



DNA barcoding reveals andropolymorphism in *Aclerogamasus* species (Acari: Parasitidae)

MIROŚLAWA DABERT¹, AGATA BIGOŚ² & WOJCIECH WITALIŃSKI^{2,3}

¹Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland

²Department of Comparative Anatomy, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland

³Corresponding author. E-mail: w.witalinski@gmail.com

Abstract

Andropolymorphism, defined as discontinuous morphological variability in males, can lead to taxonomic confusion when different male morphs are determined and described as separate species. This study addresses this issue in two occasionally sympatric mite species *Aclerogamasus similis* (Willmann, 1953) and *A. holzmannae* (Micherdziński, 1969) collected in Poland. The females of these two taxa are morphologically indistinguishable but males are quite different, and could be either separate species or one species with two male morphs. We address this question by performing molecular assays, testing variation in a fragment of the mtDNA COI gene and the D2 region of 28S rDNA. Molecular analysis of populations revealed very low variation in the studied gene fragments. All sequences of the D2 region of 28S rDNA (size 375 bp) were identical. Only two COI haplotypes were found, differing by two out of 644 nucleotide positions (0.3% K2P distance). The variant haplotype was found in one *A. similis* male, and probably represents intraspecific variability. The results strongly suggest that all studied females and males belong to only one species with dimorphic males. This finding confirms some earlier opinions on synonymy of both species. Therefore, *Aclerogamasus holzmannae* (Micherdziński, 1969) should be regarded as a junior synonym of *Aclerogamasus similis* (Willmann, 1953). As andropolymorphism has been rarely observed in gamasid mites, we briefly discuss its possible origins and consequences.

Key words: DNA barcode, COI, D2 region of 28S rDNA, polymorphism, gamasid mites, synonymy

Introduction

Sexual dimorphism is well developed in several groups of mites, motivating taxonomists to construct separate keys for males and females. If only one sex of a new species is available for description, there is a considerable risk of synonymous description of an already known species. Similarly, the presence of two or more female or male morphs (gyno- and andropolymorphism, respectively) can increase taxonomic confusion. This study reports a case of andropolymorphism in Gamasida, a phenomenon rarely observed in this group.

Our study concerns two species of gamasid mites, *Aclerogamasus similis* (Willmann, 1953) and *A. holzmannae* (Micherdziński, 1969) (Parasitidae). The original description of *A. similis*, as *Pergamasus similis*, was based only on male specimens (three specimens are present on slides nos. 344-14, 344-16, 232-17 in Willmann's Collection in Zoologische Staatssammlung München, München, Germany, but none is designated as a type) from Salzburg, Austria. The female described by Willmann as *P. similis* did not actually belong to this species. Schmölzer (1953) found this species in Carinthia, and subsequently also in other Austrian localities (Schmölzer, 1995). Athias-Henriot (1967) completed the species description with details of a female from Admont (Styria, Austria), and also found this species in the Sierra de Cazorla (Jaén, Spain). Micherdziński (1969) then described a new species *Pergamasus* (= *Aclerogamasus*) *holzmannae* from Ojców near Krakow, southern Poland.

The males of *A. similis* and *A. holzmannae* are morphologically distinct, but the females are practically identical. In 1971 the following synonyms were proposed, without the use of numerical or molecular data: *A. holzmannae* female = *A. similis*; *A. holzmannae* male = *A. bicalliger* (Athias-Henriot, 1967) by Athias-Henriot (1971); and *A. holzmannae* = *A. similis*, by Karg (1971). Since the males of *A. bicalliger* (Athias-Henriot, 1967; Juvara-Bals,

1977) differ distinctly in key characters from *A. holzmannae* (ventral seta on genu II in a deep concavity and tibia II with additional distal tubercle), it is unlikely that this synonymy is valid. Karg (1971) also gave an additional two synonyms for *A. similis*: *Gamasus (Pergamasus) decipiens var. germanicus* Berlese, 1906 and *Pergamasus ologamasoides* (Holzmann, 1969). In both above mentioned taxa, however, only the females have been described, which leaves the problem of morphologically distinct males still open.

