




Molecular and morphological characterization of the predatory mite *Amblyseius largoensis* (Acari: Phytoseiidae): surprising similarity between an Asian and American populations

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Abstract

The accurate characterization of biological control agents is a key step in control programs. Recently, *Amblyseius largoensis* from Thailand were introduced in Brazil to evaluate their efficiency for the control of the red palm mite, *Raoiella indica*. The aim of this study was to confirm their identification and to characterize the population from Thailand, comparing it to populations of the Americas and Indian Ocean islands. In addition, a population of *A. largoensis* from New Caledonia, Oceania, of which DNA sequences were available, was included in phylogenetic analyses. Morphometric data obtained for the population of *A. largoensis* from Thailand were compared to those of populations from Reunion Island and the Americas through univariate and multivariate analyses. Two DNA fragments were amplified and sequenced: the nuclear ribosomal region ITSS and the mitochondrial 12S rRNA. Haplotypes (12S rRNA) and genotypes (ITSS) were identified and phylogenetic analyses using both fragments were conducted separately and combined using maximum likelihood and the Bayesian information criterion. The integrative approach reveals morphometric and molecular variabilities among populations of *A. largoensis* and shows that the population identified as *A. largoensis* collected in Thailand, as well as that from New Caledonia, are conspecific to the populations of the Americas and Indian Ocean islands. Populations from the Americas and Asia are more related to each other than with that from the Indian Ocean islands. Hypotheses to explain this clustering are proposed. Data on the molecular intraspecific variability of this predatory mite from remote areas will be helpful for the development of molecular diagnosis.

Keywords Integrative taxonomy · Thailand · Classical biological control · *Raoiella indica* · Molecular systematics

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Introduction

One of the main causes of failures in biological control programs derives from errors or inaccuracy in the identification of the organisms involved (pests and/or natural enemies) (de Moraes 1987; Zucchi 1990, 2002; Stiling 1993; Gordh and Beardsley 1999). Traditionally, taxonomy has relied on morphological data to separate species. However, morphological parameters alone may not be sufficient for correct species identification or may be difficult to visualize, especially for minute organisms as, for instance, the predatory mites of the family Phytoseiidae (Tixier et al. 2006a, b, 2012a, 2013). As a consequence, cryptic species have been reported in this family, difficult to distinguish morphologically but exhibiting molecular, biological and/or behavior differences (Mahr and McMurtry 1979; McMurtry et al. 1976; McMurtry and Badii 1989; Beard 1999; Tixier et al. 2003, 2004, 2006b, 2008, 2010; Sourassou et al. 2012; Bowman and Hoy 2012). An example is the identification of the species *Typhlodromus (Typhlodromus) phialatus* Athias-Henriot and *Typhlodromus (Typhlodromus) exhilaratus* Ragusa, morphologically very similar and only distinguishable by the shape of the spermatheca (insemination apparatus) (Tixier et al. 2006a). To determine whether this character was diagnostic and whether these species were synonyms or not, molecular analyses were carried out. Thus, taxonomy should rely on integrative approaches, combining several characters (information) to conclude on the species status (e.g. Grismer et al. 2013; Miller et al. 2013; Zhang et al. 2014; Oca et al. 2016; Petrova et al. 2016).

In addition to cryptic species associated to different biological and behavioral features, biological differences among populations of a single species may be significant in the selection of biological control agents. Cavalcante et al. (2015) demonstrated that *Amblyseius swirskii* Athias-Henriot populations from geographically isolated areas differed regarding their ability to control *Bemisia tabaci* (Gennadius) biotype B. Tixier et al. (2010) also showed genetic divergence among four populations of *Phytoseilus longipes* Evans, associated to different feeding behaviors—some fed and developed on *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae), whereas others did not.

