

The predatory mite *Neoseiulus paspalivorus* (Phytoseiidae) in Brazil: taxonomic status, reproductive compatibility and morphological and molecular variability

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Abstract The predatory mite *Neoseiulus paspalivorus* (De Leon) is often found in association with the coconut mite, *Aceria guerreronis* Keifer. The identification of natural enemies is essential for the definition of biological control strategies. Therefore, the present study aimed to confirm whether the mite populations from different Northeastern Brazilian states identified as *N. paspalivorus* belong to the same species. This determination was accomplished through the study of morphometric variability in 33 anatomical characters and of molecular variability in two DNA fragments: Internal Transcribed Spacer (ITS) rDNA and cytochrome c oxidase subunit I (COI) mtDNA. This study also determined whether there is reproductive isolation between the two most morphologically distinct populations (Rio Grande do Norte and Paraíba). Intraspecific morphometric variability was observed among the five populations of *N. paspalivorus*. Despite this variability, the crosses and backcrosses of the most morphologically distinct populations did not show reproductive incompatibility. The molecular analysis indicated the absence of genetic differences among the *N. paspalivorus* populations for the ITS fragment. Three haplotypes were identified for the COI fragment, and the genetic distance ranged from 0 to 0.2 %. Despite the morphometric differences, the results of the molecular and biological analysis corroborate the previous identification of *N. paspalivorus* for all of the studied populations. The present study contributes to the systematics of Phytoseiidae predatory mites and to the biological control of *A. guerreronis* by the accurate identification and characterization of one of its main natural enemies along extensive areas in Brazil.

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Introduction

The coconut mite, *Aceria guerreronis* Keifer (Eriophyidae), is one of the most important arthropod pests of coconut in the world (Moore and Howard 1996; Navia et al. 2013). Several studies aimed at finding predatory mites that could be used in the management of *A. guerreronis* populations have been conducted in Africa (Negloh et al. 2008, 2010), America (Lawson-Balagbo et al. 2008; Reis et al. 2008; Silva et al. 2010) and Asia (Haq 2001; Ramaraju et al. 2002; de Moraes et al. 2004; Fernando and Aratchige 2010).

Aceria guerreronis develops on the fruit perianth (Moore and Howard 1996; Nair 2002; Lawson-Balagbo et al. 2007; Navia et al. 2013), which is the space between the coconut fruit surface and the set of bracts that links the fruit to the spikelet. The distance between the distal end of the bracts and the fruit surface is large enough for *A. guerreronis* penetration into the perianth but not large enough for the access of other mites (Lawson-Balagbo et al. 2007; Lima et al. 2012), including most of the predatory mites. *Neoseiulus paspalivorus* (De Leon) and *N. baraki* (Athias-Henriot) (Phytoseiidae) are the species that stand out among the predatory mites frequently found in association with *A. guerreronis* in the perianth of coconut fruit in Brazil (Lawson-Balagbo et al. 2008; Lima et al. 2012; Reis et al. 2008), Africa (Negloh et al. 2008, 2010) and Asia (de Moraes et al. 2004). These *Neoseiulus* species have reduced size, a short legs and mainly flattened body (Chant and McMurtry 2003; de Moraes et al. 2004) when compared with other predatory mite species that are also found in association with *A. guerreronis*, such as *Proctolaelaps bickleyi* (Bram), *P. bulbosus* Moraes, Reis and Gondim Jr. (Melicharidae), and the phytoseiids *Amblyseius largoensis* (Muma) and *Euseius alatus* De Leon, which are larger body size (Lima et al. 2012; Melo et al. 2015). Those characteristics make *N. paspalivorus* and *N. baraki* the most promising species as biological control agents of *A. guerreronis* (Lima et al. 2012; Melo et al. 2015).

The phytoseiid *N. paspalivorus* was first described in a grass species of the genus *Paspalum* in Florida, USA, in 1956 (De Leon 1957). Later, this mite was reported in Asia (Ghai and Gupta 1984; Schicha and Corpuz-Raros 1992; de Moraes et al. 2004), Africa (Negloh et al. 2010; Famah Sourassou et al. 2011) and in America (Denmark and Muma 1978; de Moraes et al. 2000; Lawson-Balagbo et al. 2008; Reis et al. 2008), always in association with *A. guerreronis* in the perianth of coconut fruit. *Neoseiulus paspalivorus* was reported in *Pluchea symphytifolia* (Miller) (Asteraceae) in Guadeloupe (de Moraes et al. 2000). *Neoseiulus paspalivorus* is widely distributed in Brazil, and this mite has been observed in the states of Alagoas, Bahia, Ceará, Maranhão, Pará, Paraíba, Pernambuco, Piauí, Rio Grande do Norte and Sergipe (Lawson-Balagbo et al. 2008; Reis et al. 2008). In this country, *N. paspalivorus* occurs predominantly in areas characterised by long periods of drought, average temperatures ranging from 27 to 30 °C, a relative humidity between 70 and 80 % and an annual rainfall of 600–1500 mm (Lawson-Balagbo et al. 2008).

The accurate identification of natural enemies is essential for biological control. The phytoseiid mites are currently identified based on morphological traits (e.g., Chant and McMurtry 1994, 2006). However it may be limiting for certain taxa. Cryptic species of

Phytoseiidae have been reported in the literature (Tixier et al. 2006a), including species in the genus *Neoseiulus* (Famah Sourassou et al. 2012). Furthermore differences in biological characteristics and predation efficiency have been reported in geographically and genetically distinct populations (de Moraes and McMurtry 1985; Yaninek et al. 1993; Tixier et al. 2006a, b, 2010; Ferrero et al. 2007; Furtado et al. 2007; Okassa et al. 2011; Domingos et al. 2013).

The use of an integrated approach, combining molecular data, crossings experiments and morphological studies, has assisted the identification of Phytoseiidae species and provided knowledge concerning differences between populations. The integration of this information in taxonomic studies has contributed to advances in the systematics of predatory phytoseiid mites, supporting establishment of synonymies, revealing occurrence of cryptic species or characterizing genetic lineages (e.g., Tixier et al. 2008a; Okassa et al. 2009, 2011; Famah Sourassou et al. 2012; Navia et al. 2014).

Some studies with phytoseiidae mites in the genus *Neoseiulus* associated with the coconut mite have already been conducted. Famah Sourassou et al. (2011) performed multivariate morphometric analysis and crossing experiments with three populations of *N. paspalivorus* (Benin, Ghana and Brazil) and concluded that the studied populations were different species, despite their morphological similarity. Famah Sourassou et al. (2012) used multivariate morphometric analysis, molecular analyses and crossing experiments with populations of *N. baraki* from Benin, Tanzania and Brazil. The results also provided evidence for the existence of cryptic species.

Considering that cryptic species or reproductive isolation have already been detected among *N. paspalivorus* populations from other countries/continents (Famah Sourassou et al. 2011); that *N. paspalivorus* populations in Brazil occurs in geographically distant areas (Lawson-Balagbo et al. 2008) and could present reproductive isolation; and that genetic differences can be related with predatory behaviour and bioecological aspects (Famah Sourassou et al. 2011), it would be important to confirming that the mite populations identified as *N. paspalivorus* from different Brazilian states belong to the same taxon; that there is no reproductive isolation between the most morphologically distinct populations; and know if there is genetic variability among the populations. For these purposes, an integrative approach was used, combining three methods: morphometrics, DNA sequences analysis, and crossing experiments.

Materials and methods

Sampling, identification, rearing and preservation of mites for analysis

Coconut fruits with damage caused by *A. guerreronis* were collected in all nine states of northeast Brazil (Alagoas, Bahia, Ceará, Maranhão, Paraíba, Pernambuco, Piauí, Rio Grande do Norte and Sergipe), covering an area of approximately 3000 km along the Atlantic coast. Approximately ten coconut palms were sampled at each location. In each state one sample was collected, except for Rio Grande do Norte e Ceará, where samples were collected in two and three localities, respectively, in a way to obtain enough number of specimens to conduct experiments. The geographical coordinates of each sampling location were obtained with the aid of a global positioning system (GPSMAP® 60CSx, Garmin, Romsey, UK).

The collected fruits were placed in plastic bags and stored in Styrofoam boxes for less than 2 days until transportation to the laboratory where they were stored in a refrigerator at approximately 10 °C for a maximum of 7 days until inspection. The phytoseiid mites were collected with a brush under a stereoscopic microscope. Approximately ten female from each sampling location were mounted on microscope slides in Hoyer's medium. The slides were placed in a drying chamber (± 60 °C) for 5 days and then observed under an Olympus optical microscope (model BX41, Olympus, Tokyo, Japan). The mites were identified using Phytoseiidae taxonomic studies (Chant and McMurtry 2003; de Moraes et al. 2004) and the original description of *N. paspalivorus* (De Leon 1957). When *N. paspalivorus* mites were found, all the fruits of the sample were inspected making possible collecting a higher number of specimens to establishing colonies and conduct experiments. *Neoseiulus paspalivorus* mites were found in five of the nine states in northeast Brazil where samples were collected: Ceará (CE), Paraíba (PB), Piauí (PI), Rio Grande do Norte (RN) and Sergipe (SE) (Fig. 1).

For each State in which *N. paspalivorus* mites were found, a colony was established with approximately 100 specimens, which were kept isolated in climatic chambers (25 ± 1 °C, 70 ± 10 % relative humidity and a daily 12 h photophase). The rearing unit consisted of a black polypropylene disk (1 mm thick), filter paper, polyethylene foam (1 cm thick) and a Petri dish, all superposed in this sequence and 16 cm in diameter. To prevent mites from escaping, the polypropylene disk was wrapped with hydrophobic cotton wool, which was kept constantly moist by daily addition of distilled water. Five fragments (approximately 1 cm³) of the epidermis of coconut fruit perianth infested with *A. guerreronis* (field collected) were supplied daily as food for the predatory mites (each fragment had approximately 80–100 specimens of different developmental stages).

One month after the colonies were established, 25 females were recovered from each colony for the morphometric analysis. Females were removed and mounted on microscope slides in Hoyer's medium. In addition fifteen females were removed, placed in absolute ethyl alcohol and subsequently kept at -20 °C for the molecular analysis.

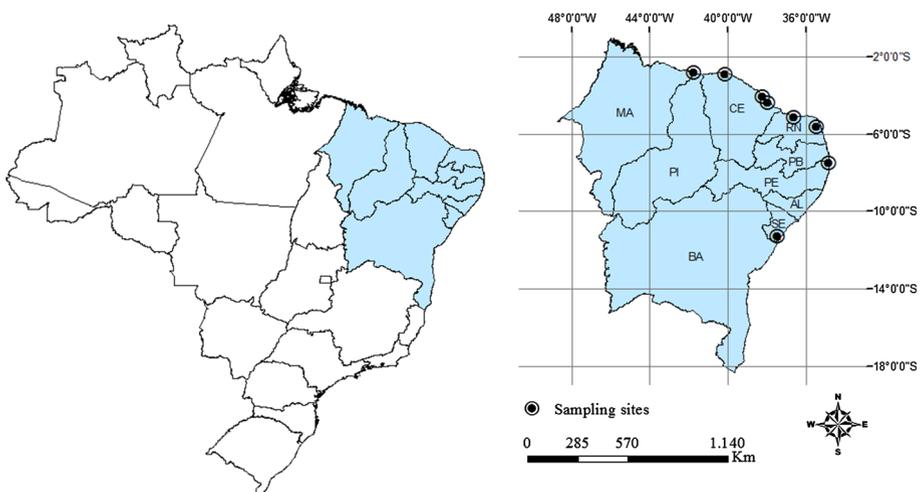


Fig. 1 Sampling locations of *Neoseiulus paspalivorus* populations in northeastern Brazil

Morphometric characterisation

The morphometric analyses were performed with specimens collected from each of the five colonies—CE, RN, PB, PI and SE States, from now considered as a “population”. Twenty females of each population were measured. The measurements were performed under an optical microscope (400× magnification) coupled to a computer for image capturing using Honestech TVR 2.5 software. Thirty-three characters that are commonly used in the identification of phytoseiid mites (e.g., Chant and McMurtry 1994, 2003, 2006) were measured (see the list of characters in Table 1). The measurements were performed with Motic Images Plus 2.0 software. The nomenclature used in the present study follows Lindquist and Evans (1965), and the measurements are given in micrometres. The measurement data were subjected to one-way analysis of variance (PROC ANOVA) followed by the Student–Newman–Keuls (SNK) test ($\alpha = 0.05$). To estimate the overlap of characters among the populations, the following equation was used: $\Delta x = \text{Min}_x - \text{Max}_x$, where Min_x is the lowest measurement for character “x” in the population that had the highest mean value, and Max_x is the highest measurement for character “x” in the population that had the lowest mean value (Tixier 2012). Multivariate analyses were also conducted, with an initial discriminant analysis performed to evaluate the re-identification of specimens based on variations in each population. Then, a canonical variable analysis (CVA) was performed to determine the morphological variation patterns and to identify the morphological characters that contribute most heavily to the morphological differentiation between populations. All of the statistical analyses were performed with SAS software (SAS Institute 2002). Measurements of studied populations were also compared with holotype measurements presented by De Leon (1957) in the species original description.

The mites measured for morphometric analysis were deposited as voucher specimens in the mite collection of the Laboratory of Acarology of the Universidade Federal Rural de Pernambuco (UFRPE), located in Recife, state of Pernambuco, Brazil, and also in the mite collection of the Empresa Brasileira de Pesquisa Agropecuária—Embrapa Recursos Genéticos e Biotecnologia, located in Brasília, Distrito Federal, Brazil.

Crosses

Reproductive compatibility tests were conducted with the *N. paspalivorus* populations from PB and RN, which were the most morphometrically distant according to the CVA. Approximately 100 females of each population were transferred to new rearing units. After 24 h, the females were removed, and the eggs were kept in the rearing units to obtain specimens of the same age. The specimens were fed in a manner similar to that previously described for the maintenance colonies. After 5 days, the resulting female deutonymphs were separated in experimental units consisting of a plastic container ($\text{Ø} = 2 \text{ cm} \times \text{H} = 1.8 \text{ cm}$), (128-well bioassay tray, Bio-Serv, Frenchtown, NJ, USA). To prevent mites from escaping, the cells were sealed with a transparent, adhesive, plastic film. From each population studied, ten unmated females were kept in isolation for 10 days to determine whether unmated females would lay eggs. At the same time, ten homogamic pairs and ten heterogamic ($\text{♂ PB} \times \text{♀ RN}$ and $\text{♂ RN} \times \text{♀ PB}$) pairs with males from the PB and RN colonies were formed. Any male that died during the oviposition period (10 days) was replaced by another male from the same original colony. Females resulting from the heterogamic crosses were backcrossed with males of the original populations (PB or RN). The pairs were evaluated for 10 days after the beginning of oviposition. Pre-oviposition

Table 1 Mean, standard error and range for 33 morphometric characters measured in adult females from five Brazilian populations identified as *Neoseiulus paspalivorus*

Morpho-metric characters	CE		PB		PI		RN		SE		Holo-type (De Leon 1957)
	Mean ± SE	Min-Max	Mean ± SE	Min-Max	Mean ± SE	Min-Max	Mean ± SE	Min-Max	Mean ± SE	Min-Max	
CED	326.0 ± 2.76b*	307.9–357.3	364.2 ± 6.69a	309.7–405.5	332.7 ± 3.26b	301.3–364.5	338.0 ± 2.41b	324.0–367.4	324.6 ± 2.18b	309.5–346.3	340
LED	131.9 ± 1.47a	121.9–146.0	135.4 ± 1.09a	125.9–144.7	132.1 ± 1.67a	119.3–145.9	135.0 ± 1.06a	126.9–142.2	131.2 ± 0.93a	125.6–140.7	140
j1	9.4 ± 0.16b	8.0–10.6	10.4 ± 0.19a	8.5–11.9	9.2 ± 0.11b	8.2–10.0	9.6 ± 0.19b	8.3–11.3	9.5 ± 0.13b	8.4–10.6	11
j3	9.1 ± 0.15b	7.2–10.1	9.9 ± 0.23a	8.0–11.8	9.3 ± 0.19ab	7.8–10.9	9.8 ± 0.14a	8.9–10.8	9.6 ± 0.11ab	8.6–10.5	11
j4	8.0 ± 0.15b	6.4–8.9	8.5 ± 0.15a	7.3–9.6	7.6 ± 0.11b	6.9–8.5	8.0 ± 0.12b	6.8–8.8	7.9 ± 0.12b	7.0–8.9	9
j5	7.8 ± 0.13a	6.9–8.9	8.2 ± 0.18a	7.1–9.8	7.3 ± 0.19b	5.3–8.8	8.1 ± 0.12a	7.0–8.9	7.9 ± 0.12a	7.1–9.1	10
j6	8.4 ± 0.14b	7.2–10.2	9.2 ± 0.14a	8.2–10.4	8.1 ± 0.18b	6.7–9.4	9.0 ± 0.13a	8.1–10.6	8.3 ± 0.15b	7.4–9.7	11
J2	8.7 ± 0.17a	7.6–10.1	9.3 ± 0.29a	6.4–11.8	8.8 ± 0.13a	7.8–9.7	9.3 ± 0.12a	8.0–10.2	9.1 ± 0.14a	7.8–10.3	11
J5	7.6 ± 0.11b	7.0–8.7	8.5 ± 0.21a	6.4–9.9	8.0 ± 0.21ab	6.8–9.9	7.8 ± 0.12b	7.1–8.8	8.0 ± 0.12ab	6.9–8.9	9
z2	9.1 ± 0.18b	7.6–10.7	9.8 ± 0.21a	7.7–11.3	8.7 ± 0.14b	7.9–10.2	9.1 ± 0.14b	7.8–10.3	8.7 ± 0.15b	7.1–9.9	11
z4	9.1 ± 0.13b	8.1–10.4	10.2 ± 0.23a	8.5–12.2	9.1 ± 0.15b	7.7–10.2	9.7 ± 0.14b	8.0–10.5	9.4 ± 0.16b	8.3–10.9	11
z5	7.7 ± 0.14bc	6.7–8.7	8.6 ± 0.17a	6.8–9.4	7.7 ± 0.11bc	6.7–8.8	8.1 ± 0.13b	7.1–9.3	7.6 ± 0.13c	6.4–8.5	9
Z1	9.2 ± 0.17b	8.2–11.1	10.1 ± 0.19a	8.9–12.2	9.2 ± 0.19b	8.0–11.1	9.6 ± 0.18b	8.0–11.4	9.1 ± 0.17b	7.5–10.7	11
Z4	13.6 ± 0.23a	11.4–15.2	14.6 ± 0.32a	11.9–16.7	12.5 ± 0.31b	8.7–14.6	14.2 ± 0.17a	12.8–15.5	13.7 ± 0.23a	11.9–15.4	17
Z5	46.8 ± 0.84b	41.9–58.6	53.6 ± 0.88a	46.3–59.4	48.2 ± 0.67b	43.9–55.7	48.3 ± 0.46b	43.2–51.3	47.2 ± 0.56b	41.0–53.2	52
s4	10.3 ± 0.20bc	8.1–12.0	11.2 ± 0.23a	8.8–12.6	9.9 ± 0.23c	7.9–11.4	10.8 ± 0.14ab	9.3–12.1	10.5 ± 0.16bc	9.0–11.4	12
S2	10.7 ± 0.16b	9.4–11.8	12.4 ± 0.25a	10.2–14.3	10.5 ± 0.20b	8.5–12.5	11.1 ± 0.12b	10.1–12.4	10.8 ± 0.15b	9.9–12.0	14
S4	12.0 ± 0.21b	9.5–13.3	13.3 ± 0.33a	9.1–15.7	12.5 ± 0.17b	10.8–13.8	12.4 ± 0.12b	11.5–13.2	12.3 ± 0.16b	10.7–13.3	15
S5	15.3 ± 0.36c	11.0–17.7	17.7 ± 0.36a	14.8–20.9	15.9 ± 0.36bc	11.0–18.5	16.5 ± 0.19b	14.6–18.0	16.4 ± 0.30b	13.5–18.9	19
r3	10.0 ± 0.18bc	8.6–11.4	11.0 ± 0.15a	9.6–11.9	9.5 ± 0.22c	8.0–12.0	10.3 ± 0.20b	7.7–11.9	10.0 ± 0.20bc	7.9–11.5	11
R1	8.1 ± 0.16bc	6.5–9.7	9.2 ± 0.23a	7.4–11.1	7.9 ± 0.16c	6.7–9.4	8.7 ± 0.14b	7.3–9.6	8.5 ± 0.15b	6.7–10.1	10
StIV	16.8 ± 0.26b	14.9–18.5	18.1 ± 0.35a	14.6–20.6	16.4 ± 0.21b	14.9–18.2	16.9 ± 0.29b	15.0–20.7	16.2 ± 0.23b	14.5–18.5	–
St1–St3	80.7 ± 0.40b	78.7–84.4	90.9 ± 1.38a	80.2–99.1	82.1 ± 0.50b	77.1–85.5	83.3 ± 0.44b	80.0–86.8	81.8 ± 0.38b	78.9–85.9	–
St2–St3	34.6 ± 0.38c	31.4–37.5	38.6 ± 0.62b	33.8–42.7	35.1 ± 0.28c	31.9–36.8	50.3 ± 0.28a	48.0–52.5	34.5 ± 0.38c	31.6–37.8	–

Table 1 continued

Morpho-metric characters	CE		PB		PI		RN		SE		Holo-type (De Leon 1957)
	Mean ^a ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	
St1–St1	38.1 ± 0.42a	35.4–41.8	38.0 ± 0.24a	36.4–40.3	38.1 ± 0.40a	35.8–43.1	37.4 ± 1.94a	1.0–41.4	38.2 ± 0.39a	33.6–41.2	–
St2–St2	49.4 ± 0.47b	45.8–52.9	49.1 ± 0.29b	46.8–51.0	50.1 ± 0.51b	45.9–54.5	53.2 ± 0.42a	49.7–56.7	49.3 ± 0.24b	46.5–50.6	–
St3–St3	51.9 ± 0.54a	47.5–56.2	52.5 ± 0.56a	49.1–58.3	52.4 ± 0.44a	47.9–55.5	35.0 ± 0.31b	32.6–38.3	52.4 ± 0.40a	49.4–55.6	–
EVA-Com	101.1 ± 1.23b	90.9–113.1	113.2 ± 2.43a	90.6–131.9	104.8 ± 1.40b	90.4–117.0	104.8 ± 1.01b	95.2–114.8	99.8 ± 1.13b	90.7–110.9	–
EVA-Ant	76.2 ± 0.86b	70.2–83.3	77.3 ± 0.77ab	69.9–82.0	78.3 ± 1.16ab	66.6–85.6	80.0 ± 0.54a	76.7–86.0	76.3 ± 0.86b	69.8–84.4	–
EVA-Post	71.4 ± 0.85b	64.0–79.2	72.3 ± 0.68ab	66.8–76.7	71.8 ± 0.90ab	64.2–77.8	74.4 ± 0.67a	69.6–80.7	70.9 ± 0.81b	65.4–78.6	–
G-G	54.3 ± 0.61b	49.6–60.5	55.3 ± 0.56ab	50.5–60.7	55.9 ± 0.56ab	50.0–59.5	56.9 ± 0.43a	52.9–60.0	55.1 ± 0.54ab	49.0–59.1	–
DM	23.1 ± 0.16bc	21.9–24.4	25.6 ± 0.42a	22.1–29.1	23.8 ± 0.26b	22.5–27.7	23.9 ± 0.18b	22.8–25.7	22.6 ± 0.21c	21.2–25.0	–
JV5	19.7 ± 0.33d	16.7–22.1	23.9 ± 0.50a	20.0–27.9	20.5 ± 0.29 cd	18.2–23.2	21.8 ± 0.31b	18.1–23.8	20.9 ± 0.28bc	19.1–24.5	–

* Means within a row followed by the same letter do not differ significantly (SNK test. $P > 0.05$)

^a Means given in micrometres

period, number of eggs per female, total egg viability and sex ratio were evaluated in the homogamic and heterogamic crosses. To evaluate the sex ratio, the pairs were transferred every 3 days to new experimental units, and the eggs were kept in the same units to obtain adults, which were then sexed. A fragment (approximately 1 cm³) of epidermis of the perianth of field collected coconut fruit infested with *A. guerreronis* (approximately 80–100 specimens of different developmental stages) was supplied daily as food. The experimental units were kept in climatic chambers at 25 ± 1 °C, 75 ± 10 % relative humidity and a daily 12-h photophase. The differences between the treatments (crosses and backcrosses) for pre-oviposition period, number of eggs per female, viability and sex ratio were evaluated by analysis of variance (PROC ANOVA) followed by the SNK test ($\alpha = 0.05$) using SAS software (SAS Institute 2002).

Molecular characterisation

Two fragments of the genome of five *N. paspalivorus* populations (CE, PB, PI, RN and SE) were sequenced: the intergenic region internal transcribed spacer (ITS), covering the ITS1 and ITS2 regions and the 5.8S gene (ITS1-5.8S-ITS2) of the nuclear ribosomal genome (rDNA), and a fragment of the cytochrome c oxidase subunit I (COI) of the mitochondrial genome (mtDNA). These fragments were chosen for the present study because these markers are independent and complementary, with a distinct evolutionary rate. The ITS region has been used in studies of the Phytoseiidae family to elucidate important aspects related to phylogeny (Kanouh et al. 2010a; Tsolakis et al. 2012) and confirm taxonomic status (e.g., Gotoh et al. 1998; Hillis and Dixon 1991; Navajas and Fenton 2000; Navajas et al. 1999) as well as to clarify synonyms and verify the occurrence of cryptic species (Tixier et al. 2006a, 2011, 2012; Navia et al. 2014). The COI region has been used in the confirmation of phytoseiid species (Tixier et al. 2008a; Okassa et al. 2009); the clarification of taxonomic problems, such as synonyms (Tixier et al. 2006a, b, 2011); and the detection of cryptic species of the genus *Neoseiulus* (i.e. Famah Sourassou et al. 2012).

DNA extraction

Fifteen *N. paspalivorus* females of each population were isolated for genomic DNA extraction, which was performed separately for each specimen using a “DNeasy Blood and Tissue Kit” (Qiagen®, USA). The extraction followed the protocol for animal cell culture, which was adapted for the extraction of total DNA from small arthropods, as described by Mendonça et al. (2011), with the adjustments proposed by Kanouh et al. (2010b). The specimens used for the DNA extractions, whenever possible, had their exoskeleton recovered from the membrane of the extraction column and mounted on microscope slides in Hoyer’s medium (Tixier et al. 2010). These slides were deposited as voucher specimens in the mite collection of Embrapa Genetic Resources and Biotechnology, Brasília, Distrito Federal, Brazil.

DNA amplification

The primers used for PCR amplification of the ITS region were 5'-AGAGGAAG TAAAAGTCGTAACAAG-3' (Navajas et al. 1999) and 5'-ATATGCTTAAATT CAGCGGG-3' (Navajas et al. 1998). The COI fragment was amplified with primers 5'-TGATTTTTTGGTCACCCAGAAG and 5'-TACAGCTCCTATAGATAAAAC (Navajas et al. 1996).

The PCR reaction used for the ITS amplification was adapted from the protocol proposed by Navia et al. (2014). The reaction had a final volume of 25 μL , containing 2.5 μL of 10X buffer (Qiagen[®]), 0.125 $\text{un}/\mu\text{L}$ (5 U/mL) Taq DNA polymerase (Qiagen[®]), 0.5 μL of dNTP (10 mM), 0.175 μL of each primer (10 μM), 1 μL of MgCl_2 (25 mM), 18.525 μL of MiliQ water and 2 μL of DNA. The thermocycler (Eppendorf Mastercycler pro S) was set with the following program: denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 15 s (denaturation), 51 °C for 45 s (annealing) and 72 °C for 1 min (extension), ending with one cycle of 72 °C for 7 min, dropping to 10 °C until the samples were removed.

The COI region was amplified following the protocol proposed by Famah Sourassou et al. (2012). The final reaction volume was 25 μL , containing 2.5 μL of 10X buffer (Qiagen[®]), 0.5 $\text{un}/\mu\text{L}$ (5 U/mL) Taq DNA polymerase (Qiagen[®]), 2 μL of dNTP (10 mM), 0.5 μL of each primer (10 μM), 1 μL of MgCl_2 (25 mM), 0.4 μL of BSA, 15.6 μL of DEPC water and 2 μL of DNA. The thermocycler (Eppendorf Mastercycler pro S) was set with the following program: denaturation at 95 °C for 2 min, 30 cycles of 92 °C for 2 min (denaturation), 45 °C for 1 min (annealing) and 72 °C for 90 s (extension), ending with a final cycle of 72 °C for 5 min, dropping to 10 °C until the samples were removed.

The amplified PCR products were subjected to electrophoresis in 1 % agarose gel stained with GelRed[®] (Biotium) and visualised in a transilluminator (BioRad) coupled to a computer with Image Lab 3.0 software.

Four to six specimens from each population were sequenced for both target fragments. The COI and ITS sequences were sequenced from the same mite individuals. Not every DNA template was sequenced for COI fragment because of problems in obtaining PCR amplification, probably resulting from the degraded state of some specimens. Therefore, the COI data set was reduced. The PCR product was sent for direct sequencing on an ABI PRISM 3730xl platform (Applied Biosystems, Lille, France).

Sequence analysis

The ITS (rDNA) and COI (mtDNA) sequences were aligned according to the multiple alignment method CLUSTALW (Thompson et al. 1994) using BioEdit software. No manual adjustments were necessary to align the sequences. The distribution and frequency of the variant sequences—the haplotypes (COI) and genotypes (ITS)—for the *N. paspalivorus* specimens were inferred for the two fragments under study and estimated in DnaSP version 5.10.1 software (Librado and Rozas 2009).

Results

Morphometric characterisation

Significant differences were observed among the five *N. paspalivorus* populations for most of the characters measured ($P < 0.05$) (Table 1), except the width of the dorsal shield (WDS), the length of the J2 setae and the distance between sternal setae St1–St1. An overlap of the variation ranges was observed for most of the characters studied, with the exception of the distance between sternal setae (St3–St3) of the population collected in RN and the other populations studied.

Table 2 Identification based on discriminant analysis of 33 morphological characters in females from five geographic populations of *Neoseiulus paspalivorus*

State	% Well-classified ^a	CE	PB	PI	RN	SE
CE	50	10	2	6	0	2
PB	65	3	13	1	0	3
PI	45	8	0	9	0	3
RN	100	0	0	0	20	0
SE	75	2	1	2	0	15
Total	67	23	16	18	20	23

^a Percentage of specimens in the population of origin that were well rated

In the discriminant analysis, the females of the RN population were well defined, showing 100 % identity with their population of origin. For the other populations, the identity percentage ranged from 45 to 75 %, with the proportionately largest errors in identification observed in the populations from CE and PI (Table 2).

The first two canonical variables (CV1 and CV2) together explained 96.4 % of the total variance (CV1, 90.5 %; CV2, 5.9 %) (Fig. 2). All of the specimens of the RN population were plotted along the positive section of the CV1 axis, whereas the remaining populations were plotted along the negative section of the same axis. Therefore, there was no overlap between the population from RN and the other studied populations, indicating the complete morphometric separation of the RN population (Fig. 2). CV2 segregated the PB population, which was plotted along the positive section of the CV2 axis, from the other studied populations, which were predominantly plotted along the negative section of the CV2 axis. Only slight overlap was observed between the population from PB and that from CE. There was considerable overlap between the populations from CE, PI and SE, indicating morphometric similarity among them (Fig. 2).

The analysis of the CV1 and CV2 weights (Table 3) indicated that the length of the dorsal setae and the distance between the sternal shield setae are the main characteristics that differentiate the *N. paspalivorus* populations. The *N. paspalivorus* population from RN differed from the other populations by presenting a greater distance between sternal setae St2–St2 and St2–St3, a greater width of the posterior part of the ventrianal shield (post-VAS) and the shortest distance between sternal setae St3–St3. The *N. paspalivorus* population from PB differed from the other populations along CV2 because of the greater length of its dorsal setae J5, r3, S4, Z4, s5 and j5 and StIV macrosetae.

Crosses

The virgin females laid no eggs during the observation period. In the homogamic and heterogamic crosses and backcrosses between the populations from PB and RN, all the females laid eggs without malformation. There was no statistically significant difference among the homogamic and heterogamic crosses and the backcrosses for the following evaluated parameters: pre-oviposition period ($F_{7,71} = 2.25$; $P = 0.05$), number of eggs per female ($F_{7,71} = 1.82$; $P = 0.09$), total viability ($F_{7,71} = 1.22$; $P = 0.30$) and sex ratio ($F_{7,71} = 1.07$; $P = 0.39$) (Table 4).

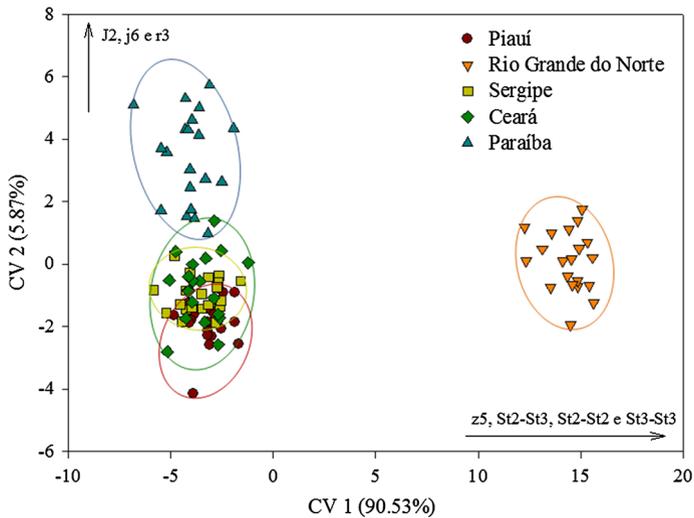


Fig. 2 Analysis of canonical variables for 33 morphological characters of females from five geographic populations of *Neoseiulus paspalivorus*, with the circles formed based on the projection of the specimens of each population for canonical variable (CV) 1 and 2. The variation explained by the two canonical variables is shown in brackets

Table 3 Analysis of canonical variables for females of five *Neoseiulus paspalivorus* populations from northeastern Brazil showing the first ten morphometric characters (eigenvectors) with higher loadings (eigenvalues) for two canonical variables (CV1 and CV2) in decreasing order of absolute values

Morphometric characters	CV1 (loadings)	Morphometric characters	CV2 (loadings)
z5	-0.5707	J2	-0.7396
St2-St3	0.5052	J5	-0.4089
St3-St3	-0.4955	j6	0.4076
St2-St2	0.4909	r3	0.4019
j3	0.2641	StIV	0.3783
R1	0.2628	S4	-0.3348
j1	-0.2534	EVA-Post	-0.2921
J5	0.2469	z4	0.2681
j6	0.2277	s4	-0.2571
EVA-Post	0.2196	j5	0.2546

Molecular characterisation

Twenty-nine sequences were obtained within the ITS region for the *N. paspalivorus* specimens from the five states in the Brazilian Northeast (CE, PB, PI, RN and SE). The length of the fragments obtained ranged from 613 to 627 bp. All of the unpublished sequences were deposited in GenBank (accession number KP250646-KP250651 for CE, KP250629-KP250634 for PB, KP250635-KP250640 for PI, KP250641-KP250645 for RN and KP250623-KP250628 for SE).

Table 4 Outcome of crosses (mean \pm SE) involving different geographic populations identified as *Neoseiulus paspaliivorus*

Cross	n	Pre-oviposition period	% ovipositing females	No. eggs/female	% deformed eggs/female	Egg hatchability (%)	Sex ratio
<i>Homogamites</i>							
PB \times PB	10	0.6 \pm 0.22a*	100	17.9 \pm 1.70a	0	75.1 \pm 5.68a	82.3 \pm 2.67a
RN \times RN	10	0.9 \pm 0.31a	100	18.5 \pm 1.16a	0	80.6 \pm 4.55a	79.8 \pm 2.60a
<i>Heterogamites</i>							
PB \times RN	10	1.0 \pm 0.15a	100	18.7 \pm 1.45a	0	76.2 \pm 4.34a	82.7 \pm 3.33a
RN \times PB	10	1.4 \pm 0.31a	100	18.9 \pm 1.47a	0	82.8 \pm 3.29a	76.5 \pm 3.46a
<i>Backcross</i>							
F1 (PB \times RN) \times PB	10	1.0 \pm 0.26a	100	18.7 \pm 1.48a	0	83.5 \pm 4.89a	82.3 \pm 3.52a
F1 (PB \times RN) \times RN	10	1.1 \pm 0.23a	100	23.5 \pm 1.18a	0	89.4 \pm 2.92a	82.3 \pm 1.84a
F1 (RN \times PB) \times PB	10	0.6 \pm 0.16a	100	21.2 \pm 1.41a	0	82.0 \pm 5.44a	87.0 \pm 2.29a
F1 (RN \times PB) \times RN	10	0.4 \pm 0.16a	100	20.5 \pm 0.76a	0	85.0 \pm 4.58a	81.2 \pm 2.34a

n number of pair crosses

* Means followed by the same letter in a column do not differ significantly (SNK test; $P > 0.05$)

Table 5 Accession numbers and attributed haplotype codes of COI sequences of the predatory mite, *Neoseiulus paspalivorus*, obtained in this study

Haplotype number	GenBank accession number	States
H1	KR492517	SE
	KR492518	SE
	KR492519	PB
	KR492520	RN
	KR492521	RN
	KR492522	RN
	KR492523	RN
	KR492524	CE
H2	KR492525	CE
H3	KR492526	CE

No genetic variability was observed among *N. paspalivorus* populations. The average mean divergence within *N. paspalivorus* studied populations was 0.0 % with a single genotype identified.

For the COI mitochondrial gene fragment, ten sequences representing the populations collected in CE, PB, RN and SE were obtained and deposited in GenBank (Table 5). The population from PI was not characterized for the ITS, since it was not possible to obtain good quality sequences of this region for their specimens. The COI fragment ranged from 390 to 450 bp and corresponded to the 712 to 1102 bp and the 712 to 1162 bp positions, respectively, of the complete mitochondrial genome of the phytoseiid mite *Phytoseiulus persimilis* Athias-Henriot, available in GenBank (accession number NC014049). As observed for *N. baraki* (Famah Sourassou et al. 2012), the COI aligned sequences of *N. paspalivorus* were similar in nucleotide composition and A–T rich (27.03 % Adenine; 45.71 % Thymine). The translation of the nucleotide sequence resulted in a sequence with 146 amino acids.

Little polymorphism was observed among the ten COI sequences, and three haplotypes were identified (H = 3). The genetic distance ranged from 0 to 0.2 %. H1 (n = 8) was present in the populations from CE, PB, RN and SE. H2 and H3 were identified only in the population collected in CE (Table 5). H2 and H3 each showed a single nucleotide change, the replacement of G by A at position 305 of H2 (n = 1) and the replacement of T by G at position 180 of H3 (n = 1).

Discussion

Morphometric variability was observed among the *N. paspalivorus* populations under study, with a clear distinction of the population from RN. Intraspecific variability has been frequently reported in phytoseiid mites (e.g., Tixier et al. 2006a, b, 2008b). Misinterpretations of intraspecific variation can lead to misidentification and likely erroneous descriptions of new species (Tixier et al. 2008b). To minimise possible identification errors, Tixier (2012) proposed a statistical approach that establishes limits to distinguish between intra- and interspecific variability based on the length of phytoseiid setae. The Tixier (2012) study used 14 species of five genera (*Euseius* Wainstein, *Neoseiulus* Hughes, *Phytoseiulus* Evans, *Kampimodromus* Nesbitt and *Typhlodromus* (*Typhlodromus*) Scheuten). The minimum difference between the average values of samples belonging to two

different species must be 10.58 μm (for setae $<65 \mu\text{m}$) and 33.99 μm (for setae $>65 \mu\text{m}$) (Tixier 2012). According to this approach, the variability found between the *N. paspalivorus* populations of the present study reflects intraspecific variability. However, in the present study, the seta length was not the characteristic that most influenced the morphometric distance of the populations.

The overlap of the character variation intervals indicates morphological similarity among the studied populations. The only morphometric character with no overlap of the variation ranges between the RN population and the other studied populations was the distance between the sternal setae St3–St3. Yet, this character has never been considered for distinguishing phytoseiid species (Chant and McMurtry 2003; de Moraes et al. 2004). The measurements of the *N. paspalivorus* populations under study were similar to the holotype (De Leon 1957) and other populations from Sri Lanka (de Moraes et al. 2004), Benin, Ghana and Brazil (Itamaracá, Pernambuco) (Famah Sourassou et al. 2011).

Several authors have reported morphometric differences between populations of phytoseiid species (e.g., Chant 1955; Ragusa and Tsolakis 1994; Tixier et al. 2006a, b, 2008a, b); however, little is known regarding the factors that can cause morphological variability. Studies have shown that arthropod populations may experience morphological variations along their geographical distribution (Saunders 1982). In eriophyid mites, cases of morphological variation have been associated with host specificity, and certain studies have shown that the involved characters vary according to the structure of the host plant (Skoracka et al. 2002). Studies with *Euseius mesembrinus* (Dean), a mite of the Phytoseiidae family, attributed the morphological variations observed in populations from Florida and Texas (USA) to geographical or nutritional factors because the morphological variations were verified only in field specimens, whereas in the laboratory under conditions in which food resources and other ecological factors were controlled, the variations did not occur (Abou-Setta et al. 1991). Furtado (1997) hypothesised that food may affect the size of characters in *Euseius citrifolius* Denmark and Muma reared under laboratory conditions. However, there was no significant difference between females reared with different food supplies. In the present study, the population from RN was the most morphometrically distant of other five populations. This Brazilian state occupies an geographic intermediate position among the sampling locations; thus, it is likely that the variability found in the population from RN is unrelated to the geographical separation of the sampling sites. Regarding nutritional factors, reports of *N. paspalivorus* in Brazil are limited to its association with *A. guerreronis* on coconut palm, with little information concerning its ecology. However, it is most likely that *N. paspalivorus* has other still unknown prey and hosts whose occurrence along the northeast coast may not be continuous and uniform because the weather along the coast, especially the precipitation, is variable. Therefore, more research on *N. paspalivorus* is needed to elucidate its ecology and the factors associated with its morphometric variability.

The lack of oviposition in unmated females of *N. paspalivorus* confirms the need for sexual reproduction in this species (Famah Sourassou et al. 2011), as in most phytoseiids (Croft 1970; de Moraes and McMurtry 1981; Noronha and de Moraes 2002, 2004). The lack of reproductive isolation between the most morphometrically distant *N. paspalivorus* populations (RN and PB) supports the hypothesis that the studied populations belong to a single species, according to the biological concept of species (Mayr 1977). Previous studies have reported reproductive incompatibility among three geographically distant, *N. paspalivorus* populations (Benin, Ghana and Brazil) because of their association with different endosymbionts. The populations from Benin and Brazil carried different strains of *Wolbachia*, whereas the population from Ghana carried *Cardinium* (Famah Sourassou et al.

2011, 2014). The absence of reproductive isolation observed in the present study, besides confirming the co-specificity of the populations, may be related to the absence of endosymbionts or the presence of the same endosymbiont species in all the populations. Therefore, as a future perspective, *N. paspalivorus* populations from the Brazilian Northeast populations should be screened for endosymbionts. This endosymbiont characterization may be useful concerning the coconut mite biological control since the introduction of populations with different strains of endosymbionts could cause reproductive incompatibility among Brazilian populations.

In addition to the reproductive compatibility observed in the present study, the molecular characterisation based on the ITS marker showed no genetic variability. For the COI marker, the genetic distance ranged from 0 to 0.2 %, which corresponds to intraspecific distances previously reported for populations of *N. paspalivorus* from Benin, Ghana and Brazil (Famah Sourassou et al. 2014). Despite the presence of two haplotypes in the population from CE, suggesting greater genetic variability among the specimens of this population, these two polymorphic sites may represent a single, isolated event. The low genetic variability observed among the studied populations collected from geographically distant areas along the Brazilian coast suggests that *N. paspalivorus* is not a native species of Brazil location, since that the center of origin of a specie is defined as the geographic area wherein the specie exhibits the highest degree of variation (Gasparich et al. 1997). Phylogenetic and phylogeographic studies including populations from different localities/continents and hosts should be conducted to determine the centre of origin of *N. paspalivorus*.

Separately morphological analysis could leave doubts on the taxonomic unit of studied populations, taking into account the complete or almost complete morphometric discontinuity among populations. However, the molecular and biological (reproductive compatibility) analyses revealed no significant differences among the studied populations, corroborating to the prior identification of *N. paspalivorus* for all of the populations studied (CE, PB, PI, RN and SE). The present study highlights the importance of the precise identification of predatory mites through an integrative approach. Molecular characterization allowed obtaining the first DNA sequences of *N. paspalivorus* and its deposit in public databases making this information available for future studies on the molecular systematics of phytoseiid mites and also for predatory mites molecular diagnosis. Obtained results also contributed to the knowledge on Phytoseiidae systematics by providing information on the morphometric and genetic variability of *N. paspalivorus* populations from a continuous and extensive geographic area in Brazil.

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