As *A. similis* and *A. holzmannae* are sympatric at several sites in Poland, the aim of our study was to verify whether *A. similis* and *A. holzmannae* are indeed separate, sibling species with different males and very similar females, or whether these two species are a single species with two forms of males. We address this problem on the basis of common DNA barcoding markers (sequences of cytochrome oxidase subunit I gene fragment and D2 region of 28S rDNA), which have proven their utility in molecular taxonomy of mites (Dabert *et al.*, 2008; Martin *et al.*, 2010; Skoracka & Dabert, 2010).

Material and methods

Material examined. Light microscopic observations were performed on males and females collected from mixed forest litter in eight distinct localities (Table 1, Fig. 1) in the years 1968–2006, and routinely mounted in Hoyer's medium on microscope slides. For molecular analysis we used material collected as for SEM observations, i.e. from mixed forest litter in Mników near Krakow, southern Poland, on 4 August 2006 (Table 2): three females (specimen code numbers 1.A, 4.1, and 5.1), five males of *A. holzmannae* (5.4, 5.5, 5.6, 5.7, and 5.8), and three males of *A. similis* (1.B, 5.2, and 5.3). Males were provisionally identified under a stereomicroscope and then placed into 70% ethanol. The left leg II was dissected from each male and mounted on microscopical slides in Hoyer's medium for precise species identification. The ethanol-preserved material was further processed.

TABLE 1. Basic collection data for *Aclerogamasus similis* and *A. holzmannae* used for morphological observations.

Sex	Male morph	Number of specimens	Locality [as marked in Fig. 1]	Collection year
F		3	Las Wolski [1]	1968, 1969, 1970
F		3	Kochanów [2]	1969
F		14	Myślenice [3]	1969, 1970, 1995, 2006
F		4	Pieniny Natl. Park: Facimiech [4]	1976
F		2	Wielkopolski Natl. Park [7]	2003
F		1	Pieniny Natl. Park: Majerz [5]	2004
F		6	Mników [8]	2006
M	holzmannae	1	Las Wolski [1]	1969
M	holzmannae	1	Kochanów [2]	1969
M	holzmannae	1	Kampinoska Forest [6]	1969
M	similis	2	Myślenice [3]	1969
M	holzmannae	8	Myślenice [3]	1969, 1970, 2006
M	holzmannae	4	Mników [8]	2006

SEM morphological study. For scanning electron microscopy (SEM) observations, several males and females collected on 4 August 2006 from mixed forest litter in Mników near Krakow, southern Poland (locality 8 on Fig. 1), were preserved in 75% ethanol, air-dried on tissue paper then glued to double-sided sticky tape on microscope stubs, coated with carbon and gold, and examined with a JEOL JSM-35 scanning electron microscope at 25 kV.

DNA-barcoding. DNA extraction was performed using a nondestructive method as described by Dabert *et al.* (2008). A ~670-bp fragment of the cytochrome oxidase subunit I (COI) gene was amplified with primers bcdF01: 5'-CATTTCCHACTAAYCATAARGATATTGG-3' and bcdR04: 5'-TATAAACYTCDGGATGNCCAAAAAAA-3' (Dabert *et al.*, 2010). Amplification of the D2 region of 28S rDNA was done with primers f1230: 5'-TGAAACT-

TAAAGGAATTGACG-3' (Skoracka & Dabert, 2010) and D1D2rev4: 5'-GTTAGACTCCTTGGTCCGTG-3' (Sonnenberg *et al.*, 2007). PCRs were carried out in 25 μ l reaction volumes containing a reaction buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P40), 1.5 mM MgCl₂, 0.1 mM dNTPs, 0.25 μ M each primer, 1.25 U HiFi *Taq* polymerase (Novazym, Poznań, Poland) and 5 μ l of DNA template using a thermocycling profile of one cycle of 3 min at 96°C followed by 35 steps of 10 sec at 95°C, 15 sec at 50°C, 2 min at 72°C, with a final step of 5 min at 72 °C. Amplicons were used for direct sequencing of the D2 region using primer D1D2fw2 (Sonnenberg *et al.*, 2007). Sequencing was performed with BigDye v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, Foster City, CA, USA). Trace files were checked and edited using FinchTV 1.3.1. (Geospiza Inc.). Low-quality base calls that are typically found near the 5'- and 3'- ends of the sequence were excluded from the final alignment. Contigs were aligned and assembled manually in GeneDoc v. 2.7.000 (Nicholas & Nicholas, 1997). Nucleotide sequence divergences were computed using the Kimura two parameter (K2P) distance model (Kimura, 1980) with MEGA 4 software (Tamura *et al.*, 2007). The final dataset comprised 644-bp for COI and 375-bp for D2 region of 28S rDNA for 11 samples.

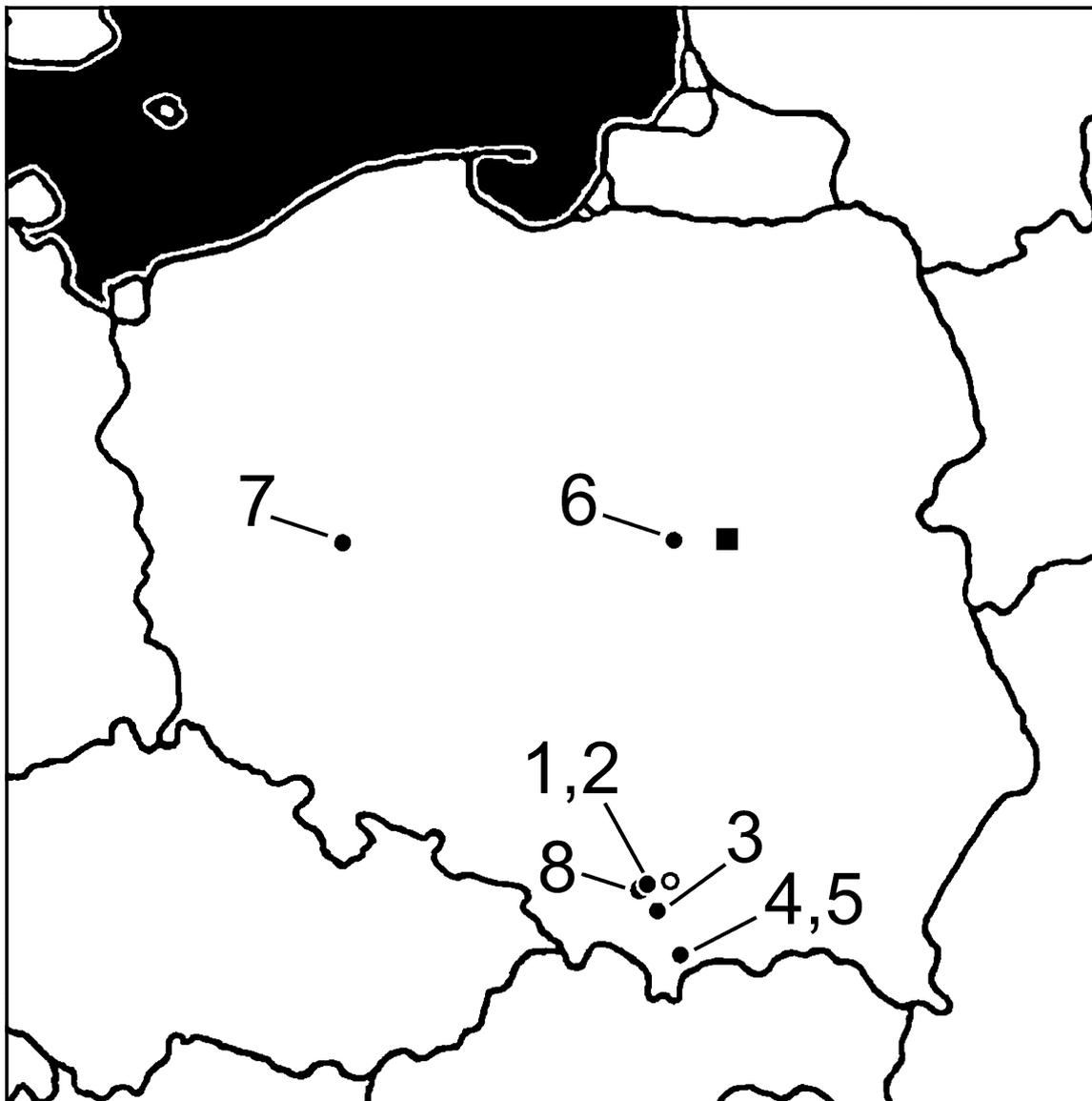


FIGURE 1. Distribution of sites where the material used in this study was collected. Black square = Warszawa, open circle = Krakow.

TABLE 2. Specimen code numbers and basic collection data for *Aclerogamasus similis* and *A. holzmannae*, used in DNA analysis.

Sex	Male morph	Specimen code	Locality [as marked in Fig. 1]	Collection year	GenBank Acc. COI	GenBank Acc. D2
F		1.A	Mników [8]	2006	FJ577978	FJ577987
F		4.1	Mników [8]	2006	FJ577975	FJ577989
F		5.1	Mników [8]	2006	FJ577977	FJ577990
M	holzmannae	5.4	Mników [8]	2006	FJ577981	FJ577993
M	holzmannae	5.5	Mników [8]	2006	FJ577982	FJ577994
M	holzmannae	5.6	Mników [8]	2006	FJ577983	FJ577995
M	holzmannae	5.7	Mników [8]	2006	FJ577984	FJ577996
M	holzmannae	5.8	Mników [8]	2006	FJ577985	FJ577997
M	similis	1.B	Mników [8]	2006	FJ577976	FJ577988
M	similis	5.2	Mników [8]	2006	FJ577979	FJ577991
M	similis	5.3	Mników [8]	2006	FJ577980	FJ577992

Results and discussion

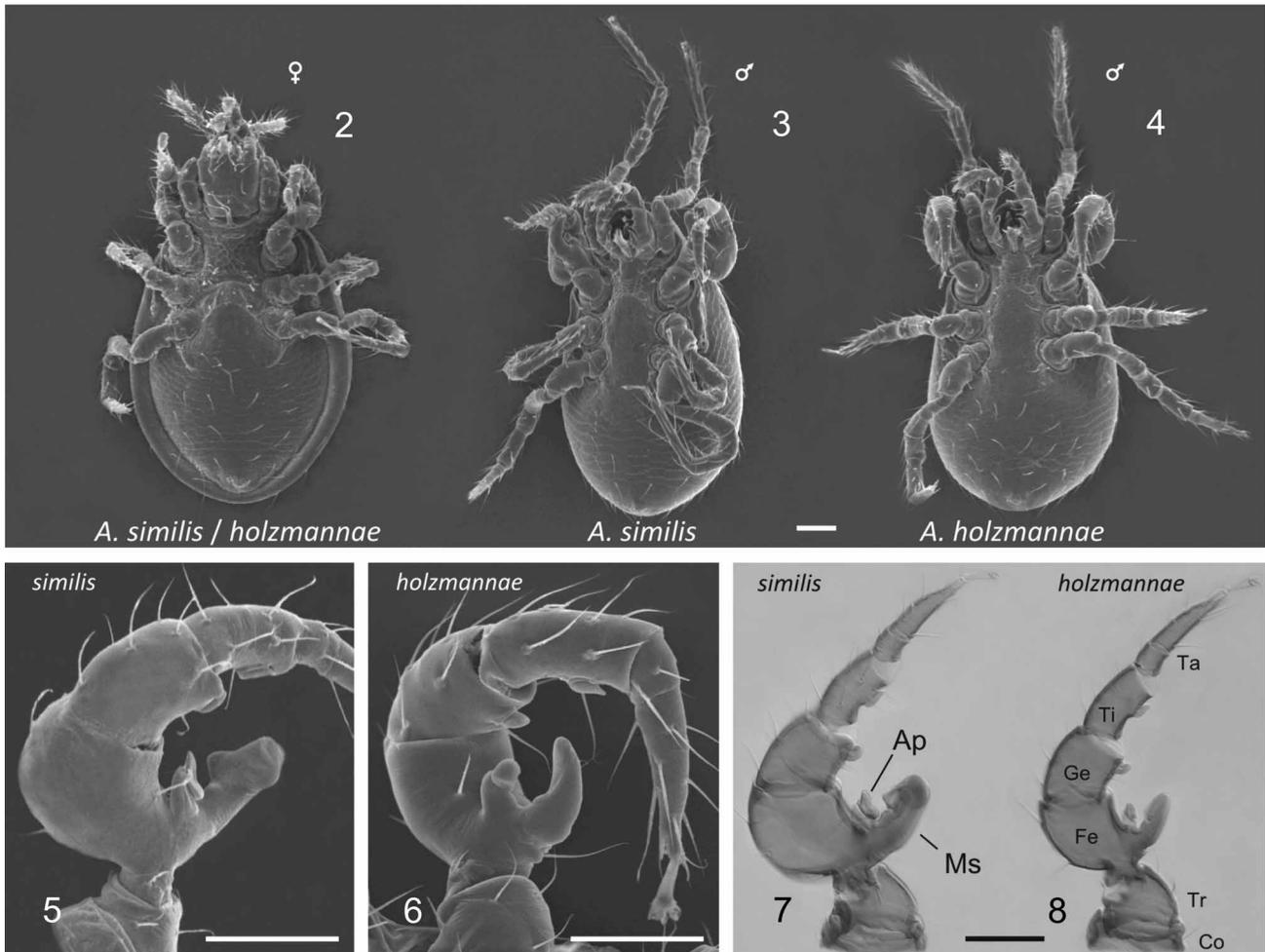
Morphology. Only males of the studied species are distinguishable; in practice, females (Fig. 2) are morphologically identical. The main key characters of *A. similis* males as compared to *A. holzmannae* (Figs 3–8), are as follows: (1) a more robust main spur, especially in its apical part, and a larger axillary spur on femur II, (2) larger spurs on genu II and tibia II, and (3) slightly larger and more sclerotized body.

Molecular assay. We obtained identical sequences for the COI gene fragment for all investigated specimens of *A. similis* and *A. holzmannae* for both sexes. The only exception was one *A. similis* male (specimen 5.2), in which we found two nucleotide substitutions: one synonymous and one nonsynonymous change that resulted in the substitution of methionine for valine. Thus, only two COI haplotypes were found in all examined specimens, representing intraspecific variability at the level of 0.3 % K2P distance. No variation was found in the nuclear DNA data, which included 375 nucleotides for the D2 region of 28S rDNA. All sequences have been deposited in GenBank (Table 2). The alignments are available upon request.

In mites, the levels of COI and D2 28S rDNA differentiation are similar to those observed in populations of the majority of other animal species (Hebert *et al.*, 2003). Genetic distance at the interspecific level in closely-related species ranges from 10 to 25% for COI and 0.2 to 7.5% for D2, depending on the substitution rate within those species, and typically is ten times higher than the intraspecific distance (Dabert *et al.*, 2008; Martin *et al.*, 2010; Skoracka & Dabert, 2010). The specimens used for molecular analysis in this study belonged to one population (see Table 2, Fig. 1) which could explain the low level of variation among analyzed sequences. However, the lack of differences in DNA barcodes from *A. similis* and *A. holzmannae* for both sexes shows that all studied specimens belong to one species, *Aclerogamasus similis* (Willmann, 1953), with *A. holzmannae* (Micherdziński, 1969) as its junior synonym.

Remarks on male polymorphism. Male polymorphism is in most cases related to special sexual behaviour and/or male reproductive strategy and, in consequence, leads to an increase of reproductive success in heteromorphic males. Morphological variability in Acari is common and known in many groups such as Astigmata and Prostigmata (Evans, 1992). The literature concerning male polymorphism in Gamasida, however, is much more limited (Vitzthum, 1943; Wiśniewski, 1979; Walter & Proctor, 1999; Gwiazdowicz, 2004), and mostly descriptive. Male polymorphism in Gamasida is also marked by continuous variability, for example in setae length or body size. On the other hand, andropolymorphism is currently defined by male phenotypes falling into two or more distinct categories (morphs) showing discontinuous phenotypes. Such variability mainly concerns morphology, but may be manifested also at other levels, such as physiology or behaviour, especially that related to reproduction. In mites, polymorphic males are best known and studied in Astigmata, e.g. in *Sancassania anomala*, *S. berlesei*, *S. boharti*, *Rhizoglyphus echinopus*, *R. robini*, *Falculifer rostratus* (Woodring, 1969; Timms *et al.*, 1980a, 1980b, 1981; Rad-

wan, 1993, 1995, 2001; Radwan & Bogacz, 2000; Witaliński, 2004), in which differences between morphs are not limited to morphology, but also include behaviour such as aggression towards other males (Radwan, 1993; Radwan *et al.*, 2000; Radwan & Klimas, 2001; Tomkins *et al.*, 2004; Unrug *et al.*, 2004; Witaliński, 2004). As natural selection should favour the best adapted morph (Moran, 1992), it is commonly accepted that andropolyorphism is maintained by variation in reproductive success of morphs depending on environmental situations, mainly density and population structure. In most cases, polymorphic males evidently confront each other when competing for a female. However, in *A. similis* such face-to-face male challenge is difficult to envisage since, as many other litter predatory gamasids, this species lives in relatively low densities and does not aggregate for feeding or reproduction. Female and male meetings are reputed to be incidental, and thus conflict between rival males seems unlikely. Moreover, we have not observed aggression between males maintained together or with females under laboratory conditions (unpublished observations).



FIGURES 2–8. *Aclerogamasus similis*. 2. Female, ventral, SEM; 3. Male, ventral, *A. similis* type, SEM; 4. Male, ventral, *A. holzmannae* type, SEM; 5. Male, leg II, antero-lateral aspect, *A. similis* type, SEM; 6. Male, leg II, antero-lateral aspect, *A. holzmannae* type, SEM; 7. Male, leg II, antero-lateral aspect, *A. similis* type, light microscope; 8. Male, leg II, antero-lateral aspect, *A. holzmannae* type, light microscope. Ap = axillary process; Ms = main spur; Co, Tr, Fe, Ge, Ti, Ta – coxa, trochanter, femur, genu, tibia and tarsus of leg II, respectively. Scale bars = 0.1 mm.

An alternative explanation may involve the specific, key differences between Parasitidae males in the development of the second pair of legs, i.e. the size, shape and distribution of spurs (e.g. Athias-Henriot, 1967; Hyatt, 1980; Hennessey & Farrier, 1989; Karg, 1993). These characters are probably under selection because the second pair of male legs is used to firmly mount the female during insemination. Both morphs in *A. similis* can be found in the same localities, suggesting a stabilizing selection on the second leg morphology at least in some populations. Local litter factors, like low humidity decreasing litter compactness, may promote the morph *A. holzmannae*, supposedly less firmly coupled with the female during mating. This explanation could be valid in southern Poland,

where the *A. similis* morph, being predominant in other European localities, is only occasionally found (unpublished observation, WW), but detailed studies on physical litter parameters in such localities have never been performed.

Finally, it must be noted that the data on distribution of both male morphs coming from the literature are fragmentary and based on occasional, uncertain findings. It is impossible to interpret records of *A. similis* or *A. holzmannae* when only the females were found, and/or the sex of studied specimens is not mentioned in source literature, as in Maraun *et al.*, 2001; Salmane, 2001; Kaczmarek & Marquardt, 2006; Stanescu, 2007; Skorupski *et al.*, 2009), making any hypothesis highly speculative. Therefore, to improve our knowledge on this species, it is recommended to define in future ecological or faunistic studies which form of *Aclerogamasus similis* male was collected in a given locality.

Male polymorphism is an interesting phenomenon showing alternative behavioural, physiological and ecological aspects of reproduction, and provides an opportunity to study the reasons leading to more than only one evolutionary pathway to increasing fitness and reproductive success. In *Aclerogamasus* mites, the origin of male dimorphism is not simply explainable in terms of reproductive advantage. The incomplete knowledge of the behaviour and ecology of these mites makes it more difficult to solve this problem. Although some interesting data can be found in several papers on *Poecilochirus*, *Parasitus*, *Parasitellus* (Parasitidae) (Schwarz & Müller, 1992; Yasui, 1997; Koulianos & Schwarz, 1999) and *Macrocheles muscaedomesticae* (Macrochelidae) (Yasui, 1994), these mites live in different conditions to *Aclerogamasus*, as they tend to aggregate, at least temporarily, to feed or reproduce, whereas *Aclerogamasus* mites are forest litter inhabitants all time sparsely distributed.

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