Testes and spermatozoa as characters for distinguishing two ant species of the genus *Neoponera* (Hymenoptera: Formicidae)

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The correct diagnosis of a species is often challenging, especially in the case of cryptic species. The misidentification can invalidate results already published, contribute negatively to the knowledge of the organism in question, and waste financial resources associated with research and control programs. Therefore, it is important to find easily visible traits that allow the diagnosis of the species under study. The external morphologies of the ant species *Neoponera inversa* (Smith) and *Neoponera villosa* (F.) (Hymenoptera: Formicidae) are very similar, and as a result, they were previously considered as the single species *N. villosa*. However, studies comparing petiole morphology and isozyme patterns (Lucas et al. 2002), chromosome number (Mariano et al. 2007), and external morphologies (Fernandes et al. 2014) revealed that they form, in fact, a complex of sympatric cryptic species. These characteristics make the distinction difficult between these species. Thus, to identify them requires a specialist and, in general, methods that are time consuming and expensive.

Morphological characteristics of the male reproductive system have been used in the systematics of insects particularly in Hymenoptera (Wheeler & Krutzsch 1992; Dias et al. 2013). In addition, spermatozoa have been used for phylogenetic analyses to determine taxonomic variations in insects (Jamieson et al. 1999; Dallai 2014). In Hymenoptera, the sperm may be free in the seminal vesicle, as reported for parasitic wasps (Lino-Neto et al. 1999) and most Aculeata (Moreira et al. 2004), or arranged in bundles as described for ants such as *Crematogaster victima* Smith (Oliveira et al. 2014) and *Lasius pallitarsis* (Provancher) (Burnett & Heinze 2014) and for sawflies (Schiff et al. 2001).

Morphological characteristics of testes and sperm may be useful in the differentiation of these sympatric, cryptic species of ants. In this study, we describe the numbers of follicles per testis and the morphometries of the spermatozoa of *N. inversa* and *N. villosa* to establish additional characters by which they can easily be distinguished.

Five colonies of *N. inversa* and 5 colonies of *N. villosa* were collected in Ilhéus, state of Bahia, Brazil, and reared for 3 mo within artificial nests in the laboratory. These nests were kept at 25 ± 4 °C with about 50 to 80% relative humidity. All colonies were fed on a mixture of honey, apple, and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae ad libitum. Our studies used in total 50 males consisting of 5 males from each colony.

To quantify the number of follicles per testis, 15 males of *N. inversa* and 15 of *N. villosa* (3 specimens per colony) were dissected, and the reproductive system was photographed with a stereoscopic microscope (Zeiss, Stemi 2000-C). For the morphometric analysis of sperm, the seminal vesicles of 2 males from each colony (totaling 10 specimens per species) were transferred to histology slides, dissected in pH 7.2 phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min, and washed in distilled water for 20 min. The spermatozoa were observed and photographed under a light microscope (Olympus, BX-60). To visualize and measure the nuclei, some slides were stained for 15 min with 4,6-diamidin-2-phenylindole (DAPI) at a concentration of 0.2 mg/mL in PBS and observed under an epifluorescence microscope (Olympus, BX-60) equipped with a BP 360–370 nm filter. For the measurements, 50 nuclei and 50 flagella of each species (10 cells per colony of both species) were randomly measured using the program Image-Pro Plus Version 4.5. The mean values were estimated and compared using the Mann–Whitney test at 5% significance.

Results showed that in the 2 species *N. inversa* and *N. villosa*, the reproductive system consisted of a pair of testes surrounded by a thin peritoneal sheath that can be broken easily (Fig. 1A, B). Each testis opened into a long and thin vas deferens, forming the seminal vesicle, and both seminal vesicles and paired accessory glands terminated into a short ejaculatory duct (Fig. 1A, B). When the peritoneal sheath was broken, 3 follicles per testis were observed in *N. inversa* (Fig. 1C), whereas 4 follicles per testis were observed in *N. villosa* (Fig. 1D).

In the seminal vesicle, the spermatozoa were individualized, long, and threadlike. The total lengths of the sperm of *N. inversa* and *N. villosa* were 155 ± 10.1 µm and 175 ± 5.4 µm, respectively (Fig. 2A, B). Lengths of flagella did not differ between the 2 species ($P = 0.3734$) and averaged 125 µm. However, the nucleus of *N. inversa* sperm was smaller than the nucleus of *N. villosa* ($P < 0.001$), with a length of 30 ± 1.6 µm and 50 ± 2.3 µm, respectively (Fig. 2C, E).

The different numbers of follicles per testis in *N. inversa* (3) and *N. villosa* (4) support the proposition that they are distinct species within the genus *Neoponera* (Lucas et al. 2002) and facilitate greatly the distinction between them. The numbers of testicular follicles vary in different groups within Hymenoptera. Three follicles per testis have been observed in Vespidae (Brito et al. 2005) and Andrenidae, Halictidae, and some species of Megachilidae (Ferreira et al. 2004). Four follicles per testis have been observed in Mellitidae, other species of Megachilidae, and Apidae sensu stricto (Roig-Alsina & Michener 1993;

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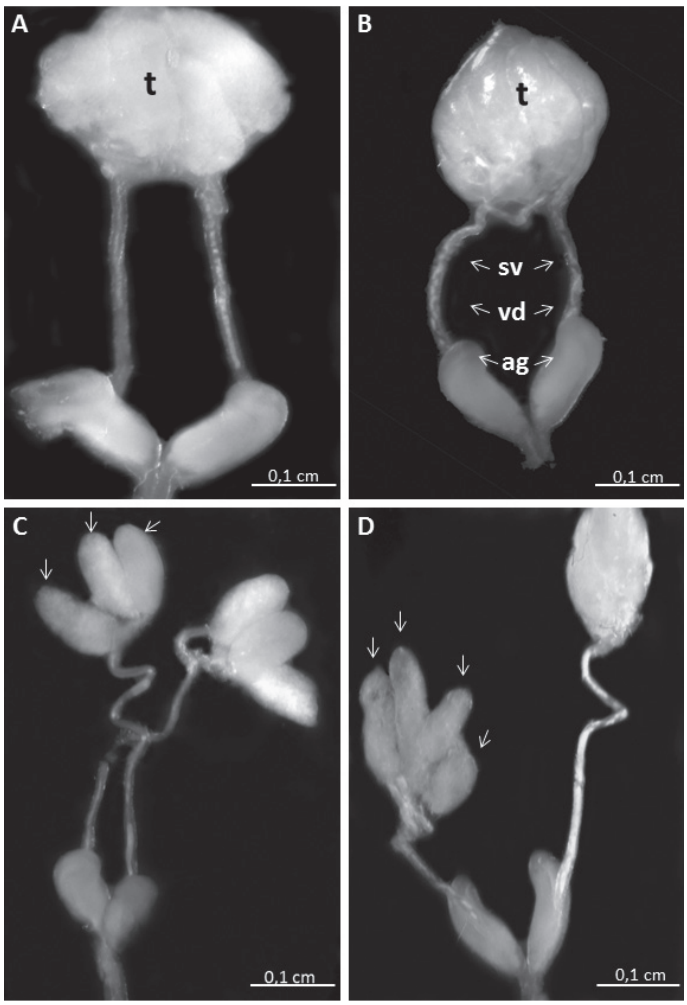

Fig. 1. Male reproductive system of (A) *Neoponera inversa* and (B) *Neoponera villosa*, showing the testes (t) united by peritoneal sheath. Seminal vesicle (sv); vas deferens (vd); accessory glands (ag). Testes of (C) *N. inversa* and (D) *N. villosa* without peritoneal sheath, showing 3 and 4 follicles (arrows), respectively.

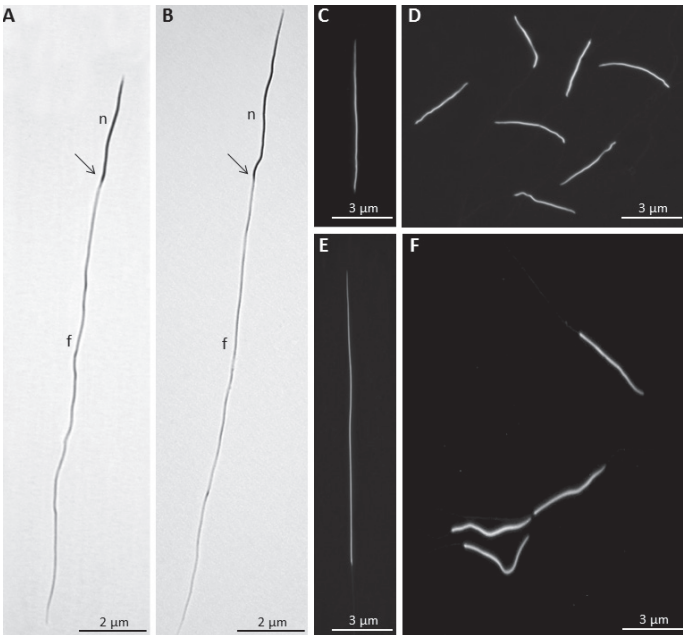

Fig. 2. Sperm of (A) *Neoponera inversa* and (B) *Neoponera villosa* in phase contrast, showing the transition (arrows) between the nucleus (n) and the flagellum (f). Nuclei stained with DAPI of (C and D) *N. inversa* and (E and F) *N. villosa* sperm.