The present study focuses on the identification of predatory mite candidates for controlling the red palm mite (RPM), *Raoiella indica* Hirst (Acari: Tenuipalpidae) through classical biological control. Red Palm Mite is an invasive pest, originated from the Western hemisphere (Dowling et al. 2012), recently reported in the Americas (Flechtmann and Etienne 2004) that causes serious problems for coconut production and that is also reported on other plants of the families Arecaceae, Musaceae, Heliconiaceae, Strelitziaceae, Zingiberaceae, Pandanaceae and Cannaceae (Kane et al. 2005; Etienne and Flechtmann 2006; Kane and Ochoa 2006; Marsaro et al. 2010; Gondim Jr. et al. 2012). In the Americas, the coconut palm is commonly grown mainly by small producers; pesticides are not easy to use, in addition to being environmentally unfriendly. Biological control emerges thus as an alternative, and the classical biological control strategy is prioritized.

The Phytoseiidae fauna has been investigated, seeking potential candidates for use as natural RPM enemies. *Amblyseius largoensis* (Muma) is the predator species most often associated with *R. indica* in the Americas, Indian Ocean islands and Asia (Gallego et al. 2003; Etienne and Flechtmann 2006; Peña et al. 2009; Taylor et al. 2012; Carrillo et al. 2012; de Moraes et al. 2012; Gondim Jr. et al. 2012; Silva et al. 2014). *Amblyseius cinctus* Corpuz-Raros & Rimando was also reported on coconut plants associated with *R. indica* in Thailand (Oliveira 2015). In Trinidad and Tobago, densities of *A. largoensis* increased after the arrival of *R. indica* (Peña et al. 2009). This predator is reported in all continents

(Çobanoğlu 1989; McMurtry et al. 1971; Gallego et al. 2003; Roda et al. 2008; Zannou et al. 2010; Carrillo et al. 2011; Bowman and Hoy 2012; Taylor et al. 2012; Gondim Jr. et al. 2012) after being first described from *Citrus aurantifolia* (Christm.) Swingle in Florida, USA (Muma 1955). A population of *A. largoensis* from Reunion Island was introduced in Brazil in 2012 (de Moraes et al. 2012). Laboratory studies indicated that this population would be more promising for RPM control than a population collected in coconut plantations in the State of Roraima, Brazil, as they displayed higher prey (RPM) consumption and a higher net reproduction rate (Domingos et al. 2013). Morphometric, molecular and cross-breeding analyses that included populations of *A. largoensis* from Indian Ocean islands (Reunion and Mauritius) and the Americas (Brazil, USA, Trinidad and Tobago) indicated that populations belong to the same species but constitute different genetic strains (Navia et al. 2014).

A population of *A. largoensis* from Thailand collected on coconut palms was officially introduced in Brazil in 2013 to control *R. indica* (Oliveira 2015). Its predation potential and biological parameters in the presence of *R. indica* are under evaluation. As differences in the performance of populations of the same phytoseiid species toward a particular prey species may be associated with molecular traits, it seems necessary to accurately identify this *A. largoensis* population.

The objective of the present work is therefore the accurate identification and/or characterization of the *A. largoensis* Asian population (Thailand) through morphological and molecular studies, compared to the previously studied populations in the Americas (Brazil, USA, Trinidad and Tobago) and Indian Ocean islands (Reunion and Mauritius). In addition sequences of *A. largoensis* from New Caledonia, Oceania, available in GenBank were included in the analysis in order to discuss its phylogenetic position. Finally, the analysis of molecular intraspecific variability of this predatory mite between populations from remote geographical areas on different continents based on nuclear and mitochondrial markers allowed to better assess intraspecific variability for a more secure molecular diagnosis.

