found on the dorsal surface of the meso- and metathorax. The pollen can be identified clearly because of its inaperturate exine, which is characteristic for *Aristolochia* (Fig. 2; Gonzalez 1999). About 50% of the investigated phorid individuals are carrying different amounts of pollen grains (3–50) (Table 2). These individuals were predominantly found during the female flower stage.

**Table 2** Number and sex of Diptera trapped in *Aristolochia pallida*, along with voucher information, stigma stage and the number of pollen grains per individual are given. Plant material has been deposited in the Herbarium Dresdense (DR) by the collection number. Arthropod specimens are deposited at the Museum für Tierkunde Dresden. [1] Morpho species of *Megaselia* are distinguished by characters given in Table 3. [2] Pollen grains per individual

| Voucher | Stigma | Dipterous taxon [1] | Number | Sex | Pollen [2] |
|---|---|---|---|---|---|
| **Phoridae** | | | | | |
| W206-br001 | dark | *Megaselia* sp. A | 1 | male | 0 |
| W206-br020 | dark | *Megaselia* sp. A | 1 | male | 20 |
| W206-br009 | pale | *Megaselia* sp. B | 1 | male | 0 |
| W206-br023 | dark | *Megaselia* sp. B | 1 | male | 0 |
| W206-br055 | - | *Megaselia* sp. B | 1 | male | 3 |
| W207-br003 | pale | *Megaselia* sp. C | 1 | male | 0 |
| W196-br011 | pale | *Megaselia* sp. C | 1 | male | 10 |
| W196-br011 | pale | *Megaselia* sp. C | 1 | male | 20 |
| W196-br011 | pale | *Megaselia* sp. C | 1 | male | 20 |
| W204-br004 | pale | *Megaselia* sp. C | 1 | male | 0 |
| W204-br005 | pale | *Megaselia* sp. C | 1 | male | 0 |
| W204-br014 | pale | *Megaselia* sp. C | 1 | male | 0 |
| W206-br017 | pale | *Megaselia* sp. D | 1 | male | 3 |
| W206-br017 | pale | *Megaselia* sp. D | 1 | male | 50 |
| **Sciaridae** | | | | | |
| W207-br016 | dark | *Bradysia rufescens-group* | 1 | male | 0 |
| W207-br008 | pale | *Corynoptera parvula-group* | 1 | male | 0 |
| W206-br017 | pale | *Corynoptera sp.* | 1 | female | 0 |
| W207-br010 | pale | *Corynoptera sp.* | 2 | female | 0 |
| W207-br035 | pale | *Epidapus microthorax-group* | 1 | male | 0 |
| W196-br001 | pale | *Pseudolycoriella morenae-group* | 1 | male | 0 |
| W204-br004 | pale | *Pseudolycoriella morenae-group* | 1 | female | 0 |
| W204-br005 | pale | *Pseudolycoriella morenae-group* | 2 | female | 0 |
| W204-br006 | dark | *Pseudolycoriella morenae-group* | 7 | female | 0 |
| W204-br006 | dark | *Pseudolycoriella morenae-group* | 1 | male | 0 |
| W204-br011 | pale | *Pseudolycoriella morenae-group* | 2 | female | 0 |
| W204-br013 | dark | *Pseudolycoriella morenae-group* | 1 | male | 0 |
| W204-br016 | pale | *Pseudolycoriella morenae-group* | 1 | female | 0 |
| W204-br021 | pale | *Pseudolycoriella morenae-group* | 1 | female | 0 |
| W204-br025 | pale | *Pseudolycoriella morenae-group* | 1 | female | 0 |
| W204-br027 | dark | *Pseudolycoriella morenae-group* | 1 | male | 0 |
| W204-br034 | dark | *Pseudolycoriella morenae-group* | 2 | female | 0 |

**Table 3** Characters of the four morphospecies of *Megaselia* (Diptera: Phoridae) found as pollinators in *Aristolochia pallida*. Terminology after Disney (1983)

| Character | Morphospecies | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Mesopleuron | five bristles in a row | six bristles irregular | bare | seven bristles irregular |
| Epandrium | one bristle beside hairs | only small mid stripe haired | nearly entire surface haired | one bristle beside hairs |
| Hypandrium | horizontal | vertical | horizontal | horizontal |
| Penis | ventral edge concave, posterior edge convex | ventral edge concave, posterior edge straight | ventral edge straight, posterior edge convex | ventral edge concave posterior edge concave |
| Halteres | dark | brownish | brownish to yellowish pale | dark |

**Fig. 2** (a) Pollen grains on the thorax of *Megaselia* sp. (b) one single pollen grain enlarged, showing a germinating pollen tube
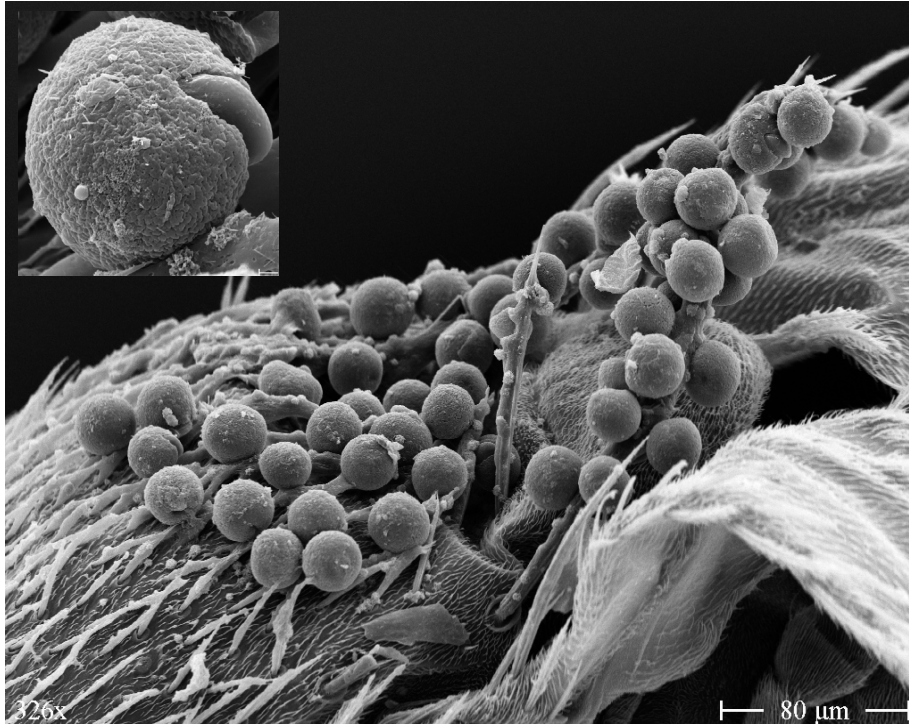


### Sciaridae (Diptera)

Sciaridae are the most frequently found arthropod group in the flowers, represented by 20 specimens of *Pseudolycoriella*, four specimens of *Cornyoptera* and one specimen of *Bradysia* and *Epidapus*, each. The Sciaridae were found in flowers during the female as well as the male stage, but none of the Sciaridae carried pollen (Table 2).

### Remaining Arthropoda

Each of the remaining arthropod groups has been found in the flowers only in small quantities. None of them carried pollen and consequently can be excluded as pollinators with high probability. It might be possible that at least some of these arthropod species have a closer relation with *Aristolochia pallida*, but their occurrence inside the flowers is probably purely accidental. Many of these remaining arthropods are found in larval stage (Table 1).

### Measurements of flower parts and pollinator size

The diameter of the syrinx has been measured from all 183 flowers. The diameter has a mean of 1.37 mm ± 0.11; minimum of 1.12 mm and a maximum of 1.68 mm (median 1.40 mm). For a total of 60 flowers, the distance between the utricle wall and

upper edge of the gynostemium was measured, as this morphological character was only recorded after dissection of the flower and preparation of the trapped insects, the number of flowers showing an intact utricle, was lower, than the original number of studied flowers. This interspace has a mean of 1.05 mm ± 0.08, a minimum of 0.90 mm, and a maximum of 1.20 mm (median = 1.00 mm) (Table 4; Fig. 3). Comparing the measurements of the flower and the size of the arthropods, the arthropods could be divided into four groups: 1) specimens bigger than the diameter of syrinx, hence they would not get through the syrinx and may block the flower tube (Chironomidae); 2) specimens bigger than the interspace between utricle wall and gynostemium with no chance to get in touch with the anthers (Empididae); 3) specimens small enough to pass the interspace but big enough to interact with gynostemium (Phoridae, Sciaridae), 4) specimens much smaller in size, than the distance between utricle wall and the gynostemium.

**Fig. 3** Comparison of arthropod size, syrinx diameter, interspace between utricle wall and gynostemium and the taxonomic groups of trapped visitors. Highlighted pale grey part with dashed lines represent insects which are recognised as potential pollinators according to size requirements
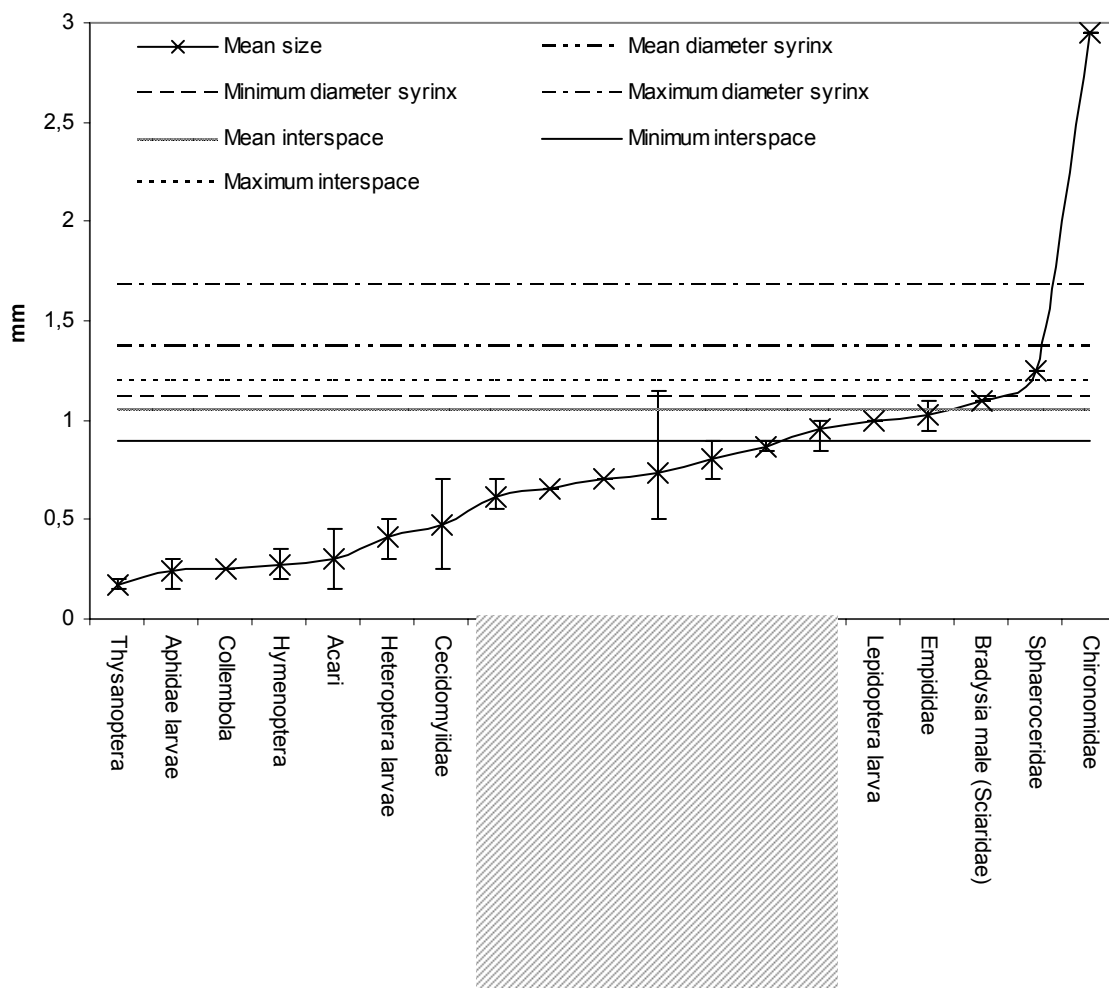
The latter are not suitable of being loaded with pollen, because no part of their body would get in contact with the anthers (all remaining arthropods trapped by the flowers)(Fig. 3). The first group contains a single Chironomidae, which got stuck in the syrinx. The second group includes representatives of the dipteran families Sphaeroceridae, Empididae and *Bradysia* species of Sciaridae. The third group contains *Megaselia* and related species and the fourth group consist of the Cecidomyiidae and all arthropods smaller than these (Fig. 3).

**Table 4** Size in millimetre of trapped arthropods, their numbers found in the flowers, along with the taxonomic group, to which the insects belong. [1] No standard deviation available if only one specimen

| Arthropod group | Mean | Minimum | Maximum | Median | Standard deviation [1] | Number |
|---|---|---|---|---|---|---|
| Thysanoptera | 0,18 | 0,15 | 0,20 | 0,18 | 0,04 | 2 |
| Aphidae larvae | 0,24 | 0,15 | 0,30 | 0,25 | 0,06 | 4 |
| Collembola | 0,25 | 0,25 | 0,25 | 0,25 | - | 1 |
| Hymenoptera | 0,28 | 0,20 | 0,35 | 0,28 | 0,11 | 2 |
| Acari | 0,30 | 0,15 | 0,45 | 0,30 | 0,11 | 6 |
| Heteroptera larvae | 0,41 | 0,30 | 0,50 | 0,40 | 0,07 | 7 |
| Cecidomyiidae | 0,48 | 0,25 | 0,70 | 0,48 | 0,32 | 2 |
| Corynoptera female | 0,62 | 0,55 | 0,70 | 0,60 | 0,08 | 3 |
| Epidapus male | 0,65 | 0,65 | 0,65 | 0,65 | - | 1 |
| Corynoptera male | 0,70 | 0,70 | 0,70 | 0,70 | - | 1 |
| Coleoptera | 0,73 | 0,50 | 1,15 | 0,55 | 0,36 | 3 |
| Megaselia male | 0,81 | 0,70 | 0,90 | 0,80 | 0,07 | 14 |
| Pseudolycoriella male | 0,86 | 0,85 | 0,90 | 0,85 | 0,02 | 4 |
| Pseudolycoriella female | 0,95 | 0,85 | 1,00 | 0,95 | 0,04 | 17 |
| Lepidoptera larva | 1,00 | 1,00 | 1,00 | 1,00 | - | 1 |
| Empididae | 1,03 | 0,95 | 1,10 | 1,03 | 0,11 | 2 |
| Bradysia male | 1,10 | 1,10 | 1,10 | 1,10 | - | 1 |
| Sphaeroceridae | 1,25 | 1,25 | 1,25 | 1,25 | - | 1 |
| Chironomidae | 2,95 | 2,95 | 2,95 | 2,95 | - | 1 |

**Discussion**

The present study reveals an assemblage of 73 arthropods in 49 *Aristolochia pallida* flowers, of which two groups, the Sciaridae and Phoridae (both Insecta, Diptera) where predominant with 37% and 19% respectively. However, only one arthropod group, the Phoridae, can be regarded as potential pollinators of this plant species as pollen has been found on the body surfaces of these insects only. All found Phoridae belong to the genus *Megaselia*.

Since the *A. pallida* flowers like all other Aristolochiaceae are protogynous, the record of a *Megaselia* individual loaded with pollen and found in a flower during its female stage is a strong prove that this insect must have been visited at least one different flower during its male stage before. Therefore, the repeated visit of one *Megaselia* specimen in flowers of *A. pallida* suggests a specific attraction, though the specific mechanisms are not known yet. In addition, this observation provides strong evidence that the flowers are cross pollinated, since the *Megaselia* specimens carry the pollen to the flower during its female stage, before it develops the pollen itself. Self-pollination has been discussed for other *Aristolochia* species (e.g., Petch 1924; Razzak et al. 1992; Trujillo and Sérsic 2006) and is generally known to occur as addition to cross pollination in endemic plant species to ensure survival (Thompson 2005). Whether self-pollination is a regular case in *A. pallida* needs further investigation.

Phoridae have repeatedly been recorded in pollination studies of *Aristolochia* species, especially from the tropics. Three phorid species associated with the flowers of *Aristolochia inflata* Kunth and *A. maxima* Jacq. are recorded from Panama (Disney and Sakai 2001; Sakai 2002). *Megaselia metropolitanoensis* Disney and *Puliciphora pygmaea* (Borgmeier) have been reared from shed *Aristolochia* flowers collected from the forest floor, but there is no evidence that these two species are also pollinators of the respective flowers. Contrarily, adults of *Megaselia sakaiae* Disney were abundantly found in flowers of *A. inflata* and *A. maxima* during anthesis. From 376 individuals found in *A. maxima*, 375 were females, as well as the 108 adults found in the flowers of *A. inflata*. In both *Aristolochia* species, the phorids were observed licking nectar secreted from the hairs on the inner surface of the utricle (Disney and Sakai 2001). In a more detailed study, 81% of the females carried pollen grains and thus are considered as pollinators of the flowers (Sakai 2002). The

females of *M. sakaiae* lay eggs inside the flowers of both species. Hatched larvae fed inside the flowers on sepals and the gynostemium and completed their development on the fallen, decaying flowers on the ground. The adults already emerged 15 days after oviposition (Disney and Sakai 2001).

Hime and Costa (1985) report 109 adult *Megaselia aristolochiae* Prado in flowers of *Aristolochia labiata* Willd. in Brazil. 102 specimens of these were females, which laid eggs inside the flowers. Later the larvae developed in cavities within the utricle wall. There is no information given about any pollen adhering to the insects.

Earlier than 1928 Brues recorded phorid flies from flowers of *Aristolochia elegans* Mast (= *A. littoralis* D. Parodi.) from Cuba: *Dohrniphora cornuta* (Bigot) (=*Phora venusta* Coquillett) and *Megaselia* (= *Aphiochaeta*) *scalaris* (Loew). Borgmeier (1925) reports hundreds of specimens of *Megaselia scalaris* (as *Apiochaeta xanthina* Speiser) again of *A. elegans* in Brasil. Hall and Brown (1993) investigated *A. elegans* in Florida. The authors collected 32 flowers, in which they found 349 phorid flies. 96% of them were males, belonging to seven species: *Megaselia scalaris*, *M. aurea* (Aldrich), *M. perdita* (Malloch) and four unidentified *Megaselia*-species. The authors provide a photograph showing a male of *Megaselia aurea* carrying a clump of *A. elegans* pollen, but no evidence is provided for correct identification of this pollen (see below).

In Argentina, Trujillo and Sersic (2006) report that flowers of *Aristolochia argentina* Griseb. attract mainly female scuttle flies of the genus *Megaselia*, carrying pollen.

Carr (1924) reports besides other fly species *Aphiochaeta dahli* Becker from the flowers of *Aristolochia macrophylla* Lam.. (= *A. sipho* L'Hér), cultivated in England. Unfortunately, no further information is provided. *A. macrophylla* is also visited by *Megaselia nigriceps* (Loew) (Schmitz et al. 1938–1981, as *Apiochaeta*; Speiser and Schmitz 1957, as *Apiochaeta projecta* (Becker)).

*A. tomentosa* Sims. is known to be visited by *Megaselia fungicola* (Coquillett) (Robertson 1928, as *Phora*).

Burgess et al. (2004) investigated *Aristolochia grandiflora* Sw. in Mexico. At the end of the first day of the female flower period they found about 454 insects per flower, 269 were phorids, 144 staphylinids, 13 calliphorids, 16 muscids, and 4 heleomyzids. During the second day and after male flower period, the number of phorids still increased to 399 and that of staphylinids to 203. In three day old flowers, the authors found about 400 phorid larvae, which however did not develop inside the flowers and

died. Though phorids where the most common insects in the flowers of *A. grandiflora*, they carried only little amount of pollen compared to the Calliphoridae. Oviposition of phorids probably happens as a result of getting trapped, which is supported by the fact that all the larvae did not develop inside the flower and subsequently died.

During a three year study on *Aristolochia baenzigeri* B. Hansen et L. Phuphathanaphong in Thailand (Bänziger and Disney 2006), 124 individuals of Phoridae, five of Agromyzidae and one of Sphaeroceridae were found inside the flowers. Phoridae were represented by 21 species from eight genera. *Dohrniphora cornuta* was the most dominant species in terms of both, proportion of all phorid individuals (39 of 92, which is 42%) as well as proportion of individuals carrying pollen (35 of 66, which is 53%). Males were carrying pollen slightly more frequent than females (20 (57%) males versus 15 (43%) females). 11 other phorid species with altogether 31 individuals were also found covered with pollen. In contrast to the results from the New World, phorids did not use the flowers as brood substrate. The cosmopolitan *Dohrniphora cornuta* breeds in a wide variety of decaying organic materials (Disney 1994). Furthermore the adults visit flowers of *Aristolochia elegans* in the New World (Brues 1928). *A. baenzigeri* belongs to the subgenus *Isotrema*, which has no trapping hairs in the tube and at the syrinx and therefore might be less selective for pollinators because this mechanism is missing. There is no evidence that the phorids pollinate the flowers of *A. baenzigeri* because it has not been shown yet that that these flies were trapped with pollen in the female flower stage (cf. Bänziger and Disney 2006).

The Mediterranean *A. sempervirens* L. (= *A. altissima* Desf.) is visited by *Megaselia pulicaria* (s.l.) and *Megaselia pumila* (Meigen) (Delpino 1869).

As mentioned above, the repeated flower visits of *Aristolochia pallida* by one *Megaselia* specimen suggests a highly specific interaction between these two organisms. However, the mechanisms selecting as well as attracting potential pollinators of *Aristolochia* species are insufficiently known. So far, two main strategies are recognised for *Aristolochia* pollination biology: (1) the micromyiophily attracting micro-diptera by small sized, bright coloured flowers, but without any strange smell recognizable to humans, and (2) the sapromyiophily attracting macro-diptera by large to giant flowers with dark colours and a smell reminding humans on animal excrements, sweat, carrion, rotten fish, old cheese or decaying plant material (Faegri

and van der Pijl 1979; Kugler 1970; Larson et al. 2001; Proctor et al. 1996). Sapromyiophily is described, e.g., for *A. grandiflora* in Mexico (Burgess et al. 2004), but might be a complex strategy as discussed by Larson et al. (2001). Its morphology as described by Bello et al. (in press) is similar to *A. pallida* concerning the trapping mechanism, but, its much bigger size allow that insects like certain Calliphoridae, Muscidae, Sepsidae and Heliomycidae (Diptera: Brachycera), with a larger body size can enter the utricle (Burgess et al. 2004).

