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A karyological study of the genus *Pnigalio* Schrank (Hymenoptera: Eulophidae): Assessing the taxonomic utility of chromosomes at the species level

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Abstract

We provide a karyological study of 12 species of the genus *Pnigalio* in an attempt to evaluate the taxonomic utility of karyotypes at the species level. For all species of *Pnigalio* examined the number of chromosome was $2n = 12$. Karyotype formulae presented mainly metacentric and submetacentric chromosomes, although a pair of acrocentrics or subtelocentrics, shorter than biarmed chromosomes, was present in some species. The analysis of karyotypes of *Pnigalio* showed frequent but not general interspecific variability of the chromosome traits. Although most of the studied species revealed concordance between morphological and karyological characters (centromeric index and relative length), two other categories have been identified: morphologically distinct species without reciprocal differences in karyotype structure, and morphologically similar species that strongly differ in chromosomal characters.

Keywords: B chromosome, cytotaxonomy, karyogram, karyotype, morphometrics, NORs

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Introduction

Chalcidoidea are one of the most abundant and taxonomically complicated groups of insects. Despite their widely recognized practical importance as biological control agents, chalcids are still poorly studied from a morphological and biological point of view. Taxonomy of Chalcidoidea, both at the species and at the genus level, is frequently hampered by a lack of reliable morphological characters to separate different biological entities (Heraty, 2004). Cytogenetic data provide information independent from morphological and biological data, revealing differences or similarities that may not be obvious at the morphological level (Gokhman & Quicke, 1995). Furthermore, karyotypic characters, unlike many morphological features, change rather independently of the environment, so they can be useful in cases where morphological characters fail to provide an unambiguous solution to taxonomic problems (Gokhman, 2006). Within Hymenoptera, data on chromosomal morphology have been successfully used for clarifying taxonomic status and species grouping, as well as for searching and recognizing sibling species. For example, karyotaxonomy has been very successful in ants, revealing groups of sibling species (Imai *et al*., 1988). Substantial progress in chromosome research for parasitic Hymenoptera has occurred over the last 20–30 years (see

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Table 1. *Pnigalio* species used for chromosome analysis.

SPECIES	HOST	PLANT	LOCALITY
Pnigalio agraules	*Tischeria ekebladella*	*Castanea sativa*	Cumiana, Italy
Pnigalio cristatus	*Trachys coruscus*	*Malva* sp.	Cariati, Italy
Pnigalio mediterraneus PM	*Phyllonorycter millierella*	*Celtis australis*	Portici, Italy
Pnigalio mediterraneus BO	*Bactrocera oleae*	*Olea europaea*	Portici, Italy
Pnigalio nemati	*Pontania proxima*	*Salix alba*	Revello, Italy
Pnigalio pectinicornis	*Trachys troglodytiformis*	*Malva* sp.	Cariati, Italy
*Pnigalio soemius*_CP	*Cosmopterix pulchrimella*	*Parietaria diffusa*	Portici, Italy
*Pnigalio soemius*_CS	*Chrysoesthia sexguttella*	*Chenopodium album*	Sessa Aurunca, Italy
*Pnigalio soemius*_HR	*Holocacista rivillei*	*Vitis vinifera*	Latina, Italy
*Pnigalio soemius*_TA	*Trypeta artemisiae*	*Artemisia vulgaris*	Usseaux, Italy
Pnigalio vidanoi	*Rhynchaenus alni*	*Ulmus minor*	Villar Dora, Italy
Pnigalio sp. 1	*Trachys coruscus*	*Malva* sp.	Cariati, Italy
Pnigalio sp. 2	unidentified lepidopteran	unknown	Niagara Falls, USA