Materials and methods

Morphometric characterization

The morphometric characterization was performed for a population of *A. largoensis* collected at Kamphaeng Saen (14°00'22"N, 99°59'78"E, 6 m altitude), Thailand. Twenty adult females were mounted on slides in Hoyer's medium and 36 characters (Table 1) were measured, currently used for the identification of phytoseiid mites (e.g., Chant and McMurtry 1994, 2005, 2007). The terminologies for chaetotaxy were those proposed by Lindquist and Evans (1965) as adapted by Rowell et al. (1978) for dorsal idiosomal setae of Phytoseiidae and by Chant and Yoshida-Shaul (1991) for ventral idiosomal setae. Observations were made through a Nikon Eclipse 80i phase contrast microscope (Nikon, Tokyo, Japan) at a magnification of 400×. The characters considered are continuous variables: length of setae on the dorsal shield, of the spermatheca and of the three macrosetae on leg IV (GeIV, StIV, StiIV), length and width of the dorsal and of the ventrianal shield, and distances between the sternal setae (*St1–St1*, *St2–St2*, *St3–St3*, *St4–St4*, *St5–St5*, *St1–St3*, *St2–St3*). All measurements are presented in micrometers. The specimens measured for morphometric analyses were deposited as voucher specimens in the mite collection at Embrapa Genetic Resources and Biotechnology, Brasília, Distrito Federal, Brazil.

Table 1 Mean (\pm SE), minimum and maximum observed values (μm) of 36 morphometric characters measured from adult females of five populations identified as *Amblyseius largoensis*

Morphological characters	Thailand			La Reunion Island ^d			Pernambuco, Brazil ^a			Roraima, Brazil ^a			Trinidad and Tobago ^a		
	Mean \pm SE	Min–Max	Mean \pm SE	Min–Max	Mean \pm SE	Min–Max	Mean \pm SE	Min–Max	Mean \pm SE	Min–Max	Mean \pm SE	Min–Max	Mean \pm SE	Min–Max	
Length of dorsal shield	364.1 \pm 2.65 b	340.0–395.0	371.1 \pm 3.50 b	337.5–397.5	362.0 \pm 3.61 b	320.0–377.5	368.8 \pm 3.60 b	312.5–387.5	383.0 \pm 3.73 a	340.0–407.5					
Width of dorsal shield	224.6 \pm 2.79 b	192.5–245.0	222.5 \pm 2.70 b	200.0–240.0	225.6 \pm 2.08 b	212.5–242.5	229.25 \pm 1.72 b	212.5–242.5	236.3 \pm 0.94 a	225.0–242.5					
Distance <i>S1</i> – <i>S1</i>	63.2 \pm 0.43 c	60.0–67.5	71.1 \pm 0.53 a	67.5–75.0	67.4 \pm 0.53 b	62.5–70.0	68.5 \pm 0.52 b	65.0–72.5	68.3 \pm 0.63 b	62.5–72.5					
Distance <i>S2</i> – <i>S2</i>	66.8 \pm 0.50 d	62.5–71.2	72.8 \pm 0.54 a	70.0–77.5	68.8 \pm 0.43 c	67.5–72.5	70.6 \pm 0.44 b	67.5–72.5	71.7 \pm 0.57 ab	67.5–75.0					
Distance <i>S3</i> – <i>S3</i>	72.0 \pm 0.67 c	65.0–76.2	76.5 \pm 0.70 a	72.5–85.0	72.4 \pm 0.50 c	70.0–75.0	74.4 \pm 0.75 b	67.5–80.0	76.7 \pm 0.51 a	72.5–80.0					
Distance <i>S1</i> – <i>S3</i>	62.9 \pm 0.50 b	58.7–67.5	67.4 \pm 0.42 a	62.5–70.0	67.6 \pm 0.38 a	65.0–70.0	67.1 \pm 0.49 a	62.5–70.0	68.0 \pm 0.53 a	65.0–72.5					
Distance <i>S2</i> – <i>S3</i>	29.8 \pm 0.35 c	27.5–35.0	30.9 \pm 0.38 bc	30.0–35.0	33.5 \pm 0.42 a	30.0–37.5	32.1 \pm 0.33 b	30.0–35.0	32.1 \pm 0.44 b	30.0–35.0					
Distance <i>S4</i> – <i>S4</i>	79.4 \pm 0.95 c	71.2 \pm 87.5	81.9 \pm 0.96 ab	75.0–87.5	76.2 \pm 0.56 c	70.0–80.0	81.1 \pm 1.41 ab	72.5–92.5	83.6 \pm 0.84 a	80.0–90.0					
Distance <i>S5</i> – <i>S5</i>	68.0 \pm 1.02 c	57.5–78.5	75.1 \pm 0.59 a	70.0–80.0	70.6 \pm 0.57 b	67.5–75.0	72.3 \pm 0.68 b	67.5–77.5	70.5 \pm 0.70 b	65.0–75.0					
Length of ventrian shield	110.4 \pm 1.02 c	102.5–122.5	118.5 \pm 1.70 a	105.0–130.0	116.4 \pm 1.26 ab	107.5–125.0	113.0 \pm 1.48 bc	100.0–122.5	120.7 \pm 1.50 a	112.5–135.0					
Width of ventrian shield (ZV2)	46.7 \pm 0.68 b	42.5–52.5	47.5 \pm 1.01 ab	40.0–55.0	50.1 \pm 0.70 a	45.0–55.0	49.3 \pm 0.81 ab	42.5–57.5	47.5 \pm 0.81 ab	40.0–52.5					
Width of ventrian shield (anus)	68.7 \pm 0.71 c	62.5–77.5	72.9 \pm 1.27 ab	62.5–82.5	70.6 \pm 0.68 bc	65.0–75.0	75.5 \pm 0.53 a	72.5–80.0	74.1 \pm 0.80 a	70.0–80.0					
Setae length <i>j</i> /	35.5 \pm 0.36 c	32.5–37.5	38.9 \pm 0.64 a	35.0–45.0	36.4 \pm 0.61 bc	32.5–40.0	34.6 \pm 0.58 c	30.0–40.0	37.6 \pm 0.30 ab	35.0–40.0					

