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Genetic Characterization of Some *Neoponera* (Hymenoptera: Formicidae) Populations Within the *foetida* Species Complex

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Abstract

The *foetida* species complex comprises 13 Neotropical species in the ant genus *Neoponera* Emery (1901). *Neoponera villosa* Fabricius (1804) , *Neoponera inversa* Smith (1858), *Neoponera bactronica* Fernandes, Oliveira & Delabie (2013), and *Neoponera curvinodis* (Forel, 1899) have had an ambiguous taxonomic status for more than two decades. In southern Bahia, Brazil, these four species are frequently found in sympatry. Here we used Bayesian Inference and maximum likelihood analyses of COI and 16S mtDNA sequence data and conventional cytogenetic data together with observations on morphology to characterize sympatric populations of *N. villosa*, *N. inversa*, *N. bactronica*, and *N. curvinodis*. Our results showed marked differences in the karyotype of these ants. Both *N. curvinodis* and *N. inversa* have chromosome number of $2n = 30$. Their chromosome composition, however, is distinct, which indicates that *N. curvinodis* is more closely related to *N. bactronica*. These four species clustered into three distinct groups. The close relationship between *N. bactronica* and *N. curvinodis* deserves further investigation since it has not been fully resolved here. Our results confirm that *N. inversa*, *N. villosa*, *N. bactronica* + *N. curvinodis* indeed represent four distinct taxa within the *foetida* species complex.

Key words: Ponerinae, cytogenetic analysis, phylogenetic analyses

In a recent study (Schmidt and Shattuck 2014), the ponerine ant genus *Neoponera* Emery (1901) comprises 57 valid species of very diverse morphology and behavior. The genus ranges from southern Texas and Northern Mexico to southern Brazil and northern Argentina (Schmidt and Shattuck 2014). Several studies have revealed that many taxa previously assigned to this genus are in fact complexes of cryptic species (Lucas et al. 2002, Ferreira et al. 2010). Cryptic species complexes were defined by Bickford et al. (2007) as "two or more species … if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable." Within *Neoponera*, such complexes can include sympatric species and species of wide geographic distribution [e.g., *Neoponera apicalis* (Latreille, 1802), *Neoponera verenae* (Forel, 1922) and *Neoponera obscuricornis* (Emery, 1890) in the group apicalis (Delabie et al. 2008); and *Neoponera foetida* (Linnaeus, 1758), *Neoponera bactronica* (Fernandes, De Oliveira and

Delabie, 2014), *Neoponera curvinodis* (Forel, 1899), *Neoponera inversa* (Smith, 1858), and *Neoponera villosa* (Fabricius, 1804) in the group foetida (Fernandes et al. 2014)]. Most of these species were grouped into complexes or even under a single species name due to the lack of characters that can be used to differentiate among them (Wild 2005, Delabie et al. 2008, MacKay and MacKay 2010).

Thirteen species comprise the *foetida* species complex (Mackay and Mackay 2010) that includes *Neoponera bactronica*, *N. curvinodis*, *N. inversa*, and *N. villosa*. The species within this complex are relatively large (1.5–2 cm), covered with silky pubescence, and arboreal like many (but not all) other *Neoponera* species. These four aforementioned species have had an ambiguous taxonomic status for more than two decades. This is mostly due to both their morphological similarity and their wide geographic distribution, many times in a sympatric situation (Fernandes et al. 2014). Most of the studies

published from 1970 to 2010 on *N. villosa* (see an extensive, but not exhaustive, list on http://www.antwiki.org/wiki/Neoponera_villosa, accessed on 29 November 2017) can refer to any of these four taxa.

More recently, morphological and biochemical studies have indicated that *N. bactronica*, *N. inversa*, *N. villosa*, and *N. curvinodis*, which occur in sympatry in northeastern Brazil, show discrete differences in the petiole morphology, in the cuticular hydrocarbon composition (Lucas et al. 2002), in morphological characteristics of the male reproductive system (Barcellos et al. 2015), and in the clypeus morphology (Fernandes et al. 2014). Moreover, preliminary cytogenetic analyses have indicated differences in chromosome number for populations of *N. villosa*, *N. inversa*, and *N. curvinodis* in the state of Bahia, Brazil (Mariano et al. 2012). Most recently, the taxonomic status of these four species in the *foetida* species complex was recently defined by Fernandes et al. (2014). The authors described two new *Neoponera* species in the complex from Brazil: *N. billemma* and *N. bactronica*. The species *N. bactronica* corresponds to the species previously named as the *nomen nudum N. 'subversa'* (Lucas et al. 2002), which is morphologically very close to both *N. inversa* and *N. curvinodis* (Fernandes et al. 2014).

The use of morphology alone for species delimitation can be particularly challenging in organisms that either display cryptical morphological characters or show a combination of low interspecific and high intraspecific variation (Rissler and Apodaca 2007, Schlick-Steiner et al. 2010). DNA-based methods have been increasingly employed and have proved useful for discovering hidden diversity for phylogenetic analyses and species identification, particularly in groups that comprise species complexes such as ants (Ross and Shoemaker 2005, Moreau et al. 2006, Ward 2007, Bacci et al. 2009, Bernasconi et al. 2011, Schmidt 2013, Hanisch et al. 2017, Yagound et al. 2017), tephritids (Smith-Caldas et al. 2001, Barr et al. 2005, 2018, Dias et al. 2016, Manni et al. 2015, Drosopoulou et al. 2017), and butterflies (Silva-Brandão et al. 2015, Bächtold et al. 2017, Janzen et al. 2017), among other insects.

Cytogenetics has also contributed significantly to the resolution of taxonomic problems and phylogenetic relationships in several insect orders (Dincă et al. 2011, Gokhman 2011). Previous such studies on ants have helped to distinguish species groups of controversial taxonomy (Crosland and Crozier 1986, Crosland et al. 1988, Mariano et al. 2006, 2012, Santos et al. 2010, Cristiano et al. 2013). In the tribe Ponerini, substantial variation in karyotype has been documented, ranging from a few metacentric large chromosomes in *Pseudoponera stigma* (Fabricius, 1804) (Hymenoptera: Formicidae) and *Neoponera unidentata* (Mayr, 1862) ($2n = 12$) (Mariano et al. 2012) to a high number of small acrocentric chromosomes in *Dinoponera* spp. (Hymenoptera: Formicidae) ($2n = 82–120$) (Santos et al. 2012).

In this study, we used molecular and cytogenetic data together with observations on morphology to characterize sympatric populations of *N. villosa*, *N. inversa*, *N. bactronica*, and *N. curvinodis*.

Materials and Methods

Sampling
Specimens used in the molecular analysis were collected in 14 localities in the states of Bahia (BA) and Espírito Santo (ES) in Brazil. Colonies used in the cytogenetic analysis were collected in seven localities solely in the state of Bahia (Fig. 1; Table 1). Voucher specimens of each colony were deposited at the Laboratório de Mirmecologia (CPDC Collection), CEPEC-CEPLAC, Ilhéus, Bahia, Brazil.

Genomic DNA Extraction, Gene Amplification, and Sequencing
For each individual, genomic DNA was extracted from legs and thorax using the DNeasyTM Tissue Kit (Qiagen Inc., Valencia, CA)

following the manufacturer's instructions. Two mitochondrial gene regions were amplified for the molecular phylogenetic analysis: cytochrome oxidase subunit I gene (COI) and 16S using primers LCO1490/HCO2198 for COI (Folmer et al. 1994) and LR13943F/LR13392R (Costa et al. 2003), respectively. DNA amplification was carried out in 25 µl volume reactions: 12.45 µl ultrapure water, 2.5 µl 10× buffer, 3.0 µl, 25 mM MgCl$_2$, 2.5 µl 100 mM dNTP, 1.25 µl of each primer (20 mM), 3 µl of DNA, and 0.3 µl Taq DNA polymerase (Promega Corporation, Madison, WI). The gene amplification consisted of an initial step at 94 °C for 3 min followed by 39 cycles (denaturation at 94°C for 1 min, annealing at 52°C [COI] for 1 min and 47°C (16S) for 1:30 min, and extension at 72°C for 1 min) and a final extension step at 72°C for 10 min. Amplifications were performed using an Applied Biosystems Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA).

Polymerase chain reaction (PCR) products were purified using exonuclease I (EXO I) and shrimp alkaline phosphatase (SAP) (Thermo Fisher Scientific Inc., Waltham, MD). EXO I-SAP reactions were carried out in 8.5 µl volumes with a final concentration of 1.6 U/µl Exo I and 0.15 U/µl SAP. EXO I-SAP reactions were carried out in 8.5 ml volumes with a final concentration of 1.6 U/ml Exo I and 0.15 U/ml SAP. The thermal-cycling regime was 15 min at 37°C followed by 15 min at 80°C. PCR products were sequenced in both forward and reverse directions using Big Dye v3.1 and run on an ABI3730 automated capillary sequencer at the Centro de Pesquisa sobre o Genoma Humano e Células-Tronco, Universidade de São Paulo. Forward and reverse sequences were analyzed using the Sequencing Analysis 5.3.1 (Applied Biosystems, Foster City, CA).

Phylogenetic Analyses
Sequences were aligned using Bioedit 7.0.9.0 (Hall 1999). MEGA 5 (Tamura et al. 2011) was used to calculate genetic distances and nucleotide composition.

Bayesian Inference (BI) and maximum likelihood (ML) were used to estimate the phylogeny of the species within the *foetida* complex from concatenated sequences of fragments of the COI and 16S mitochondrial genes. The best fit model was selected using Partition Finder v1.1.1 (Lanfear et al. 2012) based on the Akaike information criterion (AIC) (Posada and Buckley 2004). For the 16S fragment the best model was HKY+G. On the other hand, for COI fragment, three subset partitions were obtained (COI-1, COI-2, and COI-3), with three different models: TIM, GTR+G, and TrN, respectively. For the BI, two independent runs of 20 million generations with four chains of Markov chain Monte Carlo iterations each were performed. The first 4,000 generations were discarded as burn-in, after which trees were sampled every 1,000 generations. This analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). To check the convergence of parameters between runs and analysis performance (effective sample size [ESS] values > 200) we used TRACER 1.5 (http://beast.bio.ed.ac.uk/Tracer) and we accepted that the results if ESS values were >200. The ML inference was run using RxML program (Stamatakis 2014) under the GTRAC model, using invariable sites and gamma distribution for each partition during the run. Node supports of the ML analyses were estimated by 1,000 bootstrap replications. The trees were visualized and edited using FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). The ant *Neoponera apicalis* was used as outgroup for both analyses.

Conventional Cytogenetic Analysis
Mitotic metaphases were obtained for *N. bactronica*, *N. curvinodis*, *N. inversa*, and *N. villosa* following Imai et al. (1988) and subsequently stained with Giemsa for the determination of chromosome
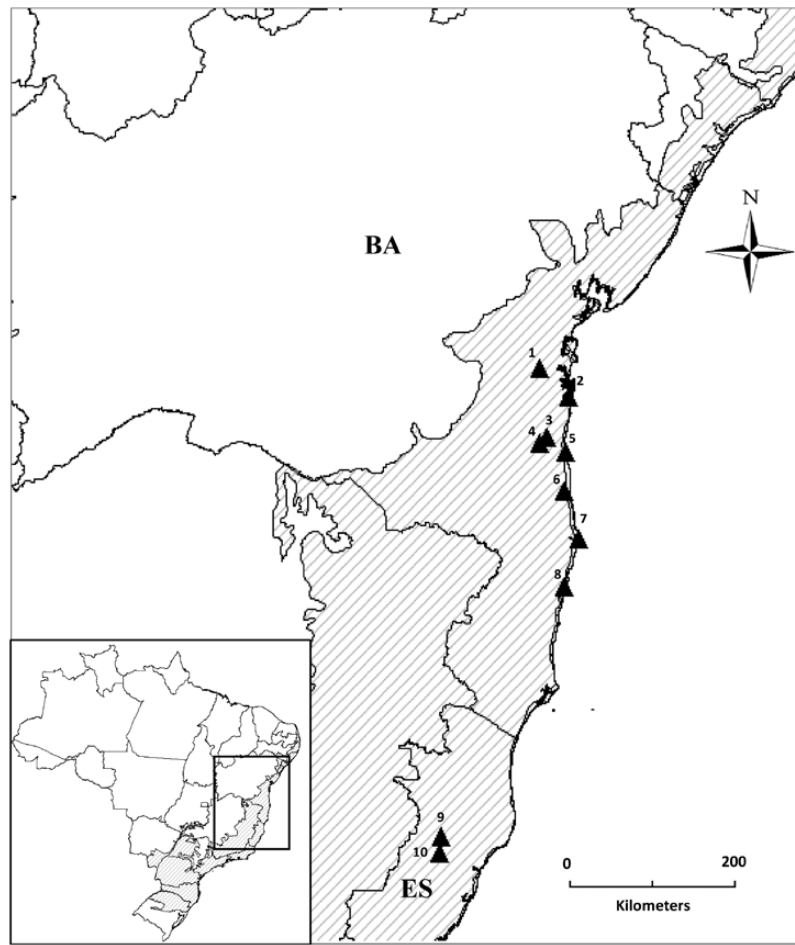
Fig. 1. Sampled localities for *Neoponera* species in Brazil. The triangles represent the collection sites in the states of Bahia (BA) and Espírito Santo (ES).

Table 1. *Neoponera* species sampled in 14 localities in the states of Bahia (BA) and Espírito Santo (ES), Brazil, with their respective geographical coordinates

Localities	Species	Coordinates
Barro Preto-BA	*N. villosa**	14° 47′ S, 39° 27′ W
Belmonte-BA	*N. villosa, N. bactronica, N. curvinodis*	15° 51′ S, 38° 52′ W
Buerarema-BA	*N. villosa**	14° 56′ S, 39° 18′ W
Ilhéus-BA	*N. bactronica**, N. curvinodis**, N. villosa**, N. inversa**	14° 47′ S, 39° 13′ W
Itajuipe-BA	*N. villosa*	14° 40′ S, 39° 22′ W
Itapinas-ES	*N. villosa*	19° 31′ S, 40° 48′ W
Jitaúna-BA	*N. villosa**	14° 01′ S, 39° 53′ W
Maraú-BA	*N. villosa*	14° 05′ S, 39° 00′ W
Piraí do Norte-BA	*N. inversa*	13° 45′ S, 39° 22′ W
Porto Seguro-BA	*N. villosa, N. inversa*	16° 26′ S, 39° 03′ W
São Roque do Canaã-ES	*N. bactronica*	19° 44′ S, 40° 39′ W
Itororó-BA	*N. villosa**	15° 07′ S, 40° 04′ W
Una-BA	*N. villosa, N. bactronica* N. inversa**	15° 17′ S, 39° 04′ W
Uruçuca-BA	*N. villosa**, N. inversa**	14° 35′ S, 39° 17′ W

Samples used just in molecular analysis have no asterisk.

*Samples used just in the cytogenetics analysis.

**Samples used in both molecular and cytogenetics analysis.

number and morphology. Metaphases were photographed using a microscope equipped with a photomicroscope Olympus BX60 with 100× objective (Olympus, Tokyo, Japan) with TMAX 35 mm film (Kodak, Rochester, NY). Chromosome classification followed Levan et al. (1964). The species *N. curvinodis* was analyzed, but the material analyzed did not allow to distinguish karyotypes clearly.

Results

Our sequence dataset comprised 35 sequences of both COI and 16S fragments (Table 2), and all sequences were deposited at GenBank (accession numbers MF509196–MF509265). Alignment of the concatenated sequences resulted in a matrix of 1,010 characters

with 255 variable sites. No premature stop codons or indels were found, and 28 nonsynonymous substitutions were detected for the COI gene.

Phylogenetic analyses using BI (Fig. 2) and ML (Supp Fig. 1 [online only]) produced trees with similar topology. Three major clades were recovered with high support values. The first clade (I) comprised all individuals of *N. villosa* with high support values (PP = 1, bootstrap = 78). The second clade (II) clustered all individuals of *N. bactronica* together with all individuals of *N. curvinodis* also with high support values (PP = 1, bootstrap = 99). The third clade (III) comprised all individuals of *N. inversa* with high support values (PP = 1, bootstrap = 100).

The cytogenetic results revealed that the four species analyzed showed distinct karyotypes: *N. bactronica* (2n = 26–28), *N. curvinodis* (2n = 30), *N. inversa* (2n = 30), and *N. villosa* (2n = 34) (Figs. 3 and 4). Intraspecific variation in chromosome number was observed in colonies of *N. bactronica*: 2n = 26 from Ilhéus and 2n = 28 for individuals collected in a colony from Una (Table 3; Fig. 3b).

The karyotypes of *N. inversa*, *N. curvinodis*, and *N. bactronica* are predominantly constituted by metacentric chromosomes, which are relatively uniform in length and heterochromatin content (Figs. 3c and 4). The variation is continuous for the number of chromosomes: 2n = 26, 28, 30. There was no pronounced difference in chromosome length for these karyotypes as it was observed for *N. villosa* (2n = 34, Fig. 3d). The diploid chromosome numbers of *N. curvinodis* and *N. inversa* are the same: 2n = 30. However, there are differences in the karyotype composition, as *N. curvinodis* (2K = 24M + 6A) has an extra pair of metacentric chromosomes compared with *N. inversa* (2K = 20M + 10A).

Neoponera villosa, the species that was found more commonly, had a karyotype with very peculiar chromosomes, having seven pairs of acrocentric chromosomes with their long arm extremely longer than the short arm and rich in heterochromatin. This karyotype comprised chromosomes of two distinct types: 14 large acrocentric chromosomes and 8 small metacentric chromosomes.

Discussion

Our phylogenetic analyses indicated that *N. bactronica* and *N. curvinodis* are very closely related, and also revealed a closer relationship between *N. inversa*, *N. bactronica*, and *N. curvinodis* relative to *N. villosa*.

Our cytogenetic analysis showed that the karyotypes of the taxa studied are also distinct and may reflect reproductive isolation. The karyotype of *N. villosa* showed chromosomes with a peculiar morphology and high heterochromatin content. Due to the visibly disproportionate length of these chromosomes (pairs 6–15), it may be argued that they have resulted from centric fissions followed by an increase in heterochromatin content, a recurring event in chromosomes of hymenopterans. This pattern has been observed in some bees (*Melipona* spp. Illiger 1806 [Hymenoptera: Apidae]) (Lopes et al. 2011) and in ants

Table 2. Number of individuals sequenced for both mitochondrial regions COI and 16S

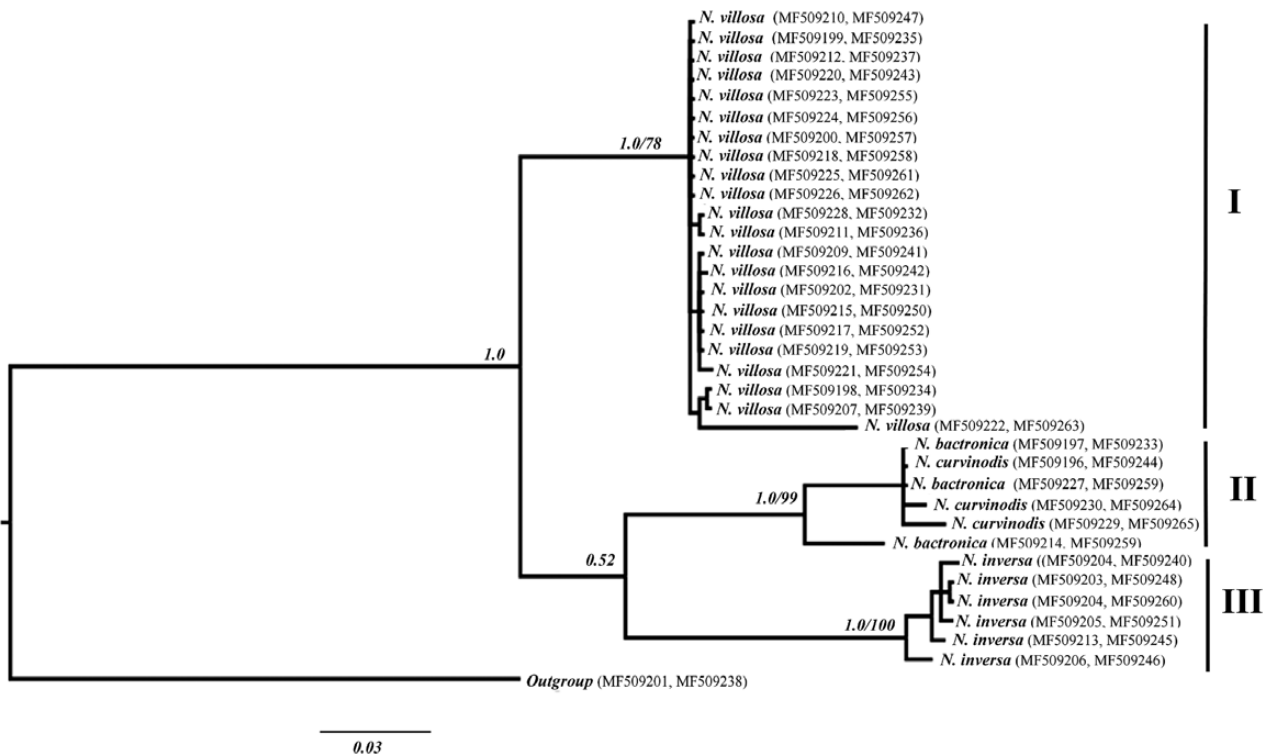| Species	No. sequences
N. villosa	22
N. inversa	6
N. bactronica	3
N. curvinodis	3
N. apicalis	1
Total	**35**

Fig. 2. BI based on 1,010 bp of COI and 16S mitochondrial genes showing three major clades recovered with high support values. Posterior probability and bootstrap values for BI and ML, respectively, are shown above each supported node. The numbers in brackets indicate the GenBank accession numbers.
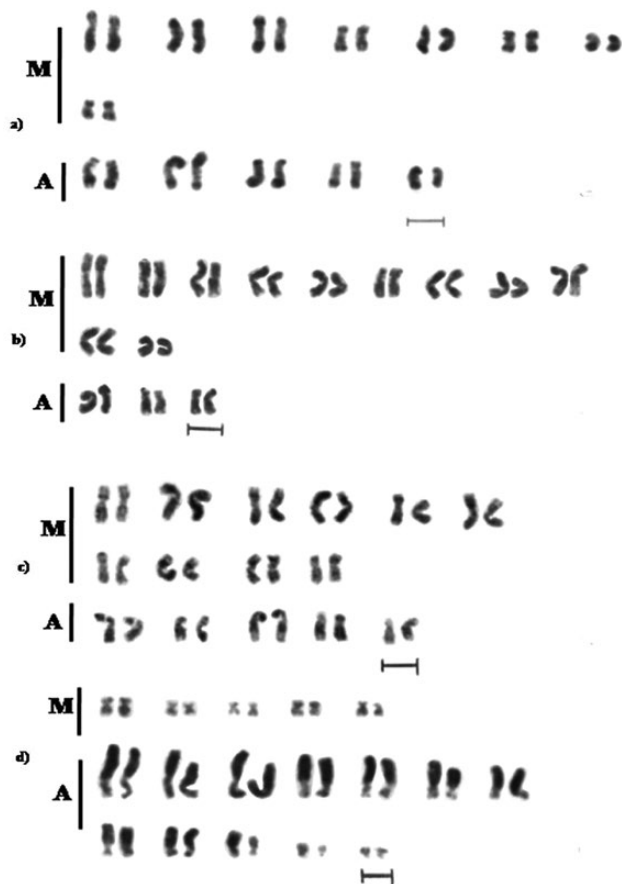
Fig. 3. Karyotype of *Neoponera* species. (a) *N. bactronica* 2*n* = 26, collected in Ilhéus-BA. (b) *N. bactronica* 2*n* = 28, collected in Una-BA. (c) *N. inversa* 2*n* = 30, collected in Ilhéus-BA. (d) *N. villosa* 2*n* = 34, collected in Ilhéus-BA.
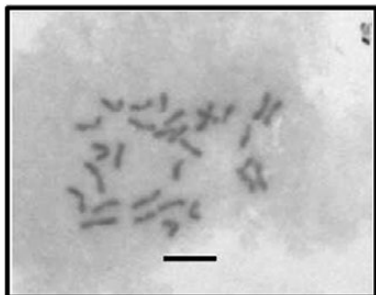

Fig. 4. Metaphase of *N. curvinodis* 2*n* = 30, collected in Ilhéus-BA.

it has been reported solely for the ponerine *Bothroponera* sp. 2 Mayr 1862 (Hymenoptera: Apidae) (Imai et al. 1984). The mechanism of centric fission may play an important role in reducing the occurrence of deleterious reciprocal translocations in ants and result in smaller and more numerous chromosomes (Imai et al. 1988, Imai 1991, Mariano et al. 2012). We have considered this mechanism as the most likely explanation due to the amount of heterochromatin that was easily observed on the submetacentric chromosomes even without the use of heterochromatin conventional staining techniques. Moreover, previous analyses have showed that the karyotypes within *Neoponera* fit well into the pattern suggested by Imai et al. (1988) of successive cycles of centric fission leading to an increase in chromosome number (Mariano et al. 2012). However, other alternative explanations could be considered

Table 3. Results obtained for the cytogenetic analysis of four species of *Neoponera* sampled in the state of Bahia

Species	Localities	N/I	2*n*, (*n*)	Karyotypes
N. bactronica	Ilhéus	2/15	26	♀ = 16M + 10A
	Una	1/10	28	♀ = 22M + 6A
N. curvinodis	Ilhéus	1/04	30	♀ = 24M + 6A
N. inversa	Ilhéus	12/40	30	♀ = 20M + 10A
	Una	2/12	30	♀ = 20M + 10A
	Uruçuca	2/15	30	♀ = 20M + 10A
N. villosa	Ilhéus	13/40	34	♀ = 10M + 24A
	Barro Preto	1/05	34	♀ = 10M + 24A
	Buer arema	2/08	34	♀ = 10M + 24A
	Itororó	3/12	(17)	♂ = 6M + 11A
	Jitaúna	1/05	34	♀ = 10M + 24A
	Uruçuca	2/04	34	♀ = 10M + 24A

N = number of nests; I = number of individuals.

as well. Some authors have indicated that high rates of recombination, by an increase in either chromosome number or the rate of intra-chromosomal recombination, are strongly favored in social insects (Sherman 1979, Seger 1983, Wilfert et al. 2007). Others have indicated that genetic drift following a population decline could lead to an increase in chromosome number, although there is no ant species with a wide geographic range where this phenomenon was observed (Ross et al. 2015).

No marked difference in chromosome length was observed in *N. bactronica*, *N. curvinodis*, or *N. inversa*. The karyotype of *N. bactronica* (2*n* = 26) showed the lowest chromosome number and the karyotype of *N. curvinodis* showed the highest number of acrocentric chromosomes (24A) within the complex. The increase of one pair of metacentric chromosomes in the karyotype of *N. curvinodis* (when compared with that of *N. inversa*, both with 2*n* = 30) may suggest that rearrangements such as centric fission and/or pericentric inversion are responsible for chromosomal alterations acting on species differentiation. Even though *N. curvinodis* and *N. inversa* have the same diploid chromosome number (2*n* = 30), the karyotype composition of *N. curvinodis* suggests that this species is more closely related to *N. bactronica*. This is due to the fact that the latter species both have more metacentric chromosomes (M) than the remaining species studied herein.

In this group of ants, speciation might have not been accompanied by significant changes in morphology. For instance, the predator morphology is maintained in both ants and ant-mimic in the spider families Clubionidae and Salticidae that occur in sympatry in Ilhéus. These spiders even possess a silvery pubescence like the one typical of several species within the *foetida* complex (Delabie 1999, Lucas et al. 2002, Lattke 2003), including those studied here. The maintenance of the "*foetida* pattern" may indicate that this model, together with some behavioral elements and ecological aspects, represent an ecological advantage (Delabie 1999, Lucas et al. 2002). Thus, it seems that the rates of differentiation in external morphology were not at all correlated with karyotypic differentiation within this complex. Morphological evolution rates and speciation have been shown to be incongruent for many species, and morphological stasis does not indicate evolutionary stasis in many cases but rather cryptic speciation (Mayr 1963, Lee and Frost 2002, Bickford et al. 2007). Recently, cryptic species complexes and sibling species following a similar pattern have been observed in other species within *Neoponera* and the related genus *Pachycondyla* Smith 1858 (Hymenoptera: Formicidae) (Lucas et al. 2002, Delabie et al. 2008, Mariano et al.

2012, Fernandes et al. 2014, Velasco et al. 2014) and even in other Ponerinae (Mariano et al. 2006). In such groups, behavior, chemical signatures, and karyotype composition may act as mechanisms of reproductive isolation, and it is rather probable that several speciation processes have successively occurred. Thus, the use of integrative taxonomy for studies on ants has been recently encouraged by several authors (Mariano et al. 2012, Cristiano et al. 2013).

Our molecular and cytogenetic results clearly showed a number of marked differences between *N. bactronica*, *N. curvinodis*, *N. inversa*, and *N. villosa*. These four species were clustered in different groups and also showed distinct karyotypes. The close relationship between *N. bactronica* and *N. curvinodis* suggests a recent speciation and deserves further investigation. Molecular markers could be useful in further studies to corroborate this inference. The results reported herein confirm that these four species within the *foetida* complex are distinct taxa. Therefore, previous studies that had indicated that these species could be distinguished from each other by subtle differences in external morphology and cuticular hydrocarbon composition are confirmed (Lucas et al. 2002, Fernandes et al. 2014).

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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