Gokhman, 2009, for an extensive review). Nevertheless, chromosomes of Chalcidoidea, in general, are still poorly studied. Based on the available data, it is clear that the use of the structural karyotype features in Hymenoptera taxonomy is more effective at the species level. One of the simplest and most widespread methods that can be successfully applied to find and document the differences between chromosomes and whole karyotypes is morphometric analysis, which, along with differential chromosome staining, has been used to solve taxonomic problems at the species level also in some genera of Chalcidoidea (Baldanza *et al*., 1999; Giorgini & Baldanza, 2004). The most studied chalcid genera include the highly speciose *Encarsia* Förster (Aphelinidae) and *Trichogramma* Westwood (Trichogrammatidae), with 16 and 12 karyotyped species, respectively. In this study, we provide karyotypes for 12 species (11 Palaearctic and one Nearctic) of the genus *Pnigalio* Schrank (Eulophidae), a genus comprising 57 species that are ectophagous parasitoids of leafminers, gall wasps, defoliators and carpophagous insects (Universal Chalcidoidea Database: Noyes, 2010). *Pnigalio* is an experimentally challenging non-model insect group, characterized by a poor diagnosis and a complicated taxonomic history (Bernardo *et al*., 2007; Gebiola *et al*., 2009, 2010). Literature shows that, in Eulophidae, chromosomal data can be used for solving taxonomic problems mostly at the tribe and genus level, whereas the karyotypes of very few species have been compared for a single genus, for example in *Aprostocetus* Westwood, *Melittobia* Westwood, *Euplectrus* Westwood (Gokhman, 2009). Here, using as a study case the genus *Pnigalio*, which includes both morphologically distinct and cryptic species (morphologically identical but genetically and biologically distinct species), we aimed at correlating the level of morphological variability between species to the level of karyological variability and assessing the usefulness of chromosomes as taxonomic characters.

Materials and methods

Analysis of the chromosome sets was performed on 13 populations from nine morphospecies (listed in table 1); four populations represented cryptic species of the *Pnigalio soemius* (Walker) species complex (Gebiola *et al*., 2007), while for the morphospecies *Pnigalio mediterraneus* Ferrière & Delucchi, we karyotyped two populations collected at the same location and time but on different hosts, the leafminer *Phyllonorycter millierella* (Staudinger) from leaves of *Celtis australis* (population PM) and the olive fly *Bactrocera oleae* (Rossi) from fruits of *Olea europaea* subsp. *europaea* (population BO). All species were identified by the authors and voucher specimens are deposited in the Insect Collection of the Dipartimento di Entomologia e Zoologia Agraria 'F. Silvestri' (DEZA) of the Università degli Studi di Napoli 'Federico II'. Females collected in the field were reared in the laboratory on their natural hosts (table 1) or on the laboratory host *Cosmopterix pulchrimella* Chambers (Lepidoptera: Cosmopterigidae) at 25°C, 60% RH and 14:10 L:D photoperiod, and their larval progeny were used to extract chromosomes. Metaphase chromosomes were obtained from single four-day-old female larvae collected while still feeding upon the hosts, except for *Pnigalio cristatus* (Ratzeburg) where also male larvae were used. Larvae were subjected to a modification of the 'scraping plus air-drying' method of Baldanza *et al*. (1999) and 2–6 individuals per population were analysed depending upon the availability of wasps. Each larva was nicked between head and thorax in 1.0 ml colchicine 0.1% in Shen solution (0.9 g NaCl, 0.042 g KCl, 0.025 g $CaCl_2$ in 100 ml distilled water) and incubated in a 1.5 ml tube at room temperature for one hour. After incubation, the colchicine solution containing the dissected larvae was centrifuged at 1300 rpm for 10 min. The supernatant was removed, 1.0 ml of hypotonic solution (sodium citrate 0.5%) was added to spread chromosomes and, after 20 min, the tube was centrifuged again at 1300 rpm for 10 min. The supernatant was removed, and 0.4 ml of fixative (glacial acetic acid: methanol 1:3) was added. After 30 min at room temperature, the tissues were broken up by sucking and pushing them repeatedly with a pipette equipped with a 0.2 ml tip. The volume of fixative was adjusted to 1 ml and the sample was pelleted by centrifugation at 1300 rpm for 10 min. The supernatant was removed, 1.0 ml of fixative was added, and a new centrifugation at 1300 rpm for 10 min was performed (total three washes). After the supernatant was removed, the pellet was resuspended in 30 µl of fixative and samples of 10 µl each were dropped on clean slides, air-dried and stained in Giemsa (4% in phosphate buffer pH 6.8) for 30 min. The karyotype was reconstructed from at least ten diploid plates for each parasitoid population, the only exception being *P. cristatus* for which three diploid and 11 haploid plates were used. Metaphase plates were photographed at 630× magnification using the optic microscope Zeiss Axiophot 2 fitted with the digital videocamera AxioCam HRc, and chromosomes were measured with the image analysis program AxioVision version 4.5. Morphometric