Table 1 (continued)

Morphological characters	Thailand		La Reunion Island ^d		Pernambuco, Brazil ^a		Roraima, Brazil ^b		Trinidad and Tobago ^a	
	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max
Setae length <i>j</i> ₃	51.5 ± 0.73 b	47.5–60.0	53.9 ± 0.46 a	50.0–57.5	53.4 ± 0.52 a	50.0–57.5	53.8 ± 0.38 a	50.0–57.5	54.7 ± 0.57 a	50.0–60.0
Setae length <i>j</i> ₄	6.4 ± 0.21 b	5.0–7.5	6.1 ± 0.29 b	5.0–7.5	8.1 ± 0.25 a	7.5–10.0	8.0 ± 0.23 a	7.5–10.0	6.6 ± 0.28 b	5.0–7.5
Setae length <i>j</i> ₅	4.5 ± 0.17 b	3.0–5.0	5.3 ± 0.13 ab	5.0–7.5	5.7 ± 0.26 a	5.0–7.5	5.90 ± 0.27 a	5.0–7.5	5.1 ± 0.13 ab	5.0–7.5
Setae length <i>j</i> ₆	7.6 ± 0.11 b	6.2–8.7	7.0 ± 0.23 b	5.0–7.5	9.1 ± 0.38 a	7.5–12.5	7.6 ± 0.13 b	7.5–10.0	7.8 ± 0.30 b	5.0–10.0
Setae length <i>J</i> ₂	9.5 ± 0.25 c	7.5–12.5	9.3 ± 0.26 c	7.5–10.0	11.8 ± 0.32 a	7.5–12.5	9.5 ± 0.40 c	7.5–12.5	10.7 ± 0.30 b	10.0–12.5
Setae length <i>J</i> ₅	9.2 ± 0.22 b	7.5–11.25	9.0 ± 0.33 b	7.5–12.5	11.0 ± 0.28 a	10.0–12.5	9.1 ± 0.27 b	7.5–10.0	10.5 ± 0.24 a	10.0–12.5
Setae length <i>z</i> ₂	10.8 ± 0.27 b	8.0–12.5	9.9 ± 0.29 c	7.5–12.5	12.0 ± 0.23 a	10.0–12.5	10.6 ± 0.25 b	10.0–12.5	10.9 ± 0.28 b	10.0–12.5
Setae length <i>z</i> ₄	9.3 ± 0.20 b	7.5–10.5	8.6 ± 0.29 b	7.5–10.0	10.8 ± 0.27 a	10.0–12.5	9.0 ± 0.28 b	7.5–10.0	9.3 ± 0.30 b	7.5–10.0
Setae length <i>z</i> ₅	6.5 ± 0.18 b	5.5–7.5	5.4 ± 0.20 c	5.0–7.5	8.5 ± 0.28 a	7.5–10.0	7.1 ± 0.20 b	5.0–7.5	6.6 ± 0.30 b	5.0–7.5
Setae length <i>Z</i> ₁	10.5 ± 0.26 b	9.7–12.5	11.3 ± 0.29 b	10.0–12.5	13.4 ± 0.13 a	10.0–12.5	10.8 ± 0.26 b	10.0–12.5	12.2 ± 0.18 a	10.0–12.5