According to these definitions, the Mediterranean *Aristolochia* species represent the micromyiophily type.

That size matters for the pollination of *Aristolochia* flowers has already been shown by Brantjes (1980) and is supported by the present study (Fig. 3). A fly larger than the diameter of the syrinx blocks the latter and blocks the entry and release of potential pollinators. Indeed, one individual Chironomidae has been observed which got stuck in the syrinx. Contrary, insects much smaller than the interspace between utricle wall and anthers cannot detach the pollen while walking on the utricle wall. The *Megaselia* specimens, who carried pollen, are slightly smaller as this interspace. However, on the one hand variability must be taken into account and on the other hand the length of legs has not been measured in stretched condition. Therefore the insects may appear smaller during measurement than *in vivo*.

However, size cannot be the only factor, selecting insects for pollination. There are too many fly species of similar size, which strongly suggests that further mechanisms must exist. This study shows that Sciaridae with a similar body size entered the flowers, but they never carried any pollen. It remains questionable whether sciarids might be potential pollinators of *A. pallida* as the sample size is still small and preservation of the specimens in ethanol may have washed off the pollen from the insect bodies.


Several studies (Hime and Costa 1985; Hall and Brown 1993; Disney and Sakai 2001; Sakai 2002; Trujillo and Sersic 2006) as well as the present study represent a high bias towards males or females trapped by *Aristolochia* flowers. This suggests that an attracting system exists, which selects one of the sexes only and this system must be different from size selection. Only the study by Bänziger and Disney (2006) offered an equal amount of males and females, suggesting that a different attraction system occurs in *A. baenzigeri* in Thailand.

After all, phorids are not the only observed pollinators of *Aristolochia* flowers. Among the numerous arthropods recorded in *Aristolochia* flowers, there are some other Diptera and in one case also Coleoptera carrying pollen. Only Ceratopogonidae loaded with pollen are recorded from *A. clematitis* and *A. bracteolata* (Daumann 1971; Razzak et al. 1992). Two specimens of Agromyzidae loaded with pollen were observed in *A. baenzigeri*, which however has been dominantly visited by pollen-loaded phorids (Bänzinger & Disney 2006). In *A. grandiflora*, representatives of a number of fly taxa were recorded, including Phoridae, but only Calliphoridae und Muscidae carried pollen (Burgess et al. 2004). Trujillo & Sersic (2006) recorded Phoridae, Lonchaeidae and Chloropidae from *A. argentina*, of which phorids make 70.7% and 62.5% of all visitors carried pollen, but the authors did not mention whether Lonchaeidae and Chloropidae are among them. In *A. maxima*, Phoridae and Drosophilidae as well as Staphylinidae were found carrying pollen, but only Drosophilidae carried 100 or more pollen grains per specimen (37% of Drosophilidae), while 99% of phorids and 82% of staphylinids carried 10 pollen grains or less (Sakai 2002). These results suggest that insects other than Phoridae have taken into account as *Aristolochia* pollinators too. However, evidence is mostly missing that all these insects carrying pollen indeed carried *Aristolochia*-pollen of the right species. In this study on *A. pallida*, it can be shown that the *Megaselia* specimens carried *Aristolochia*-pollen, which is characteristically globular and inaperturate (Fig. 2). However, these morphological characters available hardly allow distinguishing between different species of *Aristolochia*, which requires further investigations in order to find out the specificity in *Aristolochia* pollination biology.

Summarizing, an *Aristolochia* pollinator needs to fulfil the following requirements: (1) be able to enter the flower, (2) be able to touch the anthers while walking on the utricle wall, (3) becomes repeatedly attracted by flowers of the same species, and (4) must be able to upload pollen. These criteria can be verified, if (1) an insect is found in an *Aristolochia* flower during its female stage, and (2) this insect is loaded with pollen of the same *Aristolochia*-species.

So far, the only study that meets these requirements is that on *A. clematitis* by Daumann (1971). However, that study has been undertaken in Central Europe where *A. clematitis* is an introduced species. Therefore, the investigation of *A. pallida*

presented here is indeed the first study on the pollination biology of an *Aristolochia* species under natural conditions in the Mediterranean, which can evidently provide information on its pollinators.

# References

APG [= Angiosperm Phylogeny Group] II, (2003). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. Bot. J. Linn. Soc. 141: 399-436.

Arias, T., Callejas R., Bornstein, A., (2006). New Combinations in *Manekia*, an earlier name for Sarcorhachis (Piperaceae). NOVON 16, (in press).

Ashe, P., Cranston, P.S., (1990). Family Chironomidae. In: Soos A, Papp, L (ed) Catalogue of Palaearctic Diptera Vol. 2 Psychodidae – Chironomidae. Elsevier, Amsterdam, pp113–355.

Ball, P.W. (1964). Gen. *Aristolochia* L.. In: Tutin TG. et al. (Eds.) Flora Europaea. Cambridge 1, 73-74.

Bänziger, H., Disney, R.H.L., (2006). Scuttle flies (Diptera: Phoridae) imprisoned by Aristolochia baenzigeri (Aristolochiaceae) in Thailand. Mitt Schweiz Entomol Ges 79:29–61.

Barriel, V., (1994). Molecular phylogenies and how to code insertion/deletion events. C.R. Acad. Sci. III 317, 693-701.

Bechett, E. (1988). Aristolochiaceae. In: Balkema (Eds.) Wild Flowers of Majorca, Minorca and Ibiza. Rotterdam, Brookfield.

Bell, C.D., Edwards, E.J., Kim, S.T., Donoghue, M.J., (2001). Dipsacales phylogeny based on chloroplast DNA sequences. Harward Papers in Botany 6, 481-499.

Bello, M.A., Valois-Cuesta, H., González, F. (in press). *Aristolochia grandiflora* Sw. (Aristolochiaceae): Desarrollo y morfología de la flor más larga del mundo. Rev Acad Colomb Cienc.

Bena, G., Prosper, J.M., Lejeune, B., Olivieri, I., (1998). Evolution of annual species of the genus *Medicago*: a molecular phylogenetic approach. Mol. Phyl. Evol. 9, 552-559.

Berry, P.E., Hipp, A.L., Wurdack, K.J., Van Ee, B. & Riina, R., (2005). Molecular phylogenetics of the giant genus Croton and tribe Crotoneae (Euphorbiaceae sensu stricto) using ITS and trnL-trnF DNA sequence data. Amer. J. Bot. 92: 1520-1534.

Benson, G., (1997). Sequence alignment with tandem duplication. J. Comput. Biol. 4, 351-367.

Blanco, M.A. (2005). Un híbrido espontáneo entre *Aristolochia gorgona* y *A. grandiflora* (Aristolochiaceae). Lankesteriana 5: 115–117.

Blume, K.L., (1830). *Zippelia* In: Roemer and Schultes (Eds.), Systema Vegetabilium 7, 1614, 1651. Cottae, Stuttgart.

Bonafe, F.B. (1978). Aristoloquiacies. In: Moll (Eds.) Flora de Mallorca. Vol II.

Bonner, A. (1985). Plants of the Balearic Islands. 2nd edition. Moll Palnade Mallorca.

Borg, J. (1927). Descriptive flora of the Maltese Islands including the ferns and flowering plants. 198-199. Malta.

Borgmeier, T. (1925). Novos subsidios para o conhecimento da familia Phoridae (Dipt.). Archivos do Museu Nacional 25:85–281.

Bornstein, A.J., (1989). Taxonomic studies in the Piperaceae I. The pedicellate Pipers of Mexico and Central America (*Piper* subg. *Arcttonia*). J Arnold Arboretum 70, 1-50.

Bornstein, A.J., (1991). The Piperaceae in the southeastern United States. J. Arnold Arbor, Suppl. Ser. 1, 349-366.

Borsch, T., Hilu, K.W., Quandt, D., Wilde, V., Neinhuis, C., Barthlott, W., (2003). Noncoding plastid trnT-trnF sequences reveal a well resolved phylogeny of basal angiosperms. J. Evol. Biol. 1: 558-76.

Borsch, T., Löhne, C., Müller, K., Wanke, S., Worberg, A., Barthlott, W., Neinhuis, C., Hilu, K.W., Quandt, D.; (2005). Towards understanding basal angiosperm diversification: recent insights using fast evolving genomic regions. Nova Acta Leopoldina 342:85-110.

Borsch, T.; (2000). Phylogeny and evolution of the Nymphaea (Nymphaeaceae). Doctoral Thesis. PhD Thesis University of Bonn.

Brantjes, N.B., (1980). Flower morphology of *Aristolochia* species and consequences for pollination. Acta Bot Neerl 29:212–213.

Brues, C.T., (1928). Some Cuban Phoridae which visit the flowers of *Aristolochia elegans*. Psyche 35:160–161.

Burger, W., (1977). The Piperales and the monocots – alternate hypothesis for the origin of Monocotyledonous flowers. Bot. Rev. 43, 345-393.

Burgess, K.S., Singfield, J., Melendez, V., Kevan, P.G., (2004). Pollination biology of *Aristolochia grandiflora* (Aristolochiaceae) in Veracruz, Mexico. Ann Missouri Bot Gard 91:346–356.

Buzgo, M., Endress, P. K., (1999). The gynoecium of *Gymnostachys* (Araceae) and *Potamogeton* (Potomagetonaceae). P. 240, in XVI International Botanical Congress: Abstracts.

Callejas, R., (1986). Taxonomic revision of *Piper* Subgenus *Ottonia* (Piperaceae). PhD Dissertation, City University of New York, New York.

Carlquist, S., (1964). Morphology and relationships of Lactoridaceae. Aliso 5, 421-435.

Carr, J.W., (1924). The Diptera pollination the flowers of *Aristolochia sipho*. Entomol Mon Mag 60:258.

Castroviejo, S., (1986). Aristolochiaceae. In: Castroviejo, S., Lainz, M., LopezGonzalez, G., Montserrat, P., Munoz Garmendia, F., Paiva, J., Villar, L. (Eds.), Flora Iberica. Vol. I. Real Jard. Bot. C.S.I.C. Madrid.

Chase, M.W., Soltis, D.E., Olmstead, R.G., Morgan, D., Les, D.H., Mishler, B.D., Duvall, M.R., Price, R.A., Hills, H.G., Qiu, Y.L., Kron, K.A., Rettig, J.H., Conti, E., Palmer, J.D., Manhart, J.R., Sytsma, K.J., Michaels, H.J., Kress, W.J., Karol, K.G., Clark, W.D., Hedrén, M., Gaut, B.S., Jansen, R.K., Kim, K.-J., Wimpee, C.F., Smith, J.F., Furnier, G.R., Strauss, S.H., Xiang, Q.Y., Plunkett, G.M., Soltis, P.S., Swensen, S.M., Williams, S.E., Gadek, P.A., Quinn, C.J., Eguiarte, L.E., Golenberg, E., Learn, G.H., Jr., Graham, S.W., Barrett, S.C.H., Dayanandan, S., Albert, V.A., (1993). Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene *rbcL*. Ann. Missouri Bot. Gard. 80, 528-580.

Chieba, T., Harada, T., Goto, S., Ishikawa, R., Niizeki, M., (1996). Transcription of tRNA genes from large-scale plastid DNA deletion clearly reveals the action of nuclear-encoded RNA polymerase in the plastid. J. Plant Physiol. 148, 652-656.

Comes, H.P., Abbott R.J., (2001). Molecular phylogeography, reticulation, and lineage sorting in Mediterranean Senecio sect. Senecio (Asteraceae). Evolution 55: 1943-1962.

Comes, H.P., Abbott, R.J., (1999). Reticulate evolution in the Mediterranean species complex of Senecio sect. Senecio: uniting phylogenetic and population level approaches. In: Hollingsworth P.M., Bateman R.M., Gornall R.J. (eds) London, Taylor & Francis: 171-198.

Constance, L., (1963). Amphitropical relationships in the herbaceous flora of the Pacific coast of North and South America: A symposium. Introduction and historical review. Quart Rev Biol 38:109-116.

Constantinidis, T., Kamari, G., Phitos, D., (1997). A cytological study of 28 phanerogams from the mountains of SE Sterea Ellas, Greece Willdenowia 27: 121—142.

Correns, C., (1891). Beiträge zur biologischen Anatomie der *Aristolochia*-Blüte. Jahrb Wiss Bot 22:161–189.

Correns, C., (1892). Beiträge zur biologischen Anatomie der Aristolochiablüte. Bot Cent bl 52:439–443.

Cronquist, A., (1981). A integrated system of classification of flowering plants. Columbia University Press, New York.

Cronquist, A., (1988). The evolution and classification of flowering plants, 2nd ed. New York Botanical Garden, Bronx.

Cummings, M.P., Nugent, J.M., Olmstead, R.G., Palmer, J.D., (2003). Phylogenetic analysis reveals five independent transfers of the chloroplast gene *rbcL* to the mitochondrial genome in angiosperms. Curr. Genet. 43, 131-138.

Dahlstedt, H., (1900). Studien über Süd-und Central-Amerikanische Peperomien, mit besonderer Berücksichtigung der brasilianischen Sippen. Kongl. Svenska Vetenskaps-Akademiens Handlingar. Band 33 No. 2.

Daumann, E., (1959). Zur Kenntnis der Blütennektarien von *Aristolochia*. Preslia 31:359–372.

Daumann, E., (1971). Zur Bestäubungsökologie von *Aristolochia clematitis* L. Preslia 43:105–111.

de Candolle, C., (1866). Mémoire sur la Famille des Pipéracees. Mém. Soc. Phys. Genève 17(2): 29 p.

de Candolle, C., (1923). Piperacearum clavis analytica. Candollea 1: 286-415.

de Figuereido, R.A., Sazima, M., (2000). Pollination biology of Piperaceae species in southeastern Brazil. Ann, Bot. 85, 455–460.

de Groot, H., Wanke, S., Neinhuis, C. (2006). Revision of the genus Aristolochia L. (Aristolochiaceae) in Africa, Madagascar, and adjacent islands. Bot. J. Linn. Soc. 151: 219–238.

Debussche, M., Grandjanny, M., Debussche, G., Mus, M., Torres, N., Arguimbau, P., (1997). Ecologie d'une espèce endémique en milieu insulaire: Cyclamen

balearicum Willk. aux îles Baléares. Anales Del Jardín Botánico de Madrid 55: 31–48.

Debussche, M., Thompson, J.D., (2003). Habitat differentiation between two closely related Mediterranean plant species, the endemic Cyclamen balearicum and the widespread C. repandum. Acta Oecologia 24: 35-45.

Delpino, F., (1868). Ulteriori osservazioni e considerazioni sulla Dicogamia nel regno vegetale. Atti Soc Ital Sci Nat 11(2):265–332.

Delpino, F., (1869). Ulteriori osservazioni e considerazioni sulla Dicogamia nel regno vegetale. Atti Soc Ital Sci Nat 12(1):21–141, 179–233.

Dickison, W.C., (1992). Morphology and anatomy of the flower and pollen of Saruma henryi Oliv., a phylogenetic relict of the   Aristolochiaceae. Bull Torrey Bot Club 119:392-400.

Disney, R.H.L., (1983). Scuttle Flies Diptera, Phoridae (except *Megaselia*). Handbk Ident Br Insects 10(6):1–81.

Disney, R.H.L., (1989). Scuttle Flies Diptera Phoridae Genus *Megaselia*. Handbk Ident Br Insects 10(8):1–155.

Disney, R.H.L., (1994). Scuttle flies: The Phoridae. Chapman and Hall, London

Disney, R.H.L., (2006). Nine new species of *Megaselia* Rondani (Diptera: Phoridae) from the Seychelles. Zootaxa 1210:1–25.

Disney, R.H.L., Sakai, S. (2001). Scuttle flies (Diptera: Phoridae) whose larvae develop in flowers of *Aristolochia* (Aristolochiaceae) in Panama. Eur J Entomol 98:367–373.

Doyle, J.A., Endress, P.K., (2000). Morphological phylogenetic analysis of basal angiosperms: comparison and combination with molecular data. Int. J. Plant Sci. 161, 121-151.

Du Jardin, P., Portetelle, D., Harvengt, L., Dumont, M., Wathelet, B., (1994). Expression of intron-encoded maturase-like polypeptides in potato chloroplasts. Curr. Genet. 25, 158-163.

Duchartre, P., (1854). Tentamen methodicae divisionis generis *Aristolochia*. Ann Sci Nat Bot ser. 4, 2:29-76.

Duchartre, P., (1854b). Sur les prétendues stipules des Aristoloches. Bull Soc Bot France 1:56-60.

Duchartre, P., (1864). Aristolochiaceae. Pages 421-498 in AP de Candolle, (ed.) Prodromus Systematis Naturalis Regni Vegetabilis. Vol 15, pt 1. Masson, Paris.

Duvall, M.R., (2000). Seeking the sister group of the monocots. In: Wilson, K.L., Morrison D.A., (Eds.), Monocots: systematics and evolution. CSIRO: Melbourne.

Ems, S.C., Morden, C.W., Dixon, K.W., (1995). Transcription, splicing, and editing of plastid RNAs in the nonphotosynthetic plant *Epifagus virginiana*. Plant Mol. Biol. 29, 621-733.

Endress, P.K., (1995). Major evolutionary traits of monocot flowers. in: Rudall PJ, Cribb PJ, Cutler D F. (eds.) Monocotyledons: systematics and evolution. Royal Botanic Gardens, Kew, 1:43-79.

Engler, A., (1893). Piperaceae. In: Engler A., Prantl K., (Eds.), Die natürlichen Pflanzenfamilien. III. 1. 1a. Leipzig: W. Engelmann.

Eriksson, O., Hansen, A., Sunding, P. (1974). Flora of Macaronesia. Checklist of vascular plants, 7. Umea.

Fabbri, F., Fagioli, A., (1971). Nummeri cromosomici per la flora italica: 19-22. Inform. Bot. Ital. 3, 51-55.

Faegri, K., van der Pijl, L., (1979). The principles of pollination ecology. Pergamon, Oxford.

Felsenstein, J., (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783-791.

Fiorini, G., (1988). Numero cromosomico di una Aristolochia raccolta in territorio jugoslavo. Inform. Bot. Ital. 19, 265-266.

Fiorini, G., Nardi, E., (1993). Mediterranean chromosome number reports 3 (177-178) Flora Mediterranea 3: 341-344.

Fournier, P., 1990 Aristolochiaceae. In: Fournier P. (eds.) Les Quatre Flores de France. Edition Lechevalier, Saint Germain, Paris.

Franjic, J., Trinajstic, I., (1999). Interspecific and intraspecific variability of *Aristolochia lutea* Desf. and *A. pallida* Willd. (Aristolochiaceae) in Croatia. Period. Biol. 101, 259-264.

Freudenstein, J.V., Chase, M.W., (2001). Analysis of mitochondrial nad1b-c intron sequences in Orchidaceae: utility and coding of length-change characters. Syst. Bot. 26, 643-657.

Frodin, D.G., (2004). History and concepts of big plant genera Taxon 53, 753-776.

Gardner, R.O., (1997). Macropiper (Piperaceae) in the south-west Pacific. New Zealand Journal of Botany 35, 293-307.

Goldstein, D.B., Schlötterer, C., (1999). Microsatellites: Evolution and Applications, Oxford University Press.

Gomez, C., Espadaler, X., (1998). Seed dispersal curve of a Mediterranean myrmecochore: influence of ant size and the distance to nest. Ecological Research 13: 347-354.

González, F., (1990). Aristolochiaceae. Flora de Colombia. Monografía No. 12. Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, 184 pp.

González, F., (1991). Notes on the systematics of *Aristolochia* subsect. *Hexandrae*. Ann Missouri Bot Gard 78: 497-503.

González, F., (1997). Hacia una filogenia de *Aristolochia* y sus congéneres neotropicales. Caldasia 19:93-108.

Gonzalez, F., (1999). A phylogenetic analysis of the Aristolochioideae (Aristolochiaceae). Ph. D. Dissertation, The City University of New York.

González, F., (1999a). A phylogenetic analysis of the Aristolochioideae (Aristolochiaceae). Ph D. Dissertation. City University of New York, New York.

González, F., (1999b). Inflorescence morphology and the systematics of Aristolochiaceae. Syst Geogr Pl 68:159-172.

González, F., (1999c). Un nuevo nombre para *Holostylis reniformis* Duchartre (Aristolochiaceae). Rev Acad Colomb Ci Exact Fís Nat 23:337-339.

González, F., (2001). Una nueva especie de *Aristolochia* (Aristolochiaceae) de Bolivia. Rev Acad Colomb Ci Exact Fís Nat 25:225-228.

González, F., Rudall, P.J., (2001). The questionable affinities of *Lactoris*: evidence from branching pattern, inflorescence morphology and stipule development. Amer J Bot 88:2143-2151.

González, F., Rudall, P.J., (2003). Structure and development of the ovule and seed in Aristolochiaceae, with particular reference to *Saruma*. Plant Syst. Evol. 241:223-244.

González, F., Rudall, P.J., Furness, C., (2001). Microsporogenesis and systematics of Aristolochiaceae. Bot J Linn Soc 137:221-242.

González, F., Stevenson, D.W., (2000a). Gynostemium development in *Aristolochia* (Aristolochiaceae). Bot Jahrb Syst 122:249-291.

González, F., Stevenson, D.W., (2000b). Perianth development and systematics of *Aristolochia.* Flora 195:370-391.

González, F., Stevenson, D.W., (2002). A phylogenetic analysis of the subfamily Aristolochioideae (Aristolochiaceae). Rev Acad Colomb Ci Exact Fís Nat 26:25-60.

Graham, S. W., Olmstead, R. G., (2000). Utility of 17 chloroplast genes for inferring the phylogeny of the basal angiosperms. Am. J. Bot. 87, 1712-1730.

Graham, S.W., Reeves, P.A., Burns, A.C.E., Olmstead, R.G. (2000). Microstructural changes in noncoding chloroplast DNA: interpretation, evolution, and utility of indels and inversions in basal angiosperm phylogenetic inference. Int. J. Plant Sci 161 (Suppl.), 83-96.

Greuter, W., (1991). Botanical Diversity, endemism, rarity, and extinction in the Mediterranean area: an analysis based on the published volumes of Med-Cecklist. Bot. Chron. 10: 63-79.

Greuter, W., McNeill, J., Barrie, F.R., Burdet, H.M., Demoulin, V., Filgueiras, T.S., Nicolson, D.H., Silva, P.C., Skog, J.E., Trehane, P., Turland, N.J., Hawksworth, D.L. (2000). International Code of Botanical Nomenclature. *Regnum Veg.* 138. Königstein, Germany.

Hale, M.L., Borland, A.M., Gustafsson, M.H.G., Wolff, K. (2004). Causes of size homoplasy among chloroplast microsatellites in closely related *Clusia* species. Journal of Molecular Evolution 58, 182-190.

Hall, D.W., Brown, B.V., (1993). Pollination of *Aristolochia littoralis* (Aristolochiales: Aristolochiaceae) by Males of *Megaselia* spp. (Diptera: Phoridae). Ann Entomol Soc Am 86:609–613.

Haslam, S.M., Sell, P.D., Wolseley, P.A., (1977). A flora of the Maltese Islands. 17 Msida, Malta.

Havelka, P., (1978). Blütenbesuchende Ceratopogoniden (Diptera) aus der Umgebung von Tübingen. Beitr Naturkd Forsch Südwestdeutschl 37:175–179.

Heckard, L.R., (1963). The Hydrophyllaceae. Quart Rev Biol 38:117-123.

Herrera, J., (1991). The reproductive biology of a riparian Mediterranean shrub, Nerium oleandr L. (Apocynaceae). Bot. J. Linn. Soc.106: 147-172.

Heywood, V.H., (1993). Flowering plants of the world. 2d ed. Oxford University Press, Oxford.

Hidalgo, O., Garnatje, T., Susanna, A., Mathez, J., (2004). Phylogeny of Valerianaceae based on *matK* and ITS markers, with reference to *matK* individual polymorphism. Ann. Bot. 93, 283-293.

Hildebrand, F., (1867). Ueber die Befruchtung von *Aristolochia clematitis* und einiger anderer *Aristolochia*-Arten. Jahrb Wiss Bot 5: 343–358.

Hill, A.W., (1907). A revision of the geophilous species of *Peperomia*, with some additional notes on their morphology and seedling structure. Ann. Bot. Vol. XXXI No. LXXXII.

Hilu, K.W., Alice, L.A., (1999). Evolutionary implications of *matK* indels in Poaceae. Am. J. Bot. 86, 1735-1741.

Hilu, K.W., Alice, L.A., Liang, H. (1999). Phylogeny of Poaceae inferred from *matK* sequences. Ann. Missouri Bot. Gard. 86, 835-851.

Hilu, K.W., Borsch, T., Müller, K., Soltis, D.E., Soltis, P.S., Savolainen, V., Chase, M, Powell, M., Alice, L.A., Evans, R., Sauquet, H., Neinhuis, C., Slotta, T.A., Rohwer, J.G., Campbell, C.S., Chatrou, L., (2003). Angiosperm phylogeny based on *matK* sequence information. Am. J. Bot. 90, 1758-1776.

Hilu, K.W., Liang, H., (1997). The *matK* gene: sequence variation and application in plant systematics. Am. J. Bot. 84, 830-839.

Hime, N.C., Costa, E.L., (1985). Sobre *Megaselia aristolochiae* n. sp. (Diptera: Phoridae) cujas larvas se criam nas flores de *Aristolochia labiata* Willd. (Aristolochiaceae). Rev Brasil Biol 45:621–625.

Hoehne, F.C., (1927). Monographia ilustrada das aristolochiaceas brasileiras. Mem Inst Oswaldo Cruz 20:67-175.

Hoehne, F.C., (1942). Aristolochiaceas. Pages 3-141 in Flora Brasílica Instituto de Botanica, Sao Paulo.

Hou, D., (1981). Florae Malesiana Praecursores LXII. On the genus *Thottea* (Aristolochiaceae). Blumea 27:301-332.

Hou, D., (1984). Aristolochiaceae. Florae Malesiana. Ser. 10, 1:53-108.

Huber, H., (1960). Zur Abgrenzung der Gattung *Aristolochia* L. Mitt Bot Staatssamml München 3:531-553.

Huber, H., (1985). Samenmerkmale und Gliederung der Aristolochiaceen. Bot Jahrb Syst 107:277-320.

Huber, H., (1993). Aristolochiaceae. In: Kubitzki K, JG Rohwer, V Bittrich. (eds.) The families and genera of vascular plants. Springer, Berlin, 129-137.

Hutchinson, J, Dalziel, J.M., (1927). Flora of West Tropical Africa, ed. 1. Royal Botanic Gardens, Kew.

Igersheim, A., Endress, P.K., (1998). Gynoecium diversity and systematics of paleoherbs. Bot. J. Linn. Soc. 127, 289-370.

Jankowiak, K., Lesicka, J., Pacak, A., Rybarczk, A., Szweykowska-Kulinska, Z., (2004). A comparison of group II introns of plastid tRNA$^{Lys}$ $_{UUU}$ genes encoding maturase protein. Cell. Mol. Biol. Letters 9, 239-251.

Jaramillo, M.A., Callejas, R., (2004a). A reappraisal of *Trianaeopiper* Trelease: convergence of dwarf habit in some *Piper* species of the Chocó. Taxon 53: 269-278.

Jaramillo, M.A., Callejas, R., (2004b). Current perspectives on the classification and phylogenetics of the genus *Piper* L. In: Dyer, L. A. Palmer, A., (Eds), *Piper*: a model genus for studies of phytochemistry, ecology and evolution. Kluwer Academic/Plenum Publisher. New York, pp. 179-198.

Jaramillo, M.A., Kramer, E.M., (2004). APETALA3 and PSTILLATA homologs exhibit novel expression patterns in the unique perianth of *Aristolochia* (Aristolochiaceae). Evolution & Development, 6, 449-458.

Jaramillo, M.A., Manos, P.S., (2001). Phylogeny and patterns of floral diversity in the genus *Piper* (Piperaceae) Am. J. Bot. 88, 706-716.

Jaramillo, M.A., Manos, P.S., Zimmer, E.A., (2004). Phylogenetic relationships of the perianth-less Piperales: reconstructing the evolution of floral development. Int. J. Plant Sci. 165, 403-416.

Johnson, L.A., Soltis, D.E. (1995). Phylogenetic inference in Saxifragaceae sensu stricto and *Gilia* (Polemoniaceae) using *matK* sequences. Ann. Missouri Bot. Gard. 82, 149-175.

Johnson, L.A., Soltis, D.E., (1994). *matK* DNA sequences and phylogenetic reconstruction in Saxifragaceae s.str. Syst. Bot. 19, 143-156.

Karol, K.G., Suh, Y., Schatz, G E., Zimmer, E.A., (2000). Molecular evidence for the phylogenetic position of Takhtajania in the Winteraceae: Inference from nuclear ribosomal and chloroplast gene spacer sequences. Ann. Missouri Bot. Gard. 87, 414-432.

Kelchner, S.A., (2000). The evolution of non-coding chloroplast DNA and its amplication in plant systematics. Ann. Missouri Bot. Gard. 87, 482-498.

Kelly, L. M., (1998). Phylogenetic relationships in *Asarum* (Aristolochiaceae) based on morphology and ITS sequences. Am. J. Bot. 85, 1454–1467.

Kelly, L.M., (1997). A cladistic analysis of *Asarum* (Aristolochiaceae) and implications for the evolution of herkogamy. Am. J. Bot. 84, 1752–1765.

Kelly, L.M., González, F., (2003). Phylogenetic relationships in Aristolochiaceae. Syst. Bot. 28, 236-249.

Kim, S.T., Yoo, M.J., Albert, V.A., Farris, J.S., Soltis, P.S., Soltis, D.E., (2004). Phylogeny and diversification of B-function MADS- box genes in angiosperms: evolutionary and functional implications of a 260-million-year-old duplication. Am. J. Bot. 91, 2102-2118.

Klotzsch, F., (1859). Die Aristolochiaceae des Berliner Herbariums. Monatsber Königl Preuss Akad Wiss Berlin 1859:571-625.

Knoche, H., (1921). Aristolochiaceae. In: Flora Balearica. Etudes phytographique sur les îles Baléares, Montpellier: Roumegous & Dehen.

Knoll, F., (1929). Die Gleitfalle als Blumentypus. Verh Zool-Bot Ges Wien 79:9–12.

Knuth, P., (1899). Handbuch der Blütenbiologie II Band Die bisher in Europa und im arktischen Gebiet gemachten Blütenbiologischen Beobachtungen 2 Teil Lobeliaceae bis Gnetaceae. Engelmann, Leipzig.

Koch, M., Dobe, C., Matschinger, M., Bleeker, W., Vogel, J., Kiefer, M., (2005). Evolution of the plastidic *trnF* (GAA) gene in *Arabidopsis* relatives and the Brassicaceae family: monophyletic origin and subsequent diversification of a plastidic pseudogene. Mol. Biol. Evol. 22, 1032-1043.

Kocyan, A., Qiu, Y.-L., Endress, P.K. and Conti, E., (2004). A phylogenetic analysis of Apostasioideae (Orchidaceae) based on ITS, *trnL-F* and *matK* sequences. Pl. Syst. Evol. 247, 203-213.

Kores, P.J., Weston, P.H., Molvray, M., Chase, M.W., (2000). Phylogenetic relationships within the Diurideae (Orchidaceae): inferences from plastid *matK* sequences. In: Wilson, K.L., Morrison, D.A., (Eds.), Monocots : systematics and evolution. CSIRO Publishing, Melbourne, Australia, pp. 449-456.

Kruckeberg, A.R., Rabinowitz D., (1985). Biological aspects of endemism in higher plants. Annual Review of Ecology and Systematics 16: 447-479.

Kugler, (1970). Blütenökologie. Fischer, Stuttgart.

Lahaye, R., Civeyrel L., Speck T., Rowe, N. P., (2005). Evolution of shrub-like growth forms in the lianoid subfamily Secamonoideae (Apocynaceae s.l.) of Madagascar: phylogeny, biomechanics, and development Am. J. Botany 92: 1381-1396.

Larson, B.M.H., Kevan, P.G., Inouye, D.W., (2001). Flies and flowers: taxonomic diversity of anthophiles and pollinators. Can Ent 133:439–465.

Lavergne, S., Thompson, J.D., Garnier, E., Debussche, M., (2004). The biology and ecology of endemic and widespread plants: A comparative study of trait variation in 20 congeneric pairs. Oikos 107: 505-518.

Leconte, T., Stevenson, D.W., (1991). Cladistics of the Magnoliidae. Cladistics 7, 267-296.

Lei, L.-G., Azhang, S.-S., Yu, Z.-Y., (1991). The karyotype and the evolution of Gymnotheca. Acta Botanica Boreali-Occidentalia Sinica 11, 41-46 (in Chinese with English abstract).

Lei, L.G., Wu, Z.Y., Liang, H.X., (2002). Embryology of *Zippelia begoniifolia* (Piperaceae) and its systematic relationships. Bot. J. Linn. Soc. 140: 49-64.

Levin, L.A., 1993 Local speciation in plants: the rule not the exception. Syst. Bot. 18: 197-208.

Li, Y.-C., Korol, A.B., Fahima, T., Nevo, E., (2004). Microsatellites within genes: structure, function, and evolution. Mol. Biol. Evol. 21, 991-1007.

Liang, H, Hilu, K.W., (1996). Application of matK sequences to grass systematics Can.J. Bot. 74:125-134.

Liang, H.X., (1995). On the evolution and distribution in Saururaceae. Acta Bot. Yunnan 17, 255–267.

Liang, H.X., Tucker, S.C., (1990). Comparative study of floral vasculature in Saururaceae. Am. J. Bot. 77, 607-623.

Liang, H.X., Tucker, S.C., (1995). Floral ontogeny of *Zippelia begoniifolia* and its familial affinities: Saururaceae or Piperaceae? Am. J. Bot. 82, 681-689.

Lobo, J.M., Castro, I., Moreno, J.C., (2001). Spatial and environmental determinants of vascular plant species richness distribution in the Iberian Peninsula and Balearic Islands. Biol. J. Linn. Soc. 73: 233-253.

Löhne, C., Borsch, T., (2005). Molecular evolution and phylogenetic utility of the *petD* group II intron: a case study in basal angiosperms. Mol. Biol. Evol. 22, 317-32.

Ma, J.S., (1989) A revision of *Aristolochia* Linn. from E. & S. Asia. Acta Phytotax Sin 27:321-364.

Ma, J.S., (1990). The Geographical Distribution and the System of *Aristolochiaceae*. Acta Phytotaxonomica Sinica 28, 345-355 (in Chinese with detailed English abstract). Marquis, R.J., 1988. Phenological variation in the neotropical

understory shrub *Piper arieanum*: causes and consequences. Ecology 69, 1552-1565.

Masters, M.T., (1875). Aristolochiaceae. Pages 77-114 in CFP von Martius, ed. Flora Brasiliensis Vol 4, pt 2. Oldenbourgh, Munich.

Mathew, P.J., Mathew, P.M., (2001). Pollen morphology of some members of Pipereaceae and its bearing on the systematics and phylogeny of the family. Rheedea 11, 65-78.

Mathews, D.H., Zuker, M., Turner, D.H., (2005). RNAstructure 4.2; http://rna.urmc.rochester.edu.

Mathews, S., Donoghue, M.J., (2000). Basal angiosperm phylogeny inferred from duplicate phytochromes A and C. Int. J. Plant Sci. 161(6, suppl.), 41-55.

Mayer, E., Greuter, W., (1985). *Aristolchia merxmuelleri*, ein neuer Serpentin-Endemit aus Südwest-Serbien. Bot. Jahrb. Syst. 107, 321-327.

Medail, F., Quezel, P., (1997). Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Annals of the Missouri Botanical Garden 84: 112-127.

Meimberg, H., Thalhammer, S., Brachmann, A., Heubl, G., (2006). Comparative analysis of the translocated copy of the trnK intron in carnivorous family Nepenthaceae. Mol. Phyl. Evol. 39, 478-490.

Meng, S.W., Chen, Z.D., Li, D.Z., Liang, H.X., (2002). Phylogeny of Saururaceae based on mitochondrial *matR* gene sequence data. J. Plant Res. 115, 71-76.

Meng, S.W., Douglas, A.W., Li, D.Z., Chen, Z.D., Liang, H.X., Yang, J.B., (2003). Phylogeny of Saururaceae based on morphology and five regions from three plant genomes. Ann. Missouri Bot. Gard. 90, 592–602.

Menzel, F., Mohrig, W., (2000). Revision der paläarktischen Trauermücken (Diptera: Sciaridae). Studia dipterologica Supplement 6(1999):1–761.

Metzgar, D., Bytof, J., Wills, C., (2000). Selection against frameshift mutations limits microsatellite expansion in coding DNA. Genome Res. 10, 72-80.

Miquel, F.A.W., (1843-1844). Systema Piperacearum. Kramer Rotterdam.

Mohr, G., Perlman, P.S., Lambowitz, A.M., (1993). Evolutionary relationships among group II intron-encoded proteins and identification of a conserved domain that may be related to maturase function. Nucleic Acids Res. 21, 4991-4997.

Montmollin, B. de, (1986). Etude cytotaxonomique de la flore de la Crète. III. Nombres chromosomiques. *Candollea* 41: 431—439.

Müller K, Quandt, D., Müller, J., Neinhuis, C., (2005). PhyDE version 0.92: Phylogenetic Data Editor, Distributed by the authors www.phyde.de.

Müller, H. (1873). Die Befruchtung der Blumen durch Insekten und die gegenseitige Anpassung beider: ein Beitrag zur Erkenntnis des ursächlichen Zusammenhanges in der organischen Natur. Engelmann, Leipzig.

Müller, J., Müller, K., (2004). TreeGraph: automated drawing of complex tree figures using an extensible tree description format. Mol. Ecol. Notes 4, 786-788.

Müller, K., (2002). GRate - relative rate tests for taxon groups using general DNA substitution models in PAUP*, Program distributed by the author, http://www.nees.uni-bonn.de/downloads/GRate.

Müller, K., (2004). PRAP - computation of Bremer support for large data sets. Mol Phylogenet. Evol 31:780-782.

Müller, K., (2005). SeqState - primer design and sequence statistics for phylogenetic DNA data sets. Applied Bioinformatics 4:65-69.

Müller, K., (2006). Incorporating information from length-mutational events into phylogenetic analysis. Mol. Phylogenet.. Evol. 38: 667–676.

Müller, K., Borsch, T., (2005a). Systematics of *Utricularia* (Lentibulariaceae) and molecular evolution of the *trnK* intron in a lineage with high mutational rates. Pl. Syst. Evol. 250, 39-67.

Müller, K., Borsch, T., (2005b). Phylogenetics of Amaranthaceae using *matK/trnK* sequence data - evidence from parsimony, likelihood and Bayesian approaches. Ann. Missouri Bot. Gard. 92, 66-102.

Müller, K., Borsch, T., Legendre, L., Porembski, S., Theisen, I., and Barthlott, W., (2004). Evolution of carnivory in Lentibulariaceae and the Lamiales. Plant Biol. 6, 477-490.

Murata, J., Ohi-Thoma, T., Wu, S., Darnaedi, D., Sugawara, T., Nakanishi, T., Murata, H., (2001). Molecular phylogeny of Aristolochia (Aristolochiaceae) inferred from *mat*K sequences. Acta Phytotax. Geobot. 52:75-83.

Murin, (1976). A. In Index of chromosome numbers of Slovakian flora. Part 5. Acta Facultatis Rerum Naturalium Universitatis Comenianae, Botanica 25: 1-18.

Nardi, E., (1983). Proposal to reject the name *Aristolochia longa* L. (Aristolochiaceae). Taxon 32, 654-656.

Nardi, E., (1984). The genus *Aristolochia* L. (Aristolochiaceae) in Italy. Webbia 38:221-300.

Nardi, E., (1984). The genus *Aristolochia* L. (Aristolochiaceae) in Italy. Webbia 38, 221-300.

Nardi, E., (1988). Renewed proposal to reject *Aristolochia longa* L. (Aristolochiaceae) Taxon 37, 978-980.

Nardi, E., (1989). De speciebus Aristolochiae pallidae gregis (Aristolochiaceae) in Graecia crescentibus. Willdenowia 18, 367-375.

Nardi, E., (1991). The genus *Aristolochia* L. (Aristolochiaceae) in Greece. Webbia 45:31-69.

Nardi, E., (1991). The genus *Aristolochia* L. (Aristolochiaceae) in Greece. Webbia 45, 31-69.

Nardi, E., Akeroyed, J.R., (1993). Aristolochia. In: T. G. Tutin, V. H. Heywood, N. A. Burges, D. H. Valentine. Flora Europaea 1: 87-89, Cambridge University Press.

Nardi, E., Arrigoni, P.V., (1983). Le piante endemiche della Sardegna. Boll. Soc. Sarda Sci. Nat. 22, 347-356.

Nardi, E., Nardi, C.N., (1987). Taxonomic and chorological notes on the genus *Aristolochia* L. (Aristolochiaceae) from the Central and Eastern Mediterranean area. Bot. Helvetica 97, 155-165.

Nardi, E., Ricceri, C., (1987). Il genere *Aristolochia* l. in Corsica. Webbia 41, 225-239.

Neinhuis, C., Wanke, S., Hilu, K.W., Müller, K., Borsch, T., 2005 Phylogeny of Aristolochiaceae based on parsimony, likelihood, and Bayesian analyses of *trn*L-*trn*F sequences. Plant Syst Evol 250:7-26.

Neuhaus, H., Link, G., (1987). The chloroplast tRNA$^{Lys}$(UUU) gene from mustard *(Sinapis alba)* contains a class II intron potentially coding for a maturase-related polypeptide. Curr. Genet. 11, 251-257.

Nickrent, D.L., Soltis, D.E., Blarer, A., Qiu, Y.-L., Soltis, P.S., Zanis, M., (2002). Molecular data place Hydnoraceae with Aristolochiaceae. Am. J. Bot. 89, 1809-1817.

Nixon, K. C., (1999). The parsimony ratchet, a new method for rapid parsimony analyses. Cladistics 15: 407-414.

Nortier, J.L., Martinez, M.C., Schmeiser, H.H., Arlt, V.M., Bieler, C.A., Petein, M., Depierreux, M.F., De Pauw, L., Abramowicz, D., Vereerstraeten, P., Vanherweghem, J.L., (2000). Urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). N. Engl. J. Med. 342, 1686-92.

Nuin, P.A.S., (2005). MTgui - a simple interface to ModelTest. Program distributed by the author. University of Toronto. http://www.genedrift.org/mtgui.php.

Oelschlägel, B., Wanke, S., Gorb, S., Neinhuis, C., (in press). Biomechanics of trap flowers – A case study within *Aristolochia rotunda*. In: Gorb S. (eds.) Biomechanics of functional surfaces. Springer, Berlin, Heidelberg.

Ohi-Toma, T., Sugawara, T., Murata, H., Wanke, S., Neinhuis, C., Murata, J., (2006). Molecular Phylogeny of Aristolochia sensu lato (Aristolochiaceae) based on Sequences of rbcL, matK, and phyA Genes, with Special Reference to Differentiation of Chromosome Numbers. Syst. Bot. 31:481–492.

Okada, H., (1986). Karyomorphology and relationship in some genera of Saururaceae and Piperaceae. Bot Mag Tokyo 99, 289-299.

Oliver, D., (1889). *Saruma henryi*. In Hooker´s Icon. Pl 19: Tab. 1895.

Omori, Y., (1982). Floral anatomy of *Saururus chinensis* (Saururaceae) and *Zippelia begoniifolia* (Piperaceae). Science Report of the Yokosuka City Museum 29: 51-61.

Orszag-Sperber, F., Butterlin, J., Clermonte, J., Colchen, M., Guiraud, R., Poisson, A., Ricou, L.E., (1993). Tortonian Palaeoenvironments (11.6-6 MA). And map. In: Dercourt, J., Ricou, L.E., Vrielynck, B. (Eds.), Atlas Tethys, Paleoenvironmental Maps. Gauthier-Villars, Paris, pp. 237-239.

Papp, L, Darvas, B., (2000). Contributions to a manual of Palaearctic Diptera Vol 1. Science Herald, Budapest.

Paulus, H.F., Gack, C., (1990). Pollinators as prepollinating isolation factors: evolution and speciation in Ophrys (Orchidaceae). Israel Journal of Botany 39: 43-79.

Petch, T., (1924). Notes on *Aristolochia*. Ann R Bot Gdns Peradeniya 8:1–108.

Pfeifer, H.Wm., (1966). Revision of the North and Central American hexandrous species of *Aristolochia* (Aristolochiaceae). Ann Missouri Bot Gard 53:115-196.

Pfeifer, H.Wm., (1970). A taxonomic revision of the pentandrous species of *Aristolochia*. Univ Connecticut Publ Series, 134 pp.

Posada, D., Crandall K. A., (1998). Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Proctor, M, Yeo, P, Lack, A., (1996). Natural History of Pollination. HarperCollins, London.

Provan, J, Powell, W., Hollingsworth, P.M., (2001). Chloroplast microsatellites: new tools for studies in plant ecology and evolution. Trends in Ecology & Evolution 16, 142-147.

Provan, J., Soranzo, N., Wilson, N.J., Goldstein, D.B., Powell, W., (1999). A low mutation rate for chloroplast microsatellites. Genetics, 153: 943-947.

Qiu, Y.L., Dombrovska, O., Lee, J., Li, L., Whitlock, B.A., Bernasconi-Quadroni, F., Rest, J.S., Davis, C.C., Borsch, T., Hilu, K.W., Renner, S.S., Soltis, D.E., Soltis, P.S., Zanis, M.J., Cannone, J.J., Gutell, R.R., Powell, M., Savolainen, V., Chatrou, L.W., Chase, M.W., (2005). Phylogenetic analyses of basal angiosperms based on nine plastid, mitochondrial, and nuclear genes. Int J Plant Sci 166:815-842.

Qiu, Y.L., Lee, J., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainen, V., Chase, M.W., (1999). The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. Nature 402, 407.

Qiu, Y.L., Lee, J., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainen, V., Chase, M.W., (2000). Phylogeny of the basal angiosperms: Analyses of five genes from the three genomes. Int. J. Plant Sci. 161, 3-27.

Quandt, D., Huttunen, S., (2004). Evolution of pendent life-forms in bryophytes. *J. Hattori Bot. Lab.* 95, pp. 207–217.

Quandt, D., Müller, K., Stech, M., Hilu, K.W., Frey, W., Frahm, J.P., Borsch, T., (2004). Molecular evolution of the chloroplast trnL-F region in land plants. Monogr. Syst. Bot. Missouri Bot. Gard. 98, 13-37.

Quandt, D., Stech, M. (2005). Molecular evolution of the *trn*$L_{UAA}$ intron in bryophytes. Molecular Phylogenetics and Evolulution, 36: 429-443.

Rafinesque, C.S., (1819). Nuttall's genera of North American plants and a catalogue of the species to the year 1817. Am Mon Mag Crit Rev 4: 184-196.

Rafinesque, C.S., (1828). *Aristolochia*. Pages 61-65 in Medicinal Flora. Atkinson & Alexander, Philadelphia.

Rahmanzadeh, R., Müller, K., Fischer, E., Bartels, D., Borsch, T., (2005). Linderniaceae and Gratiolaceae are further lineages distinct from Scrophulariaceae (Lamiales). Plant Biol. 7, 67-78.

Raven, P.H., (1963). Amphitropical relationships in the floras of North and South America. Quart Rev Biol 38:151-177.

Razzak, M.A., Ali, T., Ali, S.I., (1992). The pollination biology of *Aristolochia bracteolata* Lamk. (Aristolochiaceae). Pak J Bot 24:79–87.

Renner, S.S., (1999). Circumscription and phylogeny of Laurales: evidence from molecular and morphological data. Am. J. Bot. 86, 1301-1315.

Renner, S.S., (2005). Variation in diversity among Laurales, early Cretaceous to Present. Biol. Skr. 55, 441-458.

Renner, S.S., Chanderbali, A., (2000). What is the relationship among Hernandiaceae, Lauraceae, and Monimiaceae, and why is this question so difficult to answer? Int. J. Plant Science 161(6 Suppl.), 109-119.

Robertson, C., (1928). Flowers and Insects. Carlinville, Illinois.

Romo, A.M., (1994). Aristolochiaceae. In: Flores Silvestres de Baleares. Rueda, Madrid.

Ronquist, F., Huelsenbeck, J.P., (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Rosenbaum, G., Lister, G.S., Duboz, C., (2002). Reconstruction of the tectonic evolution of the western Mediterranean since the Oligocene. In: Rosenberg, G., Lister, G.S., Reconstruction of the evolution of the Alpine-Himalayan Orogen. Journal of the Virtual Explorer 8, 107-126.

Rowe, N., Speck, T., (2005). Plant growth forms: an ecological and evolutionary perspective. New Phytologist 166: 61-72.

Rulik, B., Wanke, S., Nuss, M., Neinhuis, C., (2006). Trapped! Pollination of Aristolochia pallida Willd. in the Mediterranean. Oecologia, submitted.

Sakai, S., (2002) *Aristolochia* spp. (Aristolochiaceae) pollinated by flies breeding on decomposing flowers in Panama. Am J Bot 89:527–534.

Sarich, V.M., Wilson, A.C., (1967). Immunological time scale for hominid evolution. Science 158, 1200-1203.

Sauquet, H., Doyle, J.A., Scharaschkin, T., Borsch, T., Hilu, K.W., Chatrou, L., Le Thomas, A., (2003). Phylogenetic analysis of Magnoliaceae and Myristicaceae based on multiple data sets: Implications for character evolution. Bot. J. Linnean Soc. 142, 125-186.

Savolainen, V., Chase, M.W., Hoot, S.B., Morton, C.M., Soltis, D.E., Bayer, C., Fay, M.F., de Bruijn, A.Y., Sullivan, S., Qiu, Y.L., (2000). Phylogenetics of flowering

plants based on combined analysis of plastid *atpB* and *rbcL* gene sequences. Syst Biol. 2000 49, 306-62.

Schmidt, O.C., (1935). Aristolochiaceae. In:A Engler, K Prantl (eds.), Die natürlichen Pflanzenfamilien. Ed. 2, 16b:204-242.

Schmitz, H., Beyer, E., Delage, A., (1938–1981). Phoridae. In: Lindner E (ed) Die Fliegen der palaearktischen Region. Vol 4 (7) 1. Schweizerbart, Stuttgart.

Semple, K.S., (1974.) Pollination of Piperaceae. Ann. Missouri. Bot. Gard. 61, 868-871.

Simmons, M.P., Ochoterena, H., (2000). Gaps as characters in sequence-based phylogenetic analyses. Syst Biol 49:369-381.

Smith, A.C., (1975). The genus *Macropiper* (Piperaceae). Bot. J. Linn. Soc. 71: 1-38.

Soltis, D.E., Soltis, P.S., (1998). Choosing an approach and an appropriated gene for phylogenetic analysis. In: Soltis, D.E., Soltis, P.S., Doyle, J.J., (Eds.), Molecular systematics of plants II – DNA sequencing. Kluwer Academic Publishers Boston, Dordrecht, London.

Soltis, D.E., Soltis, P.S., Case, M.W., Mort, M.E., Albach, D.C., Zanis, M., Savolainen, V., Hahn, W.H., Hoot, S.B., Fay, M.F., Axtell, M., Swensen, S.M., Price, L.M., Kress, W.J., Nixon, K.C., Farris, J.S., (2000). Angiosperm phylogeny inferred from 18S rDNA, *rbcL,* and *atpB* sequences. Bot. J. Linn. Soc. 133, 381-461.

Speck, T., Rowe, N.P., Civeyrel, L., Classen-Bockhoff, R., Neinhuis, C., Spatz, H-C., (2003). The potential of plant biomechanics in functional biology and systematics. In: Stuessy TF. Mayer V., Hörandl E., eds. Deep morphology: towards a renaissance of morphology in plant systematics. Königstein, Germany: Koeltz, 241-271.

Speiser, P., Schmitz, H., (1957). Verzeichnis der Phoriden von Ost- und Westpreußen. Natuurhist maandbl 46:82–85.

Sprengel, C.K., (1793). Das entdeckte Geheimnis der Natur im Bau und in der Befruchtung der Blumen. In: Knuth P (ed) Ostwald's Klassiker der exakten Naturwissenschaften, 48–51, 1894. Engelmann, Leipzig.

Steele, K.P., Vilgalys, R., (1994). Phylogenetic analyses of Polemoniaceae using nucleotide sequences of the plastid gene matK. Syst. Bot. 19:126-142.

Stuessy, T.F., Crawford, D.J., Anderson, G.J., Jensen, R.J., (1998). Systematics, biogeography and conservation of Lactoridaceae. Persp. Plant Ecol. Evol. Syst. 1, 267-290.

Sugawara, T., J. Murata, Sugong Wu, T. Ohi, T. Nakanishi, Murata, H., (2001). A cytological analysis of 24 taxa in Aristolochia subgenera Siphisia and Aristolochia (Aristolochiaceae). Acta Phytotax. Geobot. 52: 149-158.

Suh, Y., Thien, L.B., Reeve, H.E., Zimmer, E.A., (1993). Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA Winteraceae. Am, J, Bot. 80, 1042-1055.

Swofford, D.L., (2002). PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer, Sunderland, Massachusetts.

Takhtajan, A., (Eds.), (1992). Anatomia seminum comparativa. Tomus 4. Dicotyledones. Dilleniidae. NAUKA, Leningrad.

Takhtajan, A., (Eds.), (1997). Diversity and Classification of Flowering Plants. Columbia University Press, New York.).

Tautz D, Trick, M., Dover, G.A., (1986). Cryptic simplicity in DNA is a major source of genetic variation. Nature, 322: 652-6.

Tebbs, M.C., (1993). Piperaceae. In Kubitzki, K., Rohwer, J.G., Bittrich, V., (Eds.), The families and genera of vascular plants. II. Flowering plants: dicotyledons, magnoliid, hamamelid and caryophyllid families. Springer, Berlin, pp. 516-520.

Thompson, J.D., (2005). Plant Evolution in the Mediterranean. Oxford University Press, New York.

Thorne, R.F., (1974). A phylogenetic classification of the Annoniflorae. Aliso 8: 147-209.

Thorne, R.F., (1992).. Classification and geography of flowering plants. Bot. Rev. 58, 225-348.

Toor, N., Hausner, G., Zimmerly, S., (2001). Coevolution of group II intron RNA structures with their intron-encoded reverse transcriptase. RNA 7, 1142-1152.

Trinajstic, I. (1990). *Aristolochia pallida* Willd. (Aristolochiaceae) in the Flora of Croatia. Acta Bot. Croat. 49, 143-149.

Trujillo, C.G., Sersic, A.N., (2006). Floral biology of *Aristolochia argentina* (Aristolochiaceae). Flora 201:374–382.

Tucker, S.C., (1975). Floral development in Saururus cernuus (Saururaceae): 1. Floral initiation and stamen development. Am. J. Bot. 62, 993-1007.

Tucker, S.C., (1981). Inflorescence and floral development in Houttuynia cordata (Saururaceae). Am. J. Bot. 68, 1017-1032.

Tucker, S.C., Douglas, A.W., Liang, H.X., (1993). Utility of ontogenetic and conventional characters in determining phylogenetic relationships of Saururaceae and Piperaceae (Piperales). Syst. Bot. 18, 614–641.

Ule, E., (1898) Beitrag zu den Blütheneinrichtungen von *Aristolochia clematitis* L. Ber Dtsch bot Ges 16:236–239.

Van den Berg, C., Goldman, D.H., Freudenstein, J.V., Pridgeon, A.M., Cameron, K.M., Chase, M.W., (2005). An overview of the phylogenetic relationships within Epidendroideae inferred from multiple DNA regions and recircumscription of Epidendreae and *Arethuseae* (Orchidaceae). Am. J. Bot. 92, 613-624.

Verlaque, R., Filona, D., (1993). Mediterranean chromosome number reports 3 (228--233) Flora Mediterranea 3: 364—367.

Vijverberg, K., Bachmann, K., (1999). Molecular evolution of a tandemly repeated *trnF*(GAA) gene in the chloroplast genomes of *Microseris* (Asteraceae), and the use of structural mutations in phylogenetic analyses. Mol. Biol. Evol, 16, 1329-1340.

Vogel, J., Hübschmann, T., Börner, T., Hess, W.R., (1997). Splicing and intron-internal RNA editing of *trnK-matK* transcripts in barley plastids: support for *matK* as an essential splice factor. J.. Mol. Biol. 270, 179-187.

Vogel, S., (1965). Kesselfallen-Blumen. Umsch Wiss Tech 65:12–17.

Wanke, S., González, F., Neinhuis, C., (2006b). Systematics of pipevines – Combining morphological and fast-evolving molecular characters to resolve the relationships within Aristolochioideae (Aristolochiaceae). Int J Plant Sci (in press).

Wanke, S., Jaramillo, M.A., Borsch, T., Samain, M.S., Quandt, D., Neinhuis, C., (2006c). Evolution of Piperales –matK gene and trnK intron sequence data reveal lineage specific resolution contrast. Mol Phylogenet Evol. DOI 10.1016/j.ympev.2006.07.007.

Wanke, S., Quandt, D., Neinhuis, C., (2006a). Universal primers for a large cryptically simple cpDNA microsatellite region in Aristolochia. Mol. Ecol. Notes doi: 10.1111/j.1471-8286.2006.01430.x.

Wanke, S., Samain, M.S., Vanderschaeve, L., Mathieu, G., Goetghebeur, P., Neinhuis, C., (2006d). Phylogeny of the genus Peperomia (Piperaceae) inferred from the trnK/matK region (cpDNA). Plant Biology 8:93-102.

Weising, K., Gardner, R., (1999). A set of universal PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome, 42: 9-19.

Willis, J.C., (1957). A dictionary of the flowering plants and ferns. Cambridge University Press, Cambridge.

Wolfe, A.D., Randle, C.P., (2004). Recombination, heteroplasmy, haplotype polymorphism, and paralogy in plastid genes: implications for plant molecular systematics. Syst. Bot. 29, 1011-1020.

Wu, C.Y., Wang, W.T., (1957). Preliminary study of the flora of subtropical areas of Yunnan. Acta Phytotaxonomica Sinica 6, 183-254.

Wu, C.Y., Wang, W.T., (1958). Preliminary study of the flora of subtropical areas of Yunnan. Acta Phytotaxonomica Sinica 7, 193-196.

Zanis, M.J., Soltis, D.E., Soltis, P.S., Mathews, S., Donoghue, M.J., (2002). The root of the angiosperms revisited. Proc. Natl. Acad. Sci. USA. 99, 6848-53.

Zavada, M.S., Taylor, T.N., (1986). Pollen morphology of Lactoridaceae. Pl. Syst. Evol. 154, 31-39.

# Curiculum Vitae

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- Mediterranean phylogeography of the genus *Aristolochia*
- Population genetics in *Aristolochia*
- Pollination biology of the genus *Aristolochia*

# Publications

# Peer-reviewed articles; printed, in press or submitted

**2006**

Samain MS., Mathieu G., Vanderschaeve L., **Wanke S**., Neinhuis C. & Goetghebeur P. 2006 Nomenclature and typification of the infrageneric taxa in the genus *Peperomia* (Piperaceae). Taxon (in press).

**Wanke S**., Gonzalez F. & Neinhuis C. 2006 Systematics of pipevines – Combining morphological and fast-evolving molecular characters to investigate the relationships within subfamily Aristolochioideae (Aristolochiaceae). Inter. J. Pl. Sci. (in press).

Oelschlägel B., **Wanke S**., Gorb S. & Neinhuis C. (2006) Biomechanics of trap flowers – a case study within *Aristolochia rotunda*. In: Gorb S. Biomechanics of Functional Surfaces. Springer, Heidelberg (in press).

**Wanke S.,** Quandt D. & Neinhuis C. 2006 Universal primers for a large cryptically simple cpDNA microsatellite region in *Aristolochia*. Mol. Ecol. Notes doi: 10.1111/j.1471-8286.2006.01430.x

**Wanke S**., Jaramillo MA., Borsch T., Samain MS., Quandt D. & Neinhuis C. 2006 Evolution of Piperales –matK gene and trnK intron sequence data reveal lineage specific resolution contrast. Mol. Phylogent. Evol. doi.org/10.1016/j.ympev.2006.07.007

Ohi-Toma T., Sugawara T., Murata H., **Wanke S.,** Neinhuis C. & Murata J. 2006 Molecular Phylogeny of *Aristolochia* sensu lato (Aristolochiaceae) based on Sequences of rbcL, matK, and phyA Genes, with Special Reference to Differentiation of Chromosome Numbers. Syst. Bot. 31:481–492.

deGroot H., **Wanke S.** & Neinhuis C. 2006 Revision of the genus *Aristolochia* L. (Aristolochiaceae) in Africa, Madagascar, and adjacent islands. Bot. J. Linn. Soc. 151:219-238.

**Wanke S**., Samain MS., Vanderschaeve L., Mathieu G., Goetghebeur & Neinhuis C. 2006 Phylogeny of the genus *Peperomia* (Piperaceae) inferred from the *trn*K/*mat*K region (cpDNA). Plant Biology 8:93-102.

## 2005

**Wanke S.** & Neinhuis C. 2005 Checkliste für die Gattung Aristolochia. In: Starmühler W. Vorarbeiten zu einer „Flora von Istrien" Teil VIII. Carinthia II 195 (115) Klagenfurt 519.

Borsch T., Löhne C., Müller K., **Wanke S.,** Worberg A., Barthlott W., Neinhuis C., Hilu K. W. & Quandt D. 2005 Towards understanding basal angiosperm diversification: recent insights using fast evolving genomic regions. Nova Acta Leopoldina 342: 85-110.

Neinhuis C., **Wanke S**., Hilu KW., Müller K. & Borsch T. 2005 Phylogeny of Aristolochiaceae based on parsimony, likelihood, and Bayesian analyses of the trnL-trnF sequences. Plant Syst. Evol. 250: 7-26.

## 2004

Vences M., **Wanke S.,** Vietes DR., Branch B., Glaw F. & Mayer A. 2004 Natural colonisation or introduction? High genetic divergences and phylogeographic relationships of house geckos (*Hemidactylus*) from Madagascar. Biol. J. Linn. Soc. 83: 115-130.

## 2002

Vences M., Galan P., Vieites DR., Puente M., Oetter K. & **Wanke S**. 2002 Field body temperatures and heating rates in a montane frog population: the importance of black dorsal pattern for thermoregulation. Ann. Zool. Fennici 39: 209-220.

**2000**

Vences M., **Wanke S.,** Odierna G., Kosuch J. & Veith M. 2000 Molecular
and karyological data on the south asien ranid genera *Indirana*,
*Nyctibatrachus* and *Nannophrys* (Anura: Ranidae). Hamadryad 25(2): 9-
16.

**Submitted (selection)**

Rulik B., **Wanke S**., Nuss M. & Neinhuis C. (2006) Trapped! Pollination of
*Aristolochia pallida* Willd. in the Mediterranean. Functional Ecology,
(submitted)

**Wanke S.,** Vanderschaeve L., Mathieu G., Neinhuis C., Goetghebeur P.,
Samain MS. (2006) Molecules make it specific: *Verhuellia* revealed as
branching from the first node of Piperaceae. (submitted).

# Abstracts

Samain M.S., Vanderschaeve L., Neinhuis C., Goetghebeur P.& **Wanke S.** 2006 Is morphology telling the truth about the evolution of the giant genus Peperomia (Piperaceae)? 17th International Symposium Biodiversity and Evolutionary Biology, Bonn

**Wanke S**., González F & Neinhuis C. 2006 Systematics of pipevines – Huber's studies on Aristolochiaceae in the light of recent morphological and molecular data. 17th International Symposium Biodiversity and Evolutionary Biology, Bonn

**Wanke S**. & Goetghebeur P. 2006 From a poorly known clade to a model group - The giant genus Peperomia (Piperaceae). 11th Nordic Meeting on Neotropical Botany, Aarhus, Denmark

Quandt D., **Wanke S**., Müller K, Hernández-Maqueda R., Stech M., Löhne C., Worberg A., Hilu KW. & Borsch T., 2006 The role of hairpins in molecular evolution. Botany 2006 California State University – Chico, USA

Neinhuis C., Gonzalez F. & **Wanke S**. (2006) Systematics and molecular phylogeny of Aristolochiaceae. Gayana Botanica 63: 25

**Wanke S.**, Borsch T., Jaramillo A. & Neinhuis C. (2005) Molecular evolution of the trnK/matK region (cpDNA) and evolution of Piperales. XVII International Botanical Congress Vienna, Austria

Samain M.S., Vanderschaeve L., Mathieu G. & **Wanke S.** (2005) Studies in Peperomia (Piperaceae): insights from molecular and morphological approaches. XVII International Botanical Congress Vienna, Austria

Quandt D., **Wanke S.**, Stech M., Müller K., Löhne C., Hilu KW. & Borsch T. (2005) Inversions and length mutations in chloroplast DNA and their phylogenetic utility. International Botanical Congress Vienna, Austria Center

**Wanke S.**, Petzold K., Prieskorn S., Kaden R & Neinhuis C. (2005) Phylogeny of the western Mediterranean species of the genus Aristolochia (Aristolochiaceae). XVII International Botanical Congress Vienna, Austria

Gonzalez F., **Wanke S.** & Neinhuis C. (2005) Phylogenetic relationships of Aristolochiaceae subfamily Aristolochioideae based on molecular and morphological data. XVII International Botanical Congress Vienna, Austria

Ohi-Toma T., Sugawara T., Murata H., **Wanke S.**, Neinhuis C. & Murata J. (2005) Molecular Phylogeny and Revision of Aristolochia Sensu Lato (Aristolochiaceae), as inferred from rbcL, matK, phyA genes. XVII International Botanical Congress Vienna, Austria

Borsch T., Müller K., Hilu KW., Soltis D., Soltis P., Quandt D., Löhne C., Worberg A. & **Wanke S.** (2004) The tree of angiosperms and microstructural changes in the matK gene. Botany 2004 Alpine Diversity: Adapted to the Peaks, Snowbird Resort; Salt Lake City, Utah, USA

**Wanke S.**, Neinhuis C., Hilu KW. & Borsch T. )2001) Peppers and pipevines: Phylogenetic relationships within Piperales. 15 Internationales Symposium: Biodiversität und Evolutionsbiologie, Bochum, Germany

Neinhuis C., **Wanke S.**, Hilu KW. & Borsch T. (2001) Peppers and pipevines: Phylogenetic relationships within Piperales. Botany 2001, plants and people, Albuquerque, New Mexico, USA

Oetter K., **Wanke S.**, Schmidt K. & Vences M. (2000) Captive breeding, reproductive behaviour and larval development of Malagasy poison frogs, genus *Mantella.* 4th International Symposium, Isolated Vertebrate Communities in the Tropics, Bonn, Germany

# Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat. Die Bestimmungen der Promotionsordnung sind mir bekannt.