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Testing molecular barcodes: Invariant mitochondrial DNA sequences vs the larval and adult morphology of West Palaearctic *Pandasyopthalmus* species (Diptera: Syrphidae: Paragini)

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Abstract. The intra- and interspecific variability in the West Palaearctic *tibialis*-group species of the subgenus *Pandasyopthalmus* (Diptera: Syrphidae: *Paragus*) was analysed. Novel immature and molecular characters were studied and the traditionally used adult characters reviewed with the aim of establishing the status of the most widespread taxa of the *tibialis*-group in the Palaearctic region. Moreover, a review of the morphology of the larvae of the subgenus *Pandasyopthalmus* is also presented and includes the first description of the chaetotaxy of the larval head of Syrphidae. The larval morphology showed a continuum between two extremes. There is intraspecific variability in the male genitalia characters typically used for diagnostic species identification in this group. Molecular characters of the mitochondrial cytochrome *c*-oxidase subunit I (*COI*) was invariant for the West Palaearctic *Pandasyopthalmus* taxa analysed. Despite the fact that no great differences were found when compared with Afrotropical *tibialis*-group individuals (uncorrected pairwise divergence 0.17–0.35%), the divergences of the West Palaearctic vs. Nearctic and Austral-Oriental *tibialis*-group taxa varied between 1.15–2.75% (uncorrected pairwise divergence). Molecular characters of the nuclear ribosomal internal transcribed spacer region (*ITS2*) revealed several molecular haplotypes of a dinucleotide repeat that was not constrained to morphospecies or to populations of the same geographic origin. The closely related and morphologically similar species of the *tibialis*-group known from the West Palaearctic region are separable in most cases only by the shape and size of male postgonites. The results of this study support the presence of a single polymorphic taxon in the West Palaearctic region (or a very recent origin of the taxa studied). Moreover larval morphology and the lack of a clear relation between *ITS2* haplotypes and the geographic distribution or adult morphology, support the taxonomic implications of barcode taxonomy based on mitochondrial DNA for this species-group of Syrphidae.

INTRODUCTION

Paragus Latreille, 1804 the only genus in the tribe Paragini, is found on all continents other than South America and Antarctica. They are small hoverflies, mainly black in colour, with a black abdomen, except tergites 3–5, which are usually red-orange to dark yellow. At the beginning of the 20th century, most authors recognized that only a few widespread species among about twenty taxa worldwide (Kertész, 1910; Bezzi, 1915; Sack, 1929).

Stuckenberg (1954) was the first to use the male genitalia in combination with more traditional characters of adult morphology to divide *Paragus* into two subgenera: *Paragus* s. str. and *Pandasyopthalmus*. Most of the former widespread *P. tibialis* (Fallén, 1817) species complex belong to the subgenus *Pandasyopthalmus*, which is divided into two species groups, *longiventris*-and *tibialis*-groups, on the basis of the shape of the hypandrium, and facial and abdominal morphology (Stuckenberg, op. cit.). As far as we know, no representatives of the *longiventris*-group exist in the Palaearctic region, but the following seven species of the *tibialis*-group are recognized: *Paragus albipes* (Gimmerthal, 1842) [= *P. abrograns* Goeldlin de Tiefenau, 1971 sensu authors; = *P. rufocinctus* (Bruneti, 1908) sensu authors], *P. ascoensis* Goeldlin de Tiefenau & Lucas, 1981, *P. coadunatus* (Rondani, 1847), *P. constrictus* Simic, 1986, *P. haemorrhous* Meigen, 1822, *P. politus* Wiedemann, 1830 and *P. tibialis* (Fallén, 1817) (Vujic et al., 1998). In the Palaearctic catalogue (Peck, 1988), the species *P. jozanus* Matsumura, 1916 is included in *Pandasyopthalmus*. This species and a few species described from the eastern Palaearctic, Oriental and Afrotropical regions, however, fit neither of the above subgenera, as noted by Thompson & Ghorpadé (1992) and Kassebeer (1999), and therefore are not considered in the present study.

Paragus haemorrhous and *P. tibialis* are the most common and widely distributed species of *Pandasyopthalmus* in the Palaearctic region. The former is the only species of this subgenus recorded for the Nearctic and Neotropical (Central America) regions and also present in the Oriental and Afrotropical regions (Stuckenberg, 1954; Vockeroth, 1986; Claussen & Weipert, 2004). The geographic distributions of the other Palaearctic species are more restricted (Dirickx, 1994; Vujic et al., 1998), except *P. politus,* which is common in the Oriental and Australian regions (Thompson & Ghorpadé, 1992) (see Table 1).

The taxonomic descriptions of male genitalia usually include figures of lateral and ventral views of the hypandrium and surstyli, but the shape and size of the postgo-

TABLE 1. *Pandasyopthalmus* species cited in this paper. 1–8 Palaearctic species currently recognized. Distribution: Au = Australian, Af = Afrotropical, N = Nearctic, Nt = Neotropical, Or = Oriental, Pa = Palaearctic. Data available in this study: Ad = adult morphology, Lv = larval morphology, Mo = molecular analysis.

Species	Distribution	Taxonomic-group	This study
1. *P. albipes*	Or, Pa	*tibialis*-group	Not analysed
2. *P. ascoensis*	Pa	*tibialis*-group	Mo
3. *P. coadunatus*	Pa	*tibialis*-group	Ad, Lv, Mo
4. *P. constrictus*	Pa	*tibialis*-group	Ad
5. *P. haemorrhous*	Af, N, Nt, Or, Pa	*tibialis*-group	Ad, Lv, Mo
6. *P. jozanus*	Pa	unplaced	Not considered
7. *P. politus*	Au, Or, Pa	*tibialis*-group	Mo
8. *P. tibialis*	Pa	*tibialis*-group	Ad, Lv, Mo
P. goeldlini Thompson	Or	*longiventris*-group	Mo
P. minutus Hull	Af	*longiventris*-group	Mo

nite (traditionally called paramere, see Sinclair, 2000) are the main morphological characters used to separate West Palaearctic *Pandasyopthalmus* species (see Vujic et al., 1998). However, in the *tibialis*-group in particular, determination is extremely difficult because of the minute morphological differences between some species (e.g. Doczkal, 1996) and the high levels of intra-specific variability (Vockeroth, 1986). Thus, the figures that appear in early monographs must be carefully viewed to determine the shape of the postgonite [e.g., a ventrally pointed hypandrium in some species in lateral view in Goeldlin de Tiefenau (1976, p. 87) does not exist in the same lateral view in other papers, e.g. Vujic et al., 1998]. The most valuable and useful character for identifying most of the species of this group is the shape of postgonites, the patterns of sclerotised and membraneous areas and the arrangement of microtrichia on their inner surfaces (Claussen & Weipert, 2004). Females can sometimes be associated with males on the basis of distribution, but most remain undescribed because they are inseparable (Goeldlin de Tiefenau, 1976).

Preimaginal stages of the majority of *Pandasyopthalmus* species have not been described, and only a few papers on *P. haemorrhous* and *P. tibialis* are available. Existing accounts are incomplete (e.g. lack descriptions of larval chaetotaxy) or confusing and do not allow species to be distinguished (Metcalf, 1911, 1913; Campbell & Davidson, 1924; Heiss, 1938; Dixon, 1960). A very brief description of the eggs of *P. tibialis* is given in Daminova (1975). The general description of *Paragus* larvae from Rotheray & Gilbert (1989) and Rotheray (1993) is based on *P. haemorrhous*. The puparium of this species is described and illustrated by Goeldlin de Tiefenau (1974) based on a single specimen. In this paper, a review of the morphology of the larvae of *Pandasyopthalmus* species, including novel features of the head chaetotaxy of Syrphidae larvae, is presented.

Recently, molecular characters were successfully used for resolving species- and generic-level relationships in the Syrphoidea (e.g., Cheng et al., 2000; Skevington & Yeates, 2000; Ståhls et al., 2003). Mitochondrial coding genes as well as nuclear non-coding regions are fast evolving and have proven informative in Diptera, even at the intraspecific level (e.g. Paskewitz et al., 1993; Simon et al., 1994; Sharpe et al., 2000). The 3' end of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*) was informative for a species-level phylogeny of the syrphid genus *Cheilosia*, and is highly conservative within species (Ståhls & Nyblom, 2000). This gene was also used to resolve species relationships in other syrphid genera (Pérez-Bañón et al., 2003a; Ståhls et al., 2003). Hebert et al. (2003a) have proposed a DNA barcoding system for animal life based on species-specific unique sequences of a 650 bp fragment of the 5'-end of cytochrome c oxidase subunit 1 (*COI*). Thus, because this gene was informative both at intra- and interspecific levels in closely related species it was used in this study. The nuclear non-coding *ITS2* (internal transcribed spacer two) is a rapidly evolving region and has proved useful for comparing closely related insect species, subspecies or populations (e.g. Paskewitz et al., 1993; Harris & Crandall, 2000; Alvarez & Hoy, 2002; Leo & Barker, 2002). We chose to elucidate the phylogenetic information in this region of the high levels of intra-individual variation described for this region in other insects.

The West Palaearctic *tibialis*-group species of subgenus *Pandasyopthalmus* are very similar morphologically and separable in most cases only by structural differences in the male terminalia, especially crucial for species recognition is the morphology of the postgonite. As the morphological differences between the species are small and gradual, establishing discrete groups for each species is difficult. This prompted us to employ a diverse array of new larval characters and molecular evidence to elucidate the status of these taxa.

MATERIALS AND METHODS

Morphological studies

To study the variability of the different taxa, the existing taxonomic literature on *Pandasyopthalmus* species was reviewed and most of the West Palaearctic *tibialis*-group species examined, especially the numerous specimens of *P. haemorrhous* and *P. tibialis* in the entomological collection of Universidad de Alicante, Spain (Table 2). As type specimens were not consulted, materials from several collections were examined (i.e., Museo Nacional de Ciencias Naturales de Madrid, Spain; Finnish Museum of Natural History, Helsinki, Finland; British Museum of Natural History, London, UK, and University of Novi Sad, Serbia & Montenegro) to confirm species identification of males. The specimens were mainly from the Mediterranean area, but material from Northern and Central Europe, Macronesia, Nearctic and Afrotropical regions was also studied.