Setae length <i>Z</i> ₄	94.5 ± 0.82 c	87.5–100.0	106.4 ± 0.7 a	102.5–112.5	97.8 ± 0.72 b	92.5–102.5	94.1 ± 1.25 c	87.5–102.5	100.1 ± 0.82 b	90.0–105.0
Setae length <i>Z</i> ₅	275.9 ± 1.52 d	260.0–287.5	285.5 ± 2.34 bc	270.0–307.5	290.8 ± 2.02 b	277.5–307.5	283.4 ± 2.90 c	262.5–320.0	301.3 ± 1.46 a	292.5–315.0
Setae length <i>s</i> ₄	95.8 ± 0.85 c	90.0–105.0	106.6 ± 1.08 a	100.0–117.5	96.8 ± 0.83 c	90.0–105.0	97.5 ± 0.85 c	90.0–102.5	100.70 ± 1.01 b	92.5–107.5
Setae length <i>S</i> ₂	12.5 ± 0.30 bc	10.0–15.0	12.5 ± 0.18 bc	10.0–15.0	15.0 ± 0.41 a	12.5–17.5	11.90 ± 0.25 c	10.0–12.5	13.4 ± 0.28 b	12.5–15.0
Setae length <i>S</i> ₄	12.1 ± 0.27 b	10.0–15.0	11.5 ± 0.28 bc	10.0–12.5	14.1 ± 0.33 a	12.5–17.5	11.13 ± 0.29 c	10.0–12.5	13.6 ± 0.30 a	12.5–15.0
Setae length <i>S</i> ₅	10.8 ± 0.34 bc	7.5–12.5	10.0 ± 0.31 c	7.5–12.5	13.1 ± 0.36 a	10.0–15.0	11.4 ± 0.29 b	10.0–12.5	11.6 ± 0.30 b	10.0–12.5
Setae length <i>r</i> ₃	11.6 ± 0.26 b	10.0–13.7	12.5 ± 0.18 b	10.0–15.0	14.1 ± 0.33 a	12.5–17.5	12.3 ± 0.40 b	10.0–15.0	13.4 ± 0.28 a	12.5–15.0
Setae length	11.4 ± 0.27 bc	10.0–13.7	11.38 ± 0.29 bc	10.0–12.5	13.1 ± 0.25 a	12.5–15.0	10.6 ± 0.25 c	10.0–12.5	12.1 ± 0.35 b	10.0–15.0
<i>R</i> ₁										
Setae length	61.7 ± 0.76 d	55.0–67.5	78.0 ± 0.88 a	70.0–87.5	69.8 ± 0.91 c	62.5–77.5	63.1 ± 0.80 d	57.5–67.5	72.2 ± 0.90 b	65.0–77.5
<i>J</i> _{V5}										
Calyx of the spermatheca	30.2 ± 0.32 b	27.5–32.5	32.1 ± 0.50 a	27.5–35.0	32.8 ± 0.68 a	30.0–37.5	33.6 ± 0.60 a	30.0–37.5	30.53 ± 0.45 b	27.5–35.0

Table 1 (continued)

Morphological characters	Thailand		La Reunion Island ^a		Pernambuco, Brazil ^a		Roraima, Brazil ^a		Trinidad and Tobago ^a	
	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max	Mean ± SE	Min–Max
Length of the macrosetae genu IV	123.3 ± 1.00 c	112.5–132.5	132.3 ± 0.91 b	125.0–137.5	133.0 ± 1.08 b	125.0–142.5	131.4 ± 1.40 b	125.0–142.5	137.5 ± 0.83 a	132.5–145.0
Length of the macrosetae tibia IV	94.1 ± 1.01 b	85.0–100.0	103.3 ± 1.00 a	97.5–112.5	100.8 ± 1.00 a	95.0–110.0	100.3 ± 0.92 a	90.0–107.5	100.5 ± 0.85 a	95.0–110.0
Length of the macrosetae tarsu IV	63.3 ± 0.76 c	57.5–68.7	72.1 ± 0.84 a	67.5–77.5	68.4 ± 0.60 b	65.0–72.5	63.3 ± 0.63 c	57.5–67.5	67.1 ± 0.64 c	62.5–72.5

Means within a row followed by different letters are significantly different (Student–Newman–Keuls test: $P < 0.05$)

^aMorphometric characters taken from Navia et al. (2014)

The morphometric data obtained for the population of *A. largoensis* from Thailand were compared to measurements of populations from Reunion Island, Brazil (Pernambuco and Roraima) and Trinidad and Tobago, previously obtained in Navia et al. (2014) (Table 1), who also measured 36 morphological trait parameters for 20 females. A univariate analysis of variance (ANOVA PROC) was performed, followed by the Newman–Keuls test ($\alpha=0.05$) for the 36 measured characters. Three multivariate statistical analyses were also carried out: (1) a principal component analysis (PCA) to reveal possible discontinuities in the morphological variation between samples from different geographic areas, (2) a canonical variable analysis (CVA) to determine the morphological variation patterns and identify the morphological features that contributed the most to the differentiation among the populations, and (3) an analysis of the discriminant function to assess whether individuals had been correctly assigned to the original populations. All statistical analyses were performed with SAS software (SAS Institute 2008).

Molecular characterization

DNA sequences of *A. largoensis* specimens from the same population used for morphometric characterization were obtained. Specimens of *A. cinctus* were also sequenced in order to genetically separate them from *A. largoensis*. There are no *A. cinctus* sequences available in the GenBank yet. Both species are found on coconut palm in Thailand. These species collected on coconut leaves infested by *R. indica* (Oliveira 2015) in Thailand were introduced by DC Oliveira (MAPA permit number 208/13) (Table 2). Specimens for molecular characterization were collected from colonies established at the Laboratory of Entomology, Embrapa Roraima.

Two DNA fragments were amplified and sequenced: the nuclear ribosomal region spanning the ITS1, 5.8S and ITS2 region (reported as ITSS) and the 12S rRNA mitochondrial fragment. These regions were sequenced in the study of populations of *A. largoensis* by Navia et al. (2014) and the 12S rRNA region was also sequenced by Bowman and Hoy (2012) for populations of *A. largoensis* collected in Mauritius and Florida, USA. The ITSS region has been used in studies dealing with Phytoseiidae phylogeny (Kanouh et al. 2010a; Tsolakis et al. 2012) and cryptic species (e.g., Gotoh et al. 1998; Hillis and Dixon 1991; Navajas and Fenton 2000; Navajas et al. 1999; Tixier et al. 2006a, 2011, 2012a; Navia et al. 2014). The 12S rRNA region has also been used successfully in clarification of synonymies within the family Phytoseiidae (Jeyaprakash and Hoy 2002; Okassa et al. 2009, 2010, 2011; Kanouh et al. 2010b; Tixier et al. 2010, 2011, 2012a, b). These markers (nuclear and mitochondrial) were chosen because they are independent and complementary, with distinct evolutionary rates, and they were considered adequate markers for diagnostic purposes (Santos and Tixier 2016).

DNA extraction The mite specimens were preserved in 100% ethanol and not crushed as reported by Navia et al. (2014). Total genomic DNA was individually extracted from 15 females per population, using a Qiagen DNeasy tissue kit (Qiagen, Hilden, Germany), according to the DNA extraction protocol ‘Purification of Total DNA from Animal Blood or Cells’ (SpinColumn Protocol). The manufacturer’s instructions were modified for DNA extraction from tiny mites, as described by Kanouh et al. (2010b) and Mendonça et al. (2011).

Table 2 Data on specimens/DNA sequences of *Amblyseius* spp. and *Neoseiulus californicus* on which phylogenetic analyses were performed: locality, host plant, haplotype/genotype and GenBank accession number for ITS and 12S rRNA

Species	Code	Country/state	Host plant Species	Genotype/haplotype		GenBank accessions		
				Family	ITS	12S rRNA	ITS	12S rRNA
<i>A. largoensis</i>	Ala TH	Thailand, Nakhon Pathom	<i>Cocos nucifera</i>	Arecaceae	G1	H6	MG242071–MG242078	MG242089–MG242095
	BR PE	Brazil, Pernambuco	<i>Cocos nucifera</i>	Arecaceae	G1	H1	KF219618	KF234098
	BR RR	Brazil, Roraima	<i>Cocos nucifera</i>	Arecaceae	G1	H2	KF219625	KF234101
	BR AP	Brazil, Amapá	<i>Cocos nucifera</i>	Arecaceae	G1	H1	KF219645	KF234118
	TT	Trinidad and Tobago, Saint George	<i>Cocos nucifera</i>	Arecaceae	G1	H1	KF219631	KF234104
	TT	Trinidad and Tobago, Nariva	<i>Cocos nucifera</i>	Arecaceae	G1	H1	KF219634	KF234107
	TT	Trinidad and Tobago, Nariva	<i>Musa</i> sp.	Musaceae	G1	H1	KF219637	KF234110
	TT	Trinidad and Tobago, Saint David	<i>Cocos nucifera</i>	Arecaceae	G1	H1	KF219639	KF234112
	RE	La Reunion Island	<i>Cocos nucifera</i>	Arecaceae	G2	H4	KF219649	KF234122
	RE	Reunion Island, Saint Joseph	<i>Cocos nucifera</i>	Arecaceae	–	H5	–	KF234129
	US	EUA, Florida (Holly-wood)	–	–	–	H7	–	GU807437
	US	EUA, Florida (Lake Worth)	–	–	–	H7, H8, H9, H10	–	GU807438, GU807440, GU807442, GU807443
	MU	Mauritius Island, Flic En Flac	–	–	–	H4	–	GU807448
	MU	Mauritius Island, North of Port Luis	–	–	–	H4, H11, H12	–	GU807452, GU807453, GU807454
	MU	Mauritius Island, Trou d'Eau Douce	–	–	–	H4, H13, H14	–	GU807460, GU807467, GU807476
	NC	New Caledonia, Nouméa	Unknown	–	G1, G3	H2, H3	KU318306, KU318307	KU318124, KU318125

Table 2 (continued)

Species	Code	Country/state	Host plant		Genotype/haplotype		GenBank accessions	
			Species	Family	ITSS	12S rRNA	ITSS	12S rRNA
<i>A. cinctus</i>	Aci TH	Thailand, Nakhon Pathom	<i>Cocos nucifera</i>	Areaceae	G4, G5, G6	H15	MG242079–MG242084	MG242101–MG242105
<i>A. herbicolus</i>	ES	Spain, Canary Island	<i>Viburnum rigidum</i>	Caprifoliaceae	G7	H16	KF219656, KU318305	KF234130, KF234135, KF234136
<i>N. californicus</i>	NC	New Caledonia, Garraria Rearing units	Unknown	–	G8	H16	KU318304	KU318122, KU318123
			–	–	G9	H17	HQ404802	HQ404836

The specimens used in DNA extraction, when possible, had their exoskeleton recovered from the membrane of the extraction column and were mounted on microscope slides in Hoyer's medium. These slides were deposited as voucher specimens in the mite collection of the Acarology Laboratory, Federal University of Pernambuco, Pernambuco, Brazil, and in the mite collection at Embrapa Genetic Resources and Biotechnology, Brasília, Brazil.

Amplification through polymerase chain reaction (PCR) The primers used for amplification of the ITSS region were 5'-AGAGGAAGTAAAAGTCGTAACAAG-3' (Navajas et al. 1999) and 5'-ATATGCTTAAATTCAGCGGG-3' (Navajas et al. 1998; Navia et al. 2014); for the 12S rRNA gene, primers were 5'-TACTATGTTACGACTTAT-3' and 5'-AAACTA GGATTAGATACCC-3' (Jeyaprakash and Hoy 2002).

The amplification reactions for ITSS were performed in 25- μ L volumes containing 2.5 μ L of a 10 \times buffer supplied by the manufacturer, 1.0 μ L $MgCl_2$ (25 mM), 0.5 μ L dNTP (0.25 mM of each base), 0.175 μ L of each primer (10 μ M), 0.125- μ L $U \mu L^{-1}$ (5 units) of Taq polymerase (Qiagen), 18.525 μ L of sterile water and 2 μ L of DNA template. A PCR for the 12S rRNA fragment was performed as described above, except that 0.4 μ L of bovine serum albumin solution (BSA) (10 mg mL^{-1} Biolabs) and 0.25- μ L $U \mu L^{-1}$ (5 units) of Taq polymerase (Qiagen) were added to the reaction, and the water volume was decreased to 18 μ L. To amplify the ITSS fragment, the thermocycler profile included initial denaturation at 94 $^{\circ}C$ for 2 min, followed by 35 cycles of 15 s denaturation at 94 $^{\circ}C$, 45 s annealing at 50 $^{\circ}C$, 1 min final extension at 72 $^{\circ}C$, and a final step of 7 min at 72 $^{\circ}C$. For 12S rRNA fragment, samples were denatured at 95 $^{\circ}C$ for 1 min, followed by 40 cycles of 30 s denaturation at 94 $^{\circ}C$, 30 s annealing at 40 $^{\circ}C$, 1 min extension at 72 $^{\circ}C$, and a final step of 5 min at 72 $^{\circ}C$. After amplification, 5 mL of the PCR reaction was analyzed by electrophoresis on a 1% agarose gel and visualized by GelRed staining. Both strands of the amplified fragments (ITSS and 12S rRNA) containing visible and single bands were directly sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems, Lille, France). No additional primers were used for sequencing.

Phylogenetic analyses The Staden Package v.1.6.0 (Staden et al. 1998) was used for editing and assembling the raw data into sequence contigs. The sequences were aligned using the CLUSTAL W multiple alignment procedure (Thompson et al. 1994) in MEGA v.7 (Kumar et al. 2016). Shared haplotypes (12S rRNA sequences) and genotypes (ITSS sequences) were identified using DnaSP v.6 software (Rozas et al. 2017). The distance matrices were elaborated using the Kimura 2-parameter model (Kimura 1980), and the standard error estimates were obtained using a bootstrap procedure (1000 replicates) in MEGA v.7. A phylogenetic analysis using both the ITSS and 12S rRNA fragments were conducted using the maximum likelihood (ML) optimality criterion. The best-fit models of nucleotide substitution for both fragments were selected using the jModeltest v.2.1.1 program (Darriba et al. 2012) based on the likelihood scores for 88 different models. The Akaike (1973) information criterion corrected (AICc) and the Bayesian information criterion (BIC) were calculated. The ML analyses were performed using the online version of the PhyML3.0 algorithm (Guindon et al. 2010). A phylogenetic tree was edited using FigTree v.1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). When identical sequences among the analyzed specimens were found in the alