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Molecular systematics of selected *Diadegma* species (Hymenoptera: Ichneumonidae: Campoplegine) important in biological control

Dissertation

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Dedicated to Lara Sophie and Anna Katharina

Table of contents

1 Introduction

The genus *Diadegma* (Hymenoptera: Ichneumonidae: Campopleginae) represents a large group of parasitoids with 201 species worldwide (Yu and Horstmann 1997). They occur in all major biogeographic regions with 131 species having a palearctic and 33 a nearctic distribution. Twelve *Diadegma* species occur in more than one region and at least one of these (*D. semiclausum* (Hellén), fig. 1.1) was introduced by man as a biological control agent into other geographical areas (Talekar and Shelton 1993). *Diadegma insulare* Cresson and *D. blackburni* Cameron might have been introduced accidentally from one region into an other (Johnson *et al.* 1988; Henneman and Memmott 2001).

Parasitoid wasps of the genus *Diadegma* represent a diverse collection of solitary endoparasitoids. Adult *Diadegma* females parasitise larvae of various lepidopteran species. The host range can be restricted to a few species such as in *D. semiclausum* that is known to parasitise Lepidoptera of the family Plutellidae (*Plutella xylostella* Linnaeus, *Prays oleae* Bernard and *Prays citri* Millière; Horstmann, pers. communication). However, the host range can also be as wide as in *D. blackburni*, where several species from eight different families (Crambidae, Gelechiidae, Geometridae, Oecophoridae, Pterophoridae, Pyralidae, Scythrididae, Tortricidae) and the superfamily Tineoidea are known as suitable hosts (Perkins 1913; Zimmerman 1978; Banko *et al.* 2002).

Fig. 1.1: *Diadegma semiclausum* (Hellén) attacking *Plutella xylostella* L. larvae.

1.1 *Diadegma* as a biological control agent of the diamondback moth *P. xylostella*

Some parasitoid species, in particular *D. insulare* and *D. semiclausum*, have gained economic importance as biological control agents of *P. xylostella* and are therefore the best known and well examined species of the genus *Diadegma*. Thus, in the following, approaches used for biological control of *P. xylostella* will be explained in more detail.

The diamondback moth, *P. xylostella*, is one of the most destructive pests of cruciferous plants with serious economic damage being reported from Argentina, Australia, New Zealand and South Africa even before the 1930s (Lim 1986). *Plutella xylostella* occurs wherever crucifers (such as broccoli, cabbage, cauliflower, Brussels sprouts, rapeseed, kale, collar or mustard) are grown. In Malaysia economic losses of more than 90 % occurred through outbreaks of *P. xylostella* regularly since the 1960s (Verkerk and Wright 1996). Chemical control methods of *P. xylostella* failed due to the development of resistance to many synthetic insecticides, as well as to *Bacillus thuringiensis* products (Syed 1992; Talekar and Shelton 1993). The widespread use of broad-spectrum pesticides in the past might be a reason for the lack of effective natural enemies in many parts of the world. However, *P. xylostella* is known to be a migrant and this might be the cause why it gets easily established in newly planted *Brassica* crops compared to its natural enemy complex (Hardy 1938; Chu 1986; Braun *et al.* 2004).

Environmental concerns, coupled with growing occurrence of insecticide resistance, have led to an increased interest in biological control in crucifer production. Generally, the search for a successful biological control agent should start in the area of origin of the pest. However, the origin of *P. xylostella* has not been established yet. Kfir (1998) suggested, mainly on the basis of the natural enemy complex (14 different species of parasitoids) and the large number of wild plant species in the Brassicaceae (175, of which 32 are exotic), that *P. xylostella* might have originated in the Cape Floral Kingdom of South Africa. Using the same arguments (rich and diverse native parasitoid fauna of *P. xylostella* and a large number of indigenous *Brassi-*

cas in China) Liu *et al.* (2000) speculated that the origin of *P. xylostella* might be in China. In contrast Hardy (1938) believed that *P. xylostella* developed in the Mediterranean region, where many of the wild and cultivated forms of Brassicaceae are growing (Tsunoda 1980). This is supported by the studies of Mustata (1992), who collected 28 parasitoids from four families (Ichneumonidae, Braconidae, Pteromalidae and Eulophidae) on *P. xylostella* in Romania. Among these 28 collected parasitoids 12 different *Diadegma* species were included, however, this list was revised by Noyes (1994), who classified 11 *Diadegma* species of Mustata's list as misidentifications or as species that are not associated with *P. xylostella*. Delvare and Kirk (1999) confirmed the great diversity of the parasitoid fauna in Romania and added several parasitoids to the list of Mustata (1992), including one species of the family Trichogrammatidae (*Trichogramma evanescens* complex).

Parasitoids and predators can attack all life stages of *P. xylostella*. Hardy (1938) recorded 43, Lim (1986) 55 egg, larval and pupal parasitoids of *P. xylostella,* respectively. However, both authors note that the number of species recorded to attack *P. xylostella* might not be accurate.

Parasitoids attacking the larval stage of *P. xylostella* are regarded as the most effective ones and many of them belong to the genera *Diadegma*, *Cotesia* and *Microplitis* (both Braconidae: Microgastrinae). *Diadromus* (Ichneumonidae: Ichneumoninae) is the most important genus of pupal parasitoids of *P. xylostella*.

For biological control, knowing the correct name of the biological control agent is necessary for any successful program. Accurate identification of the natural enemies has traditionally been done using morphological characters. However, it is well known that the diagnostic characters of the subfamily Campoplegine are very variable. It is therefore not surprising that many misidentifications of the genus *Diadegma* have been perpetuated in the literature. The following examples illustrate the confusion in the taxonomy of *Diadegma*: In 1953, *Diadegma insulare* (Cresson) from Kenya was imported and released in Hawaii, but did not get

established (Johnson *et al.* 1988). In 1998, the *Diadegma* species from Kenya was identified as *D. semiclausum* by the Natural History Museum, London. However, in 2000 all African *Diadegma* species were classified as *D. mollipla* (Holmgren), a known parasitoid of the potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) (Azidah *et al.* 2000). Fitton and Walker (1992) listed ten putative species of *Diadegma* from *P. xylostella* whereas Azidah *et al.* (2000) assorted all *Diadegma* to seven different species: *D. insulare*, *D. leontiniae* (Brèthes), *D. semiclausum*, *D. fenestrale* (Holmgren), *D. mollipla*, *D. novaezealandiae* (Azidah, Fitton and Quicke) and *D. rapi* (Cameron).

In the catalogue of world Ichneumonidae of Yu and Horstmann (1997) *D. niponicum* Kusigemati, *D. varuna* Gupta and *D. fenestrale* are listed as three different species. Azidah *et al.* (2000) named all three species as *D. fenestrale,* and *D. niponica* and *D. varuna* are now regarded as synonyms.

Azidah *et al.* (2000) used common morphological characters in their morphometric studies on *Diadegma* species attacking *P. xylostella* (e.g. right fore wing, propodeum, apical segments of metasoma, length of the ovipositor). The key provided for identification was an important step forward in bringing light in the very confusing and unclear taxonomy of the genus *Diadegma*. However, the discrimination requires considerable taxonomic expertise and time.

Beside the classical taxonomy, crossing experiments between two species are often useful to determine a species. *Diadegma fenestrale* and *D. semiclausum* are occurring in the same geographical region and parasitising the same host. Hardy (1938) reported mating studies of both species where the F1 generation showed characters of both species. This shows that there is a high degree of similarity between *Diadegma* species.

With the need for accurate identification of biological control agents and the increased lack of expertise in classical taxonomy, alternative methods using molecular techniques have been introduced to systematics over the past decade (Hoy 1994; Hillis *et al.* 1996).

Natural enemies used for biological control are often going through genetic bottlenecks during collection, rearing and the following establishment in the field. Such bottlenecks decrease genetic variability, which might be a reason for the poor adaptation of the exotic parasitoids to the new environment. In Hymenoptera, reduced genetic variability causes an additional problem: the production of diploid males from fertilized eggs as a consequence of the sex-determination mechanism (Stouthamer *et al.* 1992). The common mode of reproduction in the order Hymenoptera is arrhenotokous haplodiploidy, in which diploid females develop from fertilised eggs if heterozygous at the 'sex locus', whereas haploid males arise from unfertilised eggs and are hemizygous. However, the presence of diploid males, which develop from fertilized eggs but being homozygous at the 'sex locus' (Whiting 1943), has been reported in more than 40 species in many hymenopteran superfamilies including members of the genus *Diadegma* (*D. semiclausum*, *D. armillata* (Gravenhorst), *D. chrysostictos* (Gmelin)) (Stouthamer *et al.* 1992; Cook 1993a; Butcher *et al.* 2000a; Noda 2000; Stahlhut and Cowan 2004), and this might be a reason for the relatively high rate of biocontrol failures in Ichneumonidae and Braconidae (Stouthamer *et al.* 1992). At least two types of models for the sex-determination – the genic balance model (Cook 1993a) and complementary sex determination involving a single codominant multiallelic locus (sl-CSD, Whiting 1943) - cause haplodiploidy. In the sl-CSD system, half of all fertilized eggs are expected to generate diploid males under inbreeding condition (i.e. the female mates with a son). Diploid males have zero fitness because of their low viability; they are effectively sterile or produce diploid sperm and sterile triploid female offspring (Whiting 1943; Cook 1993a; Krieger *et al.* 1999). The mortality of diploid males is higher than in females (Ross and Flechter 1986). Diploid males can be detected with isoenzymes (Hedderwick *et al.* 1985; Packer and Owen 1990; Butcher *et al.* 2000b; Noda 2000), DNA fingerprinting (random amplified polymorphic DNA-single-strand conformation polymorphism (RAPD-SSCP) analysis, Halloway *et al.* 2000), DNA microsa-

tellite markers (Stahlhut and Cowan 2004), cytologically, morphologically and cytometrically (Butcher *et al.* 2000a, b), and in response of inbreeding (Cook 1993b).

1.2 Molecular systematics

Molecular systematics employs genetic markers to make assumptions about population processes and phylogeny. At the same time it produces large databases (e.g. http://www.ncbi.nlm.nih.gov or http://www.ebi.ac.uk/embl) for specific genes, gene fragments and proteins, which can be also used for taxonomic purposes. The application of the genetic markers was reviewed by Caterino *et al.* (2000). The most commonly analysed regions for resolving genetic relationships within the species-rich insect group Hymenoptera have been the *cytochrome* c *oxidase* subunits (COI and COII), the 16S ribosomal DNA, the cytochrome *b* from the mitochondrial DNA (mtDNA), as well as the internal transcribed spacers (ITS1 and ITS2) and the 18S and 28S region of the nuclear ribosomal DNA (rDNA) (e.g. Cameron *et al.* 1992; Campbell *et al.* 1993; Carpenter and Wheeler 1999; Mardulyn and Whitfield 1999; Dowton and Austin 2001; Rokas *et al.* 2002; Ashfaq *et al.* 2005). Since the event of the first compilation of molecular biology techniques applied to insects (Hoy 1994) the list of available methodologies has considerably increased (see Hillis *et al.* 1996 for an overview).

Molecular DNA methods have not, as yet, been applied to infer phylogenetic relationships within the genus *Diadegma*. In the present study a fragment of the mitochondrial COI gene and the ITS2 of rDNA were analysed. The two genetic markers were chosen since they appear to have a different mode of evolution and transmission (Hillis and Dixon 1991; Simon *et al.* 1994).

1.2.1 Mitochondrial DNA

Mitochondrial DNA has a number of positive characteristics for phylogenetic and systematic studies including (i) maternal inheritance with little or no recombination, (ii) general conservation of gene order and composition, (iii) small size compared with the nuclear DNA and (iv) the lack of introns. Several genes of the mitochondrial genome are used to assess phylogenetic relationships among taxa (see Simon *et al.* 1994 for a review). In particular, the COI gene consists of highly conserved and also variable regions (Lunt *et al.* 1996; Zhang and Hewitt 1996) and is therefore suitable for analysis of different phylogenetic and taxonomic questions. Within the Hymenoptera the complete genome of the mitochondria has been sequenced in *Apis mellifera* Linnaeus (Crozier and Crozier 1993). The COI region was used for identification purposes in the Ichneumonidae *Mesochorus* sp. (Ashfaq *et al.* 2005) and in phylogenetic studies of apocritan wasps including the Ichneumonidae *Ichneumon promissorius* (Erichson), *Venturia canescens* (Gravenhorst) and *Xorides praecatorius* (Fabricius) (Dowton and Austin 2001). *Pimpla aequalis* Provancher., *Habronyx* sp. and *Dusona egregia* (Viereck) of the Ichneumonidae were also analysed in phylogenetic studies (Carpenter and Wheeler 1999).

1.2.2 Nuclear DNA

The three rDNA genes (fig. 1.2) encoding the 18S, 5.8S and 28S subunits of the ribosome are organized in clusters of tandem repeated units separated by the internal transcribed spacers 1 (ITS1 between the 18S and the 5.8S gene) and 2 (ITS2, between 5.8S and 28S gene) and an external transcribed spacer (ETS) at the 5' end (Hillis and Dixon 1991).

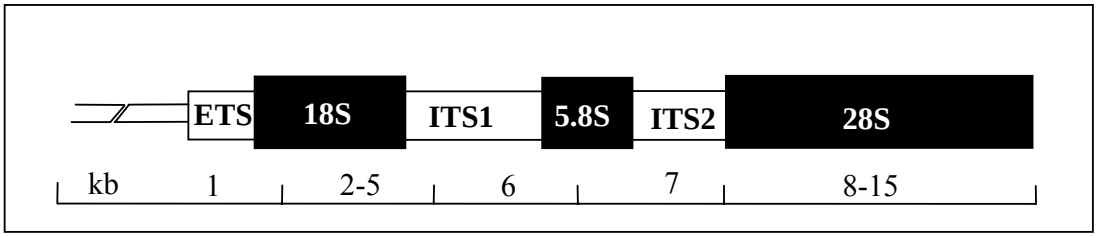

Fig. 1.2: Schematic organization of a ribosomal DNA array of an eukaryote (after Hillis and Dixon 1991). Spacer ITS2 was used in this study.

The genes are transcribed into RNA but not translated into protein. The internal spacers, which serve no further purposes, are under very little selection pressure and can accumulate substitutions very quickly (Hillis and Dixon 1991).

Ribosomal DNA is easy to amplify in polymerase chain reaction (PCR, Saiki *et al.* 1988) because it is present in multiple copies. Each copy of an rDNA array evolves very similar to the other copies within individuals or species and low variation among rDNA arrays within individuals and/or species indicates that the multiple copies are homogenized, a process of concerted evolution (Dover 1982). Nonetheless, the utility of ITS2 sequences as phylogenetic markers may be restricted due to high intra-specific variation (Rich *et al.* 1997; Harris and Crandall 2000) or low inter-specific variation (Kuperus and Chapco 1994). However, in Hymenoptera the ITS2 region has successfully been used to determine closely related species or populations such as *Diadegma* (Wagener *et al.* 2004a, b), *Mesochorus* (Ashfaq *et al.* 2005), *Aphelinus* (Prinsloo *et al.* 2002), *Trichogramma* (Stouthamer *et al.* 1999a) and *Ageniaspis* species (Alvarez and Hoy 2002) or in phylogenetic studies of pteromalid wasps (Campbell *et al.* 1993) and gallwasps (Rokas *et al.* 2002).

1.3 Aim of the study

A surprisingly low parasitism rate of <15 % (Seif and Löhr 1998) of the parasitoid complex (*Diadegma* sp., *Oomyces sokolowskii* (Kurdjumov) and *Diaplazon laetatorius* (Fabricius)) in unsprayed *P. xylostella* fields in eastern and southern Africa (Kenya, Tanzania, Uganda, Malawi, Mozambique, Zambia and Zimbabwe) was the starting point for the development of a biological control project for *P. xylostella* which was implemented by the International Centre of Insect Physiology and Ecology (ICIPE), Kenya. One output of this project was to compare the *Diadegma* species associated with *P. xylostella* in eastern and southern Africa with the exotic parasitoid *D. semiclausum* using molecular markers and cross breeding experiments to clarify the taxonomic status of the native and exotic parasitoid wasps.

In the due course the examination of several economically important *Diadegma* species was undertaken with molecular methods in order to add more information to the already existing morphological key of Azidah *et al.* (2000) and to investigate the phylogenetic relationships of different species of the genus *Diadegma*.

More specifically, the following subjects were investigated:

(i) Cross breeding experiments between the native parasitoid of eastern and southern Africa, *D. mollipla,* and the exotic parasitoid, *D. semiclausum*.

(ii) Detection of diploid males in *Diadegma* species.

(iii) Development and establishment of a molecular method (PCR-restriction fragment length polymorphism, PCR-RFLP) to identify important *Diadegma* species attacking *P. xylostella*.

(iv) Examination of the phylogenetic relationships of several *Diadegma* species with two different genetic markers (a fragment of the COI gene of mitochondrial DNA and ITS2 of ribosomal DNA).

2 Materials and methods

2.1 Insects

2.1.1 Sampling and localities

All *Diadegma* samples were collected between 2000 and 2003 either in the field or taken directly from laboratory cultures. Larvae or pupae of *P. xylostella* were collected from different cruciferous host plants (cabbage (*Brassica oleracea* Linnaeus. var. *capitata*), kale (*B. oleracea* var. *acephala*), broccoli (*B. oleracea* var. *italica*), Chinese cabbage (*B. campestris* var. *pekinensis*), and mustard (*Brassica* sp.)), larvae of *Yponomeuta cagnagellus* Zeller (Lepidoptera: Yponomeutidae) were collected from *Evonymus europaeus* Linnaeus. Larvae of *Cydia* sp. and *Ephestia kuehniella* Zeller were collected from unknown host plants. Additionally, five specimens of *D. mollipla* used had emerged from *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) collected from *Solanum tuberosum* Linnaeus. All larvae or pupae were reared until emergence of the parasitoids in the laboratory. The emerging *Diadegma* wasps were killed in 70-99.6 % ethanol and transported to the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, where they were stored at 4 ºC until further investigations. Voucher specimens were deposited at ICIPE with exemption of *D. rapi* where only one specimen was shipped. Table 2.1 provides information on the collection sites, latitude and longitude, the year of collection, collector and the EMBL accession numbers and fig. 2.1 shows the distribution of the nine *Diadegma* species examined in this study. Table 2.2 shows the different *Diadegma* populations and numbers of specimens examined with two different genetic markers (COI and ITS2) in the taxonomic studies (PCR-RFLP analyses). For the taxonomic/phylogenetic analyses *Meloboris* sp. reared from *P. xylostella* on *B. oleracea* var. *capitata* as host plant served as comparison or out-group.

Tab. 2.1: Specimens used in this study, hosts, localities and accession numbers.

Nos. of individuals investigated	Species with abbreviation of country and host[a]	Locality[b]	Latitude	Longitude	Year of collection	Collector[c]	EMBL accession nos.	
							ITS2	COI
1	*D. armillata*-F1[1]	Anduze, France	44.05 °N	3.98 °E	2003	FH	AJ877010	AJ888013
2	*D. armillata*-F2[1]	Saint-Hippolyte-du-Fort, France	43.97 °N	3.85 °E	2003	FH	AJ877011	AJ888014
3	*D. blackburni*-H1[2]	Mauna Kea, Hawaii, USA	19.82 °N	155.47 °W	2002	JS	AJ876632	AJ888020
4	*D. blackburni*-H2[2]	Mauna Kea, Hawaii, USA	19.82 °N	155.47 °W	2002	JS	AJ876633	AJ888021
5	*D. chrysostictos*-UK13[3]	Nantwich, UK	53.08 °N	2.45 °W	1998	RB	AJ877004	AJ888022
6	*D. chrysostictos*-UK2[3]	Leeds, UK	53.80 °N	1.58 °W	1996	RB	AJ877003	AJ888023
7	*D. fenestrale*-N1[4]	Wageningen, Netherlands*	51.98 °N	5.70 °E	2002	TB	AJ876416	AJ888011
8	*D. fenestrale*-N2[4]	Wageningen, Netherlands*	51.98 °N	5.70 °E	2002	TB	AJ876417	AJ888012
9	*D. insulare*-USA1[4]	Weslaco, USA	26.16 °N	97.98 °E	2001	MH	AJ877006	AJ888015
10	*D. insulare*-USA2[4]	Rosemount, USA*	44.73 °N	93.13 °W	2002	JL	AJ877007	AJ888016
11	*D. insulare*-USA3[4]	Sanford, USA*	28.79 °N	81.28 °E	2002	GL	AJ877005	AJ888017
12	*D. leontiniae*-B1[4]	Brasilia, Brazil	15.78 °S	47.92 °W	2001	MCB	AJ877009	AJ888018
13	*D. leontiniae*-B2[4]	Goiania, Brazil	16.70 °S	49.20 °W	2002	MCB	AJ877008	-
14	*D. mollipla*-K2[4]	Kisii, Kenya	0.67 °S	34.78 °E	2001	OM	AJ508221	-
15	*D. mollipla*-K1[4]	Maragua, Kenya	0.80 °S	37.13 °E	2002	RG	AJ876415	AJ888006
16	*D. mollipla*-R[4]	Piton Hyacinthe, Réunion	20.95 °S	55.30 °E	2001	PR	AJ876414	AJ888009
17	*D. mollipla*-SA[4]	Cape Region, South Africa	32 °S	37 °E	2001	SMW	AJ508222	AJ888008
18	*D. mollipla*-SH[4]	St. Helena	15.93 °S	5.72 °W	1999	RK	AJ876413	AJ888010
19	*D. mollipla*-T[4]	Ilkidinga, Tanzania	3.33 °S	36.71 °E	2002	WM	AJ876412	AJ888007
20	*D. mollipla*-U[4]	Kabale, Uganda	1.41 °S	29.99 °E	2002	FN	AJ876411	AJ888005
21	*D. rapi*-Au[4]	Adelaide, Australia	34.58 °S	138.36 °E	2001	MK	AJ877012	AJ888019
22	*D. semiclausum*-A[4]	Seibersdorf, Austria	48.22 °N	13.07 °E	2002	AK	AJ885183	AJ885191
23	*D. semiclausum*-Au[4]	Virginia, Australia	34.67 °S	138.57 °E	2001	MK	AJ885180	AJ885188

Tab. 2.1: Continued.

Nos. of individuals investigated	Species with abbreviation of country and host[a]	Locality[b]	Latitude	Longitude	Year of collection	Collector[c]	EMBL accession nos.	
							ITS2	COI
24	*D. semiclausum*-I[4]	Central Region, Israel	31 °N	34 °E	2003	ST	AJ885184	AJ885195
25	*D. semiclausum*-J[4]	Matsudo, Japan*	35.78 °N	139.90 °E	2001	MH	AJ885181	AJ885189
26	*D. semiclausum*-K[4]	Nairobi, Kenya*	1.22 °S	36.89 °E	2002	RG	AJ508223	AJ885194
27	*D. semiclausum*-NKo[4]	Pujon, DPR Korea*	40.48 °N	127.62 °E	2002	CP	AJ885185	AJ885196
28	*D. semiclausum*-M[4]	Cameron Highlands, Malaysia	4.48 °N	101.45 °E	2002	RB	AJ885187	AJ885198
29	*D. semiclausum*-NZ[4]	Pukekohe, New Zealand	37.20 °S	174.95 °E	2001	GW	AJ885182	AJ885190
30	*D. semiclausum*-SW[4]	Sugiez, Switzerland*	46.97 °N	7.10 °E	2002	MG	AJ885186	AJ885197
31	*D. semiclausum*-TW2[4]	Shanhua, Taiwan*	23.14 °N	120.28 °E	2000	NST	AJ508224	AJ885193
32	*D. semiclausum*-TW1[4]	Shanhua, Taiwan*	23.14 °N	120.28 °E	2000	NST	-	AJ885192
33	*Meloboris* sp.[4]	Alemaya, Ethiopia	9.43 °N	42.03 °E	2001	GA	AJ888025	AJ888024

[a] Lepidopteran hosts: [1] *Yponomeuta cagnagellus*; [2] *Cydia* sp.; [3] *Ephestia kuehniella*; [4] *Plutella xylostella.*

[b] The asterisk marks individuals obtained from laboratory cultures.

[c] Abbreviations of collectors: AK: A. Kirk, CP: CABI-PPRI Focus Group, FH: F. Hérard, FN: F. Nawaga, GA: G. Ayalew, GL: G. Leibee, GW: G. Walker, JL: J. Lee, JS: J. Slotterback, MCB: M. Castelo Branco, MG: M. Grossrieder, MH: M. Haseeb,MK: M. Keller, NST: N.S. Talekar, OM: O. Mfugale, PR: P. Ryckewaert, RB: R. Butcher, RG: R. Gathu, RK: R. Kfir,,SMW: S.M. Waladde, ST: S. Tam; TB: T. Bukovinsky, WM: W. Mwaiko.

Fig. 2.1: Worldwide distribution of nine *Diadegma* species examined in the present study. 1: *D. blackburni*, 2: *D. insulare*, 3: *D. leontiniae*, 4: *D. chrysostictos*, 5: *D. armillata*, 6: *D. fenestrale*, 7: *D. mollipla*, 8: *D. semiclausum*, 9: *D. rapi.*

Tab. 2.2: Geographic location, year of collection, collector and number of individuals of *Diadegma* species used for PCR-RFLP analyses.

Species	Country and collection site(s)[a,b,c]	Latitude	Longitude	Year of collection	Collector[d]	Individuals analysed			
							COI		ITS2
						P3/P4	P3/P5	P3/P8	
D. fenestrale-N	Wageningen, Netherlands[a]	51.98 °N	5.70 °E	2002	TB		1	4	**3**, 8
D. insulare-USA1	Weslaco, USA[a]	26.16 °N	97.98 °E	2001	MH			1	7
D. insulare-USA2	Rosemount, USA[a]	44.73 °N	93.13 °W	2002	JL				2
D. insulare-USA3	Sanford, USA[a]	28.79 °N	81.28 °E	1999	GL			1	2
D. leontiniae-B1	Brasilia, Brazil[a]	15.78 °S	47.92 °W	2001	MCB		**3**		**6**
D. leontiniae-B2	Goiania, Brazil[a]	16.70 °S	49.20 °W	2002	MCB				**4**
D. mollipla-E	Nazareth, Ethiopia[b]	8.55 °N	39.27 °E	2001	GA	**3**			**3**
D. mollipla-K1	Maragua, Kenya[a]	0.80 °S	37.13 °E	2002	RG			**1**	**3**
D. mollipla-K2	Kisii, Kenya[a]	0.68 °S	34.77 °E	2001	OM	**2**			**2**
D. mollipla-K3	Kapsabeth, Kenya[a]	0.20 °N	35.10 °E	2000	RG	**4**			**3**
D. mollipla-K4	Maralal, Kenya[a]	1.10 °N	36.70 °E	2001	RG	**1**			**1**
D. mollipla-K5	Meru, Kenya[a]	0.05 °N	37.65 °E	2001	RG	**1**	**1**	**1**	**4**
D. mollipla-K6	Embu, Kenya[a]	0.53 °S	37.45 °E	2002	RG		**3**	**1**	**2**
D. mollipla-K7	Murang'a Kenya[a]	0.72 °S	37.15 °E	2002	RG				**2**
D. mollipla-K8	Limuru, Kenya[a]	1.10 °S	36.65 °E	2001	RG	**15**		**1**	**15**
D. mollipla-K9	Wundanyi, Kenya[a]	3.40 °S	38.37 °E	2001	RG	**2**			**2**
D. mollipla-K10	South Kinangop, Kenya[b]	0.72 °S	36.65 °E	2002	BW	**2**			**2**
D. mollipla-R1	Piton Hyacinthe, Réunion[a]	20.95 °S	55.30 °E	2001	PR	**1**	**1**	**2**	**8**
D. mollipla-R2	Bassin Martin, Réunion[a]	21.30 °S	55.52 °E	2001	PR		**1**		**2**
D. mollipla-SA1	Cape Region, South Africa[a,c]	32 °S	37 °E	2001	SMW	**6**	**1**	**1**	**10**
D. mollipla-SA2	Pretoria, South Africa[a]	25.71°S	28.22 °E	2001	RK	8	1	1	9
D. mollipla-SH	St. Helena[a,c]	15.93 °S	5.72 °W	1999	RK				**3**
D. mollipla-T1	Ilkidinga, Tanzania[a]	3.33 °S	36.71 °E	2002	WM	**3**	**1**	**2**	**3**
D. mollipla-T2	Malindi, Tanzania[a]	4.69 °S	38.29 °E	2000	WM	**1**			**2**

Tab. 2.2: Continued.

Species	Country and collection site(s)[a,b,c]	Latitude	Longitude	Year of collection	Collector[d]	Individuals analysed			
						COI			ITS2
						P3/P4	P3/P5	P3/P8	
D. mollipla-T1	Ilkidinga, Tanzania[a]	3.33 °S	36.71 °E	2002	WM	**3**	**1**	**2**	**3**
D. mollipla-T2	Malindi, Tanzania[a]	4.69 °S	38.29 °E	2000	WM	**1**			**2**
D. mollipla-U1	Kabale, Uganda[a]	1.41 °S	29.99 °E	2002	FN		**1**		**2**
D. mollipla-U2	Kabale, Uganda[a]	1.15 °S	29.26 °E	2001	FN		**1**		**1**
D. mollipla-U3	Kabale, Uganda[a]	1.21 °S	29.94 °E	2001	FN			**1**	**1**
D. mollipla-U4	Wakiso, Uganda[a]	0.43 °N	32.44 °E	2002	FN			**1**	**1**
D. rapi-Au	Victoria, Australia[a]	37.83 °S	145.00 °E	2002	MK				**1**
D. semiclausum-A	Seibersdorf, Austria[a]	48.22 °N	13.07 °E	2002	AK		**1**	**1**	**3**
D. semiclausum-Au	Virginia, Australia[a]	34.67 °S	138.57 °E	2001	MK	**1**		**1**	**3**
D. semiclausum-I	Central Region, Israel[a]	31 °N	34 °E	2003	ST				3
D. semiclausum-J	Matsudo, Japan[a]	35.78 °N	139.90 °E	2001	MK	2		1	2
D. semiclausum-K	Nairobi, Kenya [a]	1.22 °S	36.89 °E	2002	BW	3		1	3
D. semiclausum-M	Cameron Highlands, Malaysia[a,c]	4.48 °N	101.45 °E	2002	RB				**3**
D. semiclausum-N	Wageningen, Netherlands[a]	51.98 °N	5.70 °E	2002	TB			1	3
D. semiclausum-NKo	Pujon, DPR Korea[a]	40.48 °N	127.62 °E	2002	CP				1
D. semiclausum-NZ	Pukekohe, New Zealand[a]	37.20 °S	174.95 °E	2001	GW		1	1	2
D. semiclausum-SW	Sugiez, Switzerland[a]	46.97 °N	7.10 °E	2002	MG		1	1	3
D. semiclausum-TW	Shanhua, Taiwan[a]	23.14 °N	120.28 °E	2000	NST	8	1		11
Meloboris sp.-E1	Serbo, Ethiopia[a]	7.70 °N	37.04 °E	2001	GA	**1**	**1**		**1**
Meloboris sp.-E2	Masha, Ethiopia[a]	7.84 °N	35.47 °E	2001	GA				**1**
Meloboris sp.-E3	Hole, Ethiopia[a]	8.27 °N	37.66 °E	2001	GA				**1**
Meloboris sp.-E4	Wolmera, Ethiopia[a]	9.06 °N	38.51 °E	2002	GA				**7**
Meloboris sp.-E5	Alemaya, Ethiopia[a]	9.43 °N	42.03 °E	2001	GA	**1**	**2**		**6**

Host: [a]*Plutella xylostella*, [b]*Phthorimaea operculella*; [c]region, [d]collector: abbreviations refer to those in tab. 2.1. in addition BW: B. Wagener; [e]bold figures: field populations, standard figures: laboratory cultures.

2.2 Biological analyses

2.2.1 Laboratory cultures and crossing experiments

2.2.1.1 Rearing techniques

2.2.1.1.1 Plants for rearing *Diadegma* hosts

Cabbage plants (*Brassica oleracea*, cultivar Gloria) used for rearing the *Diadegma* host (*P. xylostella*) were grown under greenhouse conditions at ICIPE headquarters in Nairobi, Kenya. Plants and/or isolated leaves from cabbage plants used for the laboratory culture in the experiments were 7-8 weeks old. When whole plants were taken for maintaining the *Diadegma* laboratory cultures, the soil was covered with aluminium foil to prevent soil borne infections. All leaves that were cut off and used for the laboratory cultures/experiments were kept in a vial with water to avoid wilting. Freshly picked and turgid leaves were used in the experiments.

2.2.1.1.2 *Plutella xylostella* culture

The *P. xylostella* colony was initiated with field-collected material from Wundanyi, Kenya (3.40 °S, 38.37 °E). The emerged adults were released into Perspex cages (40x20x20 cm). Perforated aluminium foil strips (approx. 4x15 cm) were placed in the cages for egg laying. The aluminium foil had previously been dipped into juice of crushed cabbage leaves and then allowed to dry at room temperature. After 24 h the strips were removed from the cage and the eggs were disinfected in 10 % hydrochloride solution for 30 min. The foil strips were rinsed for 10 min under running water to wash off the disinfectant. The foil sheets were kept in plastic containers at 8-10 °C. When the eggs changed colour to a dark brown, they were placed on about six leaves per cabbage plant in batches of 30 eggs per leaf. The emerged larvae fed on the plants for 12-14 days at room temperature (approx. 23±2.0 °C, relative humidity (rH) 40-

60 %, 12 h light (L): 12 h dark (D) photoperiod) until they pupated. When most of the larvae had pupated, the whole plant was cut off and placed into a 60 l black plastic drum. The drum had a hole (∅15 cm) in the top above which a Perspex cage (40x20x20 cm) without bottom was placed. Emerging adults accumulated in this cage and were kept there for egg laying as described above. A 5 % sugar solution served as food source and was exchanged regularly. Second and third instar *P. xylostella* larvae (second generation, F2) were used for the experiments.

2.2.1.1.3 *Diadegma* cultures

Three different *D. mollipla* cultures, two of *D. mollipla* East Africa (DM-K1 and DM-K2) and one of *D. mollipla* South Africa (DM-SA), were initiated. The first *D. mollipla* culture (DM-K1) was started with parasitised *P. xylostella* larvae collected during a survey in central Kenya in 2001. This culture was maintained until June 2002. The second culture (DM-K2) was started with parasitised *P. xylostella* larvae collected at Rumuruti, Kenya (0.27 °N, 36.53 °E) in October 2003. A third colony of *D. mollipla* (DM-SA) was started in December 2001 from 50 *D. mollipla* pupae of a laboratory culture imported from the Plant Protection Research Institute (PPRI) in Pretoria, South Africa (25.71 °S, 28.22 °E). The latter culture was maintained at the quarantine laboratory at ICIPE.

In the laboratory, parasitised pupae (dark brownish colour) were placed in a Perspex cage (20x20x40 cm) to allow the newly emerged parasitoids to mate. Out of the parasitised larvae collected in Rumuruti (DM-K2) 11 females and six males emerged, the colony of DM-SA started with eight females and 32 males. Data of *Diadegma* wasps emerged from parasitised host larvae collected in central Kenya in 2001 (for DM-K1) are not available. After mating, the parasitoids were allowed for 24 h to parasitise second and third instar *P. xylostella* host

glarvae (20-30 per leaf), which were feeding on cabbage leaves. Parasitised larvae were fed with cabbage leaves in ventilated lunch boxes (21x14.5x8 cm) containing paper tissue to avoid moisture condensation until pupation. The parasitoid pupae were collected with a fine brush from the cabbage leaves and the wall of the boxes and kept in smaller plastic containers at 12 °C and a L: 12 h D: 12 h photoperiod in an incubator (Rumed; Rubarth Apparate GmbH, Laatzen, Germany). A 20 % honey solution was provided as food source. DM-K1 was regularly supplemented with field-collected individuals from Wundanyi whereas DM-K2 was kept strictly in generations. The intra-specific crossing experiments between DM-K1 and DM-SA were executed in the quarantine laboratory while for the inter-specific crosses (DM-K2 x *D. semiclausum*) *D. mollipla* of the sixth generation were used.

The *D. semiclausum* (DS) culture was started in the quarantine unit in 2001 with pupae from a laboratory culture provided by the Asian Vegetable Research and Development Centre, Shanhua, Taiwan (23.14 °N, 120.28 °E). The culture was moved from the quarantine unit to a separate rearing room, where only *D. semiclausum* was kept, after permission was received fromthe Government of Kenya. *Diadegma semiclausum* was reared in a similar way as *D. mollipla*. Thus, when used for the experiments in January 2004 the culture was already optimised for mass rearing. To avoid male bias in the culture it was taken care that females could mate in a separate plastic container (20x20x25 cm) for 24 h and ten mated females were afterwards transferred to cages (45x45x45 cm) containing three cabbage plants.

2.2.1.2 Intra- and inter-specific crossings

To determine whether gene flow could occur between the two populations of *D. mollipla* (intra-specific crossing, DM-K1 x DM-SA) and between *D. mollipla* (DM-K2) and *D. semiclausum* (DS) (inter-specific crossing) crosses and backcrosses (if necessary) between these populations were performed.

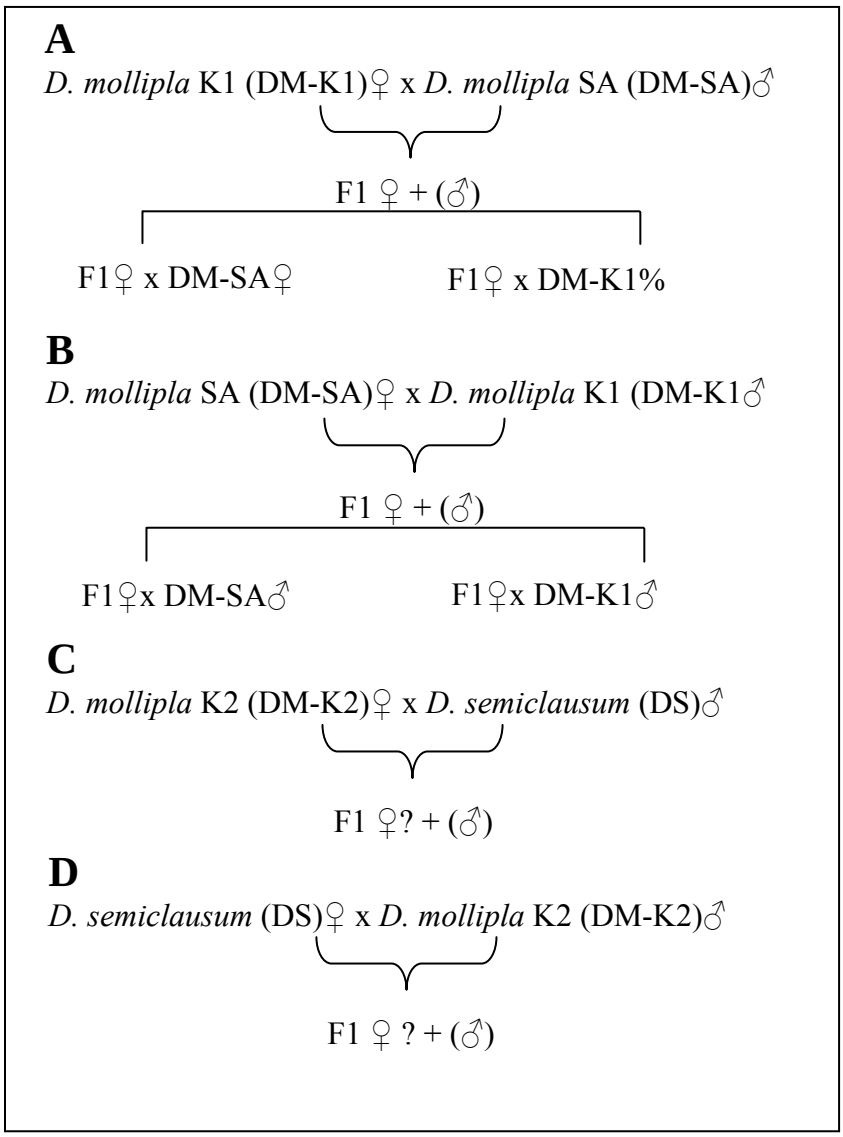

Fig. 2.2: Schematic illustration of the intra- and inter-specific crossing experiments performed between two *D. mollipla* populations and *D. mollipla* and *D. semiclausum*. **A** and **B**: Intra-specific and reciprocal crossings with *D. mollipla* populations; **C** and **D**: Inter-specific crossings between *D. mollipla* and *D. semiclausum*.

Figure 2.2 illustrates the crossing schemes and the reciprocal crossings performed during the present study.

Parasitised dark pupae of DM-K1 and DM-SA were separated individually in plastic vials (2.5 cm diameter x 6.5 cm height) and kept at room temperature until emergence. Each female was transferred to a plastic container (fig. 2.3) with a male either from the same or the different population for 3 days and with 20 second and third instar *P. xylostella* host larvae feeding on a cabbage leaf. The parasitoids were fed with ~20 % honey solution.

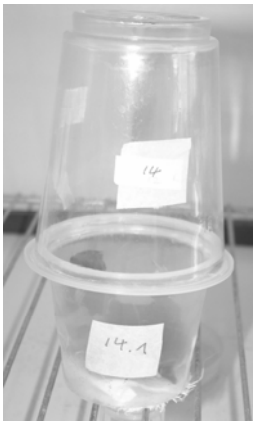

Fig. 2.3: Plastic container with a *Diadegma* pair, *P. xylostella* host larvae on a cabbage leaf and honey solution used for crossing experiments.

Leaves with the parasitised host larvae were removed after 24 h. Fresh larvae for parasitism were exposed for three consecutive days. After exposure the larvae were placed in a plastic container (10.5 cm diameter x 6.5 cm height) containing paper tissue to avoid moisture condensation and closed with mesh to maintain ventilation. Every second day the parasitised larvae were given fresh cabbage leaves as food. After pupation the pupae were kept individually in plastic vials until emergence. Reciprocal crosses were performed by mating individual F1 females with a male of each of the two parent cultures.

The cross breeding experiment between *D. mollipla* (DM-K2) and *D. semiclausum* (DS) followed the same procedure as described above with the exemption that the emerged female and the male were kept in a plastic vial (2.5 cm diameter x 6.5 cm height) for 24 h to ensure mating. In these experiments 25 pairs of each combination were tested. No backcrosses were necessary.

2.2.1.3 Crossings between mothers and sons of *D. mollipla* (DM-K2) and *D. semiclausum* (DS)

Experiments to examine the occurrence of diploid males were necessary because laboratory cultures especially DM-SA, DM-K2 and DS showed a male-biased sex ratio. Diploid males have been recorded in several *Diadegma* species (e.g. *D. armillata*, *D. chrysostictos*) including *D. semiclausum* (Butcher *et al.* 2000a,b; Noda 2000). To examine this phenomenon in *D. mollipla* and *D. semiclausum* the following experiment (after Noda 2000; slightly modified) was undertaken. The schematic procedure of the crossing experiment of *D. mollipla* is illustrated in fig. 2.4. *Diadegma semiclausum* were crossed similarly.

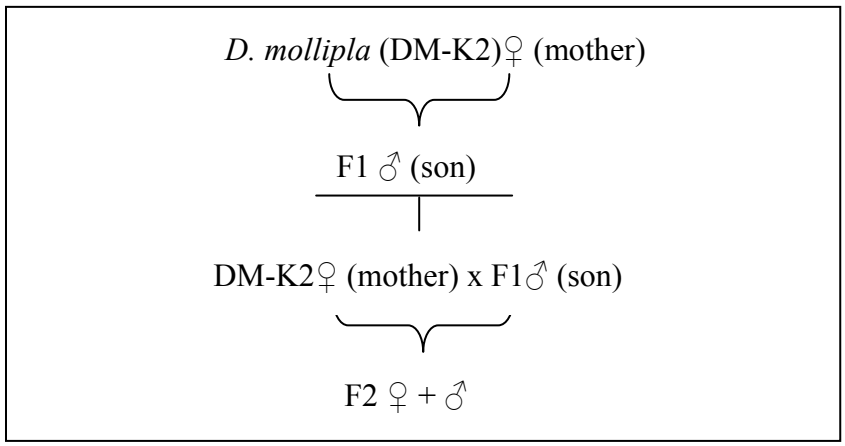

Fig. 2.4: Schematic procedure of the mother and son crossing experiment of *D. mollipla* to detect diploid males.

Five virgin females of both *D. semiclausum* and *D. mollipla* were given separately 30 second and third instar *P. xylostella* larvae feeding on cabbage for parasitism as described above. The females were removed after 24h and kept in an incubator (Rumed; Rubarth Apparate GmbH, Laatzen, Germany) at 15 °C, L: 12 h D: 12 h photoperiod until F1 emerged. Honey solution (20 %) served as food source. One son (haploid) of the F1 generation was taken to mate with his mother in a plastic vial (2.5 cm diameter x 6.5 cm height) with honey solution (20 %) for 24 h. After mating the female was allowed to parasitise 25 second and third instar *P. xylos-*

tella larvae for 24 h. Fresh larvae feeding on cabbage leaves were provided for 3 days. The parasitised host larvae were reared as described above until the adults emerged. Out of five females of each *Diadegma* species investigated, only one mother of *D. mollipla* and two of *D. semiclausum* showed diploid banding patterns when analysed with isoenzymes, respectively. The F2 generation of these diploid mothers crossed with their sons had males and females in the resulting brood and were examined with isoenzymes.

2.3 Molecular techniques

The chemicals (including suppliers) used in this study are listed in Appendix 1. All buffers were produced with deionised water. Composition of the most frequently used buffers and solutions is presented below. All chemicals (if necessary) and utensils were autoclaved at 1.1 bar, 120 °C for 20 min prior to use.

10 x *Taq* buffer with $(NH_4)_2SO_4$ (MBI Fermentas, St. Leon-Rot, Germany)	0.75 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$, 0.1 % Tween 20, pH 8.8
10 x *Taq* buffer with KCl (MBI Fermentas)	0.1 M Tris-HCl, 0.5 M KCl, 0.08 % Nonidet P40, pH 8.8
50 x TAE-buffer	242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH 8.0
10 x TE	10 mM Tris-HCl, 10 mM EDTA, pH 8.0
Loading dye (MBI Fermentas)	10 mM Tris-HCl, 0.03 % bromphenol blue, 0.03 % xylene cyanol FF, 60 % glycerol, 60 mM EDTA, pH 7.6

2.3.1 Analyses of isoenzymes

The presence or absence of diploid males in *D. mollipla* and *D. semiclausum* as an indicator for the sl-CSD was examined using isoenzym variations as genetic marker.

Haploid/diploid males and diploid females of *D. semiclausum* were analysed by polyacrylamide gel electrophoresis for one enzymatic system (phosphoglucomutase, PGM) (Butcher *et al.* 2000b; Noda 2000). The slightly modified protocols used were taken from Murphy *et al.* (1996) and Wendel and Weeden (1989).

2.3.1.1 Preparation of starch gels

A starch gel (14 %, w/v) was prepared with gel buffer in an Erlenmeyer flask. After melting the starch in the microwave the melted gel was connected to an aspirating shield to apply vacuum for ~15 sec. The hot gel was poured into a gel mold (APELEX, Massy cedex, France) and cooled to ambient temperature for 45 min. After wrapping the gel with plastic food wrap it was placed in a refrigerator for 1 h.

Gel buffer	5 mM L-histidine HCl, 2.5 mM NaCl, pH 7.0

2.3.1.2 Tissue homogenisation

Diadegma specimens were killed at -20 °C for 30 min prior to homogenisation. Each individual was then macerated with a Teflon pistil in an ice-cold ceramic mortar and a drop of ice-cold deionised H_2O. After grinding the mixture was held on ice.

2.3.1.3 Gel loading

The starch gel was cut vertically ~ 4cm from the edge of the gel mold.

The samples were adsorbed onto 2x4 mm Whatman (3 mm) filter paper wicks and loaded with forceps in the vertical cut of the gel. Samples of freshly homogenized seeds of cow pea (*Vigna unguiculata* (Linnaeus) Walpers) with a known genotype served as positive control. An additional sample consisting only of tracking dye (bromphenol blue, 0.03 %) was added.

2.3.1.4 Starch gel electrophoreses

The gels were assayed in the citrate/histidine buffer system (Pasquet 1999) and were run in a horizontal electrophoresis chamber (APELEX, Massy cedex, France) for 3-4 h at 200 V and 4 °C until the front line of the loading dye was 7 cm from the origin. The gels were subsequently cooled using plastic bags filled with ice. Contact between the gel and the electrode buffer was established using folded Whatman filter paper (3 mm).

Electrode buffer	0.41 M citric acid trisodium salt dehydrate, pH 6.0

After electrophoresis was completed the gels were cut horizontally with a fine plastic string in three equal slices of 1 mm thickness and placed in staining boxes.

2.3.1.5 Enzyme visualisation

Three staining systems were chosen for preliminary screening because they had been successfully used with other Hymenoptera species to detect diploid males: Esterase (acronym EST: non specific enzyme detected in *Halictus poeyi* Lepeletier (Hymenoptera: Halictidae), Zayed and Packer 2001); isocitrate dehydrogenase (acronym IDH: Enzyme Commission (EC) 1.1.1.42 detected in *Augochlorella striata* Provancher (Hymenoptera: Halictidae), Packer and

Owen 1990); phosphoglucomutase (acronym PGM: EC 5.4.2.2 detected in *Diadromus pulchellus* Wesmael (Hymenoptera: Ichneumonidae), Hedderwick *et al.* 1985 and *D. semiclausum,* Noda 2000). The PGM system was selected because this enzyme system was easy to handle and it could identify differences between *D. mollipla* and *D. semiclausum* in males and females.

The enzyme-specific staining was performed according to Wendel and Weeden (1989).

PGM	10 mg/ml α-D-glucose-1-phosphate, 10 mg/ml nitro blue tetrazolium chloride, 1 mg/ml phenazine methosulfate, 5 mg NADP, 25 U glucose-6-phosphate dehydrogenase, 50 mg/ml $MgCl_2$, 12 ml Tris-HCl (0.5 M, pH 8.5) and 15 ml of 2 % agarose

After applying the agar overlay containing the stain the gels were incubated in the dark at 37 °C until the bands appeared (approx. 15 min). Afterwards the gels were rinsed in water and were then photographed with a digital camera (Nikon COOLPIX995, Nikon Corporation, Tokyo, Japan). The images were transferred to a PC and different banding patterns were scored manually.

2.3.2 Nucleic acids

2.3.2.1 Extraction of nucleic acids

Diadegma specimens from eastern and southern Africa were identified to species level according to morphological characters (Azidah *et al.* 2000) prior to DNA extraction.

Total genomic DNA was extracted from single *Diadegma* specimens using a slightly modified protocol from Baruffi *et al.* (1995), originally developed for Mediterranean fruit fly (*Ceratitis capitata* Wiedemann). The insects were washed in deionised water for one second

and air dried on filter paper. Wasps were grinded with a plastic pistil in a 1.5 ml Eppendorf tube containing 200 µl extraction buffer and 0.1 mg/ml Proteinase K.

Extraction buffer	0.1 M NaCl, 0.2 M Sucrose, 50 mM EDTA, 0.5 % w/v SDS, 0.1 M Tris-HCl, pH 9.1

After incubation of the homogenate at 65 °C for 30 min, 8 M potassium acetate was added to a final concentration of 1 M, and the mixture was kept on ice for 30 min. After centrifugation for 15 min at 10,000 g, the supernatant was washed with 2 volumes of ice-cold absolute ethanol. The tubes were kept at -20 °C for at least 1 h or overnight to ensure that even little amounts of DNA were resolved. After another centrifugation step (15 min at 10,000 g) the supernatant was washed twice with 70 % ice-cold ethanol for 20 min. The pellet was dried and resuspended in 20-50 µl TE buffer (pH 8.0) and DNA preparations were stored at -20 ºC until further investigations.

2.3.2.2 Precipitation of nucleic acids

In some cases it was necessary to concentrate and purify the DNA samples. The DNA was precipitated by combining the DNA sample (1 volume) with 5 M NH_4-acetate (1 volume) and 2.5 volumes of absolute ethanol. The mixture was incubated for 30min at room temperature. After centrifugation for 20 min at 10,000 g the small whitish DNA pellet was washed twice with ice-cold ethanol (70 %), air-dried and resuspended in 20-30 µl of TE buffer.

2.3.2.3 Purification of nucleic acids

Genomic DNA used for sequence analyses must be pure and free of contaminations such as RNA, protein, residual organic solvents, primer-dimers or agarose. Two commercial kits (DNA Cleaning Kit (MBI Fermentas) and QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany)) were used according to the manufacture's instructions. Both kits utilize the modified glass beads protocol of Vogelstein and Gillespie (1979). In the presence of high-salt buffer, DNA binds to specially prepared glass particles (silica powder or silica-gel membrane). The glass beads are centrifuged and washed to remove salt and impurities. The DNA was eluted with 30 µl of purified PCR-water and stored at -20 ºC until subsequent application.

2.3.2.4 Polymerase chain reaction (PCR)

All PCR reactions of genomic and mitochondrial DNA were performed in a PCR-100 Thermocycler (MJ Research Inc., Watertown, MA, USA) including a positive and a negative control (without DNA template). When this study was started the Thermocycler did not have a heated lid and the use of mineral oil to protect the samples from evaporation was necessary. Later on a heated lid was installed and the condensation of the samples was inhibited and the use of mineral oil was discontinued.

2.3.2.4.1 PCR of rDNA

The PCR was carried out in 25 µl reaction volumes containing 0.075 M Tris-HCl (pH 8.8), 0.02 M $(NH_4)_2SO_4$, 0.01 % Tween, 1.5 mM $MgCl_2$, 17.5 pmoles of each primer, 50 nmoles of each dNTP, 1 µg of bovine serum albumin (BSA), 1 U of *Taq*-Polymerase (MBI Fermentas) and 1 µl of template DNA. The oligonucleotide primers used for PCR amplification were as

follows: 5'-ATA TGC TTA AAT TCA GCG GG-3' (ITS2-1) and 5'-GGG TCG ATG AAG AAC GCA GC-3' (ITS2-2). These primers anneal to highly conserved sequences in the 5.8 S and the 28 S rDNA genes flanking the ITS2 region (Navajas *et al.* 1998, see fig. 1.2). The PCR reaction was performed by the following cycling parameters: one cycle of 4min at 95 °C followed by 35 cycles of 1 min at 92 °C, 1 min at 51 °C, and 1 min 30 sec at 72 °C.

2.3.2.4.2 PCR of mtDNA

The amplification of a fragment of the COI gene was not always successful, even after several rounds of independent PCR set-ups. Therefore, different primer combinations were tested. Several universal primers developed by Simon *et al.* (1994) were employed. The names and sequences of the primers are listed in tab. 2.3. All primers published by Simon *et al.* (1994) anneal to the *Drosophila yakuba* Burla mitochondrial genome (Clary and Wolstenholme 1985). Primer 8 was developed for *Diadegma* species with the computer programs OLIGO (version 3.3, Borland International 1987) and Primer Designer Software (version 2.0, Scientific and Educational Software 1990/1991). Figure 2.5 illustrates the complete *cytochrome oxidase* gene with the binding positions of primers used during this study in the COI region.

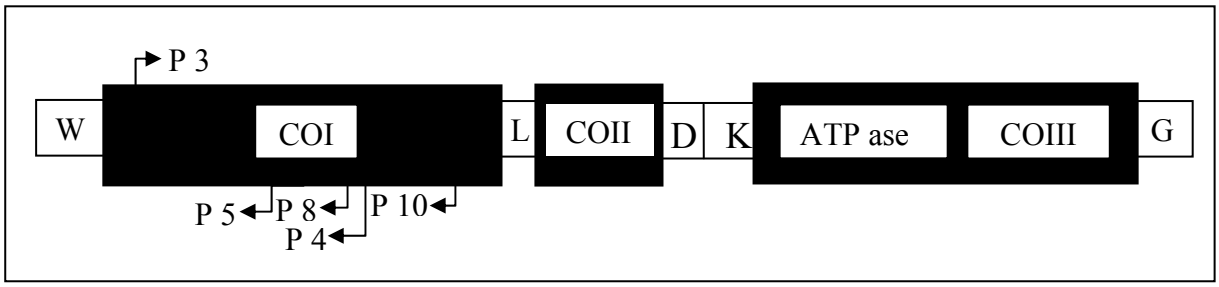

Fig. 2.5: Schematic illustration of the complete protein-encoding gene *cytochrome oxidase* of mitochondrial *Apis mellifera* DNA with the subunits I, II and III. Genes for tRNAs are indicated by one-letter code for their corresponding amino acids (W: Trytophan, L: Leucine, D: Aspartate, K: Lysine, G: Glycine). The COI region was investigated during the present study and the primer binding positions are illustrated. Abbreviations of the primers (codes) are listed in tab. 2.3.

The amplification was performed in a 25 µl reaction volume with the following primer combinations: (i) P3 and P4, (ii) P3 and P5, (iii) P3 and P8, (iv) P3 and P10. The reaction mixtures for all primer combinations (i)-(iv) contained 0.01 M Tris-HCl (pH 8.8), 0.05 M KCl, 0.08 % Nonidet P40, 1.5 mM $MgCl_2$, 15 pmoles of each primer, 50 nmoles of each dNTP, 1 µg of BSA, 1 U of *Taq*-Polymerase (MBI Fermentas) and 1 µl of template DNA.

Tab. 2.3: Sequences and codes of primers to amplify a fragment of the COI gene (mtDNA).

Primer	Code	Sequences 5'-3'
C1-J-1718	P3	GGA GGA TTT GGA AAT TGA TTA GTT CC
C1-N-2329	P4	ACT GTA AAT ATA TGA TGA GCT CA
C1-N-2191	P5	CCC GGT AAA ATT AAA ATA TAA ACT TC
Primer 8	P8	GCC AAT GGT TAA TAT TGC A
C1-N-2735	P10	AAA AAT GTT GAG GGA AAA ATG TTA

The PCR for all primer combinations (P3/P4, P3/P5, P3/P8 and P3/P10) had the following cycling parameters: 95 °C for 2 min; 35 cycles of 94 °C for 45 sec, 53 °C for 1 min, 72 °C for 1 min, and a final extension step of 7 min. Afterwards, reactions were cooled down to 4 °C. The PCR products were stored at -20 ºC.

2.3.2.5 Agarose gel electrophoreses

Prior to any PCR the quality of the extracted DNA was checked on a 1 % agarose gel in TAE-buffer along with a DNA standard. Electrophoresis was carried out in a horizontal electrophoresis chamber (Wide Mini-Sub Cell GT, Bio-Rad, Hercules, CA, USA) at 6 V/cm for 1 h, gels were stained with ethidium bromide (0.5 µg/ml) for 15 min and distained in water for another

15 min. The gels were photographed under UV light (Nikon COOLPIX995 Digital camera). The digital photographs were saved on a PC.

In addition, an aliquot of the PCR products was checked for correct size on a 1.5-2 % agarose gel in TAE buffer along with a size marker (50 bp or 100 bp ladder) (MBI Fermentas). Electrophoreses was conducted as described above.

2.3.2.6 Detection of restriction fragment length polymorphisms (RFLPs)

When the Kenyan biocontrol project for *P. xylostella* started in the year 2000 little was known about the indigenous parasitoids, especially the *Diadegma* species, associated with *P. xylostella* and their geographical distribution. In addition to the morphological analyses a molecular method (PCR-RFLP) was developed to distinguish the eastern and southern African *Diadegma* species from the exotic *D. semiclausum*, which was introduced in 2001 to Kenya and released in 2002 at two pilot sites (Wundanyi and Limuru). In addition, this approach was transferred to all *Diadegma* species (*D. semiclausum*, *D. insulare*, *D. fenestrale*, *D. rapi*, *D. leontiniae*, *D. mollipla*) associated with *P. xylostella*, except for *D. novaezea-landiae* that is native to New Zealand (Azidah *et al.* 2000) and could not be obtained.

PCR products were cleaved with several different restriction endonucleases of type II to distinguish the six *Diadegma* species. Table 2.4 shows the endonucleases with suppliers, the restriction side and the genetic region analysed.

Tab. 2.4: Fifteen restriction endonucleases with isoschizomers, supplier, recognition sequence and the amplified genetic region of different *Diadegma* species where the restriction enzyme was used.

Endo nucleases	Isoschizomer	Supplier	Recognition quence 5'-3'	se-Genetic region
*Alu*I		MBI Fermentas	AG↓CT	ITS2, COI[a]
*Bse*XI	*Bbv*I, *Bst*V1I	MBI Fermentas	GCAGC(N)8/12↓	ITS2
*Bsp*143I	*Bfu*CI, *BstEN*II, *Dpn*II, *Kzo*91, *Mbo*I, *Nde*II, *Sau*3AI	MBI Fermentas	↓GATC	ITS2, COI (3/4)
*Bsu*RI	*Bsh*FI, *Hae*III, *Pa*II, *Pho*I	MBI Fermentas	GG↓CC	ITS2
*Cfo*I	*Asp*LEI, *Bst*HHI, *Hha*I	Roche Diagnostics	GCG↓C	ITS2, COI (3/4)
*Dra*I		Roche Diagnostics	TTT↓AAA	COI (3/4;3/5)
*Eco*RV	*Eco*32I	MBI Fermentas	GAT↓ATC	ITS2
*Eco*RI	*Fun*II	MBI Fermentas	G↓AATTC	COI (3/4)
*Hinf*I		MBI Fermentas	G↓ANTC	COI (3/4;3/8)
*Msp*I	*Bsi*SI, *Hap*II, *Hpa*II	MBI Fermentas	C↓CGG	ITS2, COI (3/4)
*Rsa*I	*Afa*I	Roche Diagnostics	GT↓AC	ITS2, COI (3/4;3/5)
*Ssp*I		MBI Fermentas	AAT↓ATT	COI (3/5)
*Taq*I		MBI Fermentas	T↓CGA	ITS2, COI (3/4; 3/5)
*Xba*I		Roche Diagnostics	T↓CTAGA	COI (3/4)

[a]: Restriction enzyme used with all three primer pair combinations. COI primer combinations indicated in brackets.

Digestions of the PCR products were performed in 20 µl reaction volumes with 13 µl of amplified PCR products, 2 µl of their corresponding restriction buffer and 1 µl of each enzyme (2-10 U). The incubation took place at 37 °C or 65 °C according to the enzyme's specifications. Furthermore, two double digestions were performed: PCR products (13 µl) were cleaved using restriction endonucleases *Tru*1I and *Bse*XI in 1.0 mM Tris-HCl (pH 8.5), 1.0 mM $MgCl_2$, 10 mM KCl, 0.01 mg/ml BSA and 1 µl of each enzyme (2 U *Bse*XI and 10 U *Tru*1I). The reactions were incubated at 65 °C for 4 h or overnight. A second double digest using *Alu*I and *Hinf*I contained 13 µl of amplicon, 3.3 mM Tris-acetate, 6.6 mM K-acetate, 1 mM Mg-acetate, 0.01 mg/ml BSA and 1 µl of each enzyme (10 U each). The incubation took place at 37 °C for 4 h or overnight.

All restriction fragments were separated by electrophoresis on a 3 % agarose gel in TAE-buffer (5 V/cm) along with a 50 bp ladder (MBI Fermentas) as size marker as described above (see 2.3.2.5).

2.3.2.7 Sequencing of PCR products

PCR products selected for sequencing analyses were purified using two commercial kits described under 2.3.2.3. The concentration of the purified DNA was estimated on a 1 % agarose gel along with a λ-DNA ladder of known concentration. The PCR products of a fragment of the COI gene and the ITS2 region were sequenced directly with the corresponding PCR primers on both strands. The sequencing of double-stranded DNA followed the Sanger (enzymatic) sequencing (Sanger *et al.* 1977) and was performed by the International Livestock Research Institute (ILRI, Nairobi, Kenya) or MWG-Biotech (Ebersberg, Germany) using the Big Dye Terminator Method in an ABI 377 DNA Sequencer/ABI 3730 XL-Sequencer. An internal primer, designed with the program oligos/toolkit of QIAGEN (available at

http://oligos.qiagen.com/oligos/toolkit.php) was used for sequencing the ITS2 region (5'-TAC GAA ACC CAA CGA CAA GCG C-3') of the sample *D. chrysostictos*-UK2. All sequences were submitted to EMBL and the corresponding accession numbers are listed in tab. 2.1.

2.4 Data analyses

2.4.1 Crossing experiments

The mean and the standard error (SE) of the number of progeny as well as the sex ratio (%, female/(female+male)) of each cross was calculated using one-way analysis of variance (ANOVA). The test for normality (Gaussian distribution) was performed using the Kolmogorov-Smirnov test. When differences were significant ($P<0.05$), means were separated with Tukey-Kramer multiple comparisons test. All analyses were implemented in GraphPad InStat version 3.06 (GraphPad Software, 1992-2003). Significant differences were indicated in superscripted letters, the F value and the degrees of freedom (df) were also recorded.

2.4.2 Sequence analyses

The sequences were aligned using ClustalW version 1.4 algorithm (Thompson *et al.* 1994) as implemented by the program BioEdit version 7.0.4.1 (Hall 1999). Some adjustments of the ITS2 sequences were made by eye. The aligned sequences were compared with the COI and ITS2 sequences of the international database using the BLAST (Basic Local Alignment Search Tool) algorithm available on the World Wide Web (htpp://www.ncbi.nlm.nih.gov/BLAST/).

For the protein coding COI gene, sequences were also translated to amino acid sequences based on invertebrate codon usage of mitochondrial DNA. MEGA version 2.1 (Kumar *et al.*

2001) was used to compute sequence statistics (nucleotide composition and nucleotide pair frequencies). To estimate the pair-wise nucleotide distances of both data sets (ITS2 and COI) the Kimura 2-parameter substitution model (equal base frequencies and two substitution rates, Kimura 1980) was chosen and computed in PAUP* version 4.0b10 (Swofford 1998). For the comparison of the divergences of the fragment of the COI gene and the ITS2 sequences only means of percentage of the distances and the means of the standard errors for each species were used.

Sequences were tested for significant phylogenetic structure using the permutation tail probability test (PTP, Faith and Cranston 1991) as implemented in PAUP* with 5,000 random matrices. The procedure tests whether the data sets have any structure that was different from random.

The comparison of nucleotide divergences: COI vs ITS2 was illustrated. The equation of the linear regression was displayed on the graph.

Homogeneity of base frequencies between taxa was examined using the chi-square test as implemented in PAUP*.

2.4.3 Phylogenetic analyses

2.4.3.2 Phylogenetic analyses within the genus *Diadegma*

Dendrograms based on sequence divergences were constructed using the neighbour-joining procedure (Saitou and Nei 1987) with the Kimura 2-parameter model (K80, Kimura 1980) as implemented in PAUP*. The divergences of COI vs. ITS2 of the nine different *Diadegma* species were compared pair-wise and the regression between COI and ITS2 was calculated.

Phylogenetic studies were accomplished using neighbour-joining option (NJ) for complete distance values (Kimura 2-parameter model), maximum parsimony (MP) and maximum like-

lihood (ML) implemented in PAUP*. Dendrogams obtained by the NJ method for a fragment of the COI gene were rooted with *Drosophila yakuba* (accession no X03240)*, Apis mellifera ligustica* (accession no L06178) and *Meloboris* sp. generated in the present study. *Nasonia vitripennis* (accession no U02960), *Trichogramma rojasi* (accession no AF282239) and *Meloboris* sp. generated in the present study, rooted the dendrogram obtained for the ITS2 region. Heuristic searches were conducted using the stepwise addition with 100 random replications for MP and 50 for ML. To assess confidence in the resulting tree topologies, bootstrap tests were performed with 1000 bootstrap replications for the MP trees and 100 replicates for ML trees (Felsenstein 1985).

Due to computational constraints only identical sequences were represented in the likelihood analyses by a maximum of two entries per species or population with a total number of 19 sequences of the COI region and 20 sequences for the ITS2 region (inclusive out-group *Meloboris* sp.). Trees using the ML analyses require an appropriate model of evolution to be specified a priori. The approach outlined by Huelsenbeck and Crandall (1997) was used to test alternative models of evolution. Maximum likelihood scores for 56 models of DNA substitutions (base composition, substitution rates, proportion of invariable sites, and gamma shape parameter) were obtained for a simple neighbour-tree based on Jukes Cantor distances with MODELTEST (version 3.5) (Posada and Crandall 1998) and PAUP* and the best fit model for both data sets was then identified. For both data sets gaps were treated as missing data. MODELTEST selected for the COI sequences the general time reversible model, including the proportion of invariable sites and gamma distribution for rate variation among sites (GTR+I+G; unequal base frequencies, six substitution rates, Gu *et al.* 1995; Rodríguez *et al.* 1990) as the model which best fits the data while for the ITS2 sequences the model which best fits the data was K80+G (Kimura 2-parameter method with gamma distribution). To identify character sets that have suffered multiple hidden substitutions, a saturation analysis of both

data partitions was undertaken in PAUP*. The pair-wise uncorrected (*p*) and the corrected distances (GTR+G+I for COI and K80 for ITS2) were estimated and plotted. The corrected distances were calculated with the ML model suggested in MODELTEST. In addition, MP and ML analyses were performed by excluding the third codon position of the COI sequences in a similar way as for the complete sequence analyses. MODELTEST identified the TIM+I model (transitional model with six free parameters and unequal base frequencies; and the proportion of invariable sites) as the best fit to the COI data (excluding the third codon position). Both data sets (COI and ITS2) were also tested for heterogeneity to assess whether a combination of COI and ITS2 data is appropriate for phylogenetic analyses using the partition homogeneity test (incongruence-length difference, IDL test, Farris *et al.* 1995). This is implemented within PAUP*. Tests were performed with 1,000 iterations and were done for both the complete COI and the ITS2 data sets as well as for the COI data set when the third codon position was excluded in combination with the ITS2 data. When the results of the IDL test were greater than 5 %, MP and ML analyses were performed with the combined data sets (18 taxa) using the same heuristic search parameters described above for the separate examination of each data set.

2.4.3.2 Phylogenetic analyses within the superfamily Ichneumonoidea

To examine the phylogenetic relationships within the superfamily Ichneumonoidea molecular sequences for the ITS2 region and COI gene of the two families Braconidae and Ichneumonidae were collected from the data base GenBank (June 2005, keywords: Braconidae ITS2 or / COI and Ichneumonidae ITS2 or / COI) to compare them with the *Diadegma* and *Meloboris* sp. sequences generated during this study. Sequences of four genera (Braconidae: *Leiphron*

and *Persitus*; Ichneumonidae: *Meloboris* and *Diadegma*) were found during the ITS2 search with 28 entries for Braconidae and five for Ichneumonidae.

Out of 270 entries of COI sequences available in GenBank, 47 different genera of both Braconidae and Ichneumonidae were found, with 38 genera belonging to Braconidae and only nine to Ichneumonidae. The phylogenetic analyses of the superfamily Ichneumonoidea was obtained for ITS2 and COI sequences with MP implemented in PAUP* similar to the previously described procedure (2.4.3.1). The MP analysis was performed for the complete ITS2 sequences of the species recorded in tab. 2.5. The accession numbers of *Meloboris* sp., *D. leontiniae*-B1, *D. rapi*-Au, *D. insulare*-USA1, *D. mollipla*-K1, *D. blackburni*-H1, and *D. semiclausum*-A are listed in tab. 2.1. The reliability of the resulting nodes was determined by 1000 bootstrap replications. Tree statistics were obtained in PAUP*. A fragment of the COI gene was used to develop the most reliable phylogenetic relationships within and between the families Braconidae and Ichneumonidae. For the MP study sequences were obtained from GenBank and are listed in tab. 2.5: The accession numbers of *Meloboris* sp., *D. chrysostictos*-UK13, *D. leontiniae*-B1 and *D. semiclausum*-NZ are recorded in tab. 2.1. Alignment of all sequences was conducted using the program BioEdit and resulted in 41 consensus sequences of 446 characters. The MP analyses were carried out in PAUP* like described for the ITS2 sequences. Because of the numbers of sequences analyzed the program was holding a maximum of five trees in memory. All characters were equally weighted.

Tab. 2.5: List of the taxonomic groups used for phylogenetic studies, accession numbers and genetic regions.

Sub-order	Superfamily	Family	Subfamily	Taxon	Accession number	Genetic region
Apocrita	Chalcidoidea	Encyrtidae	Encyrtinae	*Ageniaspis citricola*	AF291441*	ITS2
	Ichneumonoidea	Braconidae	Euphorinae	*Leiophron argentinensis*	AY590817	
				Leiophron uniformis	AY170223	
				Peristenus howardi	AY170221	
				Peristenus pseudopallipes	AY519660	
Apocrita	Ichneumonoidea	Ichneumonidae	Anomaloninae	*Habronyx* sp. JC139	AF146684	COI
			Campopleginae	*Dusona egregia*	AF146682	
			Campopleginae	*Venturia canescens*	VCU59221	
			Ichneumoninae	*Ichneumon promissorius*	AF379997	
			Mesochorinae	*Mesochorus* sp. SK-CA-2004a,	AY579637	
			Pimplinae	*Pimpla aequalis*	AF146681	
			Xoridinae	*Xorides praecatorius*	AF379998	
		Braconidae	Aphidiinae	*Aphidius ervi*	AY427886	
			Aphidiinae	*Lysiphlebus testaceipes*	AY207566	
			Cardiochilinae	*Cardiochiles fuscipennis*	AY044207	
			Cardiochilinae	*Toxoneuron abdominalis*	AF379996	
			Cheloninae	*Ascogaster* sp. M161	AF379988	
			Cheloninae	*Chelonus* sp. SLB-2003	AY165727	
			Doryctinae	*Spathius generosus*	AY920290	
			Helconinae	*Diospilus* sp. M123	AF379989	
			Microgastrinae	*Alphomelon talidicida*	AY044206	
			Microgastrinae	*Apanteles nephoptericis*	AF102720	
			Microgastrinae	*Cotesia sesamiae*	AY934823	
			Microgastrinae	*Cotesia plutellae*	AM087128	

Tab. 2.5: Continued.

Sub-order	Superfamily	Family	Subfamily	Taxon	Accession number	Genetic region
Apocrita	Ichneumonoidea	Braconidae	Microgastrinae	*Dasylagon* n. sp. 'Mardulyn and Whitfield'	AF102719	COI
			Microgastrinae	*Diolcogaster cf. xanthaspis* 'W.-Y. Choi'	AY044209	
			Microgastrinae	*Fornicia* sp. 'J.B. Whitfield'	AY044210	
			Microgastrinae	*Microgaster canadensi*	AF102708	
			Microgastrinae	*Microplitis* n. sp. 2 'Annette K. Walker'	AY044212	
			Microgastrinae	*Glyptapanteles porthetriae*	AF448812	
			Microgastrinae	*Jarra maculipennis*	AF379991	
			Microgastrinae	*Parapanteles paradoxus*	AF102709	
			Microgastrinae	*Promicrogaster munda*	AY044215	
			Microgastrinae	*Snellenius* n. sp. 'Mardulyn and Whitfield'	AF102701	
			Miracinae	*Mirax* sp. 'Mardulyn and Whitfield'	AF102722	
			Rhyssalinae	*Dolopsidea* sp. M141	AF379990	
			Sigalphinae	*Sigalphus* sp. DLJQ-AC	AF379995	
			Trachypetinae	*Megalohelcon ichneumonoides*	AF379992	
	Chalcidoidea	Agaonidae	Agaoninae	*Pegoscapus grandii*	AY967999*	
			Sycophaginae	*Apocryptophagus* sp. GW841A	AF364534*	
Aculeata	Apoidea	Apidae		*Bombus lapidaries*	AJ577257*	

* Individual served as out- group.

3 Results

3.1 Morphology

The dorsal view of the propodeum, the dorsal view of the head, the shape of the fore wing and the length of the ovipositor were used to examine *Diadegma* species. No morphological differences were detected between the individuals of *D. mollipla* from eastern and southern Africa. With the morphological key provided by Azidah *et al.* (2000) *D. mollipla* and *D. semiclausum* could be distinguished. Nevertheless, the differentiation of males of both taxa was in some cases very difficult even for experienced taxonomists, indicating that other methods (e.g. molecular methods) for identification are needed.

3.2 Intra- and inter-specific crosses between *Diadegma* populations and species

3.2.1 Intra-specific crossing between *D. mollipla* populations

Crosses and backcrosses between these parasitoid wasps were conducted to determine whether gene flow can occur between individuals of two geographically separated populations of *D. mollipla* (East Africa vs South Africa). The laboratory culture of *D. mollipla* (DM-K1) was supplemented with field-collected individuals to avoid loss of fitness under laboratory conditions, whereas the *D. mollipla* laboratory culture from South Africa (DM-SA) was build up with only eight females (out of 50 pupae) and was highly male biased. The sex ratio ranged between 16.0 % in *D. mollipla* (SA) and 37.2 % in *D. mollipla* (Kenya) and between 27.5 % and 29.3 % in the experimental crosses (tab. 3.1). There were no significant differences in the total number of progeny per female and the number of male offspring between all crosses. Mating between both populations was observed several times (tab. 3.1).

Tab. 3.1: Progeny production and sex ratio from crosses among *D. mollipla* East Africa (DM-K1) and *D. mollipla* South Africa (DM-SA).

Cross		*N*[1]	Total no. of progeny/female[2]	Female	Male[2]	Sex ratio (%)[2]
Female	Male					
DM-K1	DM-K1	18	24.0±3.0	10.4±2.0[a]	13.6±1.5	37.2±5.4
DM-SA	DM-SA	20	22.8±2.5	3.6±0.9[b]	19.2±2.3	16.0±3.8
DM-K1	DM-SA	18	18.3±1.9	5.8±1.6[a]	12.5±1.7	29.3±6.3
DM-SA	DM-K1	8	17.3±1.8	4.6±2.1[a]	12.6±2.4	27.5±12.2
F value			1.457	3.789	2.796	2.526
df			3, 60	3, 60	3, 60	3, 60
P value			0.2353	0.0148	0.0478	0.066

Data are shown as mean ± SE. Means followed by the same letters in the same column are not significantly different ($P < 0.05$, Tukey-Kramer multiple comparisons test). [1]Numbers of pairs examined. [2]No significant differences between all crosses.

Hybrid female progeny resulting from both reciprocal crosses were reproductively compatible with males of both parental lines (tab. 3.2), although, the total number of pairs tested was very small due to the poor performance of the DM-SA culture, where relatively few females were available. Therefore, no statistical analyses were performed.

Tab. 3.2: Progeny production and sex ratio from backcrosses of hybrid *D. mollipla* South Africa, male (DM-SA♂) and *D. mollipla* East Africa, female (DM-K1♀) and *D. mollipla* East Africa, male (DM-K1♂) and *D. mollipla* South Africa, female (DM-SA♀).

Cross		*N*[1]	Total no. of progeny/female	Female	Male	Sex ratio (%)
Female	Male					
(DM-SA♂ x DM-K1♀)	DM-K1	10	24.9±2.0	13.9±3.5	11.0±1.8	49.6±10.8
(DM-SA♂ x DM-K1♀)	DM-SA	2	15.0±1.0	5.5±2.5	9.5±1.5	35.7±14.3
(DM-K1♂ x DM-SA♀)	DM-K1	2	45.0±6.0	22.5±13.5	22.5±7.5	46.8±23.8
(DM-K1♂ x DM-SA♀)	DM-SA	2	35.0±14.0	17.5±5.5	17.5±8.5	52.0±5.1

[1]Number of pairs examined.

In conclusion, intra-population crosses were observed with no significant differences in the sex ratio (tab. 3.1). Similarly, F1 females mated with males originating from either parental population produced F2 female adults (tab. 3.2). Thus, no genetic incompatibility was apparent between the two *D. mollipla* populations.

3.2.2 Inter-specific crossings between *D. mollipla* and *D. semiclausum*

Inter-specific crosses did not yield female offspring (tab. 3.3). In addition, no mating between both taxa could be observed.

Tab. 3.3: Progeny production and sex ratio from crosses among *D. mollipla* (DM-K2) and *D. semiclausum* (DS).

Cross		*N*[1]	Total no. of progeny/female	Female	Male	Sex ratio (%)
Female	Male					
DM-K2	DM-K2	26	38.9±2.7[a]	6.8±1.4[a]	32.1±2.3[a]	17.1±3.3[a]
DS	DS	30	35.6±2.5[a]	13.4±2.0[b]	22.2±2.5[b]	37.5±4.5[b]
DM-K2	DS	24	37.5±3.1[a]	0[c]	37.5±3.1[a]	0[c]
DS	DM-K2	25	55.8±2.3[b]	0[c]	55.8±2.3[c]	0[c]
F value			12.092	24.104	30.672	36.036

Means differ significantly at $P < 0.0001$ (ANOVA), df = 3, 101. Data are shown as mean ± SE. Means followed by the same letters in the same column are not significantly different ($P < 0.05$, Tukey-Kramer multiple comparisons test). [1]Number of pairs examined.

3.3 Variation in isoenzyme banding patterns

Diadegma mollipla and *D. semiclausum* could be distinguished on a 14 % starch gel by different migration of the PGM. In *D. semiclausum* the female showed two different sex alleles (heterozygote, S- and F-band). The female of *D. mollipla* was homozygote (but diploid) showing only the S-band. The males of both taxa were haploid (or homozygote) and the F- or the S-band was detected (fig. 3.1).

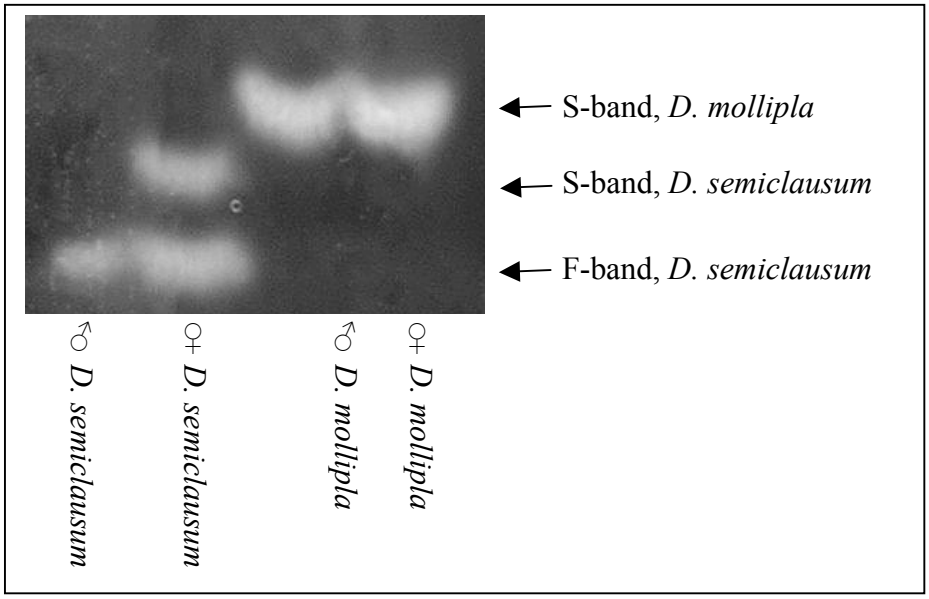

Fig. 3.1: Electrophoretic banding patterns of phosphoglucomutase (PGM) detected on a 14 % starch gel in *D. semiclausum* and *D. mollipla*.

In total, five different isoenzyme experiments were conducted (tab. 3.4). Experiments 1-3 were needed to optimise the experimental conditions and to ensure the detection of heterozygote females or males in *D. semiclausum* and *D. mollipla*. In experiment 4 the parents of the mother-son crossings were screened to identify crosses which involved females being heterozygous at the PGM loci. Only one pair of the mother-son crossings in *D. mollipla* and two pairs of the mother-son crossings of *D. semiclausum* were used to investigate if diploid males can be observed. In total, one female and 19 males of *D. mollipla* and 11 females and ten males of the first and six females and 33 males of the second pair of *D. semiclausum* were analysed (tab. 3.4, fig. 3.2).

Tab. 3.4: Detection of S-, SF- and F- bands in *D. mollipla* (DM-K2) and *D. semiclausum* (DS) by isoenzyme variations of PGM on 14 % starch gels.

Experiment	DM-K2 ♀			DM-K2 ♂			DS ♀			DS ♂		
	S-band	SF-band	F-band	S-band	SF-band	F-band	S-band	SF-band	F-band	S-band	SF-band	F-band
1	1	-	-	4	-	-	-	1	-	-	-	1
2	4	-	3	1	-	-		2		1	-	5
3	-	2	3	4	-	2	-	-	-	-	-	-
4	6	1	-	5	-	-	2	2	1	2	-	3
5	1	-	-	10	1	8	9	8	-	27	-	16
Total (∑)	12	3	6	24	1	10	11	13	1	30	-	25
Total %	57.1	14.3	28.6	68.6	2.9	28.6	44.0	52.0	4.0	54.5	-	45.5

Only one diploid male could be detected in *D. mollipla* and none in *D. semiclausum* (fig. 3.2). Out of a total of 21 females of *D. mollipla* analysed only 14.3 % were diploid/heterozygote at the PGM loci and 57.1 % were females with the S-band or 28.6 % were females with the F-band (diploid/homozygote). 68.6 % of the *D. mollipla* males showed the S-banding patterns (diploid/homozygote or haploid) and only 28.6 % the F-bands (diploid/homozygote or haploid), respectively.

In contrast, 52 % of the analysed females of *D. semiclausum* showed the diploid/heterozygote banding patterns and 44 % the S-band (diploid/homozygote) whereas the distribution between the S-band (diploid/homozygote or haploid) and the F-band (diploid/homozygote or haploid) was 54.5 and 45.5 % in the *D. semiclausum* males, respectively.

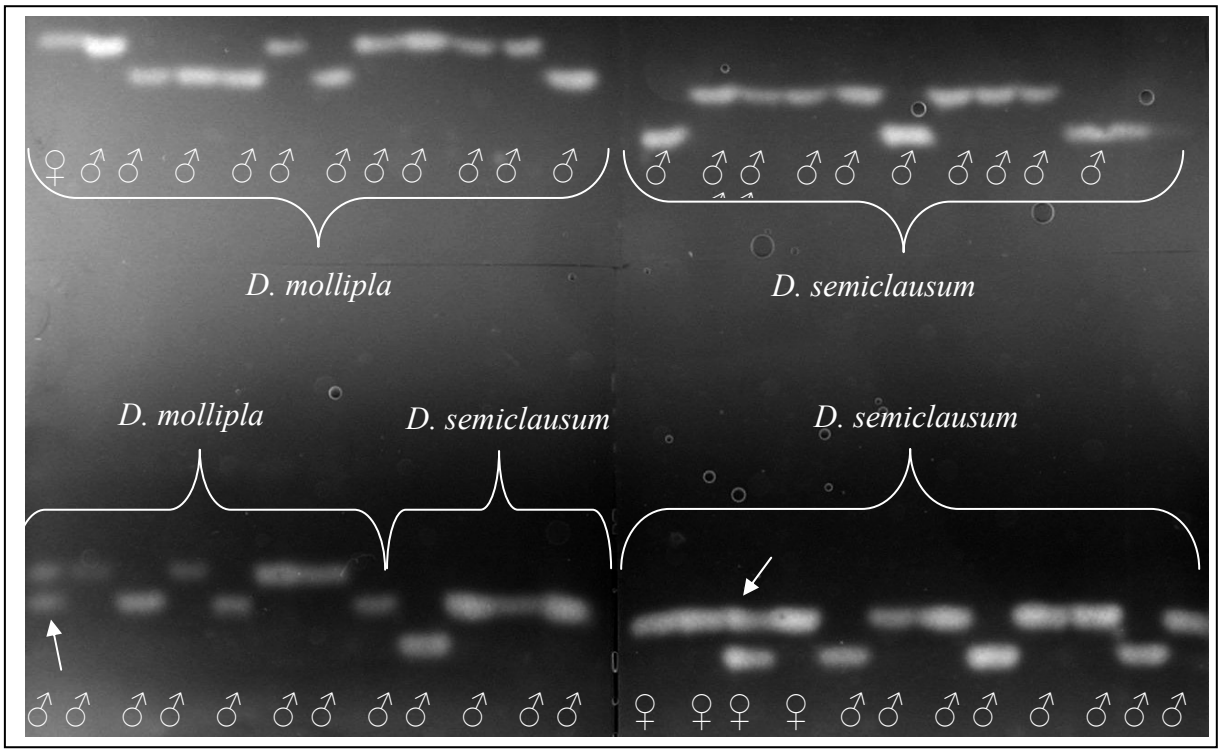

Fig. 3.2: Isoenzym banding patterns of *D. mollipla* and *D. semiclausum* detected by isoenzyme variations of PGM on a 14 % starch gel. Individuals were the progeny of the mother and son crossings. Arrows mark one diploid male of *D. mollipla* and one diploid female of *D. semiclausum.*

3.4 Molecular characterisation of *Diadegma* species

3.4.1 Amplification of DNA

DNA from all nine *Diadegma* species was successfully amplified with both molecular markers (COI of mtDNA and ITS2 of nuclear DNA). The mtDNA off all *Diadegma* species examined could be amplified with the primer combination P3/P5 yielding a short PCR product of ~540 bp. This size of amplicon was assumed to be too small for the following analyses such as RFLP for taxonomic purposes or sequencing for the subsequent phylogenetic analysis. The length of the PCR products for the following primer combinations ranged from (i) ~630 bp (P3/P8) to (ii) ~660 bp (P3/P4) and to (iii) ~1100 bp with P3/P10, respectively. Thus, primer pairs P3/P4 and P3/P8 were the most frequently used primers for molecular characteri-

sation of all *Diadegma* species except for *D. leontiniae* where the primer pair P3/P10 was required in addition.

In contrast, amplification with the primers ITS2-1 and ITS2-2 resulted in three differently sized PCR products. In *D. insulare*, *D. mollipla*, *D. fenestrale*, *D. semiclausum and D. blackburni* a ~800 bp fragment was obtained, in *D. armillata* a ~830 bp fragment was visible on a 2 % agarose gel, while PCR products of the ITS2 region of *D. leontiniae, D. chrysostictos* and *D. rapi* had a length between 840 bp and 850 bp, respectively. The ITS2 fragment of *Meloboris* sp. from Ethiopia, which served as out-group, had a length of ~780 bp.

3.4.2 PCR-RFLP

All PCR-RFLP analyses of the mitochondrial DNA (COI) and the ITS2 region of the ribosomal DNA were performed empirically without prior knowledge about the sequences of both genetic regions. In addition, restriction fragments which were <100 bp were not detected on 3 % agarose gels and were consequently not included in the analyses.

3.4.2.1 PCR-RFLP with mtDNA

DNA of *D. mollipla*, *D. semiclausum* and *Meloboris* sp. was amplified with the primer combination P3/P5. Species-specific restriction patterns were generated from digestions with five different restriction enzymes (tab. 3.5, fig. 3.3, Appendix 2A).

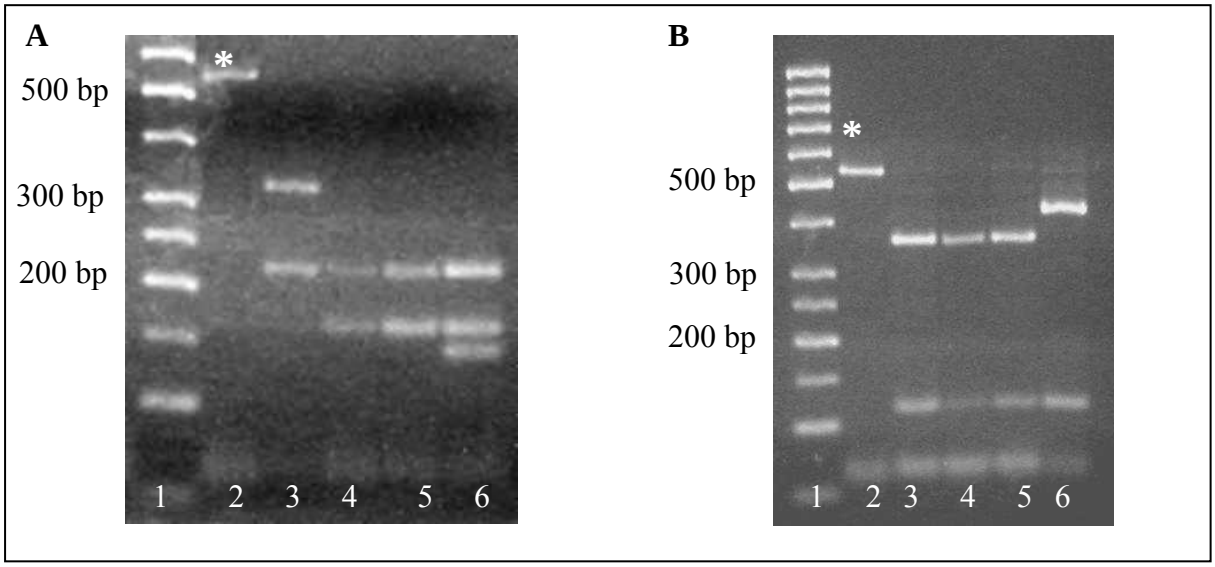

Fig. 3.3 **A**: Amplicons generated with primer combination P3/P5 (mtDNA, COI) digested with *Alu*I on a 3 % agarose gel, fragments <100 bp are not shown, * amplicon not digested.
Lane 1: size marker
Lane 2: *Meloboris* sp., Ethiopia
Lane 3: *D. mollipla*, Kenya
Lane 4: *D. mollipla*, Réunion
Lane 5: *D. mollipla*, South Africa
Lane 6: *D. semiclausum*, Taiwan

Fig. 3.3 **B**: Amplicons generated with primer combination P3/P5 (mtDNA, COI) digested with *Taq*I, 3 % agarose gel, fragments <100 bp are not shown, * amplicon not digested.
Lane 1: size marker
Lane 2: *Meloboris* sp., Ethiopia
Lane 3: *D. mollipla*, Kenya
Lane 4: *D. mollipla*, Réunion
Lane 5: *D. mollipla*, South Africa
Lane 6: *D. semiclausum*, Taiwan

Figures 3.3 **A** and 3.3 **B** show the undigested PCR product of *Meloboris* sp. (~540 bp). When digested with *Alu*I, *D. mollipla* (Kenya) had two fragments of 330 bp and 210 bp whereas *D. mollipla* (South Africa) showed two fragments of 210 bp and 160 bp. Three restriction patterns (210 bp, 160 bp and 140 bp) were detected in *D. semiclausum* (fig. 3.3 **A**). With restriction enzyme *Taq*I only *D. mollipla* and *D. semiclausum* could be distinguished by different restriction patterns. Two different fragments were detected in *D. mollipla* (370 bp and 120 bp) and *D. semiclausum* (420 bp and 120 bp), respectively.

Tab. 3.5: Examination *D. mollipla*, *D. semiclausum* and *Meloboris* sp. with five different restriction enzymes after amplification of a fragment (~540 bp) of the COI gene (mtDNA).

Species (no. of individuals examined)	*Alu*I	*Dra*I	*Rsa*I	*Ssp*I	*Taq*I
D. mollipla, eastern Africa (7)	A	nd	A	A	A
D. mollipla, southern Africa (4)	B	A	A	A	A
D. semiclausum (4)	C	A	A	A	B
Meloboris sp., Ethiopia (3)	nd	A	nd	-	nd

Different letter: unique pattern, same letter: Shared pattern, nd: amplicon not digested, -: species not analysed.

When DNA was amplified with the primer pairs 3/4 (size of resulting PCR product: ~660 bp) and 3/8 (size of resulting PCR product: ~630 bp) and afterwards digested with restriction enzymes (*Alu*I and *Hinf*I), the resulting restriction patterns did not vary much and therefore will be described together as primer pair 3/4.

Restriction enzyme *Alu*I was suitable to distinguish both *D. mollipla* populations (from eastern and southern Africa) and *D. semiclausum*. The restriction profiles obtained resulted in two fragments with the sizes of 450 bp and 210 bp in *D. mollipla* (eastern Africa), in three fragments with the sizes of 150 bp, 210 bp and 300 bp for *D. mollipla* (southern Africa), and in three fragments with the sizes of 150 bp, 210 bp and 280 bp for *D. semiclausum*, respectively. The restriction enzymes *Dra*I, *Hinf*I and *Msp*I could be used to distinguish between *D. mollipla* from eastern and southern Africa (tab. 3.6, Appendix 2B, 2C). Restriction enzyme *Rsa*I showed identical restriction patterns in both *D. mollipla* populations and in *D. semiclausum* and was therefore not suitable for taxonomic differentiation of the *Diadegma* species, whereas restriction enzymes *Sau*3AI and *Taq*I separated *D. mollipla* and *D. semiclausum*. The PCR products of the examined *Diadegma* were not digested with the restriction enzymes *Cfo*I, *Eco*RI, and *Xba*I. All restriction fragments (bp) generated from the digestion of the PCR products (COI region) of the different *Diadegma* are listed in Appendix 2.

Tab. 3.6: Examination of *D. mollipla* and *D. semiclausum* with seven different restriction enzymes after amplification with primer pair P3/P4 (~660 bp) of a fragment of the COI gene (mtDNA).

Species (no. of individuals examined)	*Alu*I	*Dra*I	*Hinf*I	*Msp*I	*Rsa*I	*Sau*3AI	*Taq*I
D. mollipla, eastern Africa (34)	A	nd	A	nd	A	A	A
D. mollipla, southern Africa (15)	B	A	nd	A	A	A	A
D. semiclausum (14)	C	A	nd	nd	A	B	B
Meloboris sp. (2)	nd	C	nd	nd	nd	-	nd

Different letter: unique pattern, same letter: shared pattern, nd: amplicon not digested, -: species not analysed.

No intra-specific variation could be observed within all *Diadegma* individuals (see tab. 2.2) examined via PCR-RFLP analysis of the fragment of COI gene except for two samples from Tanzania that showed restriction patters of the southern African *D. mollipla* population.

The five *D. mollipla* individuals reared from potato tuber moth (*P. operculella*) showed the same restriction profiles as the *D. mollipla* reared on *P. xylostella.*

3.4.2.2 PCR-RFLP with rDNA

The PCR-RFLP analyses of rDNA were performed with the species *D. semiclausum*, *D. insulare*, *D. fenestrale*, *D. rapi*, *D. leontiniae*, and *D. mollipla*, which were all associated with *P. xylostella.* Species-specific restriction patterns were generated from digestions with 13 different restriction enzymes. The results are presented in tab. 3.7 and Appendix 3. The PCR product of the single specimen of *D. rapi* was only digested with enzyme *Cfo*I and with a double digest of *Alu*I/*Hinf*I and *Bse*XI/*Tru*1I, because only 20 µl of template DNA was available.

A digest of the PCR products with restriction enzyme *Cfo*I resulted in a diagnostic pattern and distinguished all *Diadegma* species tested as well as the two populations of *D. mollipla* from eastern and southern Africa (fig. 3.4, tab. 3.7).

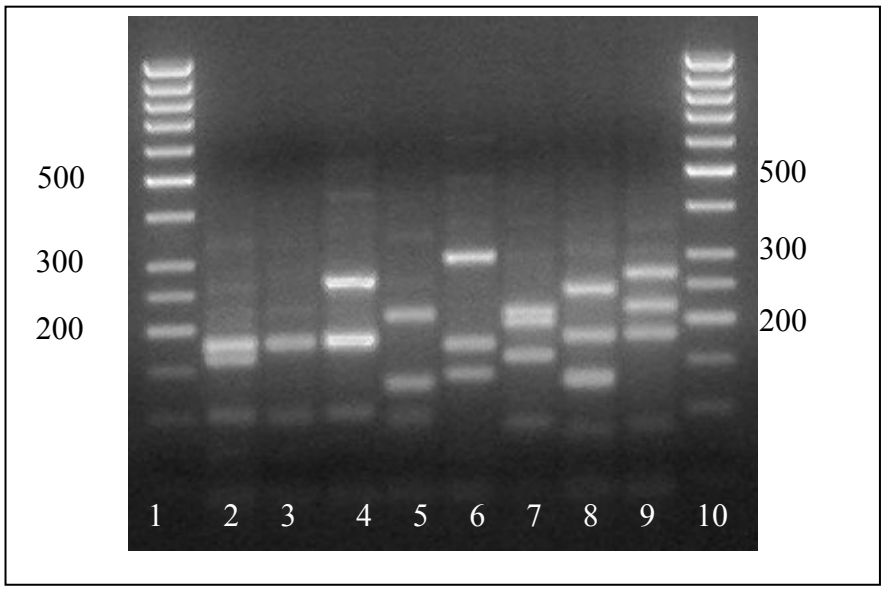

Fig. 3.4: Restriction fragment patterns of the ITS2 region (rDNA) of different *Diadegma* species produced by *Cfo*I separated on a 3 % agarose gel. DNA ladder: 50 bp steps until 300 bp, then 100 bp steps.

Lane 1: DNA ladder
Lane 2: *D. mollipla*, eastern Africa
Lane 3: *D. mollipla*, southern Africa
Lane 4: *D. semiclausum*
Lane 5: *D. insulare*
Lane 6: *D. fenestrale*
Lane 7: *Meloboris* sp. (out-group)
Lane 8: *D. leontiniae*
Lane 9: *D. rapi*
Lane 10: DNA ladder

All African *Diadegma* species (*D. mollipla* from eastern and southern Africa, and *D. semiclausum* introduced from Taiwan to Kenya in 2001) could also be digested with a double digest of *Bse*XI/*Tru*1I (fig. 3.5). With this enzyme combination the differences between the two populations of *D. mollipla* were easier to visualise than with *Cfo*I, where the two bands of 160 bp and 180 bp generated in *D. mollipla* from eastern Africa are very closely together (fig. 3.4).

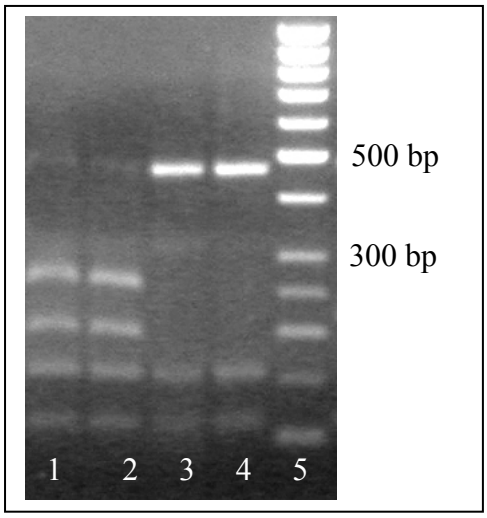

Fig. 3.5: Restriction fragment patterns of the ITS2 region (rDNA) of *D. mollipla* from two different locations produced by a double digest with *Bse*XI/*Tru*1I and separated on a 3 % agarose gel.
Lane 1: *D. mollipla*, Réunion
Lane 2: *D. mollipla*, South Africa
Lane 3: *D. mollipla*, Kenya
Lane 4: *D. mollipla*, Tanzania
Lane 5: DNA ladder: 50 bp steps until 300 bp, then 100 bp steps

Fragments resulting from a digest with *Alu*I ranged in size from 390 bp to <100 bp. *Diadegma insulare* and *D. fenestrale* showed the same *Alu*I restriction patterns as well as both populations of *D. mollipla*. With a double digest using restriction enzymes *Alu*I/*Hinf*I, *D. insulare* and *D. fenestrale* could be differentiated, however both populations of *D. mollipla* still revealed the same restriction patters (fig. 3.6).

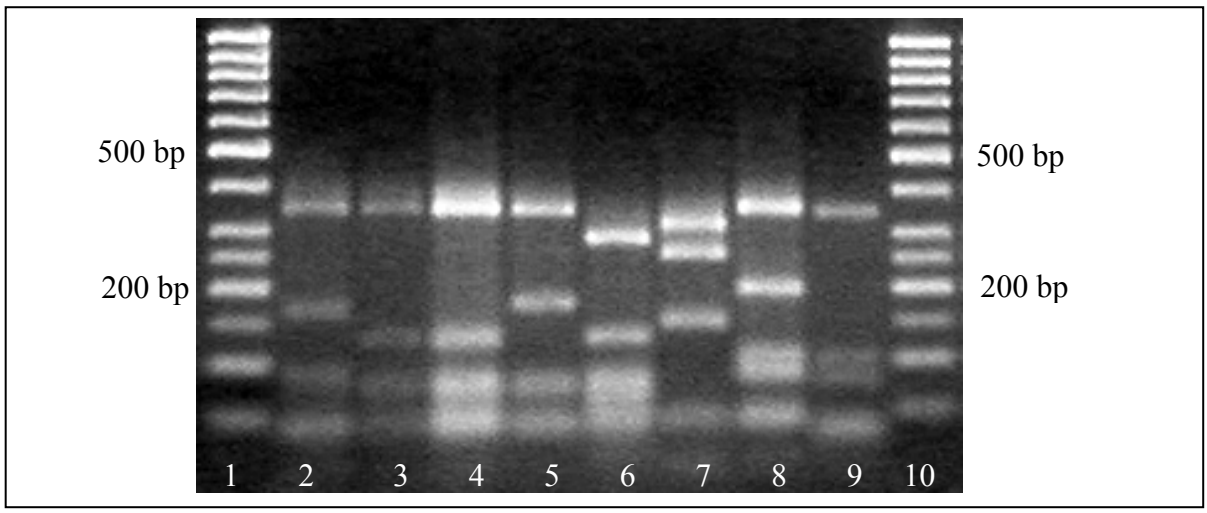

Fig. 3.6: Restriction fragment patterns of the ITS2 region (rDNA) of different *Diadegma* species produced by a double digest of *Alu*I/*Hinf*I and separated on a 3 % agarose gel. DNA ladder: 50 bp steps until 300 bp, then 100 bp steps.

Lane 1: DNA ladder
Lane 2: *D. insulare*
Lane 3: *D. mollipla*, eastern Africa
Lane 4: *D. mollipla*, southern Africa
Lane 5: *D. fenestrale*
Lane 6: *D. semiclausum*
Lane 7: *Meloboris* sp. (out-group)
Lane 8: *D. leontiniae*
Lane 9: *D. rapi*
Lane 10: DNA ladder

When the amplicons were digested with restriction enzymes *Taq*I and *Bsp*143I, the resulting fragments were <200 bp (*Taq*I), and <280 bp (*Bsp*143I) in length, and the restriction profiles were difficult to differentiate on a 3 % agarose gel. Restriction enzyme *Eco*RV cleaved *Meloboris* sp. but not *D. semiclausum*, *D. mollipla*, *D. insulare*, *D. leontiniae* and *D. fenestrale*. *Dra*I and *Eco*RI were not suitable to differentiate between the *Diadegma* species and *Meloboris* examined (Appendix 3). A digest with *Rsa*I resulted in the same restriction fragments in *D. mollipla*, *D. semiclausum* and *D. fenestrale*, and was only useful for differentiating *D. leontiniae*. PCR products of *D. insulare* were not digested with *Rsa*I.

Intra-strain polymorphism was not observed in most of the *Diadegma* samples analysed from different geographic origins with any of the restriction enzymes tested (tab. 2.2). However, three of the 19 individuals of *D. mollipla* from South Africa showed restriction patterns of *D.*

mollipla from eastern Africa. No intra-strain variation of *D. mollipla* (southern Africa) could be observed in the populations from St. Helena and Réunion (total of 13 individuals, tab. 2.2). *Diadegma mollipla* reared from *P. operculella* (five individuals) and *D. mollipla* reared from *P. xylostella* showed no differences in the restriction patterns.

Tab. 3.7: Restriction enzymes used for PCR-RFLP (ITS2 of rDNA) analyses to distinguish *Diadegma* species associated with *P. xylostella.*

	D. mollipla eastern Africa (49)[a]	*D. mollipla* southern Africa (32)[a]	*D. semiclausum* (37)[a]	*D. fenestrale* (11)[a]	*D. insulare* (11)[a]	*D. leontiniae* (10)[a]	*D. rapi* (1)[a]
*Alu*I	A	A	B	C	C	D	-
*Bse*XI	A	B	A	-	-	-	-
*Bsp*143I	A	A	B	C	D	E	-
*Bsu*RI	A	A	B	A	A	C	-
*Cfo*I	A	B	C	D	E	F	G
*Eco*RV	nd	nd	nd	nd	nd	nd	-
Hinf I	A	A	A	A	B	C	-
*Msp*I	A	A	B	A	A	C	-
*Rsa*I	A	A	A	A	nd	B	-
*Taq*I	A	A	B	-	-	C	-
*Tru*1I	A	A	B	B	C	D	-
*Alu*I/*Hinf* I	A	A	B	C	D	E	F
*Bse*XI/*Tru*1I	A	B	C	C	D	E	F

[a] Numbers in brackets refer to individuals analysed as listed in tab. 2.2.

Different letter: unique pattern; same letter: shared pattern; nd: amplicon not digested; -: sample not amplified.

3.4.3 Sequencing

The phylogenetic relationships between nine *Diadegma* species were investigated through analyses of DNA sequences. After amplification the resulting amplicons were extracted out of an agarose gel, and PCR products were directly sequenced in both directions. The sequencing was carried out first at ILRI (Kenya) and later at MWG (Germany). The results were sent as fasta-files and the chromatograms as pdf/scf-files.

3.4.3.1 Sequence analysis of mtDNA

A consensus length of 527 bp was directly sequenced from a fragment of the COI gene (nine different *Diadegma* species, a total of 30 individuals, tab. 2.1). Alignment was straightforward because no deletions or insertions of bases were observed in any of the sequences. All sequences were homogenous between positions 499 and 527 and therefore these bases were excluded from the following analysis.

Nucleotide substitutions among *Diadegma* sequences were found at 120 sites (24.1 %) of which 95 (19.1 %) were parsimony informative. Of the parsimony informative sites, 77 (81.1 %) occurred in the third-base position of codons, 16 (16.8 %) in the first and 2 (2.1 %) in the second. Nucleotide frequencies over the 498 bp segment showed a strong A+T bias (74.1 %) ranging from 72.5 % to 74.9 % among taxa. The biases were much stronger for third base positions (91.6-97.6 %) than for first (62.0-65.0 %) and second base positions (62.1-63.3 %). The mean base compositions of the 30 sequences were as follows (ranges in parentheses): 40.2 % T (39.6-41.2 %), 33.9 % A (32.9-35.1 %), 14.6 % C (13.7-15.7 %), 11.3 % G (11.0-12.0 %).

Intra-specific variation occurred only between *D. mollipla* from Kenya/Uganda and from Tanzania/South Africa/Reunion/St. Helena with nine transitions (Ti) and two transversions

(Tv). The *D. mollipla* specimen from Tanzania had one unique mutation at position 354 (A-T to Kenya/Uganda or A-C to South Africa/Reunion/St. Helena).

The COI amino acid sequence contains 25 structural regions with five structural classes (12 transmembrane helices (M1-M12), six external loops (E1-E6), five internal loops (I1-I6) and carboxy- (COOH) and amino (NH2) terminals) (Lunt *et al.* 1996). The 498 bp sequence of the COI gene obtained in the present study was translated into 166 amino acids according to the invertebrate mitochondrial code. The 498 bp segment started in internal loop I (I1), a region that is highly conserved, and ended in the third internal loop (I3) including M3, E2, M4, I2, M5, E3, and M6. The most variable parts of the analysed structural regions were I2, E2, M3 and M4. Sequence variation did result in 11 variable amino acid sites, which separated all nine *Diadegma* species (tab. 3.8). Amino acid sequence variability did not occur in M5, M6, E3 and I3. No intra-specific variation could be observed.

Tab. 3.8: Variations of amino acids within a 498 bp fragment of the COI gene (mtDNA) for nine different *Diadegma* species. Numbers in row one refer to the position of the amino acid sequences; one letter abbreviations of amino acid: I= Isoleucine, K=Lysine, L= Leucine, M=Methionine, N=Asparagine, S=Serine, T=Threonine, V=Valine. Amino acid variations are marked bold.

	17	18	21	26	44	45	53	74	75	78	85
D. armillata	I	L	I	T	**M**	S	L	T	T	N	N
D. blackburni	I	L	I	T	I	S	L	**S**	T	N	N
D. chrysostictos	I	L	I	T	I	S	L	T	**S**	N	N
D. fenestrale	I	**S**	I	T	I	S	L	T	T	N	N
D. insulare	I	L	I	T	**V**	S	L	**S**	T	N	N
D. leontiniae	**L**	**M**	I	**S**	I	S	L	T	T	N	N
D. mollipla	I	L	I	T	I	S	L	T	T	**I**	**K**
D. rapi	**L**	L	**L**	**L**	**V**	**N**	**T**	T	T	N	N
D. semiclausum	I	L	I	T	I	S	L	T	T	N	N

The nucleotide distances were corrected with the Kimura 2-parameter method (Kimura 1980) and ranged from 0.2 % to 2.5 % in *D. mollipla* (intra-specific variation). The distances of all individuals analysed and the corresponding standard errors are listed in Appendix 4. The greatest nucleotide divergence was observed between *D. rapi* and *D. chrysostictos* (16.8 %) and the lowest value (3.9 %) between *D. semiclausum* and *D. fenestrale* (tab. 3.9).

The PTP indicated the presence of significant covariance in the data set ($P = 0.0002$). Since the data have significant cladistic structure, the phylogenetic analyses were performed.

No significant differences were detected between the taxa in terms of base composition ($\chi^2 = 7.70$, df = 87, $P = 1.00$).

Tab. 3.9: Pair-wise nucleotide distances (%) (lower-left matrix) and standard errors (%) (upper-right matrix) calculated with the Kimura 2-parameter model of the mitochondrial COI sequences among nine *Diadegma* species.

Species		1	2	3	4	5	6	7	8	9
1	*D. armillata*		1.05	1.29	1.18	1.38	1.52	1.33	1.83	1.09
2	*D. blackburni*	5.20		1.21	1.11	1.41	1.46	1.31	1.85	0.99
3	*D. chrysostictos*	7.61	6.74		1.15	1.47	1.50	1.31	2.01	1.15
4	*D. fenestrale*	6.50	5.66	6.09		1.49	1.48	1.27	1.90	0.91
5	*D. insulare*	8.73	8.98	9.65	9.89		1.96	1.50	1.83	1.39
6	*D. leontiniae*	10.32	9.63	10.10	9.86	11.73			1.70	1.32
7	*D. mollipla*	8.09	7.94	7.86	7.42	10.14	10.90			1.83
8	*D. rapi*	14.34	14.58	16.82	15.32	14.34	12.20	12.66		
9	*D. semiclausum*	5.63	4.59	6.08	3.92	9.19	8.73	8.01	14.45	

3.4.3.2 Sequence analysis of rDNA

The complete ITS2 region with portions of the flanking 5.8S and 28S rDNA genes was successfully amplified and directly sequenced in 31 *Diadegma* specimens (tab. 2.1). The ITS2

boundaries of the 5.8S and 28S regions were estimated by comparison with the sequences of different hymenopteran insects available in databases, such as the *Nasonia* species complex (accession nos: U02956, U02960), *Melittobia digitata* Westwood (accession no: U02950) (Campbell *et al.*1993) and *Trichogramma minutum* Riley (accession no: AY37440). Variability in the entire ITS2 length was observed only between taxa and sequence lengths was 613 bp in *D. mollipla*, 615 bp in *D. insulare*, 616 bp in *D. fenestrale*, 621 bp in *D. semiclausum*, 634 bp in *D. blackburni*, 638 bp in *D. armillata*, 658 bp in *D. rapi* and 700 bp in *D. chrysostictos*.

An alignment of all 31 *Diadegma* ITS2 sequences yielded 782 characters when considering insertion/deletion events of which 290 characters were variable and 242 being parsimony informative. There was no marked bias in the nucleotide compositions (mean G+C content of 53.3 %).

No intra-specific variation was observed among *D. semiclausum* from nine different locations (Switzerland, Austria, Israel, Malaysia, DPR Korea, Japan, Australia, New Zealand and Kenya) and only one Tv (pos. 270 of the aligned ITS2 sequence) between all *D. semiclausum* sequences and the *D. semiclausum* sequence from Taiwan was recorded. There was one Ti and one Tv (pos. 117 and 469 of the aligned ITS2 sequence) between the four ITS2 sequences of *D. mollipla* from East Africa (Kenya, Uganda and Tanzania) and the three sequences from southern Africa (Réunion, South Africa and St. Helena). An additional transition (pos. 765 of the aligned ITS2) occurred in *D. mollipla* from Maragua (Kenya, *D. mollipla*-K1) showing the same base (G) as *D. mollipla* from southern Africa; however, the other samples from eastern Africa (*D. mollipla*-K2, *D. mollipla*-T and *D. mollipla*-U) were lacking this point mutation. One transition (pos. 35 of the aligned ITS2 sequence) differentiated both ITS2 sequences of *D. blackburni* and four point mutations (three transitions at pos. 259, 305 and 720 and one Tv at pos. 510 of the aligned sequence) appeared between both *D. armillata* specimens. No

intra-specific variation could be observed in *D. leontiniae*, *D. insulare*, *D. fenestrale* and *D. chrysostictos*.

The nucleotide distances were corrected using the Kimura 2-parameter substitution method. The intra-specific variation was 0.2-0.5 % in *D. mollipla*, 0.6 % in *D. blackburni* and 0.2 % in *D. armillata* and *D. semiclausum* (Appendix 5). Among all taxa examined (inter-specific variation) the percentage of sequence divergence ranged from 5.5 % between *D. fenestrale* and *D. blackburni* to 32.6 % between *D. chrysostictos* and *D. leontiniae* (tab. 3.10).

Tab. 3.10: Pair-wise nucleotide distances (%) (lower-left matrix) and standard errors (%) (upper-right matrix) calculated with the Kimura 2-parameter model of the ribosomal ITS2 sequences among nine *Diadegma* species.

Species	1	2	3	4	5	6	7	8	9
1 *D. armillata*		1.20	1.35	1.12	1.67	2.62	1.09	2.64	0.99
2 *D. blackburni*	8.22		1.33	0.98	1.52	2.63	1.07	2.64	1.25
3 *D. chrysostictos*	11.42	9.93		1.05	1.75	2.77	1.26	2.75	1.56
4 *D. fenestrale*	7.00	5.51	6.28		1.53	2.57	1.03	2.50	1.21
5 *D. insulare*	13.80	11.90	15.37	11.94		2.48	1.53	2.49	1.70
6 *D. leontiniae*	28.90	28.90	32.60	28.13	26.32		2.56	2.01	2.75
7 *D. mollipla*	6.61	6.44	8.83	6.01	11.94	27.87		2.49	1.28
8 *D. rapi*	28.71	28.69	31.90	26.69	26.27	20.40	26.57		2.76
9 *D. semiclausum*	5.62	8.66	12.93	7.96	14.17	30.86	8.89	30.71	

The PTP indicated the presence of significant covariance in the data set ($P = 0.0002$). Since the data have significant cladistic structure, the phylogenetic analyses were performed.

No significant differences were detected between the taxa in terms of base composition ($\chi^2 = 11.13$, df = 90, $P = 1.00$).

3.4.3.3 Comparison of nucleotide divergences: COI vs ITS2

The results shown in tabs. 3.9 (COI) and 3.10 (ITS2) are combined in fig 3.7. The ITS2 divergences were generally higher than the COI divergences. The COI divergences were 1.4-1.5 % higher than the ITS2 divergences in three cases only (*D. mollipla* vs *D. armillata*, *D. mollipla* vs *D. blackburni* and *D. mollipla* vs *D. fenestrale*). The divergences of the ITS2 and the COI sequences were almost similar in *D. semiclausum* vs *D. armillata*, *D. fenestrale* vs *D. blackburni* and *D. fenestrale* vs *D. chrysostictos* (<0.2 %, respectively).

The slope of the regression between COI and ITS2 indicates that the ITS2 evolves 3.85 times faster than the COI.

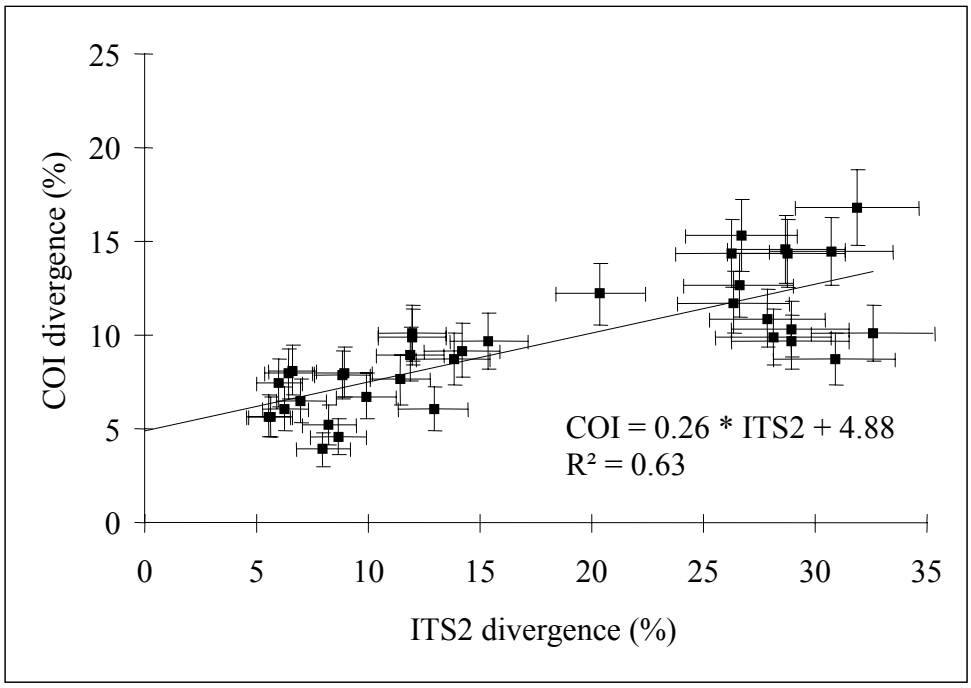

Fig. 3.7: COI vs ITS2 divergences in pair-wise comparison of nine *Diadegma* species (tabs. 3.9 and 3.10). Standard errors are indicated at each data point.

3.4.3.4 Sequence saturation and homoplasy

A qualitative test for saturation and homoplasy was used to compare uncorrected divergences with corrected pair-wise divergences. Figure 3.8 shows the uncorrected divergence vs the estimated substitutions for the ITS2 and COI loci (with the ML parameters estimated from MODELTEST). The degree of which points differ from the x = y line indicates saturation and the degree of homoplasy (Zamudio *et al.* 1997). The fragment of the COI gene clearly got more quickly saturated than the ITS2 locus.

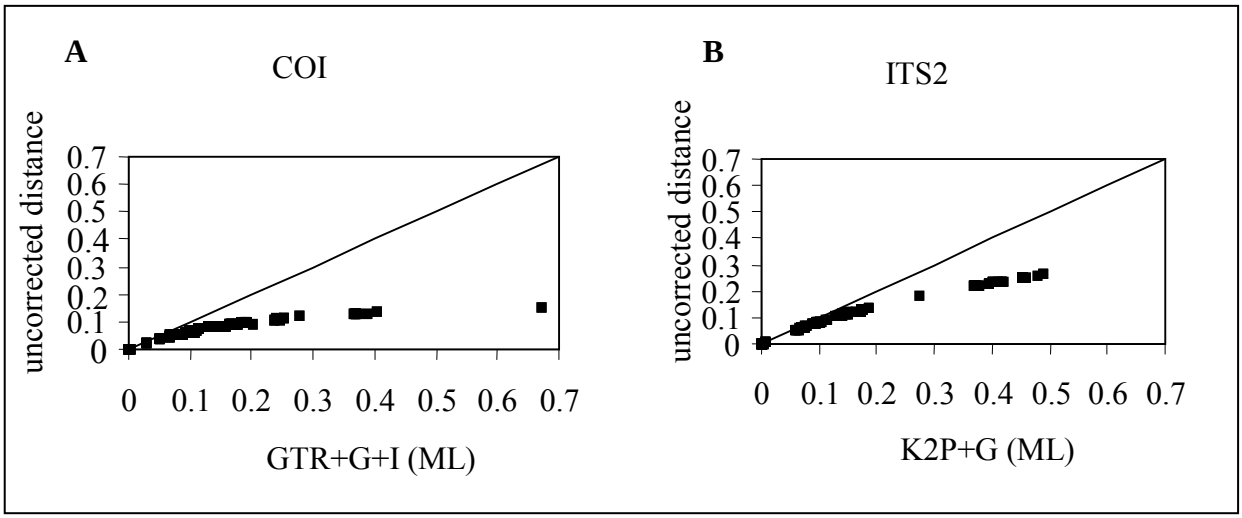

Fig. 3.8: Saturation plots of two different genetic loci: (**A**) fragment of the COI gene, mtDNA. (**B**) ITS2 region, rDNA. Pair-wise uncorrected (y axis) and corrected distances (x axis) were estimated using PAUP*, with the ML model suggested by MODELTEST.

3.4.3.5 Phylogenetic analyses

3.4.3.5.1 Phylogenetic analyses of the genus *Diadegma*

3.4.3.5.1.1 COI sequences

The neighbour-joining option implemented in PAUP* was used to construct a dendrogram for complete distance values using the Kimura 2-parameter model (fig. 3.9). The dendrogram (fig. 3.9) shows monophyly of the *Diadegma* species examined, *D. leontiniae* and *D. rapi* being sister groups as well as *D. insulare* and *D. mollipla*. The next clade grouped *D. black-*

burni, *D. armillata* and *D. chrysostictos* as sister groups whereas *D. semiclausum* and *D. fenestrale* are grouped together with moderate bootstrap support.

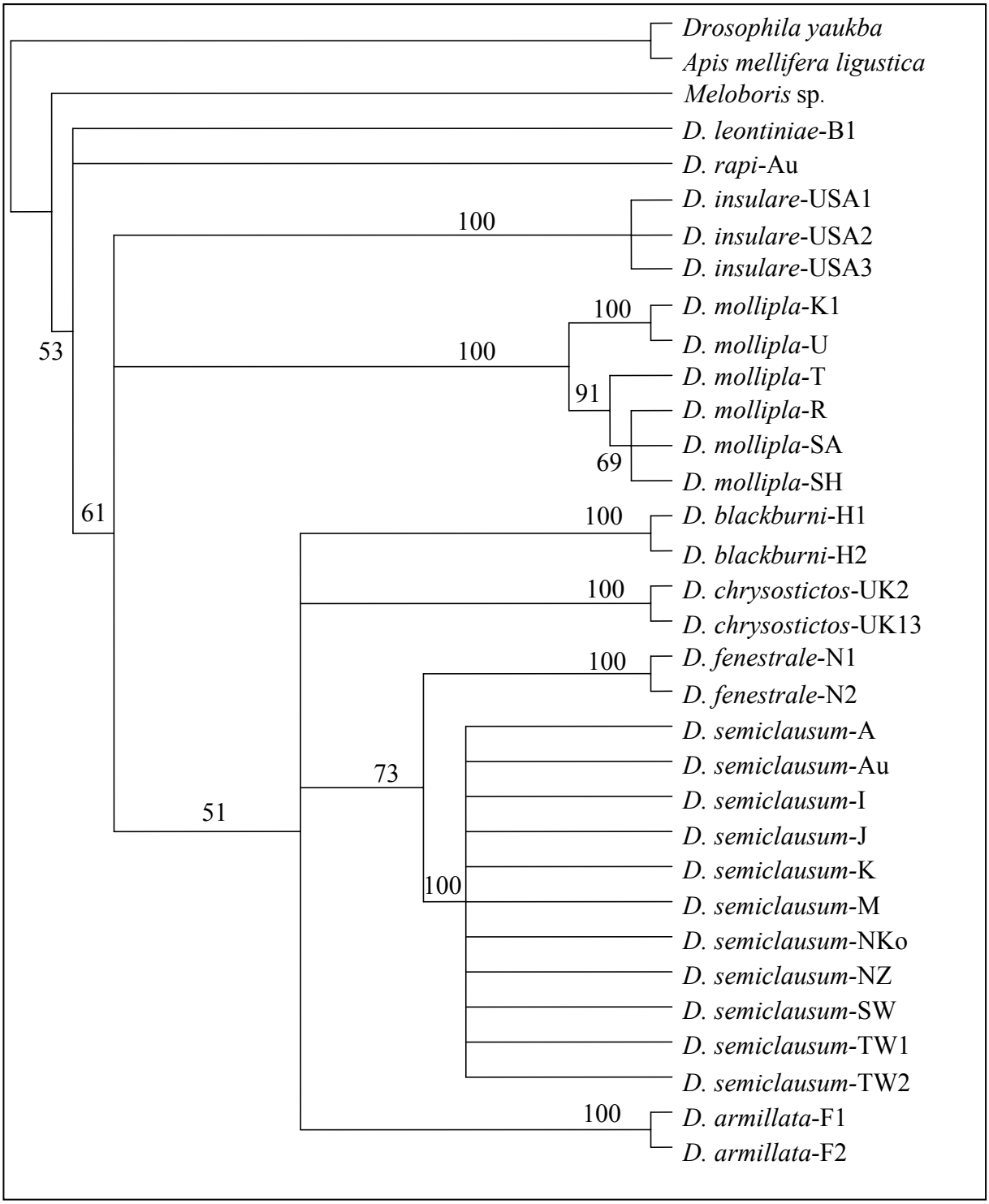

Fig. 3.9: Neighbour-joining dendrogram (Kimura 2-parameter distances) of 30 *Diadegma* individuals and three out-group taxa based on the analysis of mitochondrial COI sequences (498 bp). Bootstrap confidence levels (%), based on 1000 replicates, are indicated at each branch.

An exhaustive search using PAUP* assuming equal weight for all characters produced four most-parsimonious trees of 291 steps (consistency index (CI) = 0.57, retention index (RI) = 0.76). A single most-parsimonious tree of these four trees is shown in fig. 3.10.

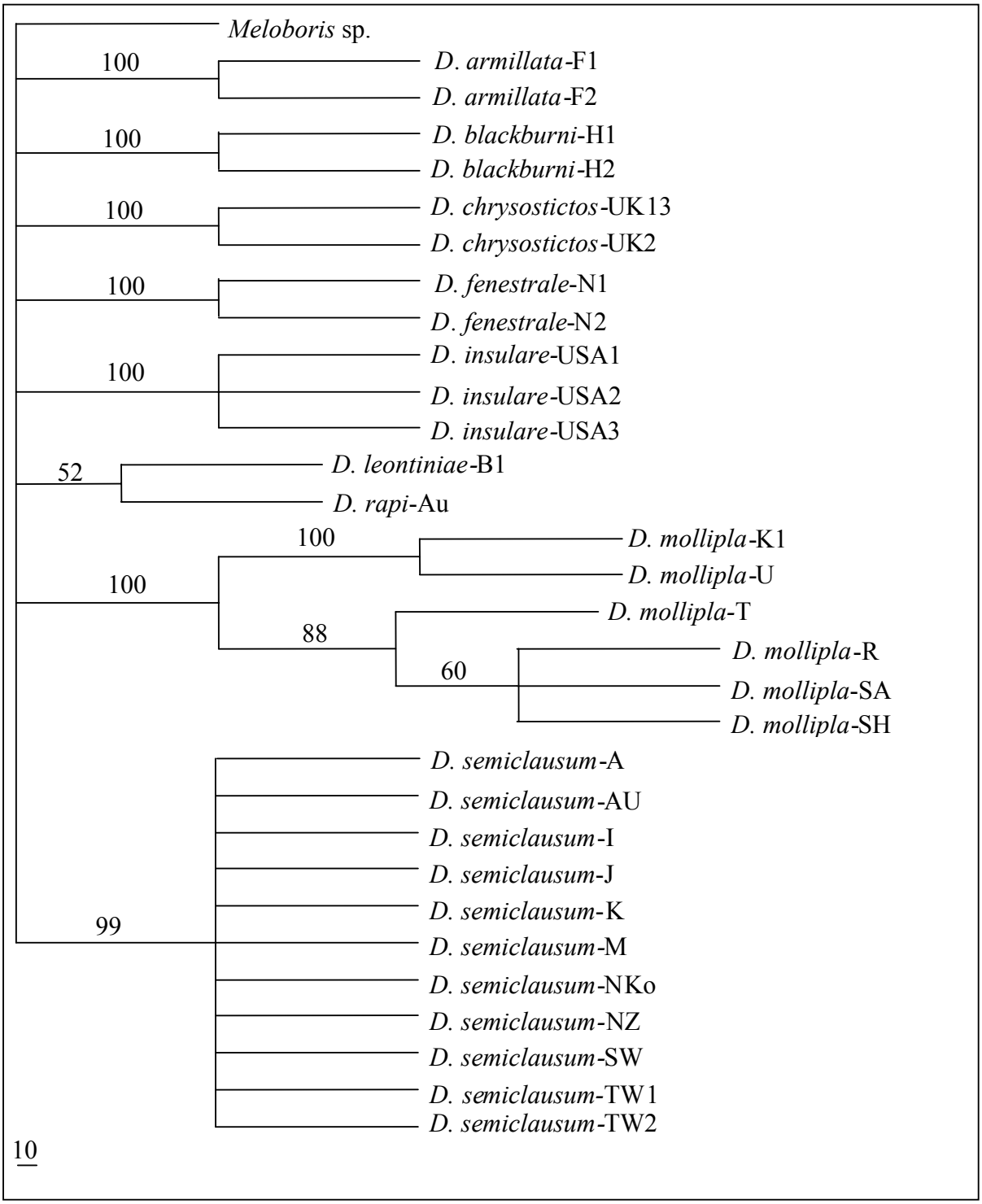

Fig. 3.10: Single most-parsimonious tree topology (291 steps; CI = 0.57, RI = 0.76) for a fragment of the COI gene (498 bp) of 30 *Diadegma* individuals and *Meloboris* sp. as out-group (tab. 2.1). Values above the branches represent bootstrap percentages greater than 50 %. Branch lengths are proportional to genetic distance (scale bar).

For the ML analysis (excluding out-group) MODELTEST selected the general time reversible model, including the proportion of invariable sites and gamma distribution for rate variation among sites (GTR+I+G; unequal base frequencies, six substitution rates) (Gu *et al.* 1995; Rodríguez *et al.*1990), as the model that best fits the data (rate matrix parameters estimated on a neighbour-joining tree were: R(a) [A-C] = $6.5x10^{6}$, R(b) [A-G] = $6.0x10^{6}$, R(c) [A-T] = $1.2 x10^{7}$, R(d) [C-G] = $1.0x10^{-4}$, R(e) [C-T] = $8.7x10^{7}$, R(f) [G-T] = 1.0). Base frequencies were A=0.34, C=0.12, G=0.12, T=0.43. The gamma distribution shape parameter (G) and the assumed proportion of the invariable sites (I) were 1.04 and 0.63, respectively. The maximum likelihood was estimated by MODELTEST as -lnL = 1595.17. Trees constructed using MP and ML were congruent and for simplicity, only the phylogenetic tree of the MP analyses is shown (fig. 3.10, Appendix 6 - tree constructed using ML). All trees consistently showed one clade with a single isolate of each *D. rapi* and *D. leontiniae* with moderate bootstrap support. All other *Diadegma* species (*D. insulare*, *D. mollipla*, *D. semiclausum*, *D. chrysostictos*, *D. fenestrale*, *D. blackburni* and *D. armillata*) were grouped according to species with very strong bootstrap support.

3.4.3.5.1.2 MP and ML analyses with the exclusion of the third codon position

There is an ongoing controversy whether multiple or hidden substitutions which are indicators for homoplasy and saturation should be included in phylogenetic analyses (Brower and DeSalle 1994, Dowton and Austin 2001, Rokas *et al.* 2002).

Because of the exclusion of the third codon position (166 characters), only 39 distinct characters were obtained for the analyses from the remaining 332 characters. An exhaustive search using PAUP* assuming equal weight for all characters produced 25 parsimonious trees

of 152 steps (CI = 0.76, RI = 0.69). Figure 3.11 shows a single most-maximum-parsimony tree of these 25 trees.

Clearly, all *Diadegma* species were monophyletic and were grouped in one clade with only *D. mollipla* and *D. chrysostictos* consisting a second subgroup with moderate bootstrap support.

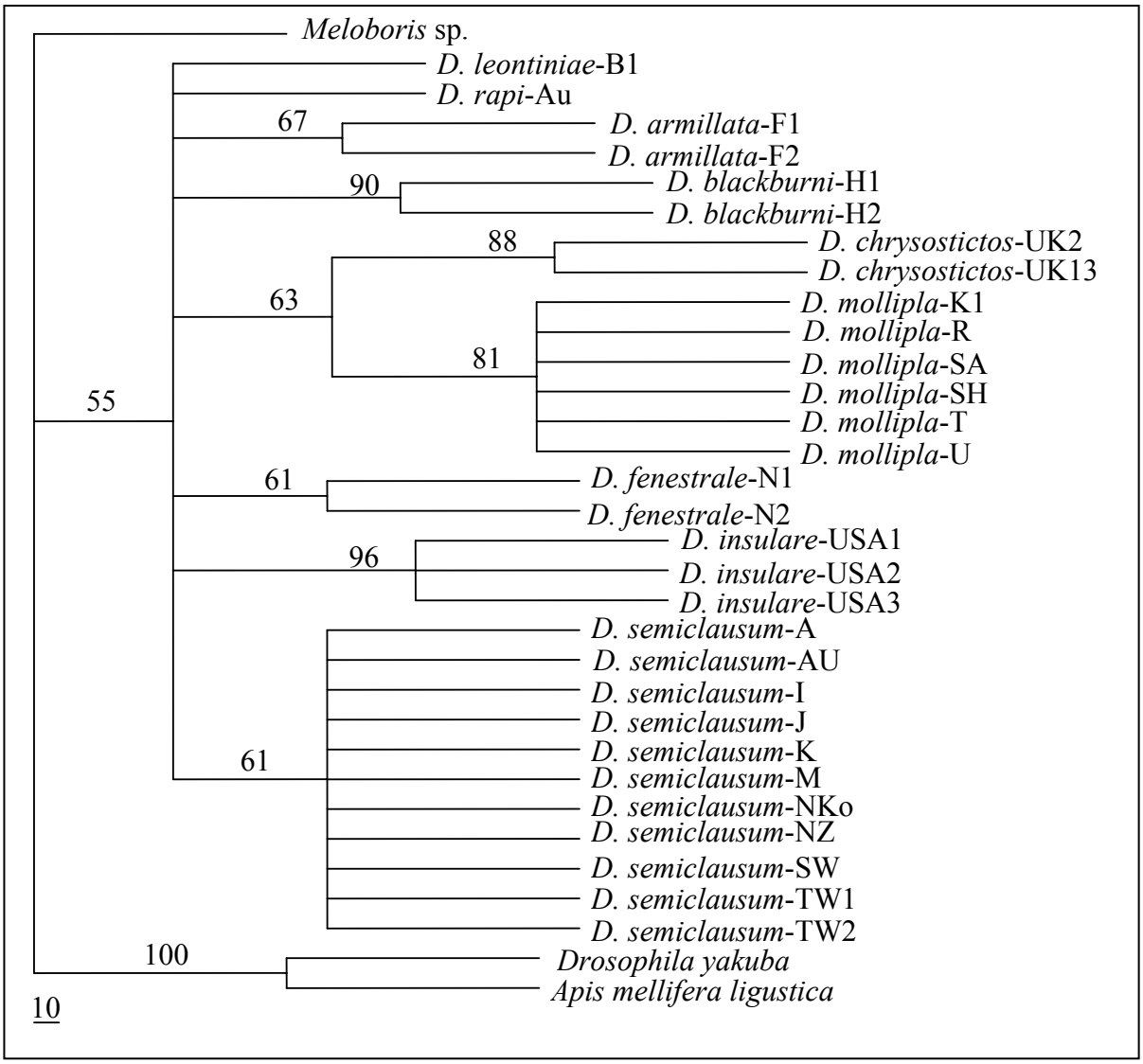

Fig. 3.11: Single most-parsimonious tree topology (152 steps; CI = 0.76, RI = 0.69) for a fragment of the COI gene of 30 *Diadegma* individuals (tab. 2.1) and three out-groups. The third codon position was excluded. Values above the branches represent bootstrap percentages greater than 50 %. Branch lengths are proportional to genetic distance (scale bar).

An additional ML analysis was performed excluding the third codon position. MODELTEST identified the TIM+I model (transitional model with six free parameters and unequal base frequencies; and the proportion of invariable sites) as the best fit to the COI data (excluding the third codon position) (fig. 3.12).

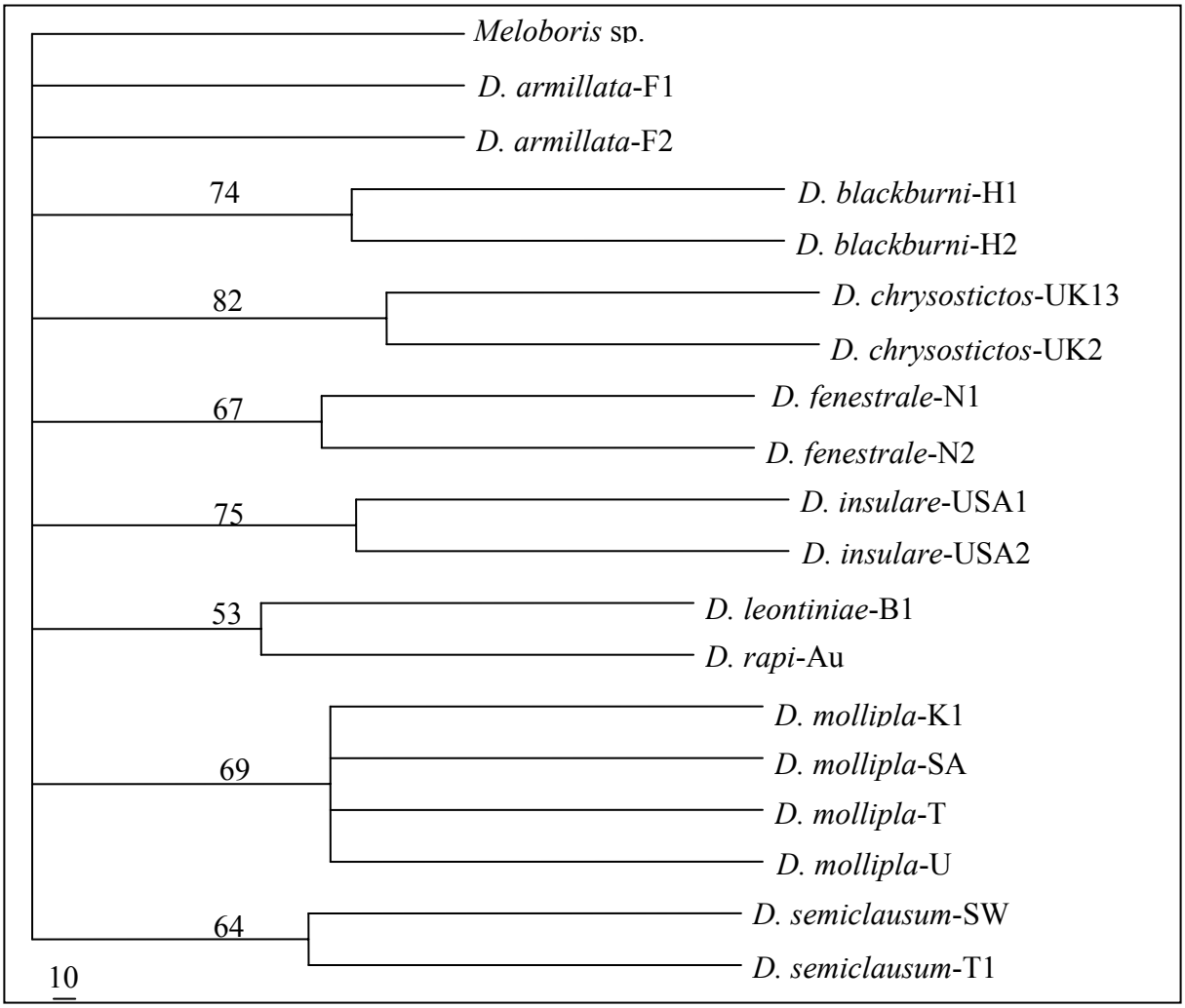

Fig. 3.12: Maximum-likelihood tree (-lnL = 647.88) for COI sequences (without third codon position) of nine *Diadegma* species and *Meloboris* sp. as out-group (tab. 2.1). The maximum likelihood analysis was conducted with TIM+I. Bootstrap confidence levels (%), based on 100 replicates, are indicated above each branch. Branch lengths are proportional to genetic distance (scale bar).

The TIM model had the following base frequencies: freqA = 0.26, freqC = 0.20, freqG = 0.16, freqT = 0.38 and a rate matrix: R(a) [A-C] = 1.00, R(b) [A-G] = $8.1x10^6$, R(c), [A-T] = $8.2x10^6$, R(d) [C-G] = $8.2x10^6$, R(e) [C-T] = $9.0x10^7$, R(f) [G-T] = 1.00. The proportion of invariable sites (I) was 0.87. The maximum likelihood was estimated by MODELTEST (without out-group *Meloboris* sp.) as -lnL = 647.89.

The phylogenetic tree showed a poor phylogenetic structure when the third codon position was excluded from the analyses. Only *D. leontiniae* and *D. rapi* were clustered together and the out-group *Meloboris* sp. was not separated from the *Diadegma* wasps examined.

3.4.3.5.1.3 ITS2 sequences

The neighbour-joining option implemented in PAUP* was used to construct a dendrogram for complete distance values using the Kimura 2-parameter model (fig. 3.13).

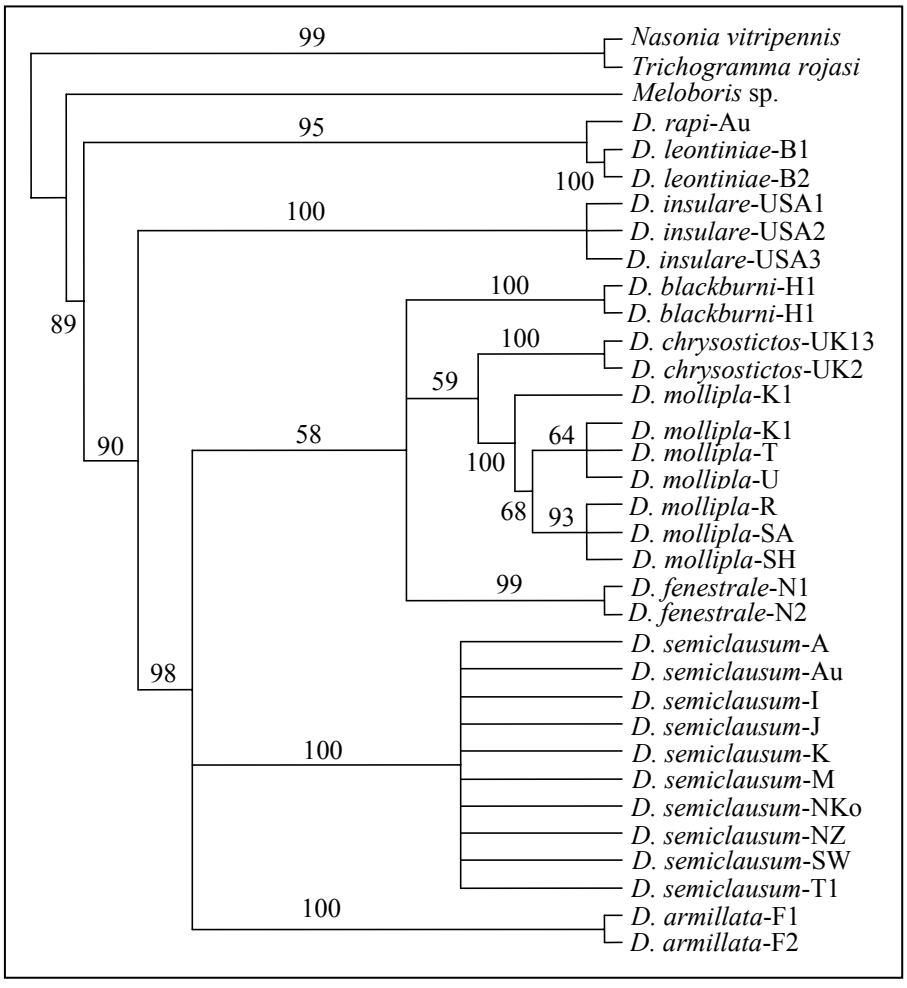

Fig. 3.13: Neighbour-joining dendrogram (Kimura 2-parameter distances) of 31 *Diadegma* individuals and three out-group taxa based on the analysis of ribosomal ITS2 sequences (821 characters). Bootstrap confidence levels (%), based on 1000 replicates, are indicated at each branch.

Figure 3.14 shows the single most-

parsimonious tree of the ITS2 sequence data for the 31 *Diadegma* specimens and the out-group examined.

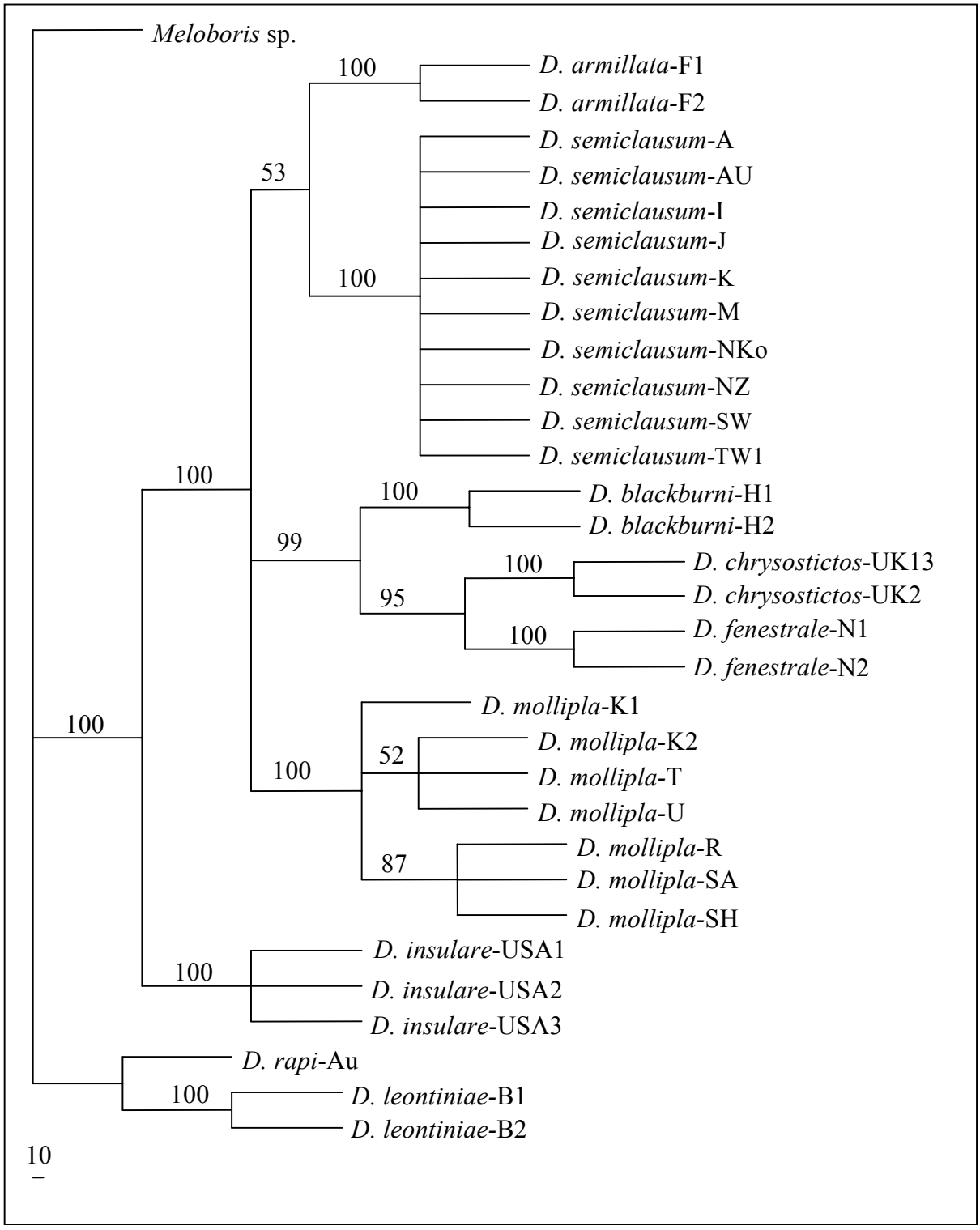

Fig. 3.14: Single most-parsimonious tree topology (610 steps; CI = 0.85, RI = 0.90) for ribosomal ITS2 sequences of nine *Diadegma* species and *Meloboris* sp. as out-group (tab. 2.1). Values above the branches represent bootstrap percentages greater than 50 %. Branch lengths are proportional to genetic distance (scale bar).

The sequences of the ITS2 region were phylogenetically informative in separating all nine *Diadegma* species as monophyletic. The total number of parsimonious trees was four with a tree length of 610 (number of steps) (CI = 0.85, RI = 0.90). The tree showed two clades with *D. rapi* and *D. leontiniae* being in clade 1 (with moderate bootstrap support), while all other *Diadegma* species *(D. insulare*, *D. mollipla*, *D. semiclausum, D. chrysostictos*, *D. fenestrale*, *D. blackburni* and *D. armillata*) were grouped in clade 2. *Diadegma insulare* formed a strong subclade of clade 2. A second subclade contained *D. armillata* and *D. semiclausum*, which clustered together with moderate bootstrap support. *Diadegma chrysostictos* and *D. fenestrale* were grouped together with strong bootstrap support and together with *D. blackburni* they formed a third subclade of clade 2 with strong bootstrap support. Another subclade of clade 2 contained *D. mollipla* samples with strong bootstrap support.

For the maximum likelihood analysis the model that best fit the data was K80+G (Kimura 2-parameter method with gamma distribution) with a Ti/Tv ratio of 1.40, a gamma distribution shape parameter of 0.58 and a maximum likelihood of –lnL = 3003.57, respectively. The phylogenetic tree is shown in fig. 3.15.

The phylogram was very similar to the MP tree (fig. 3.14) but differentiates *D. chrysostictos*, *D. fenestrale* and *D. blackburni* with strong bootstrap support in single branches. Subclade two had therefore only one subclade (*D. semiclausum* and *D. armillata*) with moderate bootstrap support.

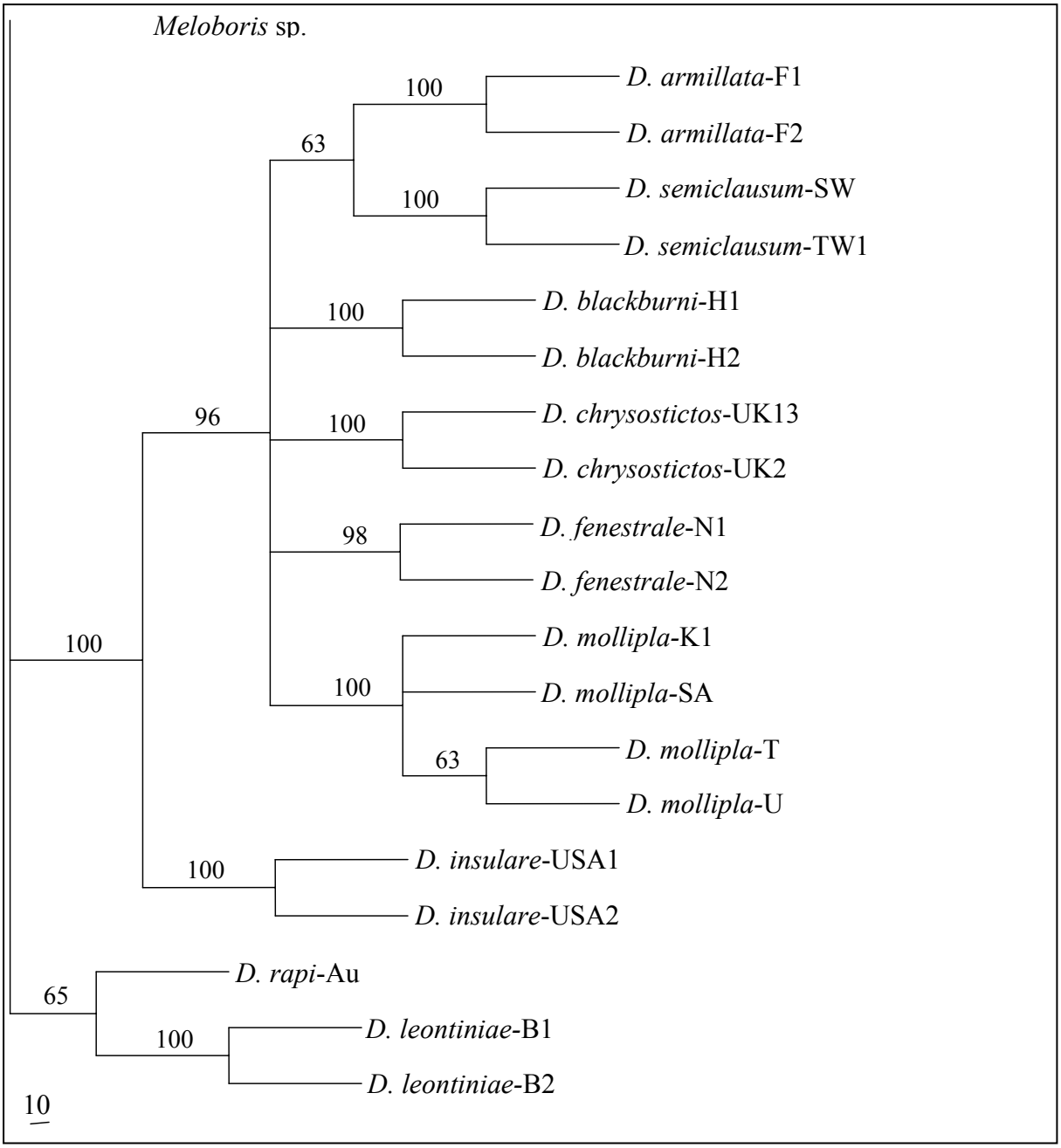

Fig. 3.15: Maximum-likelihood tree (-lnL = 3003.57) for ribosomal ITS2 sequences of nine *Diadegma* species and *Meloboris* sp. as out-group (tab. 2.1). The maximum likelihood analysis was conducted with K80+G (G = 0.58). Bootstrap confidence levels (%), based on 100 replicates, are indicated above each branch. Branch lengths are proportional to genetic distance (scale bar).

3.4.3.5.1.4 Phylogenetic analyses with combined molecular data sets (ITS2 and COI)

The partition homogeneity test was conducted under the optimal criterion of parsimony. The combined data set of 18 *Diadegma* individuals resulted in 1275 characters where 865 were constant and 114 parsimony-uninformative. The number of parsimony-informative characters was 296. The partition homogeneity test between COI and ITS2 sequences rejected the null hypothesis (P=0.015) and significant differences from either data set or the random partition of the pooled data were detected. When the third codon position of the fragment of COI gene was excluded from the data set, the IDL test showed no significant incongruence between both data partitions (P=0.08). The test was conducted with 1109 characters, where 98 were parsimony-uninformative, 219 parsimony-informative, and 792 characters were constant. An additional phylogenetic analysis (MP and ML) of the combined COI (excluding third codon position) and ITS2 partitions was therefore performed to examine the robustness of the phylogenetic trees.

The maximum parsimony and the maximum-likelihood analyses were performed with 18 different *Diadegma* individuals. The observed most parsimonious tree (fig. 3.16) had a length of 679 steps with CI=0.82 and RI=0.80.

The phylogenetic tree showed two clades with *D. rapi* and *D. leontiniae* being in clade 1 (with strong bootstrap support), while all other *Diadegma* species *(D. insulare*, *D. mollipla*, *D. semiclausum*, *D. chrysostictos*, *D. fenestrale*, *D. blackburni* and *D. armillata*) were grouped in clade 2. *Diadegma insulare* formed a strong subclade of clade 2. A second subclade of clade 2 contained *D. chrysostictos* and *D. fenestrale* as one subclade with strong bootstrap support whereas *D. armillata*, *D. semiclausum*, *D. blackburni* and *D. mollipla* formed a moderately supported subclade of clade 2. This subclade clustered only *D. armillata* and *D. semiclausum* together with strong bootstrap support, *D. blackburni* and *D. mollipla* are sister groups.

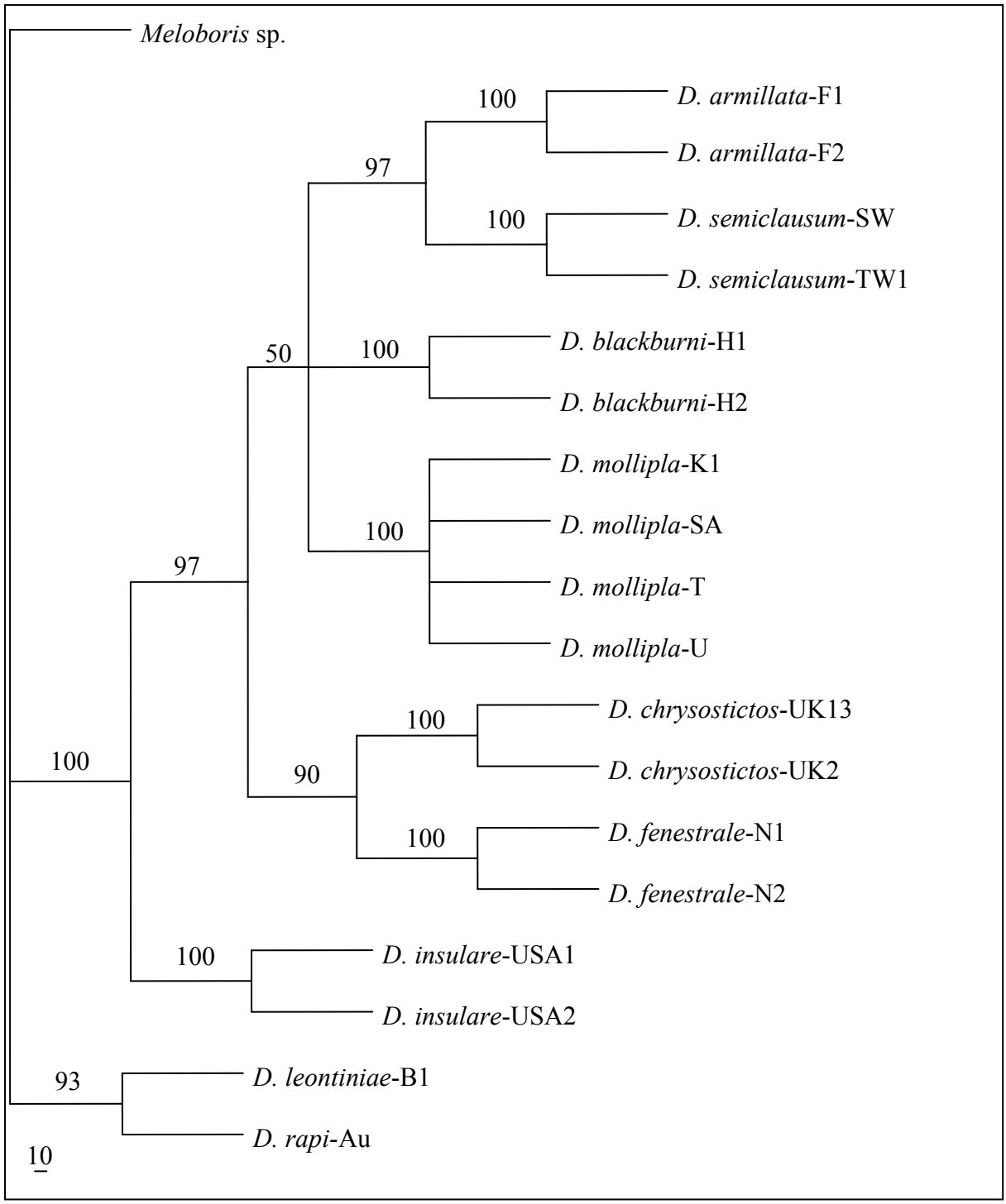

Fig. 3.16: Single most-parsimonious tree topology (679 steps; CI = 0.816, RI = 0.802) for the combined data partitions of nine *Diadegma* species and *Meloboris* sp. as out-group (tab. 2.1). The third codon of COI gene was excluded. Values above the branches represent bootstrap percentages greater than 50 %.

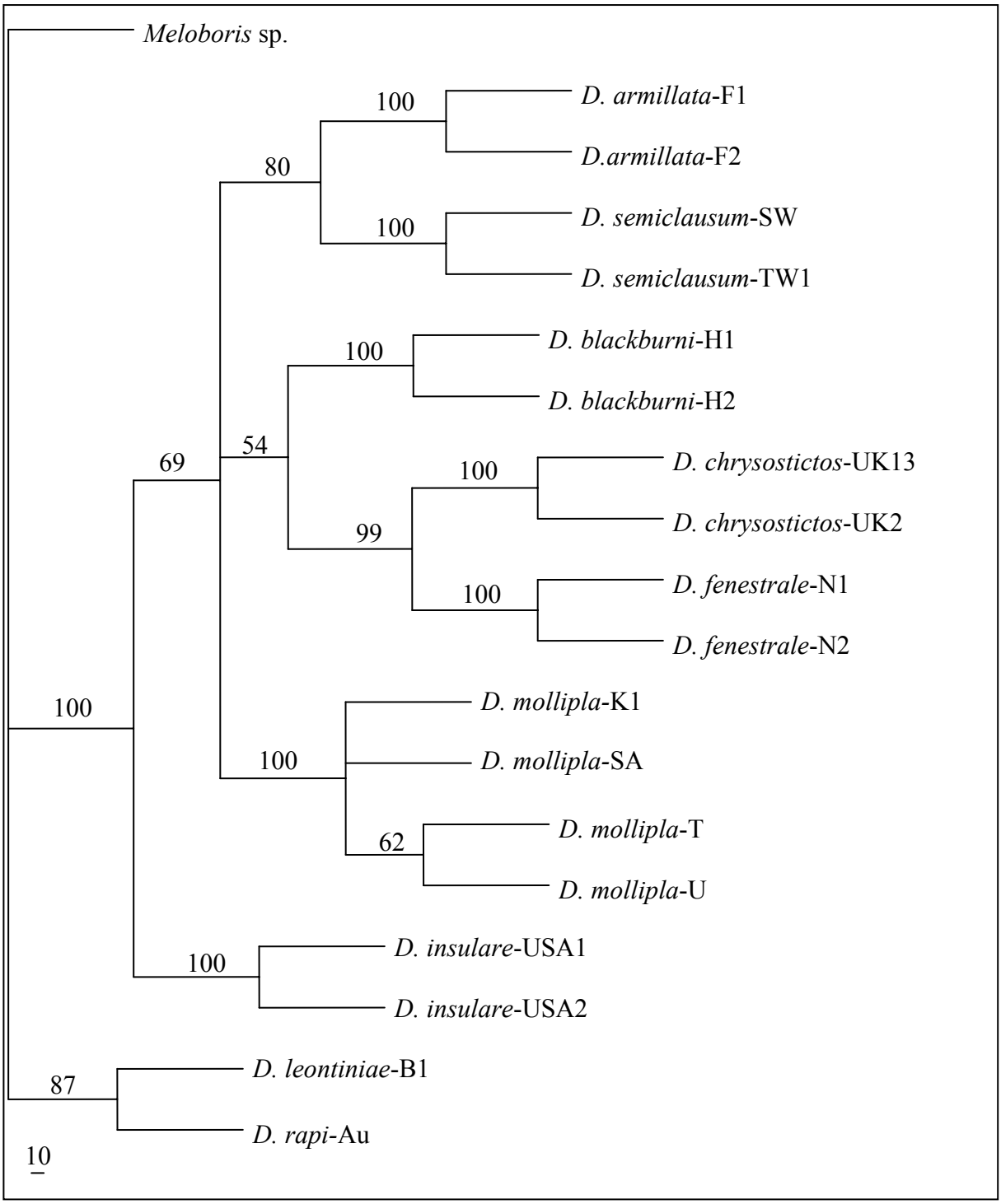

Fig. 3.17: Maximum likelihood tree topology for the combined data partitions of nine *Diadegma* species and *Meloboris* sp. as out-group (tab. 2.1). The third codon of COI gene was excluded. Values above the branches represent bootstrap percentages greater than 50 %. Branch lengths are proportional to genetic distance (scale bar).

The mode of evolution identified by MODELTEST for the combined data set (without outgroup) was the TrNef+G model (Tamura and Nei 1993) with equal base frequencies. The rate matrix had the following substitution model with four constant and two free parameters: R(a) [A-C] = 1.00, R(b) [A-G] = 2.37, R(c) [A-T] = 1.00, R(d) [C-G] = 1.00, R(e) [C-T] = 4.00, R(f) [G-T] = 1.00. The gamma distribution shape parameter was G = 0.31 and the maximum likelihood –lnL = 3751.11, respectively. The phylogenetic tree of the ML analysis is shown in fig. 3.17.

The phylogenetic tree showed also two clades when the ML tree building methodology was used. *Diadegma rapi* and *D. leontiniae* were in clade 1 (with strong bootstrap support), while all other *Diadegma* species *(D. insulare, D. mollipla*, *D. semiclausum*, *D. chrysostictos*, *D. fenestrale, D. blackburni* and *D. armillata*) were grouped in clade 2. The second clade was again divided into two subclades, where *Diadegma insulare* formed a strong subclade of clade 2. The second clade of clade 2 had three subclades containing *D. armillata*, and *D. semiclausum* as sister groups. *Diadegma mollipla* were separated in the two populations and formed a strong subclade of clade 2 whereas *D. chrysostictos*, *D. fenestrale* and *D. blackburni* were grouped together. Out of this clade *D. chrysostictos* and *D. fenestrale* formed a sister group.

3.4.3.5.2 Phylogenetic analyses of the superfamily Ichneumonoidea

The two families Braconidae and Ichneumonidae form the clade of the superfamily Ichneumonoidea which is monophyletic based on morphological evidence (Sharkey and Wahl 1992). Two phylograms were constructed using first COI sequences and second ITS2 sequences.

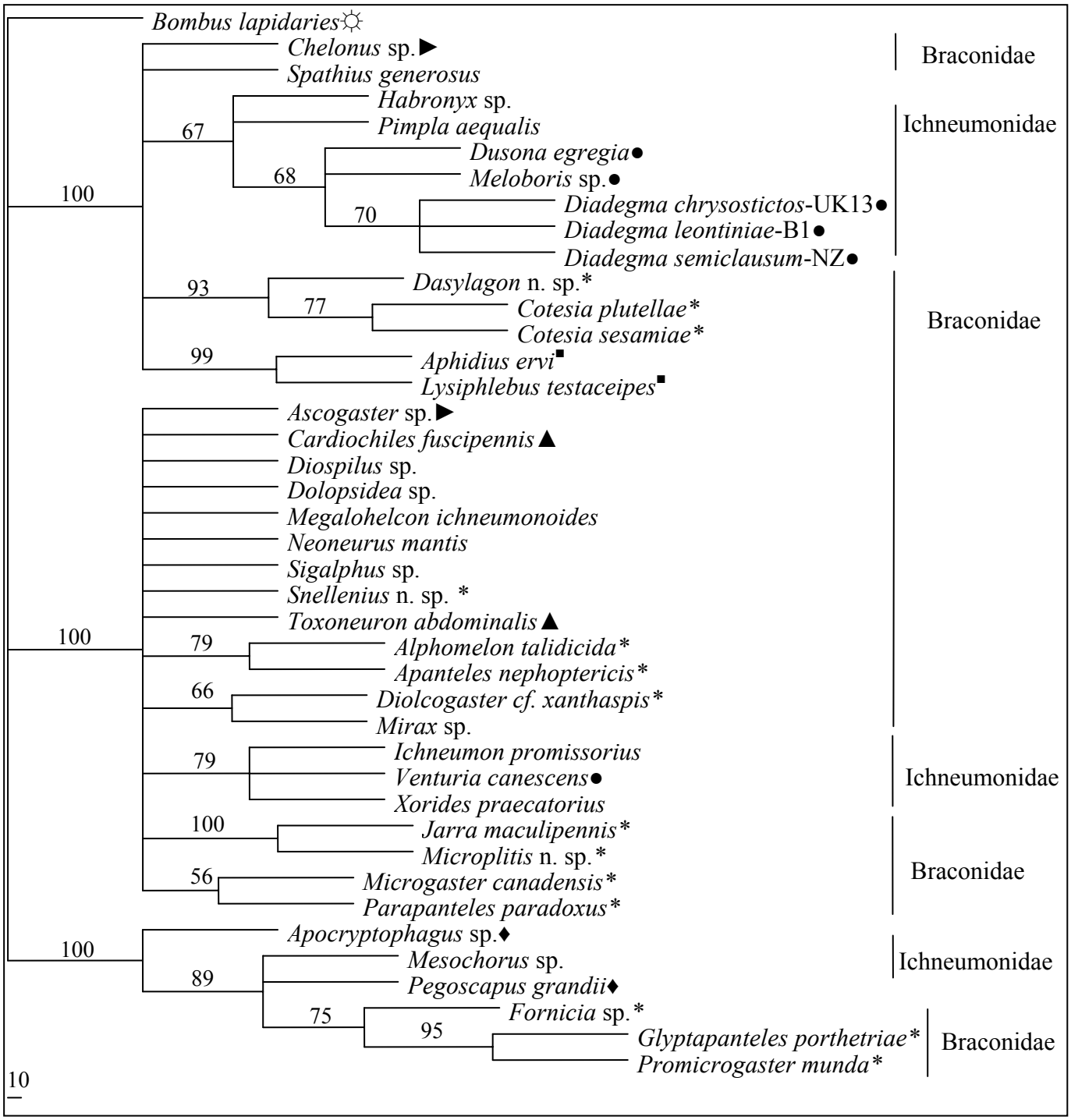

Fig. 3.18: Single most-parsimonious tree topology of 11 shortest trees (2359 steps; CI = 0.38, RI = 0.69) for COI gene sequences of 38 species (Braconidae and Ichneumonidae) of the superfamily Ichneumonoidea. Values above the branches represent bootstrap percentages greater 50 %. *Braconidae; Microgastrinae, ▲Braconidae; Cardiochilinae, ►Braconidae; Cheloninae, ■Braconidae; Aphidiinae, ●Ichneumonidae; Campopleginae, out-groups: ♦Chalcidoidea; Agaonidae; Agaoninae, ☼Aculeata; Apoidea; Apidae.

Of the 446 characters, 12 were constant, 33 variable but parsimony uninformative and 401 characters were parsimony informative. All characters had equal weight. The number of distinct trees was eleven. The number of bootstrap replications was 1000. Only five trees were held.

The phylogram of the COI sequences showed three clades with Braconidae and Ichneumonidae clustered in all three groups (fig. 3.18). On the basis of 446 COI characters Braconidae and Ichneumonidae are not monophyletic. The genus *Diadegma* showed monophyly.

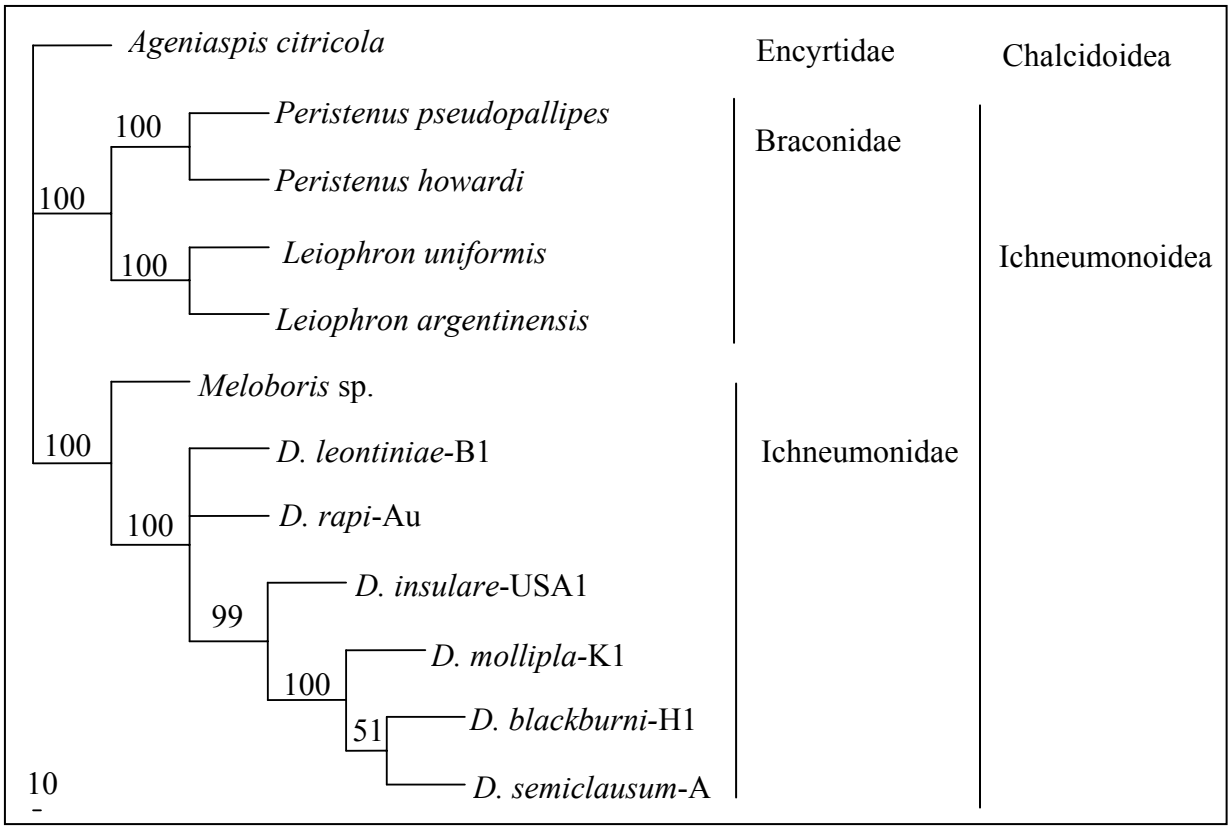

Fig. 3.19: Maximum parsimony analysis of the ITS2 region of the superfamily Ichneumonoidea. Strict consensus of two shortest trees (1411 steps, CI = 0.80, RI = 0.80). Values above the branches represent bootstrap percentages greater than 50 %. *Ageniaspis citricola* served as out-group. Branch lengths are proportional to genetic distance (scale bar).

The ITS2 sequences of similar sizes were aligned successfully and the phylogenetic inferences examined for two genera of Braconidae, two genera of Ichneumonidae and one genera of Encyrtidae (fig. 3.19). Out of 756 total characters, 169 characters were parsimony uninformative and 517 characters were parsimony informative. Seventy characters were constant. The monophyly of the genera *Diadegma*, *Meloboris*, *Leiophron* and *Peristenus* of the superfamily Ichneumonoidea could be illustrated and all genera were separated with high bootstrap support.

4 Discussion

4.1 Molecular methods used in the study

4.1.1 Extraction of DNA for molecular work

The quality of the DNA is of major importance in the successful amplification of a genetic region and depends on the extraction method used. Different extraction methods are suitable to extract DNA from insects for different applications. All methods have in common an extraction with extraction buffer, centrifugation steps and the precipitation of DNA mostly with absolute ethanol at -20 °C to remove salts, which might disturb following procedures. Some methods can be time-consuming (Favia *et al.* 1994); other methods involve hazardous chemicals (e.g. phenol/chloroform) (Harrison *et al.* 1987). The method used in this study (modified after Baruffi *et al.* 1995) was fast and therefore suitable for routine work without the usual extraction step with phenol/chloroform or phenol/chloroform/isoamylethanol. However, in some cases it was necessary to concentrate and purify the DNA samples prior to amplification.

4.1.2 Polymerase chain reaction (PCR)

Before the start of this study no information on DNA sequences within the genus *Diadegma* was available. Therefore, two different genetic regions (a fragment of COI gene and the ITS2 region) were chosen, which evolve differently and were already known to resolve taxonomic questions in insects and mites (see e.g. Campbell *et al.* 1993; Langor and Sperling 1997; Lee and Lee 1997; Navajas *et al.* 1998; Brown *et al.* 1999; Mardulyn and Whitfield 1999).

In general, the PCR technique has become a standard tool in molecular systematics. An overview of the methodology, the applications and the limitations is given by Palumbi (1996).

Briefly, one PCR cycle is subdivided into three major steps: denaturation, annealing and extension. During the first step, the DNA will be denatured from double to single strand. In the next step the temperature is lowered so that the oligonucleotide primers can bind to the appropriate sites at the template DNA. During the extension the *Taq*-polymerase synthesizes the target DNA fragment. The PCR is normally finished after 35-40 cycles. This technique sounds easy but remains often very unreliable when the PCR amplification works at one time and does not the next time. Considered that the DNA template is of good quality, the quality of the *Taq*-polymerase, which can be very costly, is frequently the reason for a failure of a PCR reaction.

During this study, the PCR conditions were adjusted whenever one component of the reaction mix was replaced. The amplification of the mitochondrial DNA was problematic due to the failure of the universal primer 4 (C1-N-2329). In the beginning of the study this primer was working properly, but later it started to fail. The reasons for this are still unknown at this stage. In order to prevent further problems, different primer combinations were used and one primer (primer 8) was newly developed. However, primer 4 was reordered from three different suppliers (see Appendix 1) and the primer provided by Invitrogen could finally be used to finish the work. This points to the fact that besides DNA and polymerase quality, the purity and quality of the primers used can also be a crucial factor in determining the success of PCR work.

In contrast, the ITS2 region was always amplified without any difficulties in all *Diadegma* specimens investigated.

4.2 Crossing experiments

Gene flow between *Diadegma* species living in the same geographical region was reported by Hardy (1938), who examined the reproductive compatibility between *D. semiclausum* (females) and *D. fenestrale* (males). The resulting F1 generation showed mixed morphological characters of both species (Hardy 1938). No detailed data were presented in that case but the possibility of inter-specific crosses between *D. semiclausum* and *D. fenestrale* is still under discussion. Delvare (2004) formulated a need for clarification concerning the taxonomic status of both species.

Two different crossing experiments were conducted to examine whether reproductive isolation exists between (i) the two geographically distinct populations of *D. mollipla* in eastern and southern Africa and between (ii) *D. mollipla*, the native parasitoid of *P. xylostella* in Kenya and *D. semiclausum*, the exotic parasitoid, which was introduced into Kenya in 2001 from Taiwan.

4.2.1 Intra-specific crosses

Both populations of *D. mollipla* (DM-K1 and DM-SA) behaved differently and the laboratory culture from Kenya, which was build up with field collected *D. mollipla*, had a more than two times higher sex ratio then the laboratory culture imported from South Africa (tab. 3.1). This might be due to the regular introduction of field material into the Kenyan culture (DM-K1) to avoid loss of fitness. The South African culture was already one year old when it was established in Kenya and it was not possible to get fresh supplements. Therefore, this culture was marked by long-time inbreeding, which then resulted in a high male-biased culture (tab. 3.1).

Highly male-biased laboratory cultures can be attributed to several reasons. One common explanation is the existence of *Wolbachia pipientis* Hertig, an endosymbiont bacterium found in

many insects (Werren *et al.* 1997; Stouthamer *et al.* 1999b). *Wolbachia* infection often results in unidirectional cytoplasmatic incompatibility: crosses between infected males and uninfected females are incompatible and show a reduction of progeny or male-biased sex ratios among offspring, whereas the reciprocal cross between uninfected males and infected females is compatible and produces normal progeny (Stouthamer *et al.* 1999b). If the culture of DM-K1 (eastern Africa) was not infected with *Wolbachia* and the culture of DM-SA (southern Africa) was, the reciprocal crosses, where the infected females of DM-SA were crossed with an uninfected male of DM-K1, should have produced normal progeny. This was not the case in the present study (tab. 3.1) and it can be suggested that the laboratory culture from South Africa was not infected with *Wolbachia.* However, further studies of *D. mollipla* should include PCR amplifications to detect the presence of *Wolbachia* as done e.g. by Mochiah *et al.* (2002).

A second possible explanation for a male-biased laboratory culture was given by Butcher *et al.* (2000a, b). These authors reported a male-biased secondary sex ratio in laboratory cultures of *D. chrysostictos* and *D. fabricianae* with a reduction of the female fecundity that stabilized after 5-6 generations and with occurrence of diploid males following in-breeding. The reversal of the male-biased sex ratio in *D. fabricianae* and *D. chrysostictos* following out-breeding suggested the operation of sl-CSD and inbreeding depression (Butcher *et al.* 2000a, b). Taking this into account it is not surprising that both *D. mollipla* cultures (DM-K1 and DM-SA) showed such a difference in the sex ratio indicating that a CSD is also operating in *D. mollipla*.

The sl-CSD results in the production of diploid males and can be detected by various techniques. Hence, a test was conducted mating the mother with a son and the progeny was tested for genetic variability via isoenzyme analyses. Results will be discussed in Chapter 4.3.

Additionally, female offspring resulted from all intra-specific crosses (tabs. 3.1, 3.2), which clearly shows that the populations of *Diadegma* from Kenya and South Africa are *D. mollipla*. This was also confirmed using morphological means by Horstmann (pers. communication).

4.2.2 Inter-specific crosses

In the inter-specific crosses between *D. mollipla* (DM-K2) and *D. semiclausum* (DS) only male offspring emerged. When comparing the total number of offspring (numbers of total progeny/female, tab. 3.3) in these incompatible crosses with the number of progeny produced by intra-specific crosses, no significant differences could be observed except for the DS& x DM-K2% where the total number of progeny (only males) in the cross was significantly higher then in the other crosses (tab. 3.3). These findings indicate that there was no substantial mortality in the incompatible crosses, which would be otherwise an indication for *Wolbachia* infection resulting in a female mortality where fertilized eggs die (Vavre *et al.* 2000). Mating between *D. mollipla* and *D. semiclausum* was also never noticed. The spermatheca of five females of these inter-specific crosses were empty, indicating absence of sperm transfer without that no gene flow between both species can occur.

4.3 Existence of diploid males in *Diadegma* species

The sex ratio of *Diadegma* species (e.g. *D. chrysostictos*) is female biased in the field but under laboratory conditions it tends to become male biased (Butcher *et al.* 2000a). This phenomenon was also observed within *D. mollipla* where the sex ratio of the collected field material was female biased and became more and more male biased under laboratory conditions unless this was prevented by regular out-breeding (e.g. DM-K1). Also, the comparison of the

D. mollipla cultures DM-K1 and DM-K2 (tabs. 3.1, 3.3) showed that a regular refreshment with field material (DM-K1) resulted in a higher sex ratio (37.2 ± 5.4), compared to 19.2 ± 3.4 in DM-K2, where no field material was added.

A male biased laboratory culture is often attributable to inbreeding where homozygosity is much higher and as a result, the proportion of diploid males increases (Cook 1993b). Diploid males can easily be detected using isoenzyme variations as a genetic marker. Noda (2000) crossed two isofemale lines of *D. semiclausum*, where one pure line was presenting the S-band of the phosphoglucomutase (PGM) electrophoretic banding pattern and the other pure line the F-band of the PGM electrophoretic banding pattern. The female of this cross (heterozygote with F- and S-band) was then crossed with her son and under the sl-CSD the detection of diploid males (showing F- and S-band) was possible. In the present study the establishment of pure isofemale lines was not possible due to time constraints. Only females having the F- and S-band were used for the mother and son crossing. Interestingly, out of 19 males (and one female) only one diploid male could be detected in *D. mollipla* and none in *D. semiclausum*. The mother-son crossing lasted for three days and the mother was allowed to parasitise 25 second and third instar larvae per day, whereas Noda (2000) reported for *D. semiclausum* that after mating with the son the mother was allowed to lay as many eggs as possible into the host larvae every day until death. The different methodologies in terms of experimental duration might have had an influence on male production. Under room temperature (25 °C) *D. mollipla* is able to live and parasitise *P. xylostella* larvae for at least three weeks with a peak female production on the third day (Sithole, pers. communication). However, these studies on *D. mollipla* were done with newly emerged females (Sithole, pers. communication), whereas the mothers of the mother-son crossing were already 15 days old before they mated with their sons and were exposed to the *P. xylostella* larvae.

In summary, the present analyses with PGM as molecular marker to detect genetic variation should be seen as a preliminary study, showing that isoenzyme analysis was suitable to detect diploid individuals in *D. semiclausum* and *D. mollipla*. The presence of diploid males in *D. semiclausum* previously reported by Butcher *et al.* (2000b) and Noda (2000) and in *D. mollipla* should be confirmed in further studies, with isofemale lines being produced prior to crossing and with increased overall duration of the experiment.

4.4 Selection of molecular markers

The best molecular marker should exist as a single copy in a haploid genome, because if several copies (multiple-copy genes) are present, sequences obtained from different individuals might be from different copies of the gene and then phylogenetic analyses become questionable. However, if multi-copy genes have the same sequences, such as mitochondrial and ribosomal genes, the amplification is much easier than single-copy genes. Thus, multiple-copy genes are most commonly used in phylogenetic studies, but generalisation about a given taxonomic level at which a genetic region might be useful should be made with caution (Cruickshank 2002).

4.4.1 Mitochondrial DNA

Insect mitochondrial DNA is a small, extrachromosomal genome, with a size of ~16 kb. In general all animal mitochondrial genomes contain the same 37 genes: two for rRNAs, 13 for proteins and 22 for tRNAs (Wolstenholme 1992). In Hymenoptera, mtDNA was used in phylogeny estimations, population genetics, and taxonomic studies (Dowton and Austin 1995; Carpenter and Wheeler 1999; Mardulyn and Whitfield 1999; Dowton and Austin 2001; Rokas

et al. 2002; Suazo *et al.* 2002). An electronic search in the NCBI GenBank data base (http://www.ncbi.nlm.nih.gov/) with the keywords: Hymenoptera COI, conducted in May 2005, revealed 2641 entries of fragments of the hymenopteran COI gene, which shows that this genetic region is very popular in Hymenoptera research.

In the more recently diverged hymenopteran groups (Apocrita) sequences have a high A+T content especially in the third codon positions of the COI gene (Dowton and Austin 1995; Rokas *et al.* 2002). The A+T frequencies of the *Diadegma* sequences (74.1 %) analysed in this study agree with the COI data of Dowton and Austin (2001) where a high A+T content was detected in parasitic wasps (74.0 ± 0.7 %). This observation is important because having only two nucleotides available at many sides, saturation and homoplasy occur much more frequently and may even influence the phylogenetic analyses (Brower and DeSalle 1994). The qualitative test for saturation and homoplasy (fig 3.9) showed that the COI (mtDNA) became more rapidly saturated than the ITS2 (rDNA). These observations correspond to the results of Reed and Sperling (1999), Dowton and Austin (2001) and Rokas *et al.* (2002). Therefore, additional phylogenetic studies were applied excluding the third codon position to reduce the noise of homoplasious silent sites.

4.4.2 Nuclear DNA

The nuclear ribosomal ITS sequences have been proven to resolve phylogenetic relationships of insects at different levels of divergence. The 176 entries of ITS2 sequences in the order Hymenoptera in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/ keywords: Hymenoptera ITS2, conducted in May 2005) belong to 12 different genera and just one analysis was so far undertaken in the genus *Diadegma* (Wagener *et al.* 2004a). The sequences of the ITS2 region

are generally considered as a highly variable marker and are therefore mainly useful to resolve relationships among closely related species.

The alignment of the ITS2 sequences of all 12 genera in an attempt to construct a broader scale phylogeny of the Hymenoptera failed due to the differences in size of the sequences of several 100 bp. In addition, the removal of regions with ambiguous alignments (Gatesy *et al.* 1993) could not achieve a better grouping for further phylogenetic analyses. However, ITS2 sequences of similar sizes were aligned successfully and the phylogenetic inferences examined for two genera of Braconidae, two genera of Ichneumonidae and one genus of Encyrtidae (fig. 3.13).

In this study, ITS2 sequences of one or two individuals per population/species were investigated, because of the assumption of low intra-genomic and intra-population rDNA diversity within a species as a result of the process known as concerted evolution (see Chapter 1.2.2). However, intra-genomic variation in ITS2 sequences has been reported in deer ticks (Rich *et al.* 1997), mosquitoes (Onyabe and Conn 1999) and *Ageniaspis* sp. (Alvarez and Hoy 2002), and may result in the development of incorrect phylogenies if the variations within individuals and between individuals in the same population differ as much as those between populations and species (Rich *et al.* 1997). Most sequences of the *Diadegma* species investigated showed little or no intra-specific variation. For example in *D. semiclausum* collected from ten different locations spanning a geographical range from Japan and New Zealand to Israel and Austria no single mutation was observed between the individuals. The only exemption was *D. semiclausum* from Taiwan where two individuals were separated by one mutation. No intra-specific variation could be detected in *D. leontiniae*, *D. insulare*, *D. fenestrale* and *D. chrysostictos*. Only one specimen of *D. rapi* was shipped and therefore intra-specific variation could not be examined.

The highest variation occurred between the two samples from two locations of *D. armillata* (4 mutations, 0.6 % divergence, Appendix 5), one mutation was found in *D. blackburni* (0.2 % divergence) and three in *D. mollipla* (0.5 % divergence). Despite the intra-specific variation, the ITS2 sequences were phylogenetically informative and separated all species in different clades (figs. 3.13-3.15).

In conclusion, when ITS2 sequences are used to examine differences in populations, cryptic species, or within genera, molecular taxonomists should be aware of intra-genomic and intra-specific variation and a minimum of two individuals per population/species should be examined so that the potential variability is considered (Alvarez and Hoy 2002). Navajas and Boursot (2003) included cloning procedures in their molecular studies of Tetranychidae to avoid undetected intra-genomic variations. This will make the analyses more reliable but also more expensive compared to other genetic makers (e.g. mtDNA) where only one sequence is needed.

4.4.3 Comparison of mitochondrial and nuclear DNA

There is an ongoing controversy about the mode of evolution among different genetic markers. Navajas *et al.* (1998) compared the inter-specific divergences of the ITS2 and COI between five *Tetranychus* species and suggested that the ITS2 evolves about 2.5 times faster than the COI. Comparable results were found in the present study with nine *Diadegma* species with the ITS2 evolving 3.85 times faster than the COI region (fig. 3.9). Interestingly, the ITS2 divergences are higher in most pair-wise comparisons than the divergences of the COI (tabs. 3.9, 3.10 and Appendices 4 and 5), which was not the case in the study of Navajas *et al.* (1998). Only the intra-specific variation in *D. mollipla* was much higher in the COI than in

the ITS2. Ancestral polymorphism in mites is generally higher for COI than for ITS2 (Navajas *et al.* 1998), however, this cannot be assumed for *Diadegma* wasps.

4.5 PCR-RFLP

PCR-RFLP analyses were developed with two different genetic markers and used to differentiate important *Diadegma* species associated with *P. xylostella* (tabs. 3.5-3.7, figs. 3.3-3.6, Appendices 2 and 3). With this method, biocontrol workers can now easily monitor the release, spread and establishment of an exotic *Diadegma* parasitoid in a new habitat. Similarly, Prinsloo *et al.* (2002) reported on the release and recovery of *Aphelinus hordei* (Kurdjumov) (Hymenoptera: Aphelinidae) in South Africa and developed a PCR-RFLP method to distinguish among three different *Aphelinus* species in order to confirm the establishment of *A. hordei* in the field.

The ITS2 region can be highly variable in length and sequence even between closely related species. Small differences in the size of the amplicons are difficult to resolve on a 2 % agarose gel and further investigations with restriction enzymes have to be carried out. In contrast, larger size differences like those detected in *D. leontiniae* or *D. rapi*, compared to other *Diadegma* species, can be easily detected by a simple PCR amplification with no additional identification step using restriction endonucleases being necessary.

The PCR-RFLP analyses for both genetic markers (ITS2 and COI) were carried out empirically because no molecular data of *Diadegma* species were available. Some restriction enzymes tested were not suitable to separate the *Diadegma* species (e.g. *Cfo*I, *Eco*RI and *Xba*I for the fragment of the COI gene (Appendix 2) and *Dra*I, *Eco*RI and *Eco*RV for the ITS2 region, tab. 3.7, Appendix 3). A quicker way to distinguish different species would be PCR-RFLP analyses in conjunction with a previous analysis of sequence data. Computer programs

are now available to search for suitable restriction enzymes to digest a given sequence (e.g. Webcutter version 2.0: http://www.firstmarket.com/cutter/cut2.html; BioEdit version 7.0.4.1). An initial comparison of suitable restriction enzymes for different sequences will then be comparatively easy and cost-efficient.

In conclusion, it is now possible to separate important *Diadegma* taxa associated with *P. xylostella* (tabs. 3.5-3.7, figs. 3.3-3.6 and Appendices 2 and 3) with the PCR-RFLP analyses of the two genetic markers ITS2 and COI. This method is relatively inexpensive and robust and provides rapid and reliable results to be used either for diagnostic purposes or for biocontrol workers who want to follow up a release of a *Diadegma* species used as a biocontrol agent (e.g. *D. semiclausum*) and can thus be used as a supplement to the morphological key in order to distinguish between morphologically similar *Diadegma* species.

4.6 What is a species?

The current taxonomy stands for a framework that has been developed over the past ~250 years, since the establishment of the binomial naming system by Linnaeus in the 1750s. In the middle of the 19th century Charles Darwin developed the theory of the origin of species leading to the development of several species concepts, which are still in debate.

At present three species concepts exist next to each other:

(i) The oldest species concept, the morphological species, distinguishes individuals by morphological characters. This concept of morphological species is still the most widely used species concept, particularly in plants.

(ii) The morphological species concept was expanded in the 1940s by Mayr to the biological species concept that identifies species, which share a more or less distinct form and are able to interbreed. Distinct geographical populations of the same “kind of organism” are usu-

ally lumped as one species. Different biological species will therefore not interbreed and there will be no significant gene flow. However, if two individuals interbreed rarely and produce viable hybrids this doesn't mean they belong to the same species (Paterson 1988). This concept is useful for species, which have a sexual reproduction but is meaningless for organisms that do not reproduce sexually.

(iii) More recently, in the 1960s, Hennig developed the phylogenetic or evolutionary species concept, where a group of organisms shares a single ancestor. The phylogenetic species concept does not include interbreeding and is less restricted than the biological species concept. Consequently, a higher number of species will be obtained under the phylogenetic species concept than under the biological species concept.

All species concepts depend on the correct identification of species. The identification is conducted by taxonomic specialists with long experience. The knowledge of classical taxonomists is often lost when they retire and the number of taxonomists has been decreasing over the last decades. With the development of molecular techniques (PCR, sequencing), the availability of universal genetic markers and the different ways of analysing of these data sets, additional data are now available, which can and should be combined with the already existing morphological and ecological knowledge to confirm or verify a species status and its assignment to higher level taxa (see Chapter 4.7 below). Tautz *et al.* (2003) discussed the development of a universal DNA-based taxonomy system to overcome the present crises in taxonomy, whereas Hebert (2003a, b) developed a DNA barcoding system for biological identifications, where molecular data, biological and ecological data sets are analysed in combination. Briefly, DNA barcoding is a technique to differentiate species using a short DNA sequence from a standardised region of the genome (e.g. COI of mtDNA) and can be used by

taxonomists to help detect, characterise and distinguish species as well as to allocate unidentified individuals to species. Right now, the obtained barcode sequences can be submitted to the public reference libraries (e.g. Barcode section of GenBank or Barcode of Life Database (BOLD)). The sequences will then be compared to a sequence of a known species. In 2004 the Consortium for the Barcode of Life (CBOL) was founded and has arranged a Data Analyses Working Group to improve the ways how DNA barcode data can be examined, presented and employed by users (see http://w.w.w.barcoding.si.edu for a review).

During this study, two questions were to be answered:

(i) Do the African *Diadegma* belong all to the same species?

(ii) Do the native parasitoid (*D. mollipla*) in Kenya and the exotic parasitoid (*D. semiclausum*) hybridise?

(i) When *D. mollipla* individuals from eastern and southern Africa were analysed with molecular methods (PCR-RFLP) differences in the restriction profiles were detected suggesting that the individuals from different geographical locations were genetically distinct (tabs. 3.5-3.7, figs. 3.3-3.6). Does this mean that *D. mollipla* from eastern and southern Africa exist as cryptic species or as subspecies? By definition, subspecies are populations within a species that are sufficiently distinct (considering the molecular differences) but are not distinct enough to prevent hybridisation where two populations come into contact (Wikipedia Encyclopedia; http://en.wikipedia.org/wiki/Subspecies).

The results of the crossing experiments (see chapter 4.2; tabs. 3.1, 3.2) clearly revealed, that interbreeding between both populations took place and fertile offspring was produced. Furthermore, *D. mollipla* from eastern and southern Africa were morphologically similar (Horstmann, pers. communication). In addition, the genetic distances (based on Kimura 2-Parameter, Kimura 1980) between *D. mollipla* from these two different geographical regions

ranged only between 0.2-2.5 % for the COI region and between 0.2-0.5 % for the ITS2, respectively (Appendices 4 and 5).

Subsequent phylogenetic analyses underlined these results by clustering all *D. mollipla* from different geographical locations together with strong bootstrap support (figs. 3.9-3.15).

When Azidah *et al.* (2000) referred to all African *Diadegma* species attacking *P. xylostella* as *D. mollipla*, a well-known parasitoid of *P. opercullella,* they however stated that "it is not impossible that there are two separate biological species". The examination of five *D. mollipla* reared from *P. opercullella* with PCR-RFLP of COI and ITS2 revealed no differences in the restriction patterns to the known restriction patterns of *D. mollipla* reared from *P. xylostella*.

In conclusion, "..the question whether there is one species or two.." (Azidah *et al.* 2000) is now resolved and the native *Diadgema* species parasitising *P. xylostella* in eastern and southern Africa should be named *D. mollipla*.

Under both species concepts - the biological species concept and the phylogenetic species concept - both populations of *D. mollipla* clearly belong to the same taxon. However, it is not clear whether *D. mollipla* from eastern Africa and southern Africa can be considered as two different subspecies.

(ii) According to the biological species concept, the inability of successful hybridisation between *D. mollipla* and *D. semiclausum* classifies them as distinct species. However, crossing studies may present problems as a diagnostic test for species differentiation. Neither sterility nor viability of offspring resulting from cross mating is always a useful criterion of species status (Paterson 1988). The existence of *Wolbachia,* a cytoplasmatically inherited bacterium can also be responsible for reproductive incompatibility as discussed in Chapter 4.2.

Morphologically, *D. semiclausum* and *D. mollipla* can be differentiated (Azidah *et al.* 2000), however, particularly males are very difficult to separate from each other. Additionally, the

genetic distances obtained by the Kimura 2-parameter model (Kimura 1980) between *D. mollipla* and *D. semiclausum* were 8.9 % for the ITS2 and 8.0 % for the COI, which clearly divided them into separate species.

4.7 Phylogenetic analyses

The relation of an organism to other organisms is build on characters which can be based on morphology, physiology, ecology, behaviour, biochemistry or molecular data. At least three main methodologies have to be considered for the development of classifications based on the three species concepts already discussed in Chapter 4.6. A comparison of the three schools of systematics: the phenetic, cladistic (phylogenetic) and classical evolutionary systematics was given by Mayr (1990). However, the debates which school of systematics is more objective or suitable are still going on. Briefly, the phenetic systematic focuses on overall similarities among organisms, whereas the cladistic systematic is based on the same ancestral origin and only monophyletic taxa are allowed. The evolutionary classification combines both criteria where not all characters are supposed to be of equal value and are weighted correspondingly (Mayr 1990).

Classifications are described in graphic figures as tree-like dichotomous branching graphs or dendrograms. A phenogram is produced from phenetic data where similarities are grouped together but the tree does not present information about feasible lines of ancestry. Classifications produced from cladistic information are represented in cladograms whereas dendrograms of both phenetic and phylogenetic data are phylogenetic trees (phylograms). These trees indicate not only the cladistic branching but also the relative amount of change that has taken place.

A variety of options are available to examine phylogenetic relationships of molecular data sets (see Swofford *et al.* 1996 for a review) but still, many controversies exist about which technique is most appropriate for inferring phylogenetic relationships.

Distance-based methods (Neighbour-Joining (NJ) or the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)) involve the transformation of sequence data into a matrix of pair-wise genetic distances, whereas maximum parsimony (MP) and maximum likelihood (ML) are character-based methods. In phylogenetics ML and MP are more widely used. However, they might not always be appropriate for a particular data set. The MP methods focus on character values for each species. These methods work by selecting trees that minimize the total tree length. In other words: between two trees, the tree requiring the fewest character state changes is the best. Maximum likelihood methods attempt to estimate the actual amount of changes (maximizing the likelihood) according to the given evolutionary history.

A good strategy to analyse phylogenetic data is to examine the molecular data sets with both ML and MP methodologies. In addition, by using the bootstrapping method (Felsenstein 1985), the reliability of various constructed branches of a phylogenetic tree can be examined.

However, there are practical limitations (computational constraints/time limitations) to using maximum likelihood, especially with large data sets. Furthermore, maximum likelihood methods are not suitable to analyse combined molecular/morphological data sets.

4.7.1 Phylogenetic relationship within the genus *Diadegma*

Sequence analyses of a fragment of the mitochondrial COI gene and the ITS2 of rDNA were phylogenetically informative and separated all nine *Diadegma* species examined. The topology of the maximum parsimony and maximum likelihood trees revealed a robust monophyly for all *Diadegma* species with high bootstrap support for each species (figs. 3.10, 3.12 and

3.13). The phylogram constructed from the mitochondrial DNA (MP and ML analyses, fig. 3.10) only clustered *D. leontiniae* and *D. rapi* together (with moderate bootstrap support) with no phylogenetic relationship being obvious to the other *Diadegma* species analysed. To avoid saturation of the data set, the third codon was excluded and the resulting phylogenetic trees showed differences in the bootstrap support that were moderate in both trees (figs. 3.11, 3.12). No phylogenetic differences were observed in MP and ML analyses between the two *D. mollipla* populations and only the MP analyses showed no cluster of *D. leontiniae* and *D. rapi* but a moderate cluster of *D. mollipla* and *D. chrysostictos*. It is noteworthy that the MP analyses analysed only 20 parsimony informative characters and the ML analyses examined only 35 distinct characters (exclusive *Meloboris* sp. as out-group). Nevertheless, the resulting phylograms of all different analyses were very similar, and except of the cluster between *D. leontiniae* and *D. rapi*, no phylogenetic structure within the genus could be observed. These results were surprising because the COI region was proven to be phylogenetically useful at genus level in many arthropods e.g. in beetles (Coleoptera: Chrysomelidae: *Diabrotica,* Clark *et al.* 2001, Coleoptera: Chrysomelidae: *Ophraella*, Funk *et al.* 1995), in Lepidoptera: Lycaenidae: *Chrysoritis* (Rand *et al.* 2000) as well as in Hymenoptera: Cynipidae: (Rokas *et al.* 2002). The poor phylogenetic structure observed in the genus *Diadegma* might be a consequence of the small size of the fragment of the COI gene (only 498 bp) examined. Larger fragments/genes of the mtDNA (e.g. COI/COII or cytochrome *b* or a combination of all) should be analysed in further studies of the genus *Diadegma*.

By contrast, MP and ML analyses of the nuclear DNA (ITS2, figs. 3.12 and 3.13) showed one cluster of all palearctic *Diadegma* species examined together with *D. blackburni* (nearctic and oceanic distribution). *Diadegma insulare* with nearctic and neotropical distribution was clustered separately as well as *D. rapi* (Australian distribution) and *D. leontiniae* (neotropical distribution). Given the different geographical origins assumed for *D. rapi* and *D. leontiniae*

(Azidah *et al.* 2000), their grouping in the current analysis is to some degree puzzling and calls for additional studies to justify their phylogenetic status. Similar analyses need to be conducted to clarify the origin of *D. blackburni*. According to the results, *D. blackburni*, originally described from Hawaii (Cameron 1883), might have originated in the palearctic region. Henneman and Memmott (2001) suggested that *D. blackburni* was accidentally introduced into Hawaii. However, the origin is still not clear.

On the basis of ITS2 sequences, all *Diadegma* species with a palearctic distribution as well as *D. blackburni* from Hawaii formed a subclade in the dendrogram, while such a differentiation was not present on the basis of COI sequence data. This might also reflect the different mode of evolution and variation in base composition previously described for both genetic regions (Simon *et al.* 1994; Lunt *et al.* 1996).

In the present study three different phylogenetic approaches were employed where the topology of the distance-based tree based on COI gene sequences (fig. 3.9) was not consistent with the phylogenetic trees developed by MP/ML analyses (figs. 3.10-3.12). In contrast, the tree topologies (figs. 3.13- 3.15) established from the ITS2 sequences showed great similarity concerning the three mayor clades of *Diadegma* species with palearctic, nearctic and neotropical/Australian distribution.

4.7.1.1 Combining of data sets for phylogenetic analyses

There is no general rule how to analyse separate data sets that represent different “classes of characters” such as morphology, mtDNA or nuclear DNA collected from hymenopteran insects and a controversy surrounding the combination of data partitions exists (Bull *et al.* 1993; Miyamoto and Fitch 1995; Huelsenbeck and Bull 1996; Belshaw *et al.* 2000; Dowton and Austin 2001). As discussed before for the genus *Diadegma*, the COI gene and the ITS2 region

evolve at different rates, and combining both data sets might be difficult for phylogenetic studies (Bull *et al.* 1993). Belshaw *et al.* (2000) and Dowton and Austin (2001) reported incongruence between nuclear and mitochondrial DNA in Hymenoptera when both genes were analysed together. This incongruence might be a result of artefacts caused by noise (selective pressure e.g. towards the AT-content in mitochondrial genes) rather than differing phylogenetic histories. However, the combining of different data sets (morphological and/or molecular data) increases the phylogenetic information and might reflect their common history. The analyses of such heterogeneous data sets most likely recover phylogenetic exactness (Belshaw *et al.* 2000, Dowton and Austin 2001).

In this study two different data partitions (fragment of COI gene (mtDNA) and ITS2 region of nuclear DNA) were tested for heterogeneity (ILD-test) before combining them for further analyses. The partition-homogeneity test did reject the null hypothesis for the complete combined data sets but when the third codon of the COI gene was excluded, no significant differences were detected between both data partitions. The MP and ML analyses of the combined data sets revealed a similar tree topology to the ML/MP topologies of the ITS2 phylogenies indicating that the analyses has revealed a robust phylogeny of the genus *Diadegma*. If it is assumed that *D. blackburni* was introduced from Europe to Hawaii as discussed above, the phylogeny of the genus *Diadegma* has a phylogeographical structure where the species with the paleacrtic distribution are separated from the neartic species. Interestingly, the species native to Brazil and Australia also form a distinct clade.

In summary, this study presents an initial step towards understanding the genetic relationship between members of the genus *Diadegma*, a taxon where no phylogenetic information existed previously. Additional sampling of *Diadegma* species and sequence analyses of other genetic regions in conjunction with morphological data are needed to confirm and extend the phylogenetic relationships developed in this study.

4.7.2 Phylogenetic relationships within the superfamily Ichneumonoidea

Dowton and Austin (2001) and Carpenter and Wheeler (1999) reported phylogenetic relationships on a higher level (e.g. family and superfamily) of apocritan wasps based on molecular sequences (nuclear (28S) and mitochondrial DNA (COI, 16S)) in combination with morphological data. The superfamily Ichneumonoidea, which is a monophyletic group based on extensive morphological evidence (Sharkey and Wahl 1992), is recognised with two extant families: the Braconidae and the Ichneumonidae.

When the COI sequences of the Ichneumonoidea were investigated in the database GenBank, 270 entries with sequences of 47 different genera of both Braconidae and Ichneumonidae were found, whereby 38 genera belong to the family Braconidae and only nine to the Ichneumonidae. The sequences of *Meloboris* and *Diadegma* obtained during this study were not yet included into this electronic search.

The observed MP topology revealed a monophyly of the *Diadegma* species, which clustered together with other Ichneumonidae. Additionally, several Braconidae were also part of this first major clade. The second clade included Braconidae as well as one subcluster of Ichneumonidae while one Ichneumonidae, three Braconidae and two Chalcidoidea (as out-group) formed the third major clade. These results are very puzzling and are in contrast to Austin and Dowton (2001) who showed on the basis of molecular sequences (simultaneous analyses of COI and 16S and 28S) that Bracconidae and Ichneumonidae are both monophyletic. However, the authors included only 12 different species while the present study involved in total 38 Braconidae and Ichneumonidae and excluded the third codon position of the COI region from the data set. Mardulyn and Whitefield (1999) discovered poor phylogenetic relationships within the subfamily Microgastrinae of the Braconidae and suggested that the COI region might be inappropriate for the level of divergence examined. This result could be confirmed in the present study were Microgastrine were found in all three major clades.

These phylogenetic data analyses were based on 446 characters, which might be too little for the construction of a comprehensive relationship between members of the superfamily Ichneumonoidea. The fact that the third codon position of the COI gene in Hymenoptera might get saturated too fast excluded this gene from the analyses of a higher-level phylogeny.

Thus, the COI region was proven to be phylogenetically useful in Hymenoptera but should be analysed together with additional genetic markers like the 16S or chytochrome *b* gene of the mitochondrial DNA.

The picture was different for the ITS2 sequences where 12 hymenopteran genera were obtained from the database GenBank (176 entries in total) and only sequences of the four genera of Braconidae and Ichneumonidae (incl. *Meloboris* and *Diadgema* obtained during this study and submitted to EMBL in 2002) could be aligned. They showed a monophyly for the genera *Diadegma, Meloboris, Leiophron* and *Peristenus* of the superfamily Ichneumonoidea (fig. 3.19) with a strong bootstrap support. *Meloboris* sp. and *Diadegma* species belonging to the subfamily Campopleginae were clustered together. Additional analyses of species classified to this subfamily (e.g. *Dusoma*) should be conducted to confirm the existence of the monophyletic group of the campoplegine wasps.

5 Conclusions and recommendations

The genus *Diadegma* is a very common genus in many natural systems, important to biological control and difficult to identify for non-specialists. *Diadegma semiclausum*, *D. insulare* and *D. armillata* gained economical value as biological control agents and have been introduced for that purpose to other geographical regions (Talekar and Shelton 1993, Unruh *et al.* 2003, Leibee, pers. communication).

How can the present study be useful for biological control?

(i) Establishment of the taxonomic status of biological control agents before release.

(ii) Detection of possible hybridisation events between native and exotic parasitoids.

(iii) Improvement of mass rearing techniques of parasitoids.

(i) The reintroduction of the biological control agent *D. insulare* in Hawaii failed due to misidentification (Johnson *et al.* 1988). Morphological characters of the subfamily Campopleginae are very variable and there are many misidentifications reported in the literature (Horstmann, pers. communication). During the present study all specimens were assessed by taxonomists to determine their identity prior to DNA extraction. A proper identification of *Diadegma* wasps is possible by relating the DNA information obtained in this study and the correct scientific name with the available biological information and its taxonomic affinities, morphology, distribution and ecological role. For further investigations voucher specimens associated with the obtained sequences (accession nos., tab. 2.1) were kept as a record at a public place (i.e. Biosystematics Unit at ICIPE, Nairobi, Kenya). With the inexpensive and rapid PCR-RFLP method a follow up of the release and recovery of the introduced insects can be easily conducted.

(ii) The amplification of genetic material of nine *Diadegma* species from different geographical locations was performed with two different genetic markers. It can be assumed that additional *Diadegma* wasps can be examined in the same manner. In addition, genetic distances between species can be calculated with the DNA sequence information, which might give a first hint about a possible hybridisation between native and exotic parasitoids.

(iii) Stouthamer *et al.* (1992) suggested that a failure of Ichneumonoidea in biological control might be caused by the occurrence of diploid males resulting from sl-CSD. Prior to a successful release, the given parasitoid has to be mass-reared. A male biased sex ratio in a laboratory culture and the reduction in the population growth can be seen as an indication of the existence of diploid males. However, even the size of the host larvae and the host food quality has an influence on the sex ratio (Fox *et al.* 1990). To avoid the reduction of the diversity of different sex alleles in a laboratory culture a clear guidance about a proper rearing of *Diadegma* parasitoids (e.g. keeping different generations of parasitoids separately in order to avoid mother and son crossing) will be necessary.

In conclusion, the success of any biological control attempt/program depends very much on the correct taxonomic status of the insect that will be introduced as well as biological (e.g. specialists vs. generalists) and physiological (e.g. climate) parameters. Precautions in the mass rearing of the biological control agents are essential to minimize failure of the biological control caused by low allelic diversity at the sex locus.

A molecular phylogeny of *Diadegma* was established on a lower (within the genus) and a higher (within the superfamily Ichneumonoidea) taxonomic level for the first time.

It can be summarized that sequences of the ITS2 region of the ribosomal DNA are useful to distinguish different species but because of the high variability of the region the sequences are difficult to align and therefore not suitable for higher phylogenetic studies.

In contrast, the COI region is known to solve lower and higher phylogenetic relationships (Navajas *et al.* 1998; Mardulyn and Whitfield 1999; Dowton and Austin 2001). However, in the present study the phylogenetic information of the fragment obtained from the COI gene within the genus *Diadegma* was very poor. This could be related to the high third codon saturation. The phylogenetic tree of the Ichneumonoidea based on the COI sequences was puzzling and differed much from previous analyses by Dowton and Austin (2001). To resolve higher taxonomic phylogenies additional markers (*cytochrome* b of mtDNA or 28S of rDNA) are recommended with a combined phylogenetic analysis of all characters. Simultaneous analyses of both molecular and morphological characters might result in the most correct phylogenetic tree of the species examined.

6 Summary

The genus *Diadegma* (Hymenoptera: Ichneumonidae: Campopleginae) represents a large group of parasitoids with 201 species worldwide. Adult *Diadegma* females parasitise larvae of various lepidopteran species and some species, in particular *Diadegma insulare* (Cresson) and *D. semiclausum* (Hellén), have gained economic importance as biological control agents of *Plutella xylostella* (Linnaeus).

A low parasitism rate of <15 % of the parasitoid complex (*Diadegma* sp., *Oomyces sokolowskii* (Kurdjumov) and *Diaplazon laetatorius* (Fabricius)) in unsprayed cabbage and kale fields infested with *P. xylostella* in eastern and southern Africa was the starting point for the development of a biological control project for *P. xylostella* which was implemented by the International Centre of Insect Physiology and Ecology (ICIPE), Kenya.

One of the objectives of the biocontrol project was to examine the taxonomic status of *Diadegma* species associated with *P. xylostella* in eastern and southern Africa and the exotic parasitoid *D. semiclausum* imported to Kenya from Taiwan (Asian Vegetable Research and Development Centre, AVRDC) by cross breeding experiments and molecular methods. Thus, two different molecular regions, a fragment of the mitochondrial *cytochrome* c *oxidase* subunit (COI) and the second internal transcribed spacer (ITS2) of ribosomal DNA were amplified utilising polymerase chain reaction (PCR) and digested afterwards with several restriction enzymes (PCR-Restriction Fragment Length Polymorphism-RFLP).

In the due course of the study examinations of several *Diadegma* species attacking *P. xylostella* were undertaken with the PCR-RFLP method developed previously for the African *Diadegma*. This molecular method could solve some taxonomic difficulties of the genus *Diadegma*. Additionally, sequence analyses were used to investigate the phylogenetic relationship of nine *Diadegma* species (*D. blackburni* (Cameron), *D. insulare*, *D. leontiniae* (Brèthes), *D.*

chrysostictos (Gmelin), *D. armillata* (Gravenhorst), *D. fenestrale* (Holmgren), *D. mollipla* (Holmgren), *D. semiclausum*, *D. rapi* (Cameron)) and the phylogenetic relationship of the genus *Diadegma* within the superfamily Ichneumonoidea.

Cross breeding experiments were carried out between two populations of *D. mollipla* from eastern and southern Africa. No significant differences in the total number of progeny per female and the number of male offspring were obtained, whereas the female progeny showed significant differences. Hybrid females resulting from both reciprocal crosses were reproductively compatible with males of both parental lines, which indicated that no genetic incompatibility was apparent between the two *D. mollipla* populations.

In contrast, crosses between *D. mollipla* and *D. semiclausum* resulted only in the occurrence of male offspring, which is typical for unfertilised progeny in *Diadegma*.

The laboratory cultures of *D. mollipla* and *D. semiclausum* were highly male biased. Inbreeding, where homozygosity is much higher, is leading to a higher diploid male production. Diploid males can easily be detected by isoenzyme variations as a genetic marker.

Heterozygote females/males of *D. semiclausum* and *D. mollipla* were identified by phosphoglucomutase (PGM) electrophoretic banding patterns. Crosses between a mother (heterozygote, diploid) and her son (homozygote, haploid) resulted in one diploid male in *D. mollipla* and none in *D. semiclausum*. Information about diploid males in *D. semiclausum* detected with PGM has already been published and different methodologies might be the reason why in *D. semiclausum* no diploid male was detected. Therefore the present analyses with PGM as molecular marker should be seen as a preliminary study.

Six *Diadegma* species (*D. leontiniae*, *D. insulare*, *D. fenestrale*, *D. semiclausum*, *D. mollipla* and *D. rapi*) attacking *P. xylostella* were differentiated with PCR-RFLP. The ITS2 region was

successfully amplified and afterwards digested with 13 different restriction enzymes. Three restriction enzymes did not cut the DNA at all whereas with restriction enzyme *Cfo*I all *Diadegma* species could be differentiated. Amplifications of the COI region were difficult and were restricted only to the African *Diadegma* species (two populations of *D. mollipla*) and *D. semiclausum*. Eleven restriction enzymes were used and with *Alu*I both *D. mollipla* populations and *D. semiclausum* were differentiated. Three restriction enzymes were not suitable to digest the PCR products. With the present study the taxonomic dilemma regarding important *Diadegma* species attacking *P. xylostella* could be solved.

The phylogenetic relationships of nine *Diadegma* species were investigated with sequences of a fragment of the COI gene and the ITS2 region. Distance-based methods (Neighbour-Joining) and character-based methods (maximum parsimony (MP) and maximum likelihood (ML)) were used for both genetic markers. In addition, to avoid saturation of the data set of mitochondrial DNA (COI region), the third codon was excluded for analyses with ML and MP.

The resulting phylograms of all different analyses of the COI region were very similar, and with the exception of one cluster of *D. leontiniae* and *D. rapi*, no phylogenetic structure could be observed. The poor phylogenetic structure observed in the genus *Diadegma* might be a consequence of the small size of the fragment of the COI gene (498 bp) examined.

In contrast, the tree topologies established from the ITS2 sequences showed great similarity concerning the three mayor clades of *Diadegma* species with palearctic, nearctic and neotropical/Australian distribution. *Diadegma blackburni*, probably accidentally introduced to Hawaii, was clustered together with the palearctic species.

The combination of different data sets increases the phylogenetic information and is most likely recovering phylogenetic exactness. In this study two different data partitions (fragment of COI gene and ITS2 region) were tested for heterogeneity (ILD-test) before combining them

for further analyses. The partition-homogeneity test did reject the null hypothesis for the complete combined data sets but when the third codon of the COI gene was excluded, no significant difference was detected between both data partitions. The MP and ML analyses of the combined data sets revealed a similar tree topology to the ML/MP topologies of the ITS2 phylogenies indicating that the analyses have revealed a robust phylogeny of the genus *Diadegma*.

The superfamily Ichneumonoidea, which is a monophyletic group based on extensive morphological evidence, is recognised with two extant families: the Braconidae and the Ichneumonidae. When the COI sequences of the Ichneumonoidea were investigated in the database GenBank, 47 different genera of both Braconidae and Ichneumonidae were found. The observed MP topology revealed a monophyly of the *Diadegma* species, which clustered together with other Ichneumonidae, however, the monophyly of Braconidae and Ichneumonidae could not be confirmed. Thus, the examined COI region might not be useful for analyses of a higher-level phylogeny.

ITS2 sequences of four genera of Braconidae and Ichneumonidae were obtained from the database GenBank. MP analyses showed a monophyly for the genus *Diadegma*. The ITS2 region is characterised by many insertions and deletions and an alignment of the sequences is sometimes impossible. Therefore, this genetic region is also not useful for analyses of a higher-level phylogeny.

7 Zusammenfassung

Die Gattung *Diadegma* (Hymenoptera: Ichneumonidae: Campopleginae) umfasst eine grosse Gruppe von Schlupfwespen mit 201 Arten weltweit. Das Wirtsspektrum beschränkt sich auf die Ordnung Lepidoptera, wo sie die Larven- und Puppenstadien parasitieren. Einige Arten, insbesondere *Diadegma insulare* (Cresson) and *D. semiclausum* (Hellén) haben ökonomische Bedeutung und werden zur biologischer Bekämpfung der Kohlmotte (*Plutella xylostella* (Linnaeus) erfolgreich eingesezt.

Im östlichen und südlichen Afrika wurde 1998 herausgefunden, dass in unbehandelten, von *P. xylostella* befallenen Kohlfeldern, die Parasitierungsleistung eines Komplexes bestehend aus *Diadegma* sp., *Oomyces sokolowskii* (Kurdjumov) und *Diaplazon laetatorius* (Fabricius) bei der Kohlmotte unter 15 % lag. Diese Erkenntnis führte zu der Entwicklung eines Projektes zur biologischen Bekämpfung der Kohlmotte im östlichen Afrika, welches seit 2000 vom International Centre of Insect Physiology and Ecology (ICIPE), Kenia, durchgeführt wird.

Im Rahmen dieses Projektes entstand die vorliegende Arbeit, wobei in erster Linie der taxonomische Status der *Diadegma* Arten aus dem östlichen und südlichen Afrika sowie des aus Taiwan (Asian Vegetable Research and Development Centre, AVRDC) nach Kenia eingeführten Parasitoiden *D. semiclausum* mit Versuchen zur genetischen Verträglichkeit (Kreuzungsversuche) und mit molekularbiologischen Methoden untersucht werden sollte. Dabei wurden mit Hilfe der Polymerasekettenreaktion (PCR) zwei sich unterschiedlich entwickelnde genetische Regionen (ein Fragment der mitochonrialen Cytochrome *c* Oxidase Untereinheit (COI) sowie der zweite „internal spacer“ (ITS2) der ribosomalen DNA) amplifiziert und anschliessend mit Restriktionsenyzmen verdaut (PCR-Restriction Fragment Length Polymorphism-RFLP).

Im Laufe der Arbeit wurde festgestellt, dass die für die Unterscheidung der afrikanischen *Diadegma* Arten entwickelte molekulare Methode (PCR-RFLP) auch auf die weiteren *Diadegma* Arten, die die Kohlmotte befallen, ausgeweitet werden sollte. Diese Methode konnte damit zur Aufklärung der taxonomischen Schwierigkeiten in der Gattung *Diadegma* beitragen. Desweiteren wurden mit Hilfe von Sequenzierungen die phylogenetischen Beziehungen zwischen neun *Diadegma* Arten (*D. blackburni* (Cameron), *D. insulare*, *D. leontiniae* (Brèthes), *D. chrysostictos* (Gmelin), *D. armillata* (Gravenhorst), *D. fenestrale* (Holmgren), *D. mollipla* (Holmgren), *D. semiclausum*, *D. rapi* (Cameron)) sowie die Beziehung der Gattung *Diagema* innerhalb der Überfamilie Ichneumonoidea aufgezeigt.

In Kreuzungsversuchen zwischen der südafrikanischen *D. mollipla* Population und der kenianischen *D. mollipla* Population sowie zwischen Rückkreuzungen wurden keine signifikanten Unterschiede zwischen den Nachkommen pro Weibchen und der männlichen Nachkommenschaft festgestellt. Signifikante Unterschiede bestanden jedoch im Auftreten der weiblichen Nachkommen. Hybride weibliche Nachkommen aus beiden reziproken Kreuzungen waren reproduktiv mit Männchen aus beiden elterlichen Populationen, was darauf zurückzuführen ist, dass keine genetische Unverträglichkeit zwischen den beiden räumlich getrennten *D. mollipla* Populationen besteht. Dagegen war eine Kreuzung zwischen *D. mollipla* und *D. semiclausum* nicht möglich; sie führte nur zu Männchen. Dies ist typisch für eine unbefruchtete Nachkommenschaft bei *Diadegma*.

Die Laborkulturen von *D. mollipla* und *D. semiclausum* waren durch Inzucht gekennzeichnet, was durch den hohen Anteil von Männchen sichtbar wurde. Inzucht führt zu einer höheren Homozygotie und dadurch zu einem stärkeren Auftreten von diploiden Männchen. Diese können leicht mit Hilfe von Isoenzym-Variationen identifiziert werden. Heterozygote Weibchen/ Männchen von *D. semiclausum* und *D. mollipla* wurden mit Phosphoglukomutase (PGM)

elektrophoretisch aufgetrennt. Bei Kreuzungen zwischen Mutter (heterozygot, diploid) and ihrem Sohn (homozygot, haploid) wurde ein diploides Männchen in *D. mollipla* und keines in *D. semiclausum* gefunden. Aufgrund der geringen Anzahl von Tieren und der kurzen Versuchsdauer sollten die vorliegenden Versuche jedoch als Vorstudien gewertet werden.

Die Differenzierung von sechs *Diadegma* Arten, welche die Kohlmotte parasitieren (*D. leontiniae*, *D. insulare*, *D. fenestrale*, *D. semiclausum*, *D. mollipla* and *D. rapi*), erfolgte mit der eigens dafür entwickelten PCR-RFLP Methode. Dabei wurde die ITS2 Region erfolgreich amplifiziert und mit 13 unterschiedlichen Restriktionsenzymen verdaut. Da diese PCR-RFLP Untersuchung empirisch erfolgte verdauten drei dieser Enzyme die DNA nicht. Jedoch konnten mit Restriktionsenzym *Cfo*I alle sechs *Diadegma* Arten unterschieden werden. Die Amplifikation der COI Region erwies sich als schwieriger und wurde daher auch nur auf die afrikanische *Diadegma* Art (*D. mollipla*) und *D. semiclausum* begrenzt. Elf Restriktionsenzyme kamen zur Anwendung und mit *Alu*I konnten die *D. mollipla* Populationen aus dem südlichen und östlichen Afrika sowie die nach Ostafrika eingeführte Schlupfwespe *D. semiclausum* unterschieden werden. Drei Enzyme konnten die DNA nicht verdauen. Mit diesen PCR-RFLP Untersuchungen konnten die Unklarheiten in der Taxonomie der afrikanischen *Diadegma* Art gelöst werden.

Die Untersuchung der phylogenetischen Beziehungen von neun *Diadegma* Arten erfolgte mit Sequenzen eines Fragments des COI Genes sowie der ITS2 Region. Folgende Methoden kamen für beide Regionen zur Anwendung: auf den genetischen Abstand (Neighbour-Joining) beruhende bzw. auf Charaktern (Maximum Parsimony (MP) und Maximum Likelihood (ML)) beruhende Methoden. Zusätzlich wurde noch die dritte Codon Position vom Datensatz der COI Region entfernt, um Sättigung und Homoplasie zu vermeiden.

Interessanterweise zeigten die resultierenden Clusteranalysen der COI Region wenige Unterschiede auf und fassten nur *D. leontiniae* and *D. rapi* zusammen. Eine phylogenetische Struktur innerhalb der Gattung konnte mit Hilfe dieser genetischen Region nicht aufgezeigt werden. Dies ist vermutlich auf die relativ kleine Grösse des untersuchten COI Fragmentes (498 bp) zurückzuführen.

Dagegen zeigten die Clusteranalysen, die mit den ITS2 Sequenzen durchgeführt wurden, drei große Cluster von *Diadegma* Arten mit palearktischer, nearktischer und neotropischer / australischer Verteilung auf. *Diadegma blackburni*, wahrscheinlich versehentlich nach Hawaii eingeschleppt, wurde zu den Arten mit palearktischer Verteilung gerechnet.

Es ist anzunehmen, dass die Kombination zweier Datensätze zu einer größtmöglichen Genauigkeit der phylogenetischen Analysen führt. Deswegen wurde ein Heterogenitätstest (IDL-Test) mit beiden Datensätzen (COI und ITS2) durchgeführt, der jedoch eine Kombination der phylogenetischen Analysen ablehnte. Als die dritte Codon Position der COI Region entfernt wurde und der Datensatz anschliessend nochmals mit den Daten der ITS2 Region verrechnet wurde, konnten keine signifikaten Unterschiede zwischen den Datensätzen mehr festgestellt werden. Die MP und ML Analysen dieser kombinierten Daten führten zu ähnlichen Clustern, wie sie bei den Analysen der ITS2 Daten entstanden waren. Dies unterstreicht die Robustheit der Clusteranalysen der ITS2 Region.

Die Überfamilie Ichneumonoidea ist eine monophyletische Gruppe basierend auf morphologischen Merkmalen und besteht aus den Familien Braconidae and Ichneumonidae.

Bei elektronischen Recherchen (Datenbank GenBank) konnten für Ichneumonoidea COI Sequenzen von 47 verschiedene Gattungen der Braconidae und Ichneumonidae gefunden werden. Die durchgeführten MP Analysen zeigten eine Monophylie für *Diadegma* Arten auf, jedoch konnte keine Monophylie für Braconidae und Ichneumonidae festgestellt werden. Dies

stellt die Nützlichkeit dieser relativ kleinen COI Region als genetischer Marker für Studien höherer phylogenetischer Beziehungen in Frage.

Die durch die elektronischen Recherchen (Datenbank GenBank) ermittelten ITS2 Sequenzen für die Ichneumonoidea lieferten nur vier Gattungen der Braconidae und Ichneumonidae. Die Monophylie für den Genus *Diadegma* konnte durch MP Analysen bestätigt werden. Aufgrund der vielen Einfügungen und Streichungen von Basenpaaren in der ITS2 Region von nahe verwandten Arten ist jedoch auch diese genetische Region für Studien höherer phylogenetischer Beziehungen nicht geeignet.

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Appendix 1

List of reagents used in the present study and their suppliers. Reagents were ordered in Germany if not indicated differently.

Reagent	Supplier
Agarose	Roche Diagnostics, Mannheim; Sigma-Aldrich Chemie, Steinheim
Bromphenol blue	Sigma-Aldrich Chemie, Steinheim
BSA	MBI Fermentas, St. Leon-Rot; Carl Roth, Karlsruhe
Citrate	Serva, Heidelberg
Citric acid trisodium salt dehydrate	Sigma-Aldrich Chemie, Steinheim
COI primers 3, 5	Integrated DNA Technologies, Coralville, IA, USA
COI primer 4	Integrated DNA Technologies, Coralville, IA, USA; MWG Biotech, Ebersberg; Invitrogen, UK
COI primer 8	EUROBIO, France
COI primer 10	MWG Biotech, Ebersberg
DNA Extraction Kit	MBI Fermentas, St. Leon-Rot
dNTP	MBI Fermentas, St. Leon-Rot
EDTA	Carl Roth, Karlsruhe; Sigma-Aldrich Chemie, Steinheim
Ethanol	Fisher Scientific, UK
Ethidium bromide	Serva, Heidelberg
Glacial acetic acid	Fisher Scientific, UK
α-D-glucose-1-phosphate	Sigma-Aldrich Chemie, Steinheim
Glucose-6-phosphate-	Sigma-Aldrich Chemie, Steinheim
HCl	BHD Labratory Supplies, UK
L-histidine mono-HCl	Sigma-Aldrich Chemie, Steinheim
Histidine	Sigma-Aldrich Chemie, Steinheim

Appendix 1: Continued.

Reagent	Supplier
Isocitrate dehydrogenase	Sigma-Aldrich Chemie, Steinheim
ITS2 primers	Integrated DNA Technologies, Coralville, IA, USA
Potassium acetate	Sigma-Aldrich Chemie, Steinheim
50 bp-ladder / 100 bp-ladder	MBI Fermentas, St. Leon-Rot
λ-DNA	MBI Fermentas, St. Leon-Rot; Invitrogen, UK
Mineral oil	Sigma-Aldrich Chemie, Steinheim
NaCl	Serva, Heidelberg
NADP	Sigma-Aldrich Chemie, Steinheim
Ammonium acetate	Serva, Heidelberg
Nitro blue tetrazolium chloride	Sigma-Aldrich Chemie, Steinheim
PCR-water	UV treated, State University of New York at Buffalo, USA
Phenazine methosulphate	Sigma-Aldrich Chemie, Steinheim
Phosphoglucomutase	Sigma-Aldrich Chemie, Steinheim
Proteinase K	MBI Fermentas, St. Leon-Rot
QIAquick Gel Extractions Kit	QIAGEN, Hilden
Restriction enzyme Typ II	Boehringer Mannheim, Mannheim; MBI Fermentas, St. Leon-Rot
Sucrose	Carl Roth, Karlsruhe
SDS	Serva, Heidelberg
Starch	University of Montpellier II, Montpellier, France
Taq buffer	MBI Fermentas, St. Leon-Rot
Taq DNA polymerase (native with BSA)	MBI Fermentas, St. Leon-Rot
Tris base	Serva, Heidelberg

Appendix 2

Restriction fragments (bp) of three fragments of the mitochondrial COI gene of several *Diadegma* species and *Meloboris* sp. as out-group produced by different restriction enzymes. Fragments < 100 bp are not visible on a 3 % agarose gel and are therefore not included, -: species not analysed, EA: Eastern Africa, SA: Southern Africa.

A: Primer pair 3/5 (~540 bp)

	*Alu*I	*Dra*I	*Rsa*I	*Ssp*I	*Taq*I
D. mollipla, EA	30, 210	540	380, 160	170, 160, 140	370, 120
D. mollipla, SA	10, 160	420, 120	380, 160	170, 160, 140	370, 120
D. semiclausum	210, 160, 140	420, 120	380, 160	170, 160, 140	420, 120
Meloboris sp.	540	420, 120	540	-	540

B: Primer pair 3/8 (~630 bp)

	*Alu*I	*Hinf*I
D. mollipla, EA	410, 210	480, 130
D. mollipla, SA	250, 210, 150	630
D. semiclausum	230, 210, 150	630

C: Primer pair 3/4 (~660 bp)

	*Alu*I	*Cfo*I	*Dra*I	*Eco*RI	*Hinf*I	*Msp*I	*Rsa*I	*Sau*3AI	*Taq*I	*Xba*I
D. mollipla, EA	450, 210	660	660	660	480, 180	660	360, 300	200, 130	250, 350	660
D. mollipla, SA	300, 210, 150	660	420, 240	660	660	520, 140	360, 300	200, 130	250, 350	660
D. semiclausum	280, 210, 150	660	420, 240	660	660	660	360, 300	330, 200	250, 390	660
Meloboris sp.	660	660	420	660	660	660	660	-	660	660

Appendix 3

Restriction fragments (bp) of fragments of 5.8S and 28S genes and the ITS2 region of the ribosomal DNA of several *Diadegma* species and *Meloboris* sp. as out-group produced by different restriction enzymes. Fragments < 100 bp are not visible on a 3 % agarose gel and are therefore not included. -: species not analysed, nd: amplicon not digested.

	D. mollipla eastern Africa	*D. mollipla* southern Africa	*D. semiclausum*	*D. fenestrale*	*D. insulare*	*D. leontiniae*	*D. rapi*	*Meloboris* sp.
*Alu*I	390, 210, 160	390, 210, 160	290, 200, 150	380, 250, 150	380, 250, 150	380, 300, 180	-	340, 270, 150
*Bse*XI	650, 120	400, 270, 120	650, 120	-	-	-	-	nd
*Bsp*143I	280, 240, 150, 120	280, 240, 150, 120	240, 150, 120	220, 120, 110	200, 120	250, 190	-	240, 210, 130
*Bsu*RI	510, 260	510, 260	510, 170, 100	510, 260	510, 260	510, 300	-	nd
*Cfo*I	180, 160, 100	180, 100	260, 180, 110	300, 180, 140	220, 130, 100	250, 180, 140	270, 220, 180	210, 200, 150
*Dra*I	nd	nd	nd	nd	nd	nd	-	nd
*Eco*RI	nd	nd	nd	nd	nd	nd	-	nd
*Eco*RV	nd	nd	nd	nd	nd	nd	-	530, 250
*Hinf*I	370, 190	370, 190	370, 190	370, 190	370, 170	480, 190, 110	-	330, 260, 180
*Hpa*II	250, 150, 100	250, 150, 100	270, 240, 100	250, 150, 100	250, 150, 100	320, 280, 150, 100	-	440, 280
*Rsa*I	550, 200	550, 200	550, 200	550, 200	nd	400, 250, 190	-	450, 280
*Taq*I	190, 120	190, 120	120	-	-	180, 120	-	200, 120
*Tru*I	460, 170, 150	460, 170, 150	470, 320	470, 320	350, 330, 110	490, 210, 160	-	320, 250, 190
*Alu*I/*Hinf*I	350, 130	350, 130	300, 130	350, 190	350, 170	350, 200, 100	340, 100	320, 260, 150
*Bse*I/*Tru*I	490, 160, 110	270, 200, 160, 110	470, 200, 110	470, 200, 110	350, 200, 110	480, 220, 110	340, 250, 110	270, 230, 170

Appendix 4

Pair-wise nucleotide distances (%) (lower-left matrix) and standard errors (%) (upper-right matrix) calculated with the Kimura 2-parameter model of the mitochondrial COI sequences among 30 *Diadegma* individuals examined. Bold numbers (1-12; 15-32) refer to individuals listed in tab. 2.1.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**15**	**16**
1		0	1.05	1.05	1.29	1.29	1.18	1.18	1.39	1.39	1.39	1.52	1.42	1.29
2	0		1.05	1.05	1.29	1.29	1.18	1.18	1.39	1.39	1.39	1.52	1.42	1.29
3	5.20	5.20		0	1.21	1.21	1.11	1.11	1.41	1.41	1.41	1.46	1.42	1.26
4	5.20	5.20	0		1.21	1.21	1.11	1.11	1.41	1.41	1.41	1.46	1.42	1.26
5	7.61	7.61	6.74	6.74		0	1.15	1.15	1.47	1.47	1.47	1.50	1.37	1.29
6	7.61	7.61	6.74	6.74	0		1.15	1.15	1.47	1.47	1.47	1.50	1.37	1.29
7	6.50	6.50	5.66	5.66	6.09	6.09		0	1.49	1.49	1.49	1.48	1.37	1.22
8	6.50	6.50	5.66	5.66	6.09	6.09	0		1.49	1.49	1.49	1.48	1.37	1.22
9	8.73	8.73	8.98	8.98	9.65	9.65	9.89	9.89		0	0	1.63	1.56	1.48
10	8.73	8.73	8.98	8.98	9.65	9.65	9.89	9.89	0		0	1.63	1.56	1.48
11	8.73	8.73	8.98	8.98	9.65	9.65	9.89	9.89	0	0		1.63	1.56	1.48
12	10.32	10.32	9.63	9.63	10.10	10.10	9.86	9.86	11.73	11.73	11.73		1.63	1.54
15	9.17	9.17	9.18	9.18	8.50	8.50	8.50	8.50	10.79	10.79	10.79	11.72		0.72
16	7.61	7.61	7.38	7.38	7.60	7.60	6.94	6.94	9.87	9.87	9.87	10.55	2.46	
17	7.61	7.61	7.38	7.38	7.60	7.60	6.94	6.94	9.87	9.87	9.87	10.55	2.46	0
18	7.61	7.61	7.38	7.38	7.60	7.60	6.94	6.94	9.87	9.87	9.87	10.55	2.46	0
19	7.39	7.39	7.16	7.16	7.38	7.38	6.72	6.72	9.64	9.64	9.64	10.32	2.46	0.20
20	9.17	9.17	9.18	9.18	8.50	8.50	8.50	8.50	10.79	10.79	10.79	11.72	0	2.46
21	14.34	14.34	14.58	14.58	16.82	16.82	15.32	15.32	14.34	14.34	14.34	12.20	13.14	12.42
22	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
23	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
24	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
25	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
25	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
27	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
28	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
29	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
30	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
31	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60
32	5.63	5.63	4.59	4.59	6.08	6.08	3.92	3.92	9.19	9.19	9.19	8.73	8.95	7.60

Appendix 4: Continued.

	17	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
1	1.29	1.29	1.27	1.42	1.83	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09
2	1.29	1.29	1.27	1.42	1.83	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09	1.09
3	1.26	1.26	1.24	1.42	1.85	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
4	1.26	1.26	1.24	1.42	1.85	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
5	1.29	1.29	1.27	1.37	2.01	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15
6	1.29	1.29	1.27	1.37	2.01	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15
7	1.22	1.22	1.20	1.37	1.90	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91
8	1.22	1.22	1.20	1.37	1.90	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91	0.91
9	1.48	1.48	1.46	1.56	1.83	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43
10	1.48	1.48	1.46	1.56	1.83	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43
11	1.48	1.48	1.46	1.56	1.83	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43
12	1.54	1.54	1.52	1.63	1.67	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39
15	0.72	0.72	0.72	0	1.74	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40
16	0	0	0.20	0.72	1.68	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
17		0	0.20	0.72	1.68	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
18	0		0.20	0.72	1.68	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
19	0.20	0.20		0.72	1.68	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26
20	2.46	2.46	2.46		1.74	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40
21	12.42	12.42	12.42	13.14		1.83	1.83	1.83	1.83	1.83	1.83	1.83	1.83	1.83	1.83	1.83
22	7.60	7.60	7.38	8.95	14.35		0	0	0	0	0	0	0	0	0	0
23	7.60	7.60	7.38	8.95	14.35	0		0	0	0	0	0	0	0	0	0
24	7.60	7.60	7.38	8.95	14.35	0	0		0	0	0	0	0	0	0	0
25	7.60	7.60	7.38	8.95	14.35	0	0	0		0	0	0	0	0	0	0
25	7.60	7.60	7.38	8.95	14.35	0	0	0	0		0	0	0	0	0	0
27	7.60	7.60	7.38	8.95	14.35	0	0	0	0	0		0	0	0	0	0
28	7.60	7.60	7.38	8.95	14.35	0	0	0	0	0	0		0	0	0	0
29	7.60	7.60	7.38	8.95	14.35	0	0	0	0	0	0	0		0	0	0
30	7.60	7.60	7.38	8.95	14.35	0	0	0	0	0	0	0	0		0	0
31	7.60	7.60	7.38	8.95	14.35	0	0	0	0	0	0	0	0	0		0
32	7.60	7.60	7.38	8.95	14.35	0	0	0	0	0	0	0	0	0	0	

Appendix 5

Pair-wise nucleotide distances (%) (lower-left matrix) and standard errors (%) (upper-right matrix) calculated with the Kimura 2-parameter model of the ribosomal ITS2 sequences among 31 *Diadegma* individuals examined. Bold numbers refer to individuals listed in tab. 2.1.

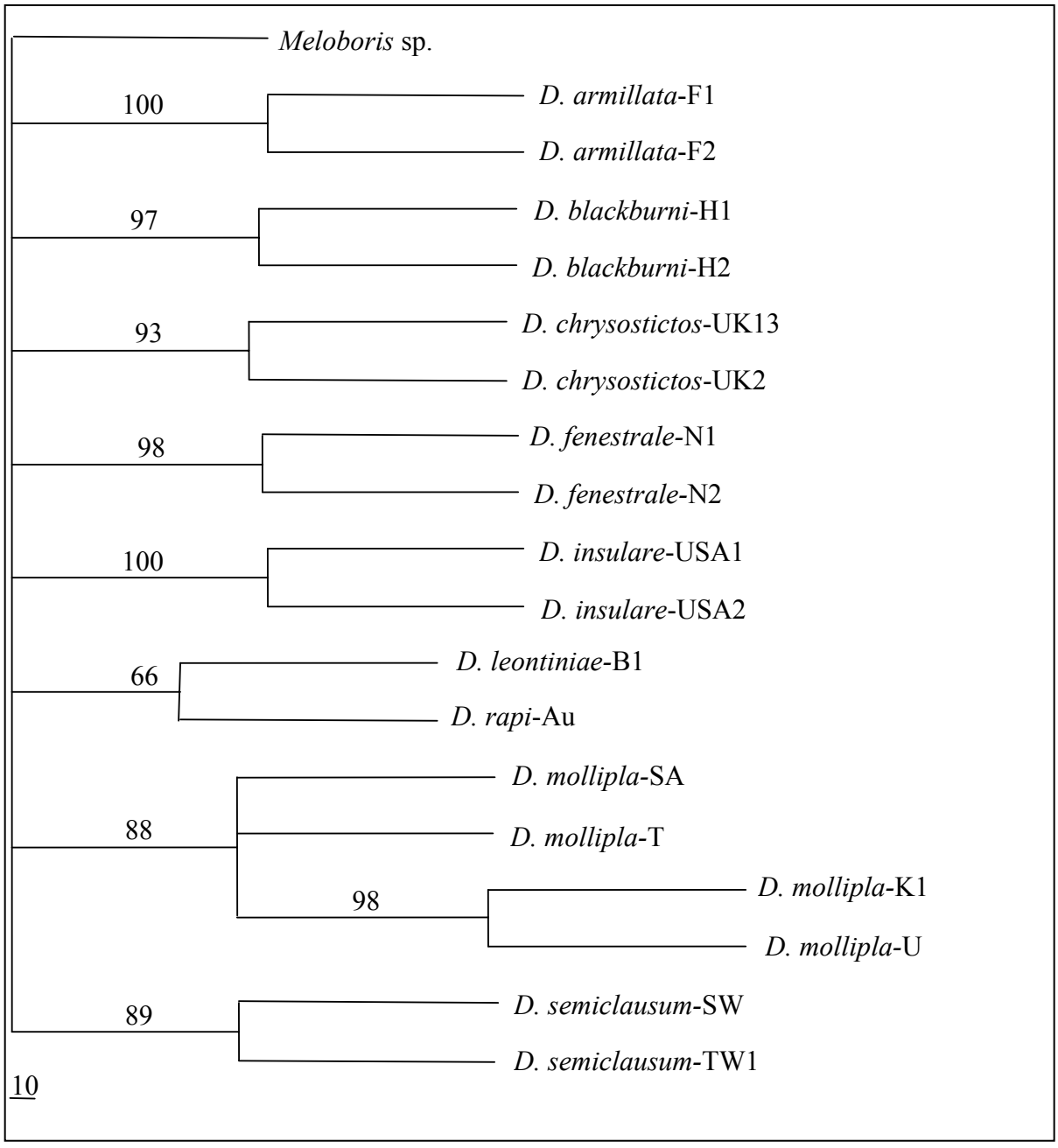
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1		0.32	1.21	1.19	1.43	1.43	1.11	1.11	1.65	1.65	1.65	2.62	2.62	1.06	1.05
2	0.63		1.23	1.21	1.44	1.44	1.12	1.12	1.69	1.69	1.69	2.63	2.63	1.10	1.08
3	8.21	8.40		0.16	1.34	1.34	0.97	0.97	1.51	1.51	1.51	2.63	2.63	1.06	1.06
4	8.03	8.22	0.16		1.33	1.33	0.99	0.99	1.53	1.53	1.53	2.63	2.63	1.08	1.08
5	11.32	11.52	10.03	9.84		0	1.05	1.05	1.75	1.75	1.75	2.77	2.77	1.25	1.25
6	11.32	11.52	10.03	9.84	0		1.05	1.05	1.75	1.75	1.75	2.77	2.77	1.25	1.25
7	6.91	7.09	15.46	5.60	6.28	6.28		0	1.53	1.53	1.53	2.57	2.57	1.02	1.02
8	6.91	7.09	15.46	5.60	6.28	6.28	0		1.53	1.53	1.53	2.57	2.57	1.02	1.02
9	13.6	14.02	11.79	12.00	15.37	15.37	11.94	11.94		0	0	2.48	2.48	1.51	1.52
10	13.6	14.02	11.79	12.00	15.37	15.37	11.94	11.94	0		0	2.48	2.48	1.51	1.52
11	13.6	14.02	11.79	12.00	15.37	15.37	11.94	11.94	0	0		2.48	2.48	1.51	1.52
12	28.77	29.01	28.89	28.89	32.6	32.6	28.13	28.13	26.32	26.32	26.32		0	2.56	2.57
13	28.77	29.01	28.89	28.89	32.6	32.6	28.13	28.13	26.32	26.32	26.32	0		2.56	2.57
14	6.37	27.18	6.35	6.53	8.67	8.67	5.86	5.86	11.66	11.66	11.66	27.84	27.84		0.16
15	6.19	6.56	6.35	6.53	8.67	8.67	5.86	5.86	11.86	11.86	11.86	28.1	28.1	0.16	
16	6.55	6.92	6.35	6.53	9.04	9.04	6.22	6.22	12.26	12.26	12.26	27.83	27.83	0.49	0.33
17	6.55	6.92	6.35	6.53	9.04	9.04	6.22	6.22	12.26	12.26	12.26	27.83	27.83	0.49	0.33
18	6.55	6.92	6.35	6.53	9.04	9.04	6.22	6.22	12.26	12.26	12.26	27.83	27.83	0.49	0.33
19	6.37	6.74	6.35	6.53	8.67	8.67	5.86	5.86	11.66	11.66	11.66	27.84	27.84	0	0.16
20	6.37	6.74	6.35	6.53	8.67	8.67	5.86	5.86	11.66	11.66	11.66	27.84	27.84	0	0.16
21	28.59	28.83	28.69	28.69	31.9	31.9	26.69	26.69	26.27	26.27	26.27	20.4	20.4	26.36	26.36
22	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
23	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
24	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
25	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
26	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
27	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
28	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
29	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
30	5.42	5.78	8.73	8.55	12.91	12.91	7.94	7.94	14.15	14.15	14.15	30.83	30.83	8.82	8.63
31	5.6	5.6	8.92	8.73	13.1	13.1	8.12	8.12	14.35	14.35	14.35	31.09	31.09	9.01	8.82

Appendix 5: Continued.

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	1.08	1.08	1.08	1.06	1.06	2.63	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.99
2	1.11	1.11	1.11	1.10	1.10	2.64	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.01	1.02
3	1.06	1.06	1.06	1.06	1.06	2.64	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.28
4	1.08	1.08	1.08	1.08	1.08	2.64	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.26
5	1.28	1.28	1.28	1.25	1.25	2.75	1.55	1.55	1.55	1.55	1.55	1.55	1.55	1.55	1.55	1.57
6	1.28	1.28	1.28	1.25	1.25	2.75	1.55	1.55	1.55	1.55	1.55	1.55	1.55	1.55	1.55	1.57
7	1.05	1.05	1.05	1.02	1.02	2.50	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.22
8	1.05	1.05	1.05	1.02	1.02	2.50	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.22
9	1.55	1.55	1.55	1.51	1.51	2.49	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.71
10	1.55	1.55	1.55	1.51	1.51	2.49	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.71
11	1.55	1.55	1.55	1.51	1.51	2.49	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.71
12	2.55	2.55	2.55	2.56	2.56	2.01	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.76
13	2.55	2.55	2.55	2.56	2.56	2.01	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.75	2.76
14	0.28	0.28	0.28	0	0	2.48	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.29
15	0.23	0.23	0.23	0.16	0.16	2.48	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26
16		0	0	0.28	0.28	2.51	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
17	0		0	0.28	0.28	2.51	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
18	0	0		0.28	0.28	2.51	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29
19	0.49	0.49	0.49	0	0	2.48	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.29
20	0.49	0.49	0.49	0	0	2.48	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.29
21	26.85	26.85	26.85	26.36	26.36		2.76	2.76	2.76	2.76	2.76	2.76	2.76	2.76	2.76	2.77
22	9.01	9.01	9.01	8.82	8.82	30.68		0	0	0	0	0	0	0	0	0.16
23	9.01	9.01	9.01	8.82	8.82	30.68	0		0	0	0	0	0	0	0	0.16
24	9.01	9.01	9.01	8.82	8.82	30.68	0	0		0	0	0	0	0	0	0.16
25	9.01	9.01	9.01	8.82	8.82	30.68	0	0	0		0	0	0	0	0	0.16
26	9.01	9.01	9.01	8.82	8.82	30.68	0	0	0	0		0	0	0	0	0.16
27	9.01	9.01	9.01	8.82	8.82	30.68	0	0	0	0	0		0	0	0	0.16
28	9.01	9.01	9.01	8.82	8.82	30.68	0	0	0	0	0	0		0	0	0.16
29	9.01	9.01	9.01	8.82	8.82	30.68	0	0	0	0	0	0	0		0	0.16
30	9.01	9.01	9.01	8.82	8.82	30.68	0	0	0	0	0	0	0	0		0.16
31	9.19	9.19	9.19	9.01	9.01	30.93	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	

Appendix 6

Maximum-likelihood tree (-ln = 1595.17) for a fragment of the COI gene (498 bp) of nine *Diadegma* species and *Meloboris* sp. as out-group (tab. 2.1). For the maximum likelihood analysis the model that best fits the data was GTR+I+G (unequal base frequencies, six substitution rates with gamma distribution shape parameter (G) = 1.04 and the assumed proportion of the invariable sites (I) = 0.63. Bootstrap confidence levels (%), based on 100 replicates, are indicated above each branch. Branch lengths are proportional to genetic distance (scale bar).



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