While some descriptions of female morphology exist in the literature, a reliable key is lacking and most female forms remain undescribed because they cannot be satisfactorily separated. Speight & Chandler (1995) indicated that males and

TABLE 2. Voucher information for the morphological studies. Each specimen is identified by a unique code; m = male, f = female. The specimens are deposited in the entomological collection of Universidad de Alicante (CEUA), Spain.

Species	Adults	Larvae	Puparia
P. coadunatus	GF-1 (f), GF-2 (f), GF-3 (m), GF-4 (m), S29 (m), S37 (m), S38 (m), ML-2 (m), ML-3 (m), ML-4 (m)	Unknown	GF-4 (m)
P. constrictus	PC-1 (m), PC-2 (f), PC-3 (m), PC-4 (m), PC-5 (f), PC-6 (m)	Unknown	Unknown
P. haemorrhous	T-653 (f), PM-2 (m), MA-1 (m), MA-2 (f), MA-3 (f), OR-537 (f), ST5113 (m), CP-3 (f), MA-14 (f), MA-15 (f), MA-16 (f), MA-17 (f), MA-18 (f), MA-19 (m), MA-183 (m), MA-213 (m), MA-802 (m), MA809 (m), ST2980 (m), S115 (m) S20 (m), S26 (m), S27 (m), S48 (m), S98 (m), S99 (m), S251 (m), S67 (m), S113 (m), SXXX (m), SXXX (m)		ST5101 (m), ST5175 (m), ST5056 (m), ST5051 (m), ST5103 (m), ST2944 (m), ST2951 (m), ST3465 (m), ST5393 (m), ST5048 (m), ST3527 (f), ST4167 (f), ST3507 (f), ST3517 (f), ST3520 (f), ST4151 (f), ST4140 (f), ST3527 (f), ST2948 (f), ST2970 (f), ST4166 (f)
P. politus	S93 (m), S95 (m), S268 (m), GF-5 (f), GF-6 (f), GF-7 (f)	Unknown	Unknown
P. tibialis	GF-8 (f), GF-9 (f), GF10 (f), PM-3 (m), MA-672 (m), MA-4 (m), MA-5 (m), S1777 (m), CP-1 (m), CP-2 (m), MA-6 (m), MB-1 (m), IR-1 (m), MA-7 (f), MA8 (f), MA-9 (f), MA-10 (f), MA-11 (f), MA-12 (f), MA-13 (f), S28 (m), S35 (m), S36 (m), S49 (m), S52 (m), S326 (m), S96 (m)		ST3486 (m), ST580 (m), ST2974 (m), ST2953 (m), ST2961 (m), ST2969 (m), SA-65-e (m), SA-65-a (m), SA-54-a (m), SA-66-c (m), ST515 (f), ST2979 (f), ST2942 (f), ST595 (f), ST518 (f), ST581 (f), ST2919 (f), ST2975 (f), ST3445 (f), SA-91-d (f)
Pandasyopthalmus spp. (West Palaearctic larvae)	—	LP-100, LP-101, LP-102, LP-103, LP-104, LP-105, LP-107, LP-108, LP-109, LP-110	—

females of *P. haemorrhous* possess abdominal transverse bands of black, reclined setae on tergites 2, 3 and often 4, and that these are absent in *P. tibialis*. Unfortunately, females of other *Pandasyopthalmus* species cannot be separated with confidence using these features. In spite of this, females identified as *P. coadunatus* and *P. constrictus,* from localities where these taxa were clearly more abundant than other species of the *tibialis*-group, were also studied. Illustrations of female terminalia were made using preserved material examined under a binocular microscope (Leica Wild M8), with an eyepiece micrometre and FSA 25 PE drawing tube.

The preimaginal morphology of *P. coadunatus*, *P. haemorrhous* and *P. tibialis* (Table 2) was also studied. Field-collected larvae from Alicante Province (Villena), Spain (30S XH 87 UTM) (*P. haemorrhou*s, *P. tibialis*; larval Figs 3A, B, 4A–D, 5A–D, 7A–D), and from Madeira Island, Portugal (*P. coadunatus*), were fed with aphids from the same colony from which they were collected. Rearing took place in a growth chamber at 16–22°C, 80 ± 5% r. h. and a constant photo period of 15L : 9D. Pupae were isolated in individual Petri dishes and inspected daily until the adults emerged.

Larvae selected for preservation were third stage and had emptied the hindgut. For permanent preservation, larvae were killed by immersion in cold water and heated slowly for about four minutes to extend them. To study the prothorax and metathorax morphology, these parts were extended by lightly pressing the first abdominal segments. Afterwards, they were preserved in 70% alcohol. The cephalopharyngeal skeleton was removed from the leading ventral edge of the interior of the puparium and placed in warm 10% potassium hydroxide (KOH) for 3–4 min. The larvae were then immediately washed in distilled water and preserved in pure glycerine prior to examination.

Descriptions are based on preserved specimens with larval characters checked against living specimens (or slides) to minimise errors due to preservation. Measurements of dimensions (mean ± standard error) were made on preserved material using a binocular microscope. The photographs were taken with a scanning electron microscope (SEM; JEOL 840, operated at 20 kV).

Terminology used for descriptions of the larvae and pupae follows Hartley (1961, 1963) and Thompson & Rotheray (1998). The positions of the sensilla were numbered sequentially from the dorsal to the ventral surface for each segment (Rotheray, 1991). In addition, included in parentheses is the morphological terminology used by Courtney et al. (2000) and Sinclair (2000), according to the current understanding of the homology of dipteran larvae and male terminalia, respectively.

Molecular studies

DNA was extracted from frozen specimens or from specimens preserved in 70–95% alcohol. The biological data of specimens and GenBank accession numbers for sequence fragments are listed in Table 3. Male genitalia were conserved for the purpose of morphological studies. DNA was extracted from single individuals by crushing and incubation at +37°C for approximately 18 h in Proteinase K (20 mg/ml), followed by 2 M sodium acetate/ethanol precipitation (96%) and re-suspension in 50–100 µl of ultra-pure water.

PCR's were carried out in 50 µl reactions containing 2 µl DNA extract, 2 µl of each primer (at 10 pmol/µl), 0.25 µl of Amplitaq DNA polymerase (5U / µl), 4 µl 2.5 mM $MgCl_2$, 5 µl 10X Buffer II (Applied Biosystems, Foster City, CA, USA) and 4 µl 200 mM dNTP (GeneAmp) and water, or in 25 µl reactions using half amounts. Thermocycler conditions were initial denaturing at 95°C 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s

TABLE 3. Taxonomic list, number of individuals, stage/reference, source, and GenBank accession numbers of specimens used in the molecular analysis. Notes. One sequence from each geographical origin was submitted to GenBank [between brackets] (*) refers to ITS2 sequences, no symbols are *COI* sequences. Male genitalia and voucher specimens are conserved in the entomological collection of University of Alicante (CEUA), Spain. NS means *COI* not submitted.

Species	Stage / ref	Source of specimens	GenBank nos
Pipiza sp. (Outgroup)	1 male / S0	Sweden, Uppland,	AY174459 [S0]
Sg. *Pandasyopthalmus* (*tibialis* group)			
P. ascoensis	1 male / Y3	Sardinia, 2003	COI & *ITS2 submitted
P. coadunatus	3 males / S29-S37-S38; 2 females/ S333-S334-S336	Malta, Msida, 1999 AY174467 [S38]	*AY217732 [S333] *AY217733 [S37] *AY217734 [S336] *ITS2 submitted [S334]
P. haemorrhous	4 males / S20-S26-S27-S48; 1 female/S14	Spain, Alicante, 2000	AY174470 [S48] *ITS2 submitted [S14]
P. haemorrhous	2 males / S98-S99	Greece, Lesbos island, 2001	AY174479 [S98] *AY217736 [S98] *AY217737 [S99]
P. haemorrhous	1 male / S251	Finland, Strömfors, 2002	AY174466 [S251] *AY217730 [S251]
P. haemorrhous	1 male / S67	Czech Republic, Rokytno, 2000	N.S. *Sequence from LCO primer only
P. haemorrhous	2 males / S113-S115	South Africa, Koop SV Drift, 2000	AY174471 [S115]
P. haemorrhous	2 males / S308-S310	Nearctic, 1999	AY275521 [S308]
P. politus	1 male / S91	Australia, Daintree National Park, 1999	AY174460 [S91]
P. nr politus	1 male / S95	Thailand, 2000	AY174461 [S95]
P. tibialis	5 males / S28-S35-S36-S49-S52; 1 female / S03	Spain, Alicante, Teruel, 2000	AY174465 [S52] *ITS2 submitted [S03]
P. tibialis	1 male / S96	Greece, Lesbos island, 2001	AY174468 [S96] *AY217735 [S96]
P. tibialis	1 male / S326	Finland, Eno, 2002	COI submitted [S326] *AY217731 [S326]
Sg. *Pandasyopthalmus* (*longiventris*-group)			
P. nr *goeldlini*	1 female / S275	West Malaysia, 2001	AY174463 [S275]
P. nr *minutus*	1 female / S286	Tanzania, 2001	AY275522 [S286]
Subgenus *Paragus*			
P. bicolor Fabricius	1 male / S110	Turkey, Sivas, 2001	AY174462 [S110]
P. quadrifasciatus Meigen	1 male / S112	Turkey, Sivas, 2001	AY174464 [S112]

annealing at 49°C, 2 min extension at 72°C, followed by a final extension of 8 min at 72°C. PCR products were purified using the GFX PCR Purification Kit (Amersham Biotech, Little Chalfont, UK) and then sequenced (with the PCR primers) in both directions using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) at one-fourth of the recommended volumes on ABI PRISM 377 and ABI 310 automated DNA sequencers. The universally conserved primers used for amplifying and sequencing the *COI* and *ITS2* fragments are listed in Table 4. Generally, the *COI* fragment was amplified using the forward primer LCO-S-1490 and the reverse primer TL2-N-3014, and sequencing was performed with amplification primers, the internal forward primer C1-S-1718 and the reverse primer C1-J-2183. The homologous *COI* sequence fragment could also be obtained by using primer combinations LCO-S-1490 + C1-J-2183 and C1-S-1718 + TL2-N-3014 and the above PCR and sequencing conditions. The *ITS2* fragment was amplified and sequenced using the same primers. The sequences were edited for base-calling errors and assembled using Sequence Navigator™ (version 1.01, Applied Biosystems).

TABLE 4. Primers used for amplifying and sequencing the COI and ITS2 fragments.

Primer	Sequence	Source
LCO-S-1490	5'-GGTCAACAAATCATAAAGATATATTGG-3'	Folmer et al., 1994
TL2-N-3014	5'-TCCAATGCACTAATCTGCCATATTA-3'	Folmer et al., 1994
C1-S-1718	5'-GGAGGATTTGGAATTGATTAGTTCC-3'	Simon et al., 1994
C1-J-2183	5'-CAACATTTATTTTGATTTTTTGG-3'	Simon et al., 1994
ITS2A (f)	5'-TGTGAACTGCAGGACACAT-3'	Beebe & Saul, 1995
ITS2B (r)	5'-TATGCTTAAATTCAGGGGGT-3'	Beebe & Saul, 1995

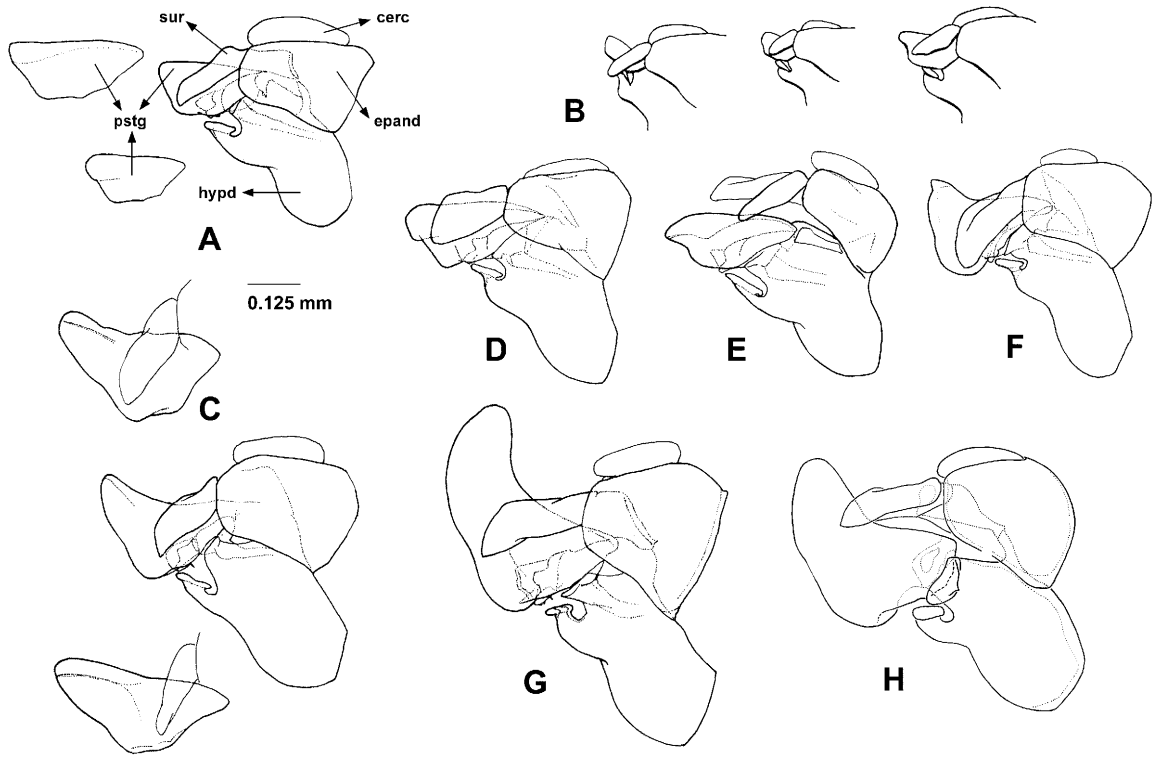

Fig. 1. Male genitalia of some West Palaearctic *tibialis*-group species, showing variability in the structure of the postgonites (lateral view). A, B – *P. haemorrhous*; C – *P. constrictus*; D – *P. albipes*; E – *P. coadunatus*; F – *P. ascoensis*; G – *P. tibialis* (Mediterranean); H – *P. tibialis* (Germany). Adapted from: A, C, D, E, F, G: Vujic et al., 1998; B: Speight, 1978; H: Doczkal, 1996. (No scale available for drawing B and H). Abbreviations: cerc – cercus, epand – epandrium, hypd – hypandrium, sur – surstylus, pstg – postgonite (= paramere).

Altogether 1128 mitochondrial nucleotide characters were obtained, spanning nucleotide positions 1563 to 2691 of the mtDNA *COI* gene (numbering is based on *Drosophila yakuba* sequence; Clary & Wolstenholme, 1985). Nucleotide sequences of the *COI* gene of multiple specimens of both groups of the subgenus *Pandasyopthalmus* (*tibialis*- and *longiventris*-groups), and a few species of the subgenus *Paragus* s. str., were produced (see Table 3). However, this study's main goal was to assess the taxonomic status of the West Palaearctic *tibialis*-group of species, not to explore the phylogenetic relationships of these species and/or subgenera of *Paragus*. The acquired sequence data, therefore, was used mainly to contrast the uncorrected pairwise sequence divergences of the sequenced taxa and explore the levels of sequence variation. As the sequences were mainly used to compare closely related species, only uncorrected sequence divergences were examined (as % sequence differences), simply because any correction algorithm will affect the *p* values when sequence divergences are >5%. ITS2 sequences for a set of twelve specimens of the West Palaearctic *tibialis*-group species, chosen to represent different morphotypes (e.g., *P. ascoensis, P. coadunatus, P. haemorrhous, P. tibialis* taxa) and different geographical regions, were produced (see Table 3).

Using the *COI* sequences of altogether 16 terminals, a parsimony analysis with equal weights was performed using the computer program Nona version 2.0 (Goloboff, 1993), spawned from WinClada version 1.00.08 (Nixon, 2002), to study the phylogenetic relationships between the included taxa. Bootstrap values (branch support) were obtained with WinClada using 500 replicates.

RESULTS

Adult morphology

Our examination of the male genitalia in a large series of the most widely distributed *Pandasyopthalmus* species, *P. haemorrhous*, confirmed the great variability in the shape and size of the postgonites, which is reported in the literature (see Fig. 1A, B, C). These differences are not correlated with the size of the adult fly and occur typically at the intraspecific level.

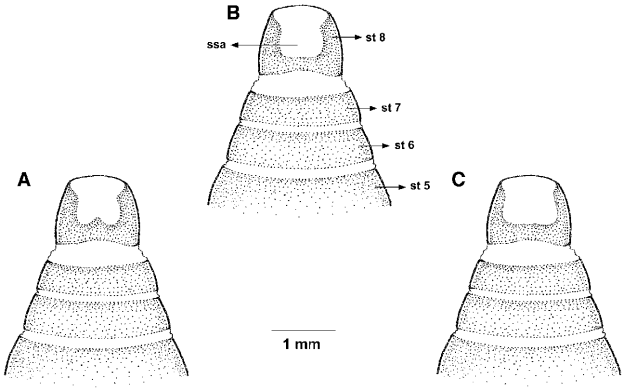

Fig. 2. Female terminalia of some West Palaearctic *tibialis*-group species. Ventral view of sternite 8 (st 8) showing the shape and size of the distal slightly sclerotized area (ssa). A – *P. haemorrhous*; B – *P. coadunatus*; C – *P. tibialis*. Epiproct and cerci are omitted.

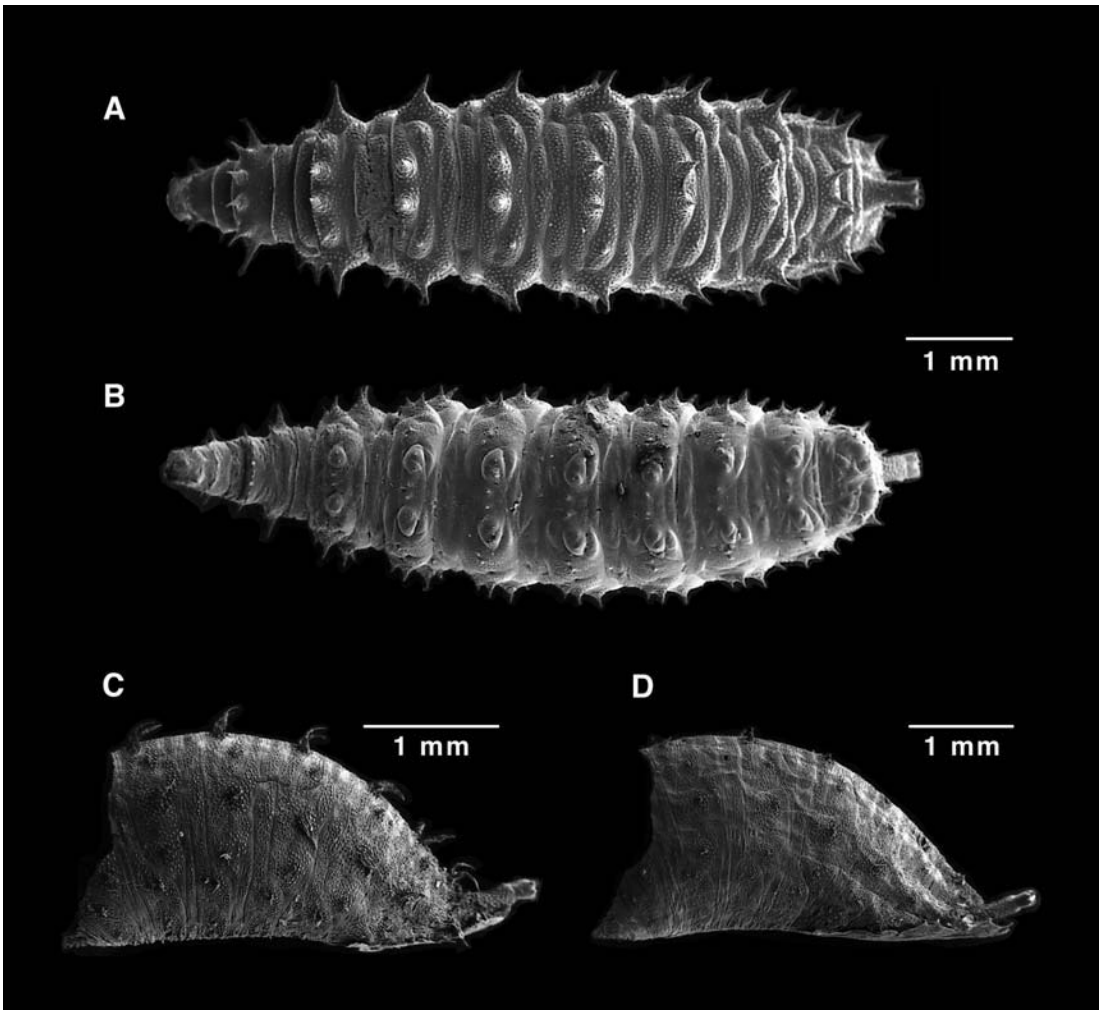

Fig. 3. Scanning electron micrographs of the immature stages of West Palaearctic *tibialis*-group species. A and B: larvae, dorsal view (A), ventral view (B); C and D: lateral view of puparium, *P. haemorrhous* (C), *P. tibialis* (D).

In the case of *P. tibialis*, small differences in the shape and size of postgonites are apparent when descriptions of material from different countries are compared. This variation occurs in other species of the Palaearctic *tibialis*-group with more restricted distributions, such as *P. constrictus* (Fig. 1, D–E).

The postabdomen of females of the exemplars studied (see Table 2) was usually entirely black, with tergites and sternites 6, 7 and 8 telescoped within the preceding sclerites. No dorsal sclerites beyond tergite 8 were found, cerci occupy a mid-dorsal position; sub-anal plate present. The females of *P. haemorrhous* and *P. tibialis* possess sternite 8, with a slightly sclerotized distal area of variable form. No intraspecific variability is apparent. However, in *P. coadunatus* the shape and size of this area is of an intermediate form (Fig. 2).

The abdominal transverse bands of black and reclined setae in *P. haemorrhous* are also present in females of *P. coadunatus*. However, this character is highly variable in males of *P. coadunatus* as well as females and males of *P. tibialis* and *P. constrictus*. These taxa either completely lack these setae, have only whitish and erect hairs, or have a small group of black setae in the middle of some tergites, which never form a continuous band. A marked variability was observed in the abdominal colour pattern of both sexes of *P. haemorrhous* and *P. tibialis*. The range is from entirely black to tergites 3, 4 and 5 being reddish, with all possible intermediates. Our speci-

TABLE 4. Primers used for amplifying and sequencing the COI and ITS2 fragments.

Primer	Sequence	Source
LCO-S-1490	5'-GGTCAACAAATCATAAAGATATATTGG-3'	Folmer et al., 1994
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C1-S-1718	5'-GGAGGATTTGGAATTGATTAGTTCC-3'	Simon et al., 1994
C1-J-2183	5'-CAACATTTATTTTGATTTTTTGG-3'	Simon et al., 1994
ITS2A (f)	5'-TGTGAACTGCAGGACACAT-3'	Beebe & Saul, 1995
ITS2B (r)	5'-TATGCTTAAATTCAGGGGGT-3'	Beebe & Saul, 1995

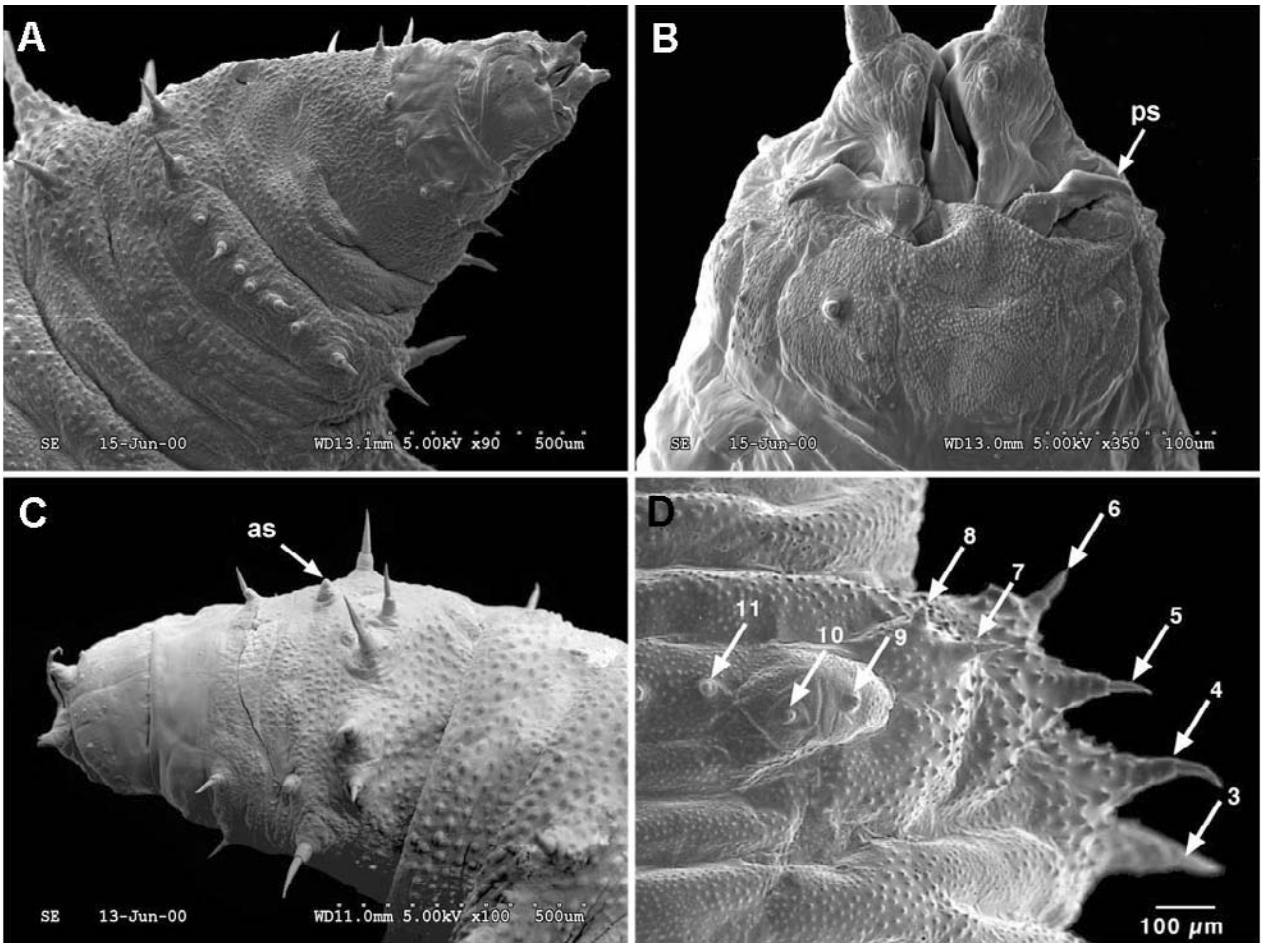

Fig. 4. Scanning electron micrographs of West Palaearctic *tibialis*-group larvae. A – head and thorax, ventral view; B – head and prothorax (ps – prothoracic sclerites); C – head and thorax, dorsal view (as – anterior spiracles); D – detail of locomotory prominences and ventral chaetotaxy (numbers) of the first abdominal segment.

mens of *P. coadunatus* had entirely black abdomens, but in the literature, other forms are also described (Goeldlin de Tiefenau, 1976).

Immature morphology

Description of the larvae

Full descriptions of the larval and puparial morphology of all the specimens studied are presented. Some specimens were reared and identified as: *P. coadunatus*, *P. haemorrhous* or *P. tibialis* (see methodology).

Overall appearance. Oval in cross-section with a flattened ventral surface, strongly tapering anteriorly and slightly tapering posteriorly (Fig. 3). Dorsal habitus serrate owing to fleshy lateral projections. Cuticle translucent when alive, creamy to dark brown after fixation. Colour pattern extremely variable, pearly white to mottled white or brown to reddish-brown. Dorsal body surface covered with dome-shaped and densely aggregated papillae, which are smaller on the ventral surface (Fig. 4A). Dorsal sensilla borne on fleshy projections (Fig. 3A). The main diagnostic feature of this taxon is the shape of posterior spiracle: at least twice as long as broad. Length 6.7 ± 0.17 mm, maximum width 2.18 ± 0.034 mm (n = 10).

Head (pseudocephalon). The head is very reduced. Mouth hooks adapted for piercing-feeding (Hartley, 1963) with the distinctive features of predacious syrphid larvae. Antenno-maxillae organs well-developed. Ultrastructure in SEM photos highlighted the occurrence of many sensilla on top of the antenno-maxillae organs, mainly chemoreceptors (styloconics and basiconics), but also mechanoreceptors (placoids), as in other aphidophagous larvae (Ngamo Tinkeu & Hance, 2002).

Thorax. Anterolateral margin of the prothorax with a pair of black triangular pointed sclerites (ps) (Fig. 4B). Vestiture on prothorax above 4th sensilla of mesothorax is reduced, giving the integument a clear shiny appearance. Anterior fold of the prothorax with longitudinal grooves and a ring (covering < 35% of dorsal surface and < 50% of ventral surface) of small, densely aggregated, backwardly directed spicules, which become progressively scarcer posteriorly (Fig. 4B). A second ring of small spicules appears immediately anterior to 4th sensilla of mesothorax. Dorsal surface of the prothorax with anterior spiracles (as) sclerotized and short (Fig. 4C).

Abdomen. Locomotory prominences small, without crochets or musculature (Fig. 4D), 7 abdominal pairs on segments 1–7 (Fig. 3B). Anal segment (anal division) bi-

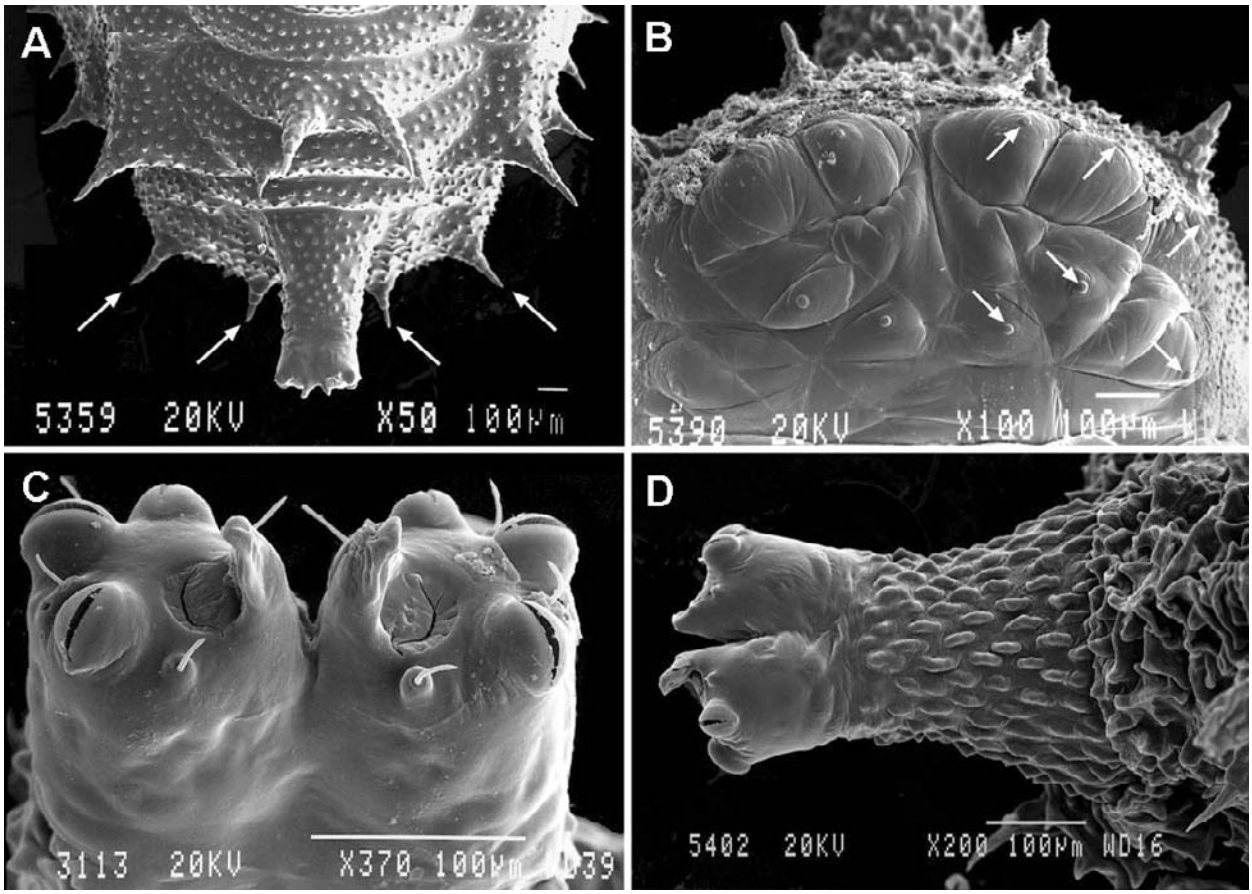

Fig. 5. Scanning electron micrographs of anal segment and posterior spiracle (ps) of West Palaearctic *tibialis*-group larvae. A–B, anal segment and chaetotaxy (arrows indicate the position of sensilla), dorsal view (2+2 sensilla) (A), ventral view (sensilla of one side) (B); C – spiracular plates of ps; D – ornamentation of ps, dorsal view.

lobed, with a row of 4 setae behind the posterior spiracle (Fig. 5A). Vestiture on ventral surface of anal segment is absent, giving the integument a clear shiny appearance (Fig. 5B). *Posterior spiracle* (ps): Length 0.48 ± 0.009 mm; width: at base 0.30 ± 0.007 mm, at tip 0.24 ± 0.003 mm; (n = 10). Lustrous, sclerotized and brown in colour. The spiracular plates are separated by a deep median groove. Dorsal spurs pointed and V-shaped, about as long as the length of the spiracular slits (Fig. 5C, D). The spiracular slits are mounted on high carinae, extending about half their length over the sides of the posterior spiracle. Carinae not differentiated in colour from the rest of the ps. Spiracular slits radial and straight, equidistant from each other. Periphery with four pairs of long interspiracular setae (peristigmatal setae) mounted on small cones (Fig. 5C). Surface of ps with a constriction about two-thirds of the distance from the base. The basal two-thirds strongly nodulate, beyond this the surface is smoother and polished (Fig. 5D).

Chaetotaxy (Fig. 6). Dorsal and dorsolateral sensilla borne on fleshy projections with one seta. The size of the setae and fleshy projections is variable, setae never longer than the fleshy projections. Head (H) with 2 pairs of sensilla of different sizes above the mouth and below the antenno-maxillae organs (Fig. 7A). Ventral sensilla without accompanying setae or projection, except on the metathorax (Fig. 7B). Prothorax (P) with 11 pairs of sensilla; mesothorax (Ms) with 8 pairs of sensilla; metathorax (Mt) with 9 pairs of sensilla belonging to the pattern of chaetotaxy. One extra pair of sensory organs near sensilla 7 on mesothorax and metathorax (Fig. 7C, D); abdominal segments 1–7 with 11 pairs (Fig. 4D); anal segment with 8 pairs (Fig. 5B).

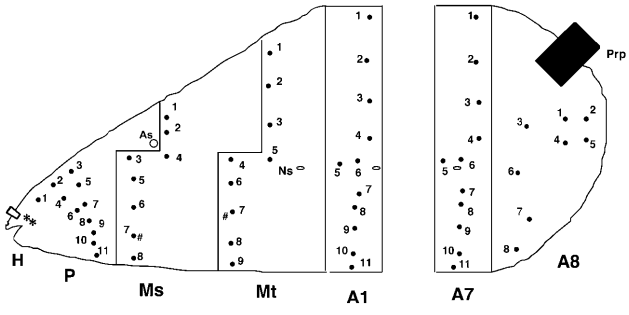

Fig. 6. Map of the chaetotaxy of third instar larvae of the *tibialis*-group species showing the positions of the groups of sensilla on: H – head; P – prothorax; Ms – mesothorax; Mt – metathorax; A1, A7 – first and seventh abdominal segments; A8 – posterior end; As – anterior spiracle; Ns – nonfunctional spiracle; Prp – posterior spiracles. Symbols: * – head chaetotaxy; # – extra pair of sensory organs.

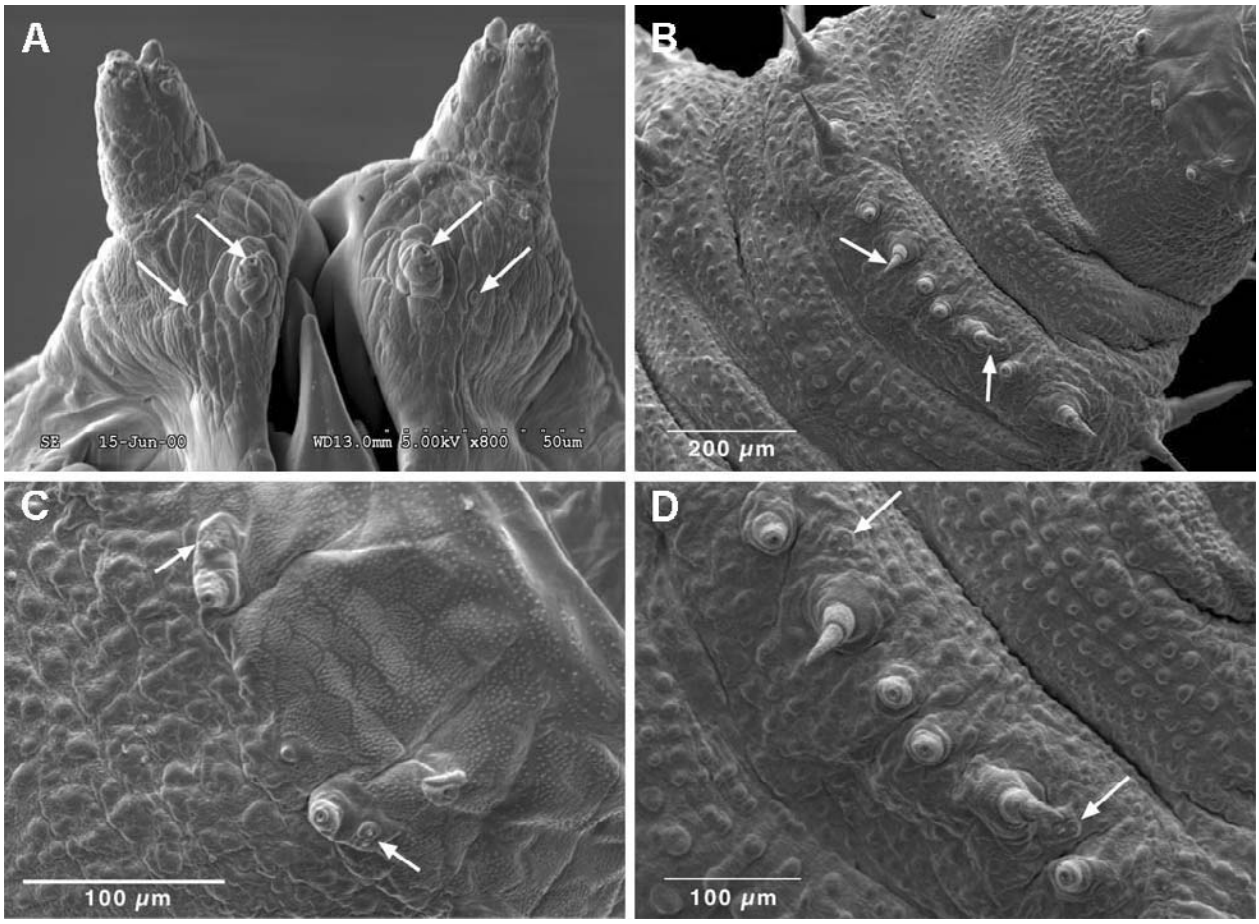

Fig. 7. Scanning electron micrographs of chaetotaxy of the third instar larva of a West Palaearctic *tibialis*-group species. A – head chaetotaxy; B – ventral sensilla of the metathorax; C and D – extra pair of sensory organs on mesothorax (C) and metathorax (D).

Description of the puparium

Pear-shaped, sub-cylindrical in cross-section. Anterior extreme truncated, tapered posteriorly and flattened ventrally (Fig. 8). Integument with larval segmentation persisting as transverse folds and wrinkles, segmental sensilla also persisting, except on the ventral surface. Colour varying from cream to dull brown. Larval characters well preserved in the puparium, such as the shape of the posterior spiracles, which are at least twice as long as broad and by which the Palaearctic *tibialis*-group puparium can be identified. Carinae supporting spiracular slits are black. Length, including posterior spiracles, 3.9 ± 0.11 mm, maximum width 1.8 ± 0.06 mm (n = 10).

Two extremes of variability in the length of sensilla were found. Puparia with long sensilla usually belonged to *P. haemorrhous* (Figs 8A, B, 9A), based on rearing and identifying the adult stages. Puparia with short sensilla were generally *P. tibialis* (Figs 8F, 9D), however, intermediates exist, which are not possible to assign to either species (Figs 8C, D, 9B, C). *Paragus coadunatus* is intermediate in variability between the two extremes (Fig. 8E).

Molecular studies

Altogether 1128 mitochondrial nucleotide characters of the mtDNA *COI* gene were identified. No alignment ambiguity was present. The nucleotide frequencies were biased toward A + T, averaging 71%. The sequences of the *COI* gene for West Palaearctic taxa of subgenus *Pandasyopthalmus* were identical. The number of parsimony informative nucleotide characters for the 15 ingroup terminals used for phylogenetic analysis of the *COI* gene was 144. The parsimony analysis resulted in two equally parsimonious trees (l = 355 steps, CI = 0.83, RI = 0.82). A strict consensus tree with the calculated support values is shown in Fig. 10.

The *ITS2* sequences were obtained for 12 specimens of the West-Palaearctic *tibialis*-group species (*P. ascoensis, P. coadunatus*, *P. haemorrhous*, *P. tibialis*) (Table 3), chosen to represent different geographic regions and different taxa. The *ITS2* sequences were assembled in a manual alignment of about 480 bp. Four different haplotypes, variations of a dinucleotide repeat (AT)6–9 (typically interpreted as a microsatellite region), were found in the specimens analyzed (Table 5). Other sequence variation was a few nucleotide changes (indicated in boldface, Table 5). As examples of variation, identical haplotypes were found in one specimen of *P. haemorrhous* from West Greece (Lesbos) and two specimens of *P. coadunatus* from Malta, and another individual of *P. haemorrhous* from West Greece had an identical haplotype with individuals of *P. tibialis* and *P. haemorrhous* from Finland, as did two individuals of *P. coadunatus* from Malta

TABLE 5. Molecular variation of the variable region of ITS2 sequences of West-Palaearctic *Pandasyopthalmus* species studied in this paper.

```
S333_coadunatus_Malta      ...TTTATAGAAAGCTATATATAATATATATATATAAATATATAAATTACGTTACGTTA...
S336_coadunatus_Malta      ...TTTATAGAAAGCTATATATAATATATATATATAAATATATAAATTACGTTACGTTA...
S003_tibialis_Spain        ...TTTATAGAAAGCTATATATAATATATATATATAAATATATAAATTACGTTACGTTA...
S096_tibialis_Greece       ...TTTATAGAAGGCTATATATA-----TATATATAAATATATAAATTATGTTGCGTTG...
S099_haemorrhous_Greece    ...TTTATAGAAGGCTATATATA-----TATATATAAATATATAAATTACGTTACGTTA...
S326_tibialis_Finland      ...TTTATAGAAGGCTATATATA-----TATATATAAATATATAAATTACGTTACGTTA...
S251_haemorrhous_Finland   ...TTTATAGAAGGCTATATATA-----TATATATAAATATATAAATTACGTTACGTTA...
S014_haemorrhous_Spain     ...TTAATAGAAGGCTATATATA-----TATATATAAATATATAAATTACGTTACGTTA...
S038_coadunatus_Malta      ...TTTATAGAAGGCTATATATA-------TATATAAATATATAAATTACGTTACGTTA...
S098_haemorrhous_Greece    ...TTTATAGAAGGCTATATATA-------TATATAAATATATAAATTACGTTACGTTA...
S334_coadunatus_Malta      ...TTTATAGAAGGCTATATATA-------TATATAAATATATAAATTACGTTACGTTA...
Y3_ascoensis_Sardinia      ...TTTATAGAAGGCTATATATA---------TATAAATATATAAATTACGTTACGTTA...
```

and one specimen of *P. tibialis* from Spain, and a specimen of *P. tibialis* from Greece had a unique haplotype, as did one specimen of *P ascoensis* from Sardinia and one specimen of *P. haemorrhous* from Spain (Table 5).

The uncorrected sequence divergences for the mitochondrial *COI* within the *Pandasyopthalmus* lineage varied between 0.0% and 7.7% (Table 6). In most cases, the West Palaearctic specimens of *tibialis*-group taxa (*P. coadunatus*, *P. haemorrhous* and *P. tibialis*) had identical sequences with a maximum of uncorrected pairwise difference of 0.35% when compared with the Afrotropical sequence of *P. haemorrhous*. However, the Nearctic specimens (*P. haemorrhous*) sequenced for the *COI* gene showed an uncorrected pairwise difference of 2.4% compared with West Palaearctic *Pandasyopthalmus*. The interspecific divergences in this group varied from 1.1% to 2.7% compared with the Australian-Oriental species of the *tibialis*-group, but this figure increased to 6.6–7.2% when species of the *longiventris*-group were considered (Table 6).

The uncorrected pairwise sequence divergences within the studied species of the subgenus *Paragus* s. str. and the *longiventris* group were 5.9% and 1.3%, respectively. The variation between the *Pandasyopthalmus* and *Paragus* s. str. was 9.3% to 11.3% (Table 6).

DISCUSSION

Adult morphology

Stuckenberg (1954) was the first to describe the intraspecific variability in the structure of the male genitalia in some species of the subgenus *Pandasyopthalmus*. He illustrated the variation in the male genitalia of *Paragus*

TABLE 6. % pairwise uncorrected sequence differences. Au = Australian, Af = Afrotropical, Ne = Nearctic, Or = Oriental, Pa = Palaearctic. Countries: Finland (Fin), Greece (Gre), Spain (Spa). Species-groups: 1–12 = *tibialis*-group; 13–14: *longiventris*-group; 15–16 = *Paragus* s. str.-group. Note: We also have a sequence from the LCO primer only of *P. haemorrhous* from the Czech Republic (see Table 3). This fragment does not differ from that of other West Palaearctic taxa analysed.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *P. ascoensis* (Pa)	-															
2 *P. coadunatus* (Pa)	0.08	-														
3 *P. haemorrhous* (Fin)	0.08	0.00	-													
4 *P. haemorrhous* (Gre)	0.08	0.00	0.00	-												
5 *P. haemorrhous* (Spa)	0.08	0.00	0.00	0.00	-											
6 *P. tibialis* (Fin)	0.08	0.00	0.00	0.00	0.00	-										
7 *P. tibialis* (Gre)	0.17	0.08	0.08	0.08	0.08	0.08	-									
8 *P. tibialis* (Spa)	0.17	0.08	0.08	0.08	0.08	0.08	0.17	-								
9 *P. haemorrhous* (Af)	0.17	0.26	0.26	0.26	0.26	0.26	0.35	0.35	-							
10 *P. haemorrhous* (Ne)	2.23	2.23	2.23	2.23	2.23	2.23	2.37	2.38	2.23	-						
11 *P. politus* (Au)	2.57	2.66	2.66	2.66	2.66	2.66	2.75	2.75	2.57	2.98	-					
12 *P.* nr *politus* (Or)	1.06	1.15	1.15	1.15	1.15	1.15	1.24	1.24	1.06	2.42	2.39	-				
13 *P.* nr *goeldlini* (Or)	7.09	7.18	7.18	7.18	7.18	7.18	7.26	7.26	7.26	5.75	7.72	7.09	-			
14 *P.* nr *minutus* (Af)	6.66	6.75	6.75	6.75	6.75	6.75	6.84	6.84	6.66	5.40	6.93	6.48	1.33	-		
15 *P. bicolor* (Pa)	10.72	10.81	10.81	10.81	10.81	10.81	10.90	10.90	10.72	9.39	10.74	10.63	9.84	9.95	-	
16 *P. quadrifasciatus* (Pa)	10.72	10.81	10.81	10.81	10.81	10.81	10.90	10.90	10.72	10.33	11.27	10.72	10.63	10.75	5.93	-

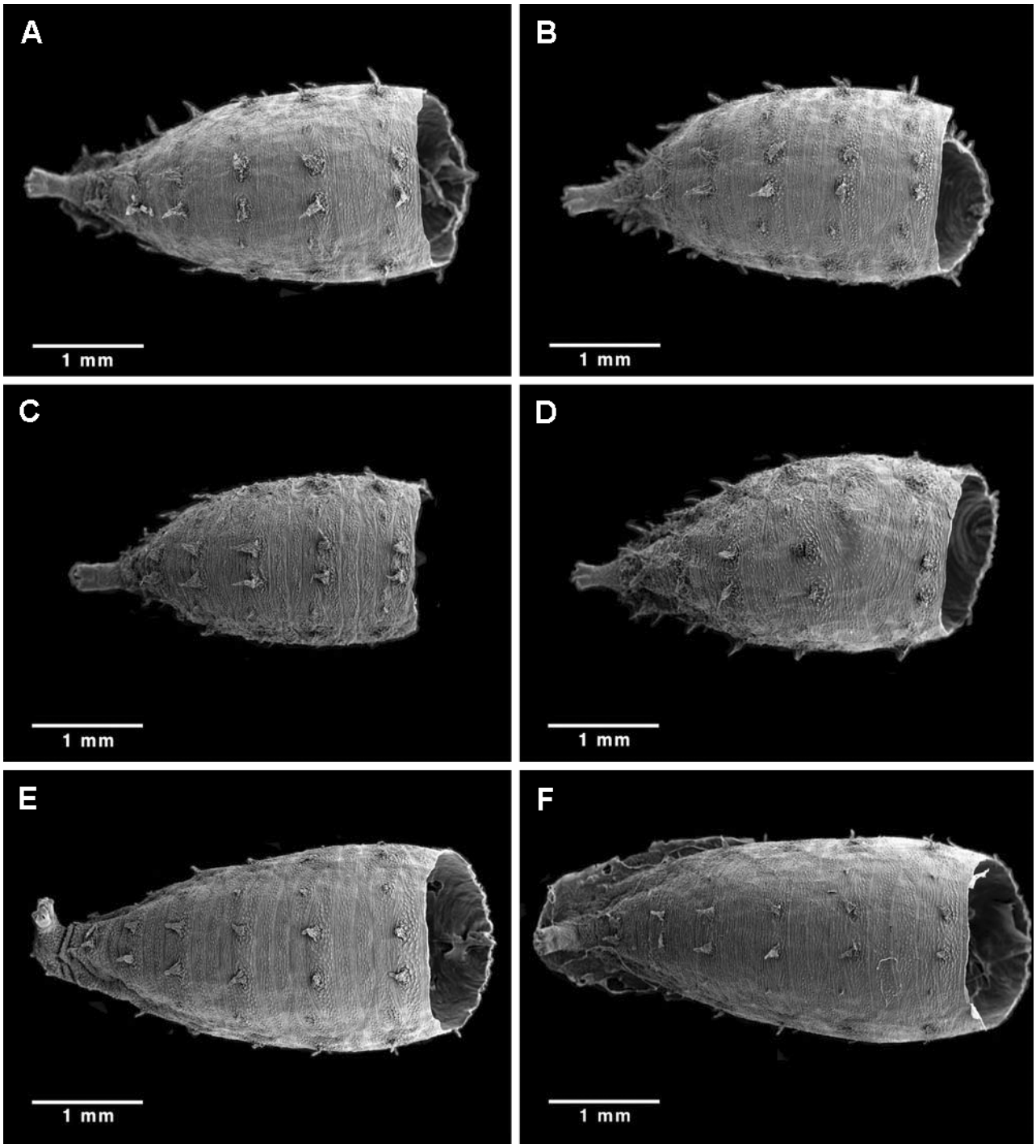

Fig. 8. Scanning electron micrographs showing the variability in the puparia of West Palaearctic *P. haemorrhous* (A, B), *P. tibialis* (C, D, F) and *P. coadunatus* (E).

longiventris Loew, 1857 (subgenus type) and indicated the more common form (pp. 108–109).

Goeldlin de Tiefenau (1976) established the main *Pandasyopthalmus* species of the West Palaearctic region, primarily using the shape and size of the postgonites. Later, Speight (1978) indicated that the range of variation exhibited by *P. haemorrhous* in the British Isles was greater than might have been expected. Vockeroth (1986) also found variability in the genitalia structures of *Paragus haemorrhous*, especially in the shape and size of the postgonites: "…the two extremes taken alone would suggest that at least two species occur in North America, but a division into two or more discrete groups appears impossible". Thompson & Ghorpadé (1992) reported that the shape of the postgonites also varies greatly in some Oriental species of the *tibialis*-group.

Our hypothesis is that *P. haemorrhous* and *P. tibialis* represent the extremes of postgonite variation in the West Palaearctic region, and the intermediates could include other *tibialis*-group species of this region (see Fig. 1).

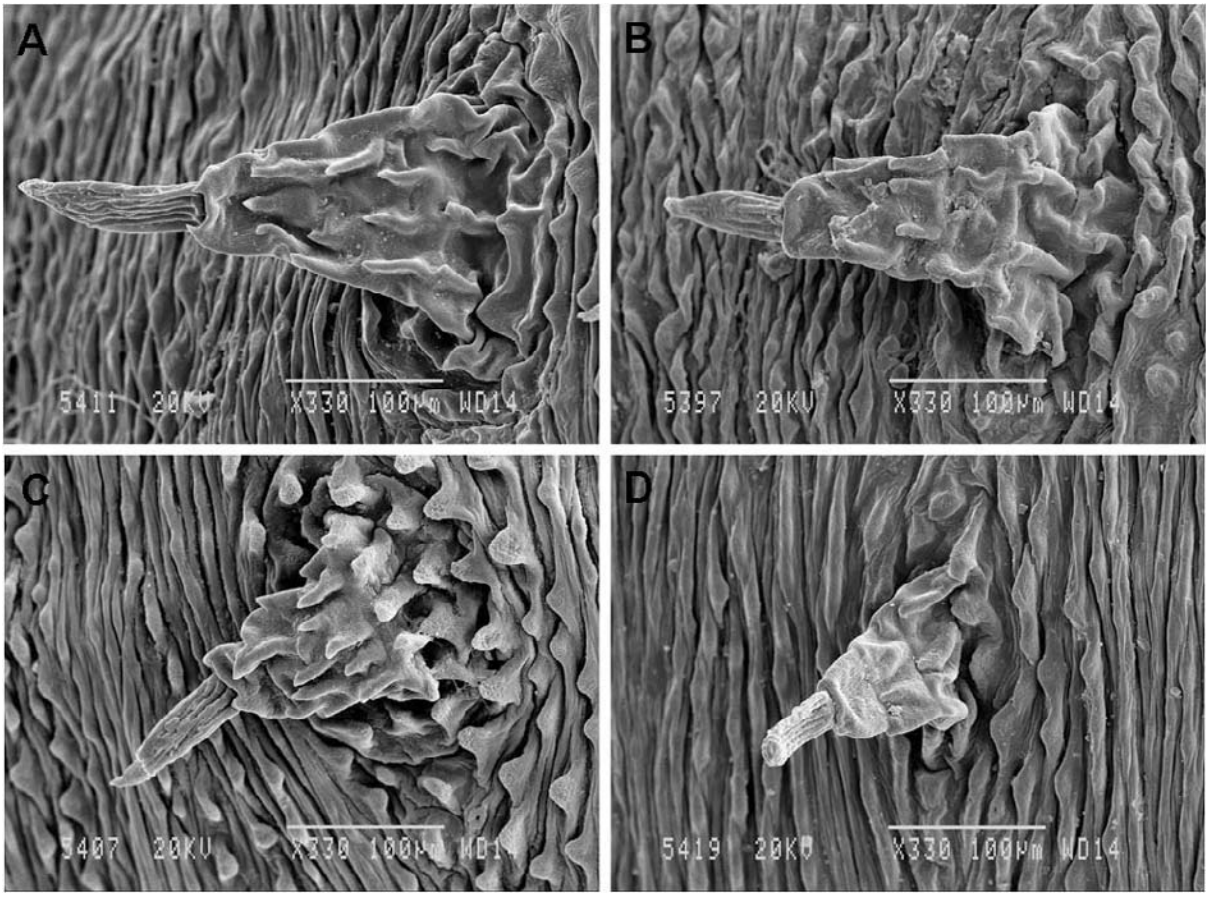

Fig. 9. Detail of sensilla from puparia of West Palaearctic *P. haemorrhous* (A, C) and *P. tibialis* (B, D).

Some European species, such as *P. albipes* and *P. coadunatus* (with small postgonites), are thus included in the variation found in *P. haemorrhous* in different parts of the world (see Speight, 1978). Similarly, larger postgonites, as in *P. constrictus*, are included in the range of variation of *P. tibialis*. In fact, Speight & Chandler (1995), in agreement with Doczkal (1996), speculated that some of the supposed variation in *P. tibialis* in certain parts of Europe is due to the inclusion of *P. constrictus*.

In the subgenus *Paragus* s. str., the size of the postgonites is clearly smaller than that of the surstyli. In this group the postgonites together with the morphology of other parts of the hypandrium provide useful characters for species identification. As stated in Dušek & Láska (1987), understanding the mating behaviour is important in evaluating the function of structures of the male genitalia during copulation. This information should be used to assess different taxonomic value of these structures.

In the case of females, Speight & Chandler (1995) indicated that *P. haemorrhous* could be separated from *P. tibialis* by the former having abdominal transverse bands of black and reclined setae. Nevertheless, this character does not allow confident separation of the females of the *tibialis*-group. According to our results, a relation seems to exist in most of the Palaearctic species between males with small postgonites and females with these setae (*P. haemorrhous / P. coadunatus*) and males with larger postgonites and females without setae (*P. tibialis / P. constrictus*), but this character is very variable.

Immature morphology

Previously, only the chaetotaxy of the thoracic and abdominal segments of syrphid larvae has been described (Hartley, 1961; Rotheray & Gilbert, 1999). This is the first time that larval cephalic setae are described for this family. As far as we know, the only published description of the chaetotaxy of *Paragus* is by Tawfik et al. (1974) for *Paragus* (s. str.) *compeditus* (Macquart, 1850). However, they apparently ignored most of the ventral setae and misinterpreted the limits of the head and thorax, and the limits of the pro-, meso- and metathorax. The result is that their reported "prothorax" is the mesothorax, and their "mesothorax" + "methatorax" [sic] are, in reality, the metathorax. Accordingly, their "cephalic setae" actually belong to the prothorax. Rotheray & Gilbert (1989) indicated that there are no setae on the ventral surface of predacious larvae, except on the metathorax of *Eupeodes* Osten Sacken, 1877 and *Scaeva* Fabricius, 1805. This condition also exists in *Paragus* larvae. There is one extra pair of sensory organs near sensilla 7 on both the meso- and metathorax not recorded in the previously reported pattern of chaetotaxy. The colour and size of sensilla of the larvae are quite variable.

We are not able to define any qualitative differences between the larvae or puparia of the species of the

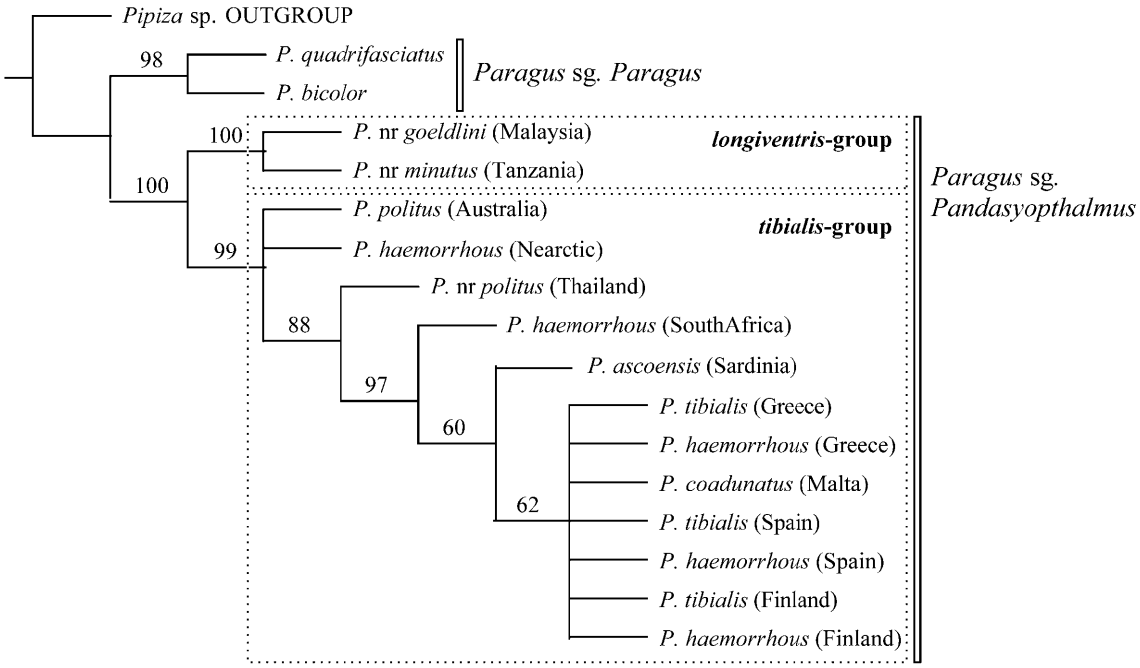

Fig. 10. Strict consensus of two equally parsimonious trees using Nona, with length = 355 steps, CI = 0.83, and RI = 0.82. Bootstrap support values indicated on nodes.

tibialis-group studied; only a gradient in sensilla size exists (Fig. 8). When attempting to distinguish *P. haemorrhous* from *P. tibialis,* two extremes of variability in the length of sensilla were found. Puparia with long sensilla belonged to *P. haemorrhous* (based on rearing and identifying the adult). Puparia with short sensilla were generally *P. tibialis*, however, intermediates exist that cannot be assigned to either species (see Fig. 8). The length of the pupal sensilla of *P. coadunatus* lies between these extremes.

There were no difference in the posterior spiracle, of the taxa studied; this feature, however, is useful for distinguishing species of subgenus *Paragus* s. str. (Goeldlin de Tiefenau, 1974; unpubl. data). Moreover, the colour of mature larvae is quite variable, with all gradations between the different patterns (see also Heiss, 1938). Based on our studies, it is impossible to establish two or more discrete groups based on larval or puparial morphology in the species of the *tibialis*-group studied, whereas larval distinction between species exist in *Paragus* s. str. or other syrphid genera (e.g. Rotheray, 1993; Pérez-Bañón & Marcos-García, 2000; Pérez-Bañón et al., 2003a,b).

Molecular variation

Molecular evidence in the mitochondrial *COI* support the monophyly of the subgenus *Pandasyopthalmus*, including the two groups of this lineage (Fig. 10). However, molecular analysis revealed no significant intra- or interspecific variation in the 1128 bp fragment of the mitochondrial *COI* between West Palaearctic individuals of *P. haemorrhous*, *P. tibialis*, *P. coadunatus* and *P. ascoensis* from different countries, and the uncorrected

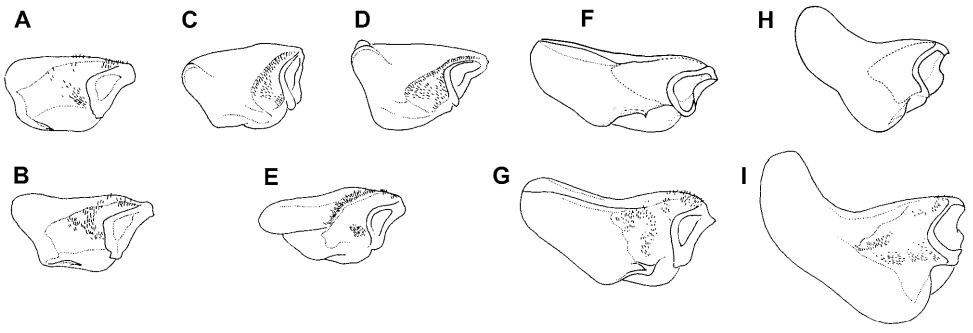

Fig. 11. Patterns of the sclerotised and membraneous areas and arrangement of microtrichia on the inner surfaces of the postgonites of some Palaearctic and Oriental *tibialis*-group species (lateral view). A, B – *P. haemorrhous* (Germany); C – *P. rufocinctus* (Vietnam); D – *P. rufocinctus* (Burna, type); E – *P. abrogans* (Turkey); F, G – *P. constrictus* (Germany); H – *P. tibialis* (Germany); H – *P. tibialis* (Turkey). Adapted from: F, H: Doczkal, 1996; A, B, C, D, E, G, I: Claussen & Weipert, 2004. (No scale available for drawings, in the originals).

pairwise divergences ranged from 1.1–2.7% when comparing *tibialis*-group species from other biogeographical regions. However, clear differences between the species of *Paragus* s. str. and those of the *tibialis*- and *longiventris*-groups of the subgenus *Pandasyopthalmus* were found (Table 6). These levels are similar to the divergences between species found in other subgenera and genera of Syrphidae (Ståhls & Nyblom, 2000; Pérez-Bañón et al., 2003a; Ståhls et al., 2003) for the same gene. The divergences from the Australian-Oriental taxa could be related to differences in hypandrium morphology, e.g., *P. politus* lacks the dorso-lateral carina, present in the Palaearctic *P. tibialis* (see Thompson & Ghorpadé, 1992). As the Nearctic *tibialis*-group specimens used for sequencing were old (1999) and not explicitly collected for DNA studies, the sequencing was difficult. Additional studies, using fresh Nearctic specimens, are necessary for a complete understanding of the existing divergences. Hebert et al. (2003b) extracted *COI* sequences for 1429 congeneric species pairs of Diptera from GenBank, and showed that 0.9% of these expressed sequence divergences between 0–1%, indicating that these low levels reflect a short history of reproductive isolation. The mean value of sequence divergence for Diptera in the above mentioned study was 9.3% between congeneric species pairs.

The nuclear *ITS2* region sequenced from twelve individuals of *P. ascoensis, P. coadunatus, P. haemorrhous* and *P. tibialis*, and from different geographic regions, revealed four different haplotypes. Dinucleotide (microsatellite) variation is generally regarded as intra-specific variation and hence exclusively used in population genetic studies. The haplotypes we found were not distributed according to geographic region or species (Table 5). If the distribution of haplotypes was an artefact of flawed species identifications, the expectation is a consistent pattern in conflict with species labels, but we are confident of the identification of *P. tibialis* and the variable haplotypes. Our results could represent a random sample of *ITS2* haplotypes present both at intra-individual and/or the intra-species level, which do not define populations (or species). When inferring phylogenetic affiliations between populations or cryptic species that (seem to) exhibit intra-genomic variation, extensive sequencing of multiple clones / individual or multiple individuals from a large geographic range should be carried out to determine the level of variability, as intra-genomic variability could affect phylogenetic conclusions.

West Palaearctic *Pandasyopthalmus*: One morphologically polymorphic species?

Based on our findings, it is suggested that the male genital characters used for species recognition in the West Palaearctic *tibialis*-group (size and shape of postgonites) are insufficient to establish species boundaries. Moreover some species are still of uncertain status (e.g., *abrogans/albipes/constrictus* complex, see Speight, 2003). Although the some authors consider that the shape of postgonites varies greatly in this group of Syrphidae (Vockeroth, 1986; Thompson & Ghorpadé, 1992), others (e.g. Doczkal, 1996; Claussen & Weipert, 2004) claim that Palaearctic and Oriental species of the *tibialis*-group, are morphologically very similar yet separable in most cases by the extent of the sclerotised and membraneous areas and arrangement of microtrichia on the inner surface of the postgonites (Fig. 11). However, the variability that we found in larval morphology overlapped when considering actual species definitions. Moreover, the molecular sequences generated indicate that the Palaearctic species studied herein represent one polymorphic taxon, as well-characterized lineages could not be obtained. Therefore, based on both morphological and molecular evidence, *Paragus haemorrhous* Meigen, 1822, *Paragus coadunatus* (Rondani, 1847) and *Paragus ascoensis* Goeldlin de Tiefenau & Lucas, 1981 appear to be synonyms of *Paragus tibialis* (Fallén, 1817). Other Palaearctic species of the *tibialis*-group, for which we do not have molecular data, might also belong to this polymorphic taxon. As the type material of the *tibialis*-group taxa was not studied, we hesitate to make actual taxonomic changes. Molecular analysis of type material is obviously impossible or very difficult. Further study of Nearctic and Palaearctic *P. haemorrhous* material might show that they are separate species (based on molecular data).

Finding a good molecular marker can be a complicated process, especially for phylogenetic studies of closely related species. We have used markers that are presently commonly used at the taxonomic levels of species and intra-specific groupings in syrphid flies (Milankov et al., 2005; Láska et al., unpubl. data), but our results are not constrained by geographic region or species. Based on those results, the existence of a single polymorphic widespread species of the *tibialis*-group at least in the West Palaearctic region is proposed, as no stable morphological characters could be found for separating the taxa and the molecular characters support this hypothesis. On the other hand, if speciation in the *tibialis*-group was very recent, with the number of lineages exceeding the number of presently described taxa, and the levels of morphological variation still very low, even the rapidly evolving *ITS2* molecular marker would not immediately reveal this variability. The levels of variation between other closely related syrphid species in *ITS2* far exceed the present level (Láska et al., unpubl. data).

Presented here is the first comprehensive study of the West Palaearctic *Pandasyopthalmus* taxa using several character systems. The interpretation of the results is, however still preliminary. Additional data is needed for an extended study, involving extensive species and specimen sampling for sequencing of rapidly evolving intron regions as well as the *ITS2*, in addition to applying more traditional allozyme markers. Despite the vague conclusions, this work is a relevant addition to the recent discussions of the merits of DNA barcoding and demonstrates a case where an understanding of the taxonomy of a particular group of very closely related species is challenged using all character systems, including *COI* barcode sequences.

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