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Genetic identity and relationship between four *Anagrus* species (Hymenoptera: Mymaridae) using RAPD analysis

ELISABETTA CHIAPPINI[1], LUCIA SORESSI[1], CORRADO FOGHER[2] and MARCO ZANIRATO[2]

[1]Istituto di Entomologia e Patologia vegetale, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy
[2]Istituto di Genetica vegetale, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29100 Piacenza, Italy

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Abstract. Four species of *Anagrus* (*A. breviphragma* Soyka, *A. incarnatus* Haliday, *A. fennicus* Soyka and *A. obscurus* Foerster sensu Soyka), that live on *Carex riparia* Curtis (Cyperaceae) in uncultivated areas along the Po river in the Piacenza province in Italy were analysed using RAPD markers in order to investigate their genetic relationships. High levels of RAPD polymorphism were found in the genus *Anagrus,* which permitted the fingerprinting of the four species. Analysis of average genetic similarities within species and comparison with average values between species confirms that *A. breviphragma*, *A. incarnatus*, *A. fennicus* and *A. obscurus* represent four genetically distinct species. A dendrogram constructed from molecular data of single families clearly clustered the four species in different groups, their mutual position reflecting morphological and biological observations.

INTRODUCTION

Four species of *Anagrus*, all belonging to the *incarnatus* species group (Chiappini, 1989), were found to live in the same microhabitat on *Carex riparia* Curtis (Cyperaceae) in uncultivated areas along the Po river in the Piacenza province in Italy: *Anagrus breviphragma* Soyka, *Anagrus incarnatus* Haliday, *Anagrus fennicus* Soyka and *Anagrus obscurus* Foerster sensu Soyka. The first three species parasitize *Cicadella viridis* (L.) (Auchenorrhyncha: Cicadellidae) eggs while the last one develops in *Anakelisia fasciata* (Kirschbaum) (Auchenorrhyncha: Delphacidae) eggs.

These species were introduced by Soyka in his taxonomic key of *Anagrus* females (1955) as new and were discriminated on the basis of a few morphological characters. Recently, Chiappini (1989), in a review of the European species of the genus, validated them and added some distinguishing characters deduced from the study of the holotypes.

A. breviphragma and *A. incarnatus* have been further investigated from ecological, ethological and biological as well as morphological and morphometric points of view (Walker, 1979; Chiappini & Curto, 1987; Moratorio, 1987; Moratorio, 1990; Moratorio & Chiappini, 1995); *A. obscurus* was probably the object of Witsack's biological and ethological research (1973) and was investigated from a morphometric point of view (Chiappini & Curto, 1987), while the holotype of *A. fennicus* was the only specimen of the species available until it was recorded from a known host and a detailed morphological description, together with biological and ecological data, was provided (Chiappini, 1996).

Therefore, *A. breviphragma* and *A. incarnatus* are well defined as species, but the specific identity of *A. obscurus* and *A. fennicus* has never been deeply investigated, especially with reference to each other and to the first two species living in the same microhabitat.

These four species can be morphologically distinguished by the presence of two setae on mesoscutum, forewing shape and ciliation and coloration. In the female, they are distinguished also by the relative length of antennal segments, presence and number of sensory ridges on funicular segments and the length of the ovipositor (Table 1).

Even when morphology seems to be enough to discriminate between species, some observations and considerations can raise doubts about the specific value of the feature used and, as a consequence, about the specific identity based on these characters only.

TABLE 1. Main morphological and biological characters for distinguishing between the four *Anagrus* species considered.

	A. breviphragma	*A. incarnatus*	*A. fennicus*	*A. obscurus*
Coloration	orange-yellow	brown	black with yellow postscutellum	brown
Mesoscutum	without setae	without setae	with two setae	with two setae
Forewing disc	with glabrous area	without glabrous area	without glabrous area	without glabrous area
Female's antenna	F2 longest of funicle segments F3 and F4 with 1 sensory ridge	F2 longest of funicle segments F3 and F4 with 1 sensory ridge	F2 as long as F3, F3 and F4 with 2 sensory ridges	F2 as long as F3, F3 and F4 with 1 or 2 sensory ridges
Ovipositor	projecting from metasoma	not projecting from metasoma	not projecting from metasoma	not projecting from metasoma
Host	*Cicadella viridis*	*Cicadella viridis*	*Cicadella viridis*	*Anakelisia fasciata*

First of all, these morphological characters have occasionally shown intermediate states between two species; second, the state of the characters could change in relation to the host parasitized, as has been shown for *Anaphes fuscipennis* Haliday (Huber & Rajakulendran, 1988).

Furthermore, in some cases (different temperature and humidity conditions), specimens develop without the typical coloration present in their parents. For example, some daughters of a typically brown *A. obscurus* female were completely light yellow (Chiappini, unpubl.).

While we cannot be sure of the real value of the morphological characters which are used to separate these species, at least for *A. breviphragma*, *A. incarnatus* and *A. fennicus*, they show, within each one of them, a constant state that obviously cannot depend on the host as the host is the same for all three.

Differently, in *A. obscurus*, which can parasitize both *A. fasciata* and *C. viridis* eggs, the number of sensory ridges on the female third and fourth funicular segments shows intraspecific variability. This variability is not host dependent as specimens with one or two sensory ridges on F3 and sometimes also on F4 were observed in the same female progeny in the same host. Therefore, when *A. obscurus* and *A. fennicus* present the same number of sensory ridges on funicular segments, it can be difficult to distinguish them. In addition, as we rely mainly on antennal female characters to distinguish *A. fennicus* from *A. obscurus*, males of these two species cannot be separated as both of them belong to the *incarnatus* species group (Chiappini, 1989) and therefore have the same kind of copulatory apparatus.

Furthermore, while we observed in the laboratory that virgin females of *A. fennicus* or *A. obscurus* did not copulate with males of either *A. breviphragma* or *A. incarnatus*, they promptly copulated when a male of their own species was substituted for the others. We observed males of *A. fennicus* copulate with *A. obscurus* females and vice versa even if we never obtained any progeny from these crossings (Fig. 1).

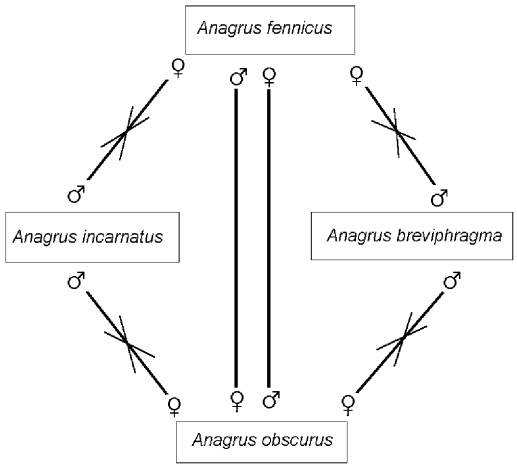

Fig. 1. Diagram illustrating sexual relationships among *A. obscurus*, *A. fennicus*, *A. breviphragma* and *A. incarnatus*: females and males of species linked by a line mated; females and males of species linked by the crossed line did not mate.

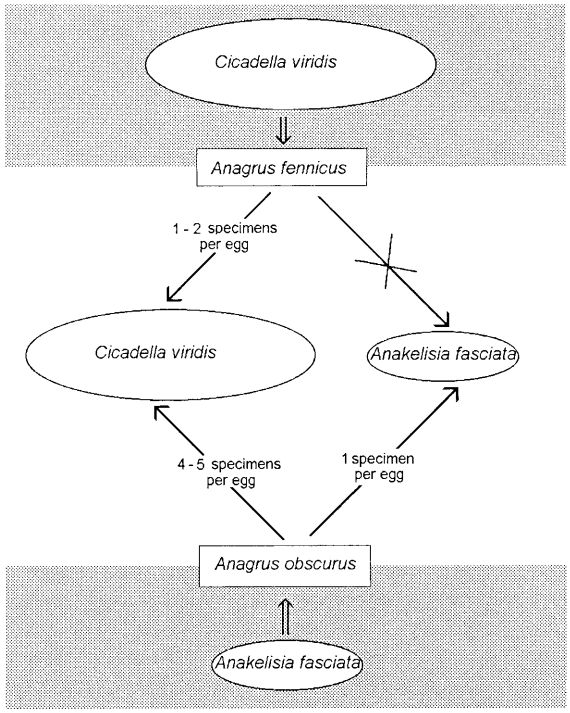

Fig. 2. Diagram illustrating relationships between *A. obscurus*, *A. fennicus* and their hosts: Shaded background indicates field conditions while white background indicates laboratory conditions; double arrows indicate the host egg the parasitoid comes from; simple arrows indicate the eggs that were parasitized, the crossed arrow indicates the eggs that were not parasitized.

These facts could allow us to conclude that these two species might in reality represent only one, showing some minor intraspecific morphological differences.

Nevertheless, some other considerations and observations could lead us to the opposite conclusion. In nature we never reared *A. obscurus* from *C. viridis* eggs. In the laboratory, when only these eggs were present, they were parasitized by *A. obscurus* females. In such a case, about four or five specimens developed per host egg while *A. fennicus* never developed in more than one or two specimens in the same egg as it killed the other larvae that were present in the same egg. Furthermore, *A. fennicus* never parasitized *A. fasciata* eggs, even if they were the only ones available (Fig. 2).

Therefore, even if we can hypothesise that these two species really exist, we need some other data to validate this hypothesis.

These facts point out the requirement to add molecular tools to the morphological characters used as diagnostic features.

Recently developed techniques, such as RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism), permit the direct analysis of DNA.

RFLP and RAPD molecular markers are not influenced by environmental conditions and can be used to estimate genetic similarities from data measured directly on DNA. These permit the comparison of parts of the genome not usually available with other techniques and provide dis-

crete data suitable for statistic analysis (Thormann et al., 1994).

RAPD markers (Welsh & McClelland, 1990; Williams et al., 1990) are produced through the amplification of DNA by the polymerase chain reaction (PCR) (Saiki et al., 1988) using arbitrary primers homologous to random target sites on the genome.

RAPD markers are cheaper and simpler to produce and to detect than RFLP as, to generate data, they do not require the use of radionuclides but only PCR amplification and electrophoresis.

RAPD markers for phylogenetic studies or comparison of species show limits in the fact that they are sometime difficult to reproduce and the genomic origin of fragments (nuclear or cytoplasmic) and the sequence homology of bands with similar mobility in a gel are unknown (Thormann et al., 1994).

In spite of that, recent research has proven that RAPD markers are appropriate for DNA fingerprinting, and are particularly useful for distinguishing morphologically similar species (Vanlerberghe-Masutti, 1994; Perron et al., 1995), for mapping (Hunt & Page, 1992; Plomion et al., 1995), for population genetic studies and intraspecific variability (Haymer & McInnis, 1994; Lenney Williams et al., 1994; N'Goran et al., 1994; Graham & McNicol, 1995) and as a support in taxonomic studies between species of the same or similar genera (Comencini et al., 1996; Millan et al., 1996).

In the present work, we analyse the genetic variability among *Anagrus* species applying RAPD techniques in order to evaluate the specific identity in comparison with classical morphological characters and verify the possibility of finding species specific molecular markers to use as fingerprints for diagnostic purpose.

The responsibilities have been divided between authors in such a way that E. Chiappini was mainly responsible for the scientific problem definition and the entomological aspect of the research, L. Soressi carried on the insect breeding and all the genetical tests, M. Zanirato and C. Fogher were mainly responsible for the supervision of the genetical aspect of the research.

MATERIAL AND METHODS

Sample collection

For RAPD analysis, the same set of primers was used to assess the differences between species and subsequently within species. In interspecies analysis, several individuals belonging to the same species were utilized to extract species specific genomic DNA; all these specimens came from field material, and were identified on ethological (host and number of specimens in one egg) and morphological bases with the help of a stereoscopic microscope (Table 1). In intraspecies analysis, laboratory parasitoid cultures were performed to obtain, for each species, families of individuals deriving from independently chosen parents. All the individuals belonging to the same family were used for DNA extraction.

A. incarnatus, *A. breviphragma* and *A. fennicus* were obtained from overwintering eggs of *C. viridis* while overwintering eggs of *A. fasciata* yielded *A. obscurus*.

Leaves of *C. riparia* bearing overwintering eggs of *C. viridis* and *A. fasciata* were collected periodically during winter months in uncultivated areas along the Po river in the Piacenza province, Italy.

The host eggs were removed from *C. riparia* leaves and isolated in Petri dishes (diameter 3.5 cm), containing absorbent paper dampened with a physiological solution, until emergence of the parasitoids.

Parasitoid cultures were obtained from eggs laid by females that had become adult in the laboratory. In this case, as soon as the adults emerged from the egg, they were moved to Petri dishes containing sucrose and a piece of leaf with unparasitized eggs. The *Anagrus* species are arrhenotokous: from virgin females allowed to oviposit, only males are obtained, while all mated females produce both male and female progeny (Moratorio & Chiappini, 1995). After parasitization, the eggs were removed from the vegetable tissue and kept until parasitoid emergence, as already described.

The *C. viridis* healthy eggs, used for *A. incarnatus*, *A. breviphragma* and *A. fennicus*, were obtained by ovipositions of the host females in *C. riparia* plants kept in the open air, in muslin covered cages during the autumn. For *A. obscurus* oviposition, we used the unparasitized eggs of *A. fasciata* left in *Carex* leaves after the removal of the parasitized ones under a stereoscopic microscope.

Genomic DNA extraction

The insects were soaked in 70% alcohol, then frozen and stored at –80°C. Total genomic DNA was extracted by a modified version of the "Lifton" method (Haymer & McInnis, 1994). Several adults (20–50 for the analysis of the populations and 2–27 for the analysis of the families) were placed in a 1.5 ml microtube and ground in liquid nitrogen (Roehrdanz & Flanders, 1993). To the same microtube were added 100 µl extraction buffer (0.2 M sucrose, 50 mM EDTA, 100 mM Tris pH 9 and 0.5% SDS), 67 µl potassium acetate (at a final concentration of 1.2 M) and 1.7 µl Proteinase K (Boheringer Mannheim) diluted to 20 mg/ml (at a final concentration of 200 µg/ml).

This solution was kept at 65°C for 35 min. The debris was pelleted by centrifugation for 15 min at 14,000 rpm, at 4°C.

The DNA was precipitated from the supernatant with 95% ice-cold ethanol and washed with 70% ice-cold ethanol, then dried under vacuum and resuspended in TE (10 mM tris pH 8.0, 0.1 mM EDTA).

The DNA solution was treated with 10–40 µg/ml RNase A (Boheringer Mannheim) for 30 min at 37°C. The DNA was quantified on ethidium bromide-stained agarose gel using 25 ng of phage lambda DNA (Gibco BRL). Samples were diluted to 4 ng/µl with sterile water treated with an ultra-pure water system (Milli-q plus-Millipore) (Sambrook et al., 1989).

PCR amplification (RAPD assay)

RAPD reactions were carried out following the methods developed by Welsh & McClelland (1990) and Williams et al. (1990). The 25 µl reactions contained 200 µM dNTPs (Boehringer Mannheim), 1 × Taq polymerase buffer (Promega), 1 unit of Taq polymerase (Promega), 8 ng of genome DNA and 25 pmoles of primer. Magnesium chloride concentration used was determined on the basis of the different concentration test results. Each reaction was overlaid with 40 µl of mineral oil.

PCR reaction preparation was carried out under a sterile hood to reduce the possibility of aerosol contamination by exogenous DNA.

Amplifications were performed in a thermal cycler Gene E (Techne, Cambridge, UK) using the touchdown PCR technique (Sutcliffe, 1978) according to the program in Table 2.

The first four cycles were repeated twice each, while the fifth cycle was repeated 35 times.

TABLE 2. PCR programs used for DNA amplification.

	Cycles 1–2	Cycles 3–4	Cycles 5–6	Cycles 7–8	Cycles 9–45
Denaturation	94°C for 2′	94°C for 20″	94°C for 20″	94°C for 20″	94°C for 2′
Annealing	40°C for 30″	39°C for 30″	38°C for 30″	37°C for 30″	36°C for 30″
Extension	72°C for 2′	72°C for 2′	72°C for 2′	72°C for 2′	72°C for 2′

The PCR products (18 µl) were electrophoresed in 2% TBE agarose gels at 100 V with a 1 Kb ladder DNA size marker (Gibco BRL), stained with 0.5 µg/ml ethidium bromide and photographed under UV light (312 nm) (Sambrook et al., 1989).

Optimization of RAPD conditions, primers selection and scoring of bands

As the validity of RAPD analysis is directly related to its reproducibility, we made preliminary experiments to identify and reduce the most important causes of pattern variability. We tested different $MgCl_2$ and DNA concentrations to find conditions that permit the consistent generation of amplification product patterns (Ellsworth et al., 1993). *A. brevipragma* DNA was used as the template for this investigation.

DNA concentrations varied from 0.1 ng to 100 ng (0.1, 5, 10, 50, 100 ng) and eight $MgCl_2$ concentrations were tested (1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mM) for each of the following 10-mer primers (Kit A, Operon Technologies):

Code	5' to 3'
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-17	GACCGCTTGT
OPA-18	AGGTGACCGT
OPA-19	GAAACGTCGG
OPA-20	GTTGCGATCC

The RAPD pattern did not vary within a range of DNA concentration between 2 ng and 20 ng of genomic DNA; we therefore used 8 ng of DNA as the optimal concentration for the amplification reaction.

For RAPD analysis of species and families we used only primers giving highly reproducible PCR amplification products (bands) (Skroch & Nienhuis, 1995) in three independent experiments.

Bold and highly repeatable bands were binary scored for presence (1) or absence (0).

Data analysis

To evaluate the genetic similarity, we used the Jaccard coefficient (Jaccard, 1908), according to Link et al. (1995).

Genetic similarity coefficients may range from 0 (different for all criteria studied) to 1 (identical).

For each species we calculated the average pairwise similarity of families belonging to the same species, defining it as the "average genetic similarity within species" (GS-within), and the average similarity between pairs of families belonging to different species, defining it as the "average genetic similarity between species" (GS-between) (Ajmone-Marsan et al., 1992).

UPGMA cluster analysis was used to construct dendrograms from the matrix of RAPD-based genetic similarity estimates. Cophenetic values (Sokal & Rolph, 1962) were calculated by using appropriate procedures of program Phylip 3.5c (Felsenstein, 1993). A simple correlation was calculated between similarity matrices and cophenetic values matrices to verify the agreement between the similarity values implied by the dendrogram and those of the original similarity matrix.

RESULTS

Material obtained from breeding

The total number of families obtained was thirteen: four of *A. breviphragma* and *A. obscurus*, three of *A. incarnatus* and only two of *A. fennicus*. Numbers of individuals obtained for each family are reported in Table 3.

TABLE 3. Families obtained by breeding of parents independently chosen.

Species	Families	Males	Females	Males + females	Total
A. breviphragma	1	5	16	21	70
	2	4	15	19	
	3	2	12	14	
	4	2	14	16	
A. incarnatus	1	27	0	27	60
	2	17	0	17	
	3	1	15	16	
A. fennicus	1	4	0	4	10
	2	6	0	6	
A. obscurus	1	2	1	3	22
	2	0	2	2	
	3	5	0	5	
	4	12	0	12	

Optimizations of conditions and primers selection

Seven primers out of the twenty tested on *A. breviphragma* DNA gave consistently bold and reproducible bands and were selected for further analyses: OPA-04, OPA-08, OPA-09, OPA-11, OPA-13, OPA-16 and OPA-18.

Highly repeatable bands were obtained with a concentration of 3.0 mM of $MgCl_2$ for all the primers utilized.

RAPD analysis

Eighty highly reproducible polymorphic bands were obtained from the RAPD analysis of the four species. An average of 11 markers per primer were detected with a range varying from a minimum of 5 to a maximum of 16 (OPA-04: 14, OPA-08: 12, OPA-09: 11, OPA-11: 5, OPA-13: 10, OPA-16: 16 and OPA-18: 12).

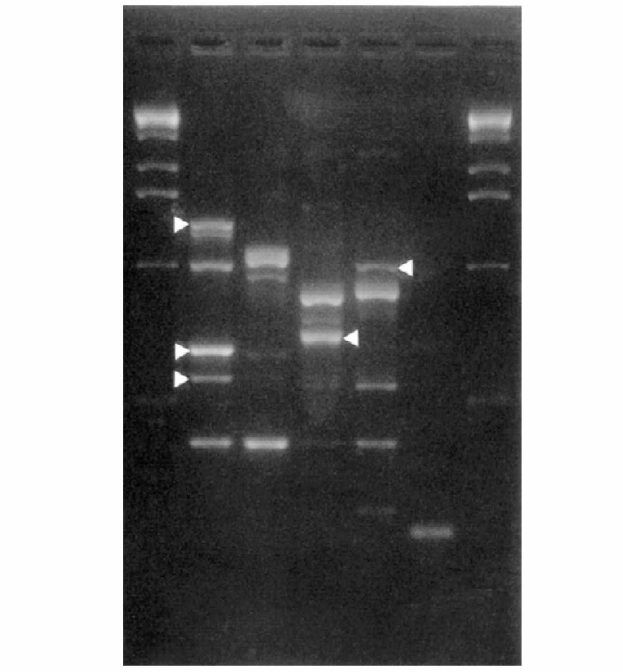

Fig. 3. RAPD patterns obtained with primer OPA-18. Lanes 1 and 7: 1Kb ladder molecular weight marker; lane 2: *A. breviphragma*; lane 3: *A. incarnatus*; lane 4: *A. fennicus*; lane 5: *A. obscurus*; lane 6: negative control.

RAPD analysis of the 13 families investigated produced a total of 88 highly reproducible bands, many of which were monomorphic between families of the same species.

The higher number of bands obtained (88 vs 80) was probably due to the fact that alleles present at high frequency in a single family, and therefore clearly amplified, may be present as rare alleles in the species and therefore not or poorly amplified in a pool of random individuals.

Fingerprinting

Among the seven primers selected, four (OPA-08, OPA-09, OPA-11 and OPA-18) gave different bands that were present in one species only (Fig. 3).

RAPD analysis at the families level, with the above primers, showed that some of these bands were present in all the families of the same species, appearing to be monomorphic inside the species, at least in the limited number of families analysed.

Therefore, these bands can be used to uniquely identify the species analysed (Table 4).

Genetic similarity

Jaccard coefficients of similarity between families are given in Table 5.

UPGMA cluster analysis, calculated from RAPD data, gave a dendrogram (Fig. 4) in which family belonging to the same species were grouped together. The four species formed two distinct clusters: *A. breviphragma* and *A. incarnatus*; *A. fennicus* and *A. obscurus*.

The matrices generated by Jaccard coefficients and cophenetic values were highly correlated (r = 0.99), indicating that the dendrogram showed a very good fit with genetic similarity data.

TABLE 4. Monomorphic bands between families of defined species used for fingerprinting. B1, B2, B3 and B4: families of *Anagrus breviphragma*; I1, I2 and I3: families of *Anagrus incarnatus*; F1 and F2: families of *Anagrus fennicus*; O1, O2, O3 and O4: families of *Anagrus obscurus*.

	B1	B2	B3	B4	I1	I2	I3	F1	F2	O1	O2	O3	O4
OPA-8_{1700}	1	1	1	1	0	0	0	0	0	0	0	0	0
OPA-8_{1200}	0	0	0	0	0	0	0	1	1	0	0	0	0
OPA-8_{700}	0	0	0	0	1	1	1	0	0	0	0	0	0
OPA-9_{1300}	0	0	0	0	0	0	0	0	0	1	1	1	1
OPA-9_{1000}	0	0	0	0	0	0	0	0	0	1	1	1	1
OPA-9_{700}	0	0	0	0	0	0	0	1	1	0	0	0	0
OPA-9_{600}	0	0	0	0	1	1	1	0	0	0	0	0	0
OPA-9_{550}	0	0	0	0	0	0	0	0	0	1	1	1	1
OPA-11_{1700}	1	1	1	1	0	0	0	0	0	0	0	0	0
OPA-11_{400}	0	0	0	0	1	1	1	0	0	0	0	0	0
OPA-18_{1400}	1	1	1	1	0	0	0	0	0	0	0	0	0
OPA-18_{1000}	0	0	0	0	0	0	0	0	0	1	1	1	1
OPA-18_{750}	0	0	0	0	0	0	0	1	1	0	0	0	0
OPA-18_{700}	1	1	1	1	0	0	0	0	0	0	0	0	0
OPA-18_{600}	1	1	1	1	0	0	0	0	0	0	0	0	0

The values of the average genetic similarity between species (GS-between) are much lower than those of the average genetic similarity within species (GS-within), demonstrating the existence of higher similarity between families of the same species than between those belonging to different species (Table 6).

The two families of *A. fennicus* showed a quite low similarity coefficient (0.31), indicating a high intraspecific variability; however, we cannot exclude a sampling effect, since only two families and ten total individuals were analysed.

CONCLUSIONS AND DISCUSSION

In this research, based on RAPD fingerprinting, we have observed a high level of molecular polymorphism in the genus *Anagrus*; this indicates the existence of high interspecific variability, similar to what was observed in the genus *Anaphes* (Landry et al., 1993) and in the genus *Tri-*

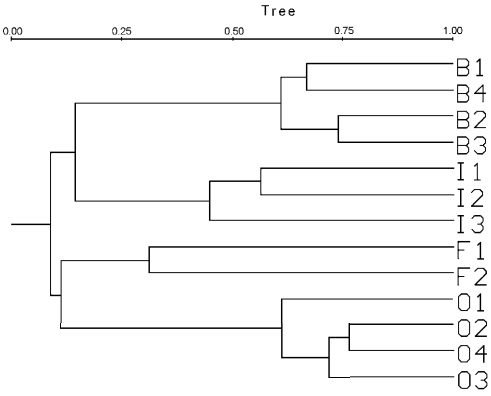

Fig. 4. Dendrogram based on UPGMA cluster analysis of genetic similarity estimates (Jaccard coefficient). B1, B2, B3 and B4: families of *A. breviphragma*; I1, I2 and I3: families of *A. incarnatus*; F1 and F2: families of *A. fennicus*; O1, O2, O3 and O4: families of *A. obscurus*.

TABLE 5. Matrix of Jaccard coefficient of similarity between the 13 families considered.

	B1	B2	B3	B4	I1	I2	I3	F1	F2	O1	O2	O3	O4
B1	1.00												
B2	0.57	1.00											
B3	0.69	0.74	1.00										
B4	0.67	0.58	0.59	1.00									
I1	0.15	0.18	0.16	0.14	1.00								
I2	0.11	0.18	0.16	0.10	0.56	1.00							
I3	0.12	0.19	0.17	0.08	0.39	0.50	1.00						
F1	0.08	0.11	0.10	0.06	0.10	0.12	0.20	1.00					
F2	0.05	0.03	0.05	0.00	0.14	0.10	0.11	0.31	1.00				
O1	0.10	0.13	0.12	0.11	0.07	0.09	0.16	0.14	0.14	1.00			
O2	0.05	0.09	0.08	0.06	0.05	0.08	0.12	0.09	0.13	0.58	1.00		
O3	0.05	0.10	0.07	0.06	0.07	0.09	0.16	0.08	0.08	0.65	0.68	1.00	
O4	0.05	0.08	0.07	0.06	0.10	0.12	0.13	0.08	0.15	0.59	0.76	0.75	1.00

chogramma (Vanlerberghe-Masutti, 1994), both belonging, like the *Anagrus*, to the superfamily of the Chalcidoidea.

Our analysis was carried out in two steps. First, assaying with RAPDs bulks of unrelated individuals belonging to some species and comparing profiles between species and then analysing bulks of genotypes derived from the same family and comparing profiles between families within and between species. This strategy allowed us to identify species specific markers, present in all the families and therefore monomorphic inside the species.

The comparison between the values of the average GS-within and those of the average GS-between confirms, also from a genetical point of view, that the families we analysed belong to four different species. In fact, the relationship between *A. breviphragma* and *A. incarnatus*, which certainly represent two specific entities, is comparable with that found between *A. obscurus* and *A. fennicus*. Therefore, the same taxonomic level should be applied.

Among the four species considered, *A. fennicus* showed the lowest average GS-within value and further investigation of a larger sample of families is needed to confirm the higher genetic variability found in this species.

RAPD analysis of the genus *Trichogramma* showed a high level of polymorphism among species (Vanlerberghe-Masutti, 1994). However, in this investigation the absence of band sharing between species did not permit the estimation of genetic similarities.

On the contrary, we found species specific RAPD markers as well as unspecific polymorphic RAPDs, shared by two or three different species. Having both kind of markers permitted us to estimate the genetic similarity between species and to construct a dendrogram.

The high level of correlation between the matrices generated by Jaccard coefficients of similarity and cophenetic values indicates that the dendrogram fit very well the genetic similarity data and thus it is possible to use it to reach some conclusions on the relationships between species.

The mutual position of the species considered in the dendrogram reflects morphological and biological observations: in *A. breviphragma* and *A. incarnatus* F2 is the longest of the funicle segments and there are no setae on the mesoscutum while in *A. fennicus* and *A. obscurus* F2 is as long as F3 and there are two setae on the mesoscutum. Furthermore, the first two species are gregarious parasitoids in *C. viridis* eggs while the other two are usually solitary parasitoids, each in its own host, *C. viridis* and *A. fasciata* respectively.

The principal objections to this type of analysis with RAPDs derive from the study of Thormann et al. (1994), which, comparing RFLP data with RAPD data, did not find a perfect correspondence between them. They could demonstrate that this was caused by scoring bands that were not homologous as identical. In spite of this, other authors (Chalmers et al., 1992; Chapco et al., 1992; Landry et al., 1993; Graham & McNicol, 1995; Black, 1996; Comencini et al., 1996; Millan et al., 1996) have used RAPDs for analysis of the relationships between species.

The presence of non homologous comigrating bands was not investigated. Therefore we cannot exclude the presence of bands, derived from different chromosomal regions, mis-scored as the same markers.

TABLE 6. Values of average genetic similarity within species (GS-within) (in bold), and values of average genetic similarity between species (GS-between).

	A. breviphragma	*A. incarnatus*	*A. fennicus*	*A. obscurus*
A. breviphragma	**0.64**			
A. incarnatus	0.14	**0.48**		
A. fennicus	0.06	0.13	**0.31**	
A. obscurus	0.08	0.10	0.11	**0.67**

However our investigation was not aimed at the estimate of the precise genetic distance between species neither at phylogenetic analysis but rather was supposed to evaluate the specific identity at the DNA level of morphologically distinct species. The eventual bias in genetic distance estimation could have inflated genetic similarity between species and therefore played against our hypothesis and conclusion.

The correspondence between bio-ethological and morphological observations and the results of the UPGMA cluster analysis on the RAPD data seems to confirm what was maintained by the authors quoted as regards the validity of the RAPD markers as an instrument for taxonomic analysis.

However, it would be interesting to widen this research to other molecular markers, such as RFLP, AFLP and DNA nucleotides sequences of 18S rRNA genes, to further verify the validity of the results we obtained.

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