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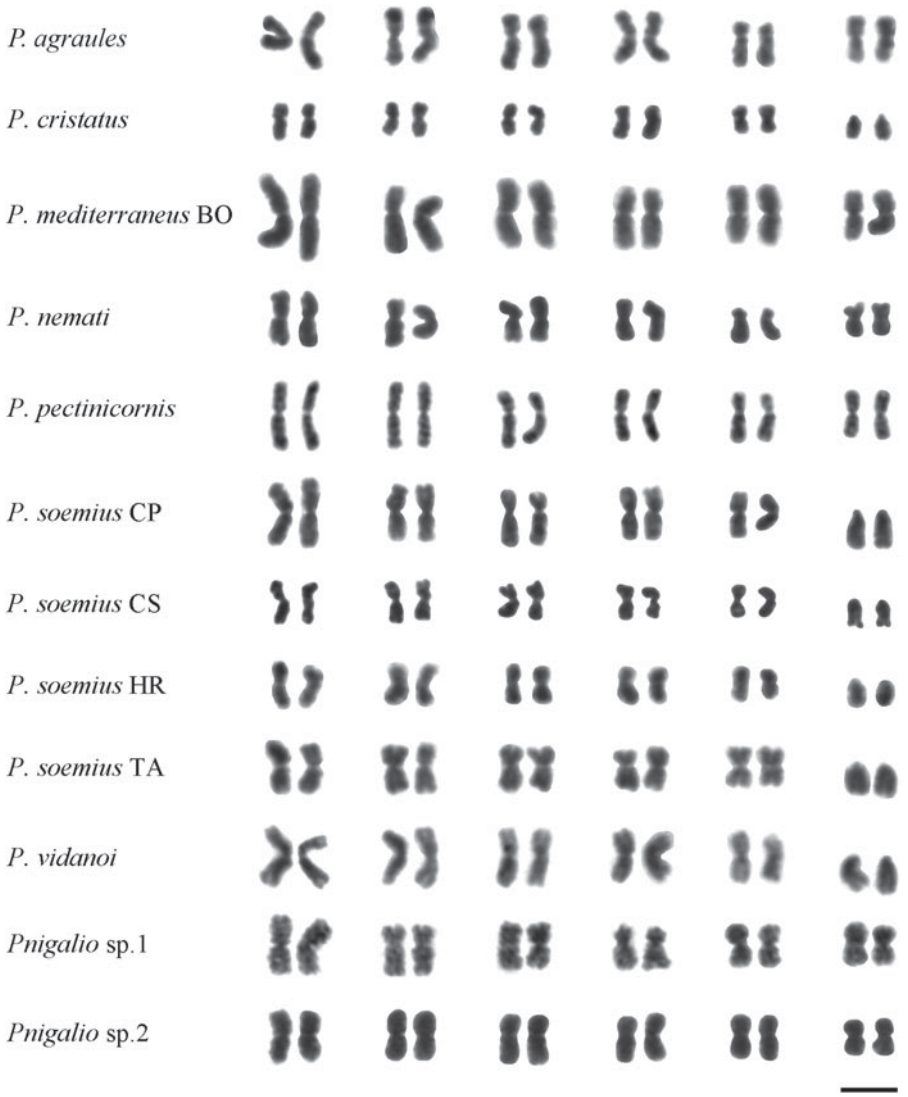

Fig. 1. Mitotic karyograms of *Pnigalio* species. Chromosomes are aligned by the centromere, and organized by decreasing relative length. Scale bar: 5 μm.

values, such as centromeric index (CI, percentage ratio of the short arm on the total length of the chromosome) and relative length (RL, percentage ratio of the absolute length of the chromosome and the total length of the haploid complement), were recorded for each couple of homologues, adopting the terminology by Levan *et al*. (1964). Nomenclature for centromere position, when the 95% confidence limits of centromeric index mean covered two chromosome categories, followed a binary terminology (Insua *et al*., 2006). The homologues in each diploid set were paired based on both RL and CI, and numbered starting from that showing the highest value of RL. Karyograms and images of metaphasic plates were obtained using the program Adobe Photoshop. In order to search for differences between karyotypes of different *Pnigalio* populations not differing for any morphometric trait, the staining of active Nucleolar Organizing Regions (NORs) was attempted. Ag-NOR staining on metaphase chromosomes was carried out according to the technique described by Maffei *et al*. (2001) after having destained the banded chromosomes first used for karyotyping.

Results

For all species of *Pnigalio* examined, the number of chromosomes was $2n = 12$. Karyograms are illustrated in fig. 1 while morphometric values are reported in table 2. Here, some features are briefly highlighted. Metacentric and meta-submetacentric chromosomes prevailed within karyotypes of *Pnigalio*, and two main classes of karyotype structures were found: one with, and the other without, a short acrocentric chromosome. Within these two main classes, differences in the morphometric values between species were recorded (table 2). In addition, two unique karyotypes were identified: one with a subtelocentric (*P. cristatus*) (fig. 2) and one with a sub-metacentric chromosome (*Pnigalio nemati* (Westwood)).

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Table 2. Chromosome morphometry and morphology of the studied *Pnigalio* species. m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric; B, B chromosome.

Chromosome pair		Relative length		Centromeric index		Classification
		Mean	SD	Mean	SD	
P. agraules $2n=8\,m+4\,m–sm$	1	19.7	0.65	43.6	3.78	m
	2	17.8	0.56	44.1	4.69	m
	3	17.1	0.55	43.1	4.97	m
	4	16.4	0.51	43.2	4.92	m
	5	15.2	0.71	39.3	5.45	m–sm
	6	13.8	0.73	43.6	6.25	m–sm
P. cristatus $2n=10\,m+2st$	1	19.7	0.60	47.0	2.81	m
	2	18.4	0.59	46.6	2.78	m
	3	17.7	0.63	44.4	4.36	m
	4	16.7	0.68	45.4	3.81	m
	5	14.3	1.04	47.5	2.00	m
	6	13.2	0.93	19.9	3.38	st
P. mediterraneus BO $2n=12\,m$	1	21.7	0.65	47.0	1.30	m
	2	18.4	0.55	44.8	5.46	m
	3	16.9	0.43	44.4	5.18	m
	4	15.6	0.57	44.8	5.01	m
	5	14.3	0.43	44.4	4.60	m
	6	13.1	0.47	47.0	3.58	m
P. mediterraneus PM $2n=12\,m+1B$	1	22.2	1.02	46.9	1.72	m
	2	18.2	0.56	43.4	5.78	m
	3	16.8	0.49	43.8	5.22	m
	4	15.5	0.42	44.5	4.66	m
	5	14.5	0.46	44.5	4.69	m
	6	12.8	0.42	47.3	2.41	m
P. nemati $2n=10\,m+2sm$	1	21.1	0.60	48.2	1.59	m
	2	18.6	0.50	44.2	5.04	m
	3	17.4	0.58	46.4	3.27	m
	4	16.3	0.72	46.0	3.06	m
	5	13.5	0.76	28.6	3.25	sm
	6	13.1	0.83	47.3	1.63	m
P. pectinicornis $2n=8\,m+4\,m–sm$	1	19.7	0.50	45.8	2.18	m
	2	18.5	0.61	47.7	1.92	m
	3	17.1	0.48	38.2	3.62	m–sm
	4	16.0	0.40	45.3	2.36	m
	5	14.7	0.50	42.5	5.35	m–sm
	6	14.0	0.58	43.9	5.31	m
P. soemius CP $2n=10\,m+2a$	1	20.8	1.08	47.0	2.54	m
	2	18.4	0.51	44.2	5.79	m
	3	17.2	0.40	44.3	4.22	m
	4	16.3	0.61	44.0	5.16	m
	5	14.2	0.74	45.7	3.80	m
	6	13.1	0.75			a
P. soemius CS $2n=10\,m+2a$	1	19.9	0.75	45.5	2.80	m
	2	18.6	0.53	45.8	3.87	m
	3	18.1	0.50	45.3	4.65	m
	4	16.8	0.74	45.0	3.82	m
	5	14.7	0.60	46.5	2.74	m
	6	11.9	0.50			a
P. soemius HR $2n=10\,m+2a$	1	19.9	0.59	46.0	2.96	m
	2	18.6	0.41	44.6	4.71	m
	3	17.7	0.39	44.8	3.86	m
	4	16.7	0.41	45.6	2.63	m
	5	14.9	0.95	46.2	2.28	m
	6	12.2	0.61			a
P. soemius TA $2n=10\,m+2a$	1	20.2	0.48	48.0	1.42	m
	2	18.5	0.67	44.0	4.20	m
	3	17.6	0.55	46.0	4.03	m
	4	16.9	0.65	44.6	4.00	m
	5	14.8	0.85	46.4	2.30	m
	6	12.0	0.76			a

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Table 2. (*Continued*)

Chromosome pair		Relative length		Centromeric index		Classification
		Mean	SD	Mean	SD	
P. vidanoi $2n=10\,m+2a$	1	21.2	0.73	46.1	2.30	m
	2	18.4	0.78	46.6	2.90	m
	3	17.4	0.78	47.1	2.17	m
	4	16.2	0.58	47.9	1.45	m
	5	14.4	0.48	47.5	2.24	m
	6	12.4	0.79			a
Pnigalio sp. 1 $2n=8\,m+4\,m–sm$	1	22.2	0.80	47.8	1.40	m
	2	17.6	0.65	43.7	5.67	m
	3	16.6	0.60	43.0	5.50	m–sm
	4	15.3	0.45	43.2	6.57	m–sm
	5	14.6	0.41	45.6	4.00	m
	6	13.7	0.48	43.5	5.28	m
Pnigalio sp. 2 $2n=10\,m+2\,m–sm$	1	19.3	0.93	47.6	2.27	m
	2	17.7	0.57	46.9	2.52	m
	3	17.3	0.57	38.1	3.61	m–sm
	4	16.4	0.46	44.7	4.28	m
	5	15.4	0.60	45.6	3.09	m
	6	13.9	0.93	44.9	4.25	m

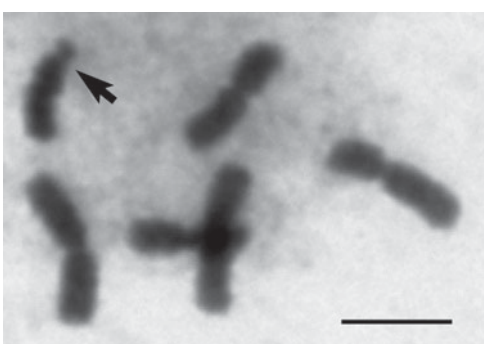

Fig. 2. Haploid mitotic metaphase plate of *P. cristatus*. Arrow indicates the subtelocentric chromosome. Scale bar: 5 µm.

The karyotype of *P. mediterraneus*, populations PM is nearly identical to population BO in terms of both CI and RL, except for the occurrence of a supernumerary (or B) chromosome with a size very smaller than the chromosomes of the regular complement and with seemingly acrocentric morphology (fig. 3). The B chromosome was found in diploid metaphase plates of four female larvae out of six observed; of these larvae, only one male (haploid) larva was analyzed showing any B chromosome.

The four different populations (cryptic species) belonging to the *P. soemius* complex and *Pnigalio vidanoi* Navone showed identical karyotype formula ($2n=10\,m+2a$; m, metacentric; a, acrocentric) and similar RL values; Ag-NOR staining did not reveal differences between the five karyotypes, showing the occurrence of one couple of NORs always located on the acrocentric pair (fig. 4).

Discussion

Comparison of the degree of morphological and karyological variability between the species of *Pnigalio* studied in this work revealed frequent but not general interspecific variability of the chromosome traits. Most of the studied species showed concordance between morphological and karyological characters, by either differing in both external morphological features and karyotype structure, or in none of them. The former case refers to *P. cristatus*, *P. nemati*, *Pnigalio pectinicornis* (Linnaeus), *Pnigalio* sp. 1 and *Pnigalio* sp. 2, which differ in morphology and karyotype reciprocally and from *Pnigalio agraules* (Walker), *P. mediterraneus*, *P. soemius* and *P. vidanoi*. The latter case concerns the *P. soemius* complex. The high biological and genetic diversity found between populations of *P. soemius* has led taxonomists to consider this entity as a complex of morphologically indistinguishable species (Gebiola *et al.*, 2007; Bernardo *et al.*, 2008). The four populations (cryptic species) studied in this paper differ for host range (monophagous vs. polyphagous) and host species,

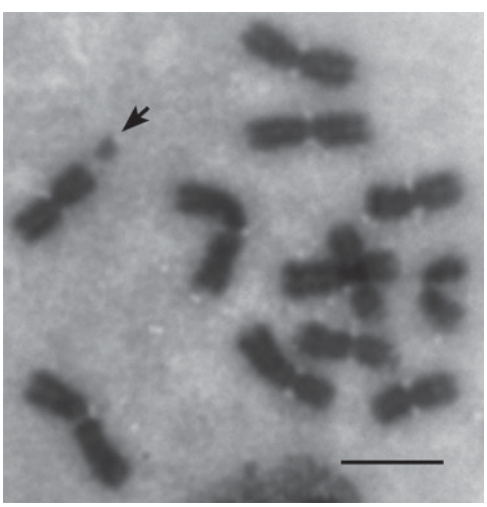

Fig. 3. Mitotic metaphase plate of *P. mediterraneus* PM. Arrow indicates the B chromosome. Scale bar: 5 µm.

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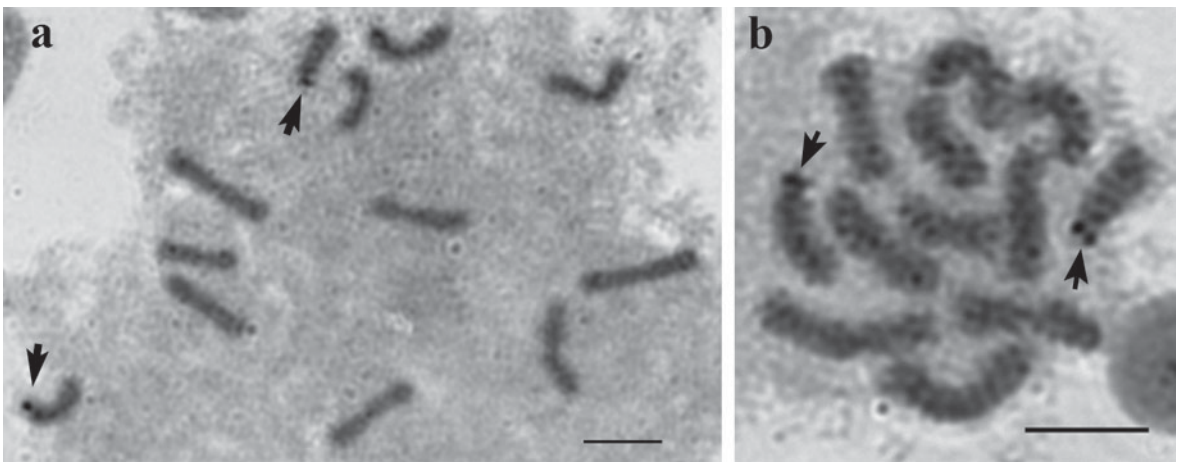

Fig. 4. NORs localization in mitotic metaphase plates of: (a) *P. soemius* CP; and (b) *P. vidanoi*. Arrows indicate NORs. Scale bar: 5 μm.

are genetically divergent (based on the mitochondrial COI gene) and engage in different reproductive modes, with the population TA characterized by thelytokous reproduction (induced by a bacterial endosymbiont: Giorgini *et al.*, 2010) rather than the typical arrhenotoky exhibited by the other populations. Notwithstanding, the four species carry the same karyotype, both in terms of morphometric traits (presence of five pairs of metacentrics and one pair of acrocentrics with reciprocally similar values of RL) and of localization of NORs, which are always located on the acrocentric chromosomes.

In contrast with the *P. soemius* complex, there are several cases reported in the literature where chromosomal data has proven critical for distinguishing cryptic species. For example, two reproductively incompatible but morphologically indistinguishable populations, belonging to the morphospecies *Encarsia sophia* (Girault & Dodd) (Hymenoptera: Aphelinidae), were found to differ in certain details of chromosomal morphometry as well as in the localization of NORs (Giorgini & Baldanza, 2004). In *Encarsia*, these karyotype differences are also supported by divergence in the fast evolving COI gene (Baldanza & Giorgini, 2001; Giorgini & Baldanza, 2004; Monti *et al.*, 2005). For the *P. soemius* species complex, the observed karyological data are not resolutive at the species level and are instead in accordance with results obtained from phylogenetic analyses of 28S-D2 rDNA. This slowly evolving gene is indeed not variable enough to resolve relationships at the species level for *P. soemius*, whilst the COI gene does (Gebiola *et al.*, 2007). Besides, the single 28S-D2 haplotype found in the *P. soemius* complex is also identical to those sequenced from *P. cristatus* (Gebiola *et al.*, 2010), yet differences between the karyotypes did emerge, with the karyotype formula of the *P. soemius* complex being $2n = 10\,m + 2a$, and that of *P. cristatus* $2n = 10\,m + 2st$ (st, subtelocentric).

Concordance between morphological and karyological differences, although in most cases obvious, does not hold for all the studied species. Indeed, two other types of relationships between different species concerning the variability of morphological and karyological characters were found, providing no evidence of a general correlation between morphological and karyotype variation among different species of *Pnigalio*.

First, morphologically well-differentiated species could not always be separated by karyotype structure. This is true between *P. vidanoi* and the populations CP, CS, HR and TA of *P. soemius*. *Pnigalio vidanoi* is easily distinguishable morphologically and genetically (Gebiola *et al.*, 2010), yet no differences were recorded either in terms of chromosomal morphometric traits or localization of NORs.

Second, morphologically cryptic species could sometimes differ strongly in their chromosomal characters. This holds for *P. mediterraneus* and *P. agraules* that were synonymized based on a lack of unambiguous traditional morphological characters discriminating between them (Askew, 1984). Gebiola *et al.* (2009) showed that the two entities are instead valid species based on both molecular data and a new morphological character, the shape of the eggs laid by females. Our chromosomal data provide a further confirmation of the species status of these two taxa, with *P. mediterraneus* showing karyotype formula $2n = 12\,m$, while *P. agraules* shows $2n = 8\,m + 4\,m–sm$ (sm, submetacentric) and the first pair of chromosomes with shorter RL. A similar pattern was also reported for two species of *Torymus* Dalman (Hymenoptera: Torymidae); *T. warreni* (Cockerell) was first synonymized with *T. californicus* (Ashmead) because they externally differ only in the body colour, but subsequently differences in karyotypes lead to the removal of the synonymy (Goodpasture & Grissell, 1975). This was the first example of application of karyological data to taxonomic decisions in Chalcidoidea.

The two populations of *P. mediterraneus* have nearly identical karyotype, except for the occurrence of a small B chromosome in the population PM. B chromosomes are additional chromosomes to the regular complement that are frequently found in natural populations of many plants and animals. They are not present in all individuals of a species, and are characterized by their dispensability, independent evolution and non-Mendelian patterns of inheritance (Camacho *et al.*, 2000). B chromosomes can have intraspecific origin from autosomes or sex chromosomes, or interspecific origin as a consequence of hybridization events (Van Vugt *et al.*, 2009). They have, in general, negative effects on the host fitness although some beneficial effect has been reported; some influence on the external phenotype has also been reported (Camacho *et al.*, 2000). B chromosomes have been poorly studied in Hymenoptera (Barth *et al.*, 2011); to date only

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three species in the superfamily Chalcidoidea have been found to harbour a single B chromosomes and only in the male sex (Baldanza *et al*., 1999; Gokhman, 2009). In two of these cases, the B chromosome has hybrid origin and behaves like paternal sex ratio (PSR) chromosome, that is, a male-biasing sex ratio distorter transmitted only via sperms (Werren, 1991; Stouthamer *et al*., 2000). In population PM, the small B chromosome was found in diploid complements of female larvae, suggesting it has no PSR role. The origin and effect of B chromosomes on the fitness of individuals bearing them and on the ecology and evolution of *P. mediterraneus* remain to be discovered.

Besides the investigation of the significance of chromosomal characters at various taxonomic levels, karyotype data currently available for parasitic Hymenoptera have been used to draw hypotheses on initial states of chromosomal characters and on the main trends and mechanisms of karyotype evolution (Gokhman, 2009). In general, it is not easy to determine the direction of chromosomal change (Sumner, 2003); and, as this study clearly shows, a deeper knowledge of single genera is necessary before any conclusion can be drawn. Indeed, even though on one hand cytotaxonomy of *Pnigalio* seems to confirm that chromosome numbers of parasitic Hymenoptera are in general relatively stable at the genus level as a result of a single evolutionary event (the so called 'genus-karyotype' concept developed by Crozier, 1975), on the other hand some unexpected results came out when compared with the data so far available for the family Eulophidae. In general, meta- and submetacentrics prevail within karyotypes of Eulophidae, although a pair of very short acro- or subtelocentrics is present in the majority of the studied species of this family, especially in those with $2n = 12$. Exceptions from this rule are *Elachertus* sp. and *Euplectrus bicolor* (Swederus), in which acrocentrics are similar in size to other elements of the sets, and *Melittobia chalybii* Ashmead, in which only biarmed chromosomes are found (Gokhman, 2009). In contrast, in the present study, we found that the karyotype with all biarmed chromosomes seems to be the most common in *Pnigalio* (seven out of 12 examined species carry it), and that the acrocentrics, when present, have a length comparable to other chromosomes of the set, like in *Elachertus* sp. and *E. bicolor*, instead of being much shorter as in most eulophids surveyed so far (Gebiola & Bernardo, 2008; Gokhman, 2009). Therefore, karyotypes with only biarmed chromosomes, which were hitherto considered exceptional, and hence apomorphic, for Eulophidae (Gokhman, 2002), are relatively abundant in *Pnigalio*. At the moment, the hypothesis that the diploid set containing five large biarmed chromosome pairs and a small acro- or subtelocentric pair is the initial state for the Eulophidae (Gokhman, 2004) still holds. Nevertheless, we are concerned that the data available could be too scarce to apply the method of outgroup comparison for inferring the ancestral family karyotype.

As for *Pnigalio*, in order to reconstruct the evolution of karyotype in this genus (towards karyotypes with acrocentric or metacentric chromosomes), we believe that a robust morphological and/or molecular phylogeny of the group on which to map the different karyotypes is first needed to test hypotheses that, based on current data, may be equally likely. This approach will also help understanding the phylogenetic placement of the unique karyotypes of *P. nemati*, a species with peculiar morphology (three-segmented clava and toothed propodeum) and biology (it only parasitizes gall wasps), as well as of *P. cristatus*.

In conclusion, the analysis of karyotypes of *Pnigalio* has shown frequent but not general interspecific variability of the chromosome traits. Although most of the studied species presented unique karyotype structure, no differences were found between two morphospecies (*P. vidanoi* and *P. soemius*) that are easily separable also by different molecular markers. Furthermore, karyological characters were not able to discriminate between cryptic species of the *P. soemius* complex. Although Ag-NOR staining was not resolutive in both cases, other banding techniques (i.e. differential chromosome staining) could be useful to discriminate apparently similar karyotypes of different species.

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