Ferreira et al. 2004). Interestingly, the number of testicular follicles can be up to 250 in *Apis mellifera* L. (Hymenoptera: Apidae) (Snodgrass 1984), whereas in ants, the number varies from 1 to 25 among different species (Wheeler & Krutzsch 1992).

In *N. inversa* and *N. villosa*, the spermatozoa were long, threadlike, linear, and free from each other in the seminal vesicle as also observed in the ant *Acromyrmex subterraneus* (Forel) (Hymenoptera: Formicidae) (Moreira et al. 2004). However, in *C. victima* (Oliveira et al. 2014) and *L. pallitarsis* (Burnett & Heinze 2014), these cells are maintained in bundles in the seminal vesicle in the mature male as reported for sawfly wasps (Schiff et al. 2001).

In Hymenoptera, the difference in lengths of the sperm can be used to differentiate cryptic species (Pereira et al. 2008). Total lengths of sperm are variable among species, for example, 13 to 577 µm in Vespidae (Quicke et al. 1992) and 80 to 1,500 µm in Apoidea (Quicke et al. 1992; Zama et al. 2004). In Formicidae, the mean length of the sperm in *Solenopsis invicta* Buren is about 70 µm (Lino-Neto & Dolder 2002), whereas in *Pseudomyrmex*, it ranges from 53 to 70 µm (Moya et al. 2007). Thus, our results showed that sperm lengths in *N. inversa* (155 µm) and *N. villosa* (175 µm) are the longest recorded to date in ants. Furthermore, we showed that the difference in the size of the sperm between *N. inversa* and *N. villosa* is due to a difference in the lengths of nuclei, with the nucleus being 40% longer in *N. villosa*. This result is consistent with a cytogenetic study on differences in chromosome number between these 2 species, with *N. inversa* showing fewer chromosomes (2n = 30) than *N. villosa* (2n = 34) (Mariano et al. 2007). Considering the importance of these reproductive cells, and that the amount of DNA in the same species is generally constant, the difference in nuclear size of the sperm of these 2 ant taxa supports that they are different species.

Although all colonies have been collected in the same region (Ilhéus, Bahia State, Brazil), the way the data were sampled and their uniformity, as well as the sample size, provide strong evidence that the number of follicles per testis and the nuclear length of sperm are species specific. Therefore, the characters cited above can be used to distinguish easily these 2 cryptic species of ants.

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Summary

We examined the morphologies of the testes and spermatozoa of the ant species *Neoponera inversa* (Smith) and *Neoponera villosa* (F.) (Hymenoptera: Formicidae), collected in Ilhéus, Bahia State, Brazil, looking for information to distinguish easily one species from another. In *N. inversa*, 3 follicles per testis occurred, and the nucleus of sperm measured 30 ± 1.6 µm in length. In *N. villosa*, 4 follicles per testis occurred, and the nucleus of sperm measured 50 ± 2.3 µm in length. The morphologies of the testes and the spermatozoa offer an easy and fast method to distinguish between the 2 sympatric, cryptic species *N. inversa* and *N. villosa*.

Key Words: *Neoponera inversa*; *Neoponera villosa*; testicular follicles; sperm morphometry

Sumário

Nós analisamos a morfologia dos testículos e dos espermatozoides das formigas *Neoponera inversa* (Smith) and *Neoponera villosa* (F.) (Hymenoptera: Formicidae), coletadas em Ilhéus, Bahia, Brasil, buscando por informações que possam facilmente distinguir uma espécie da outra. Em *N. inversa* ocorre 3 folículos por testículo e o núcleo dos espermatozoides medem 30 ± 1,6 µm de comprimento, já em *N. villosa* são 4 folículos e o núcleo dos espermatozoides medem 50 ± 2,3 µm. Portanto, a morfologia dos testículos e dos espermatozoides oferece um método fácil e rápido para distinguir *N. inversa* de *N. villosa*, duas espécies crípticas simpátricas.

Palavras Chave: *Neoponera inversa*; *Neoponera villosa*; folículos testiculares; morfometria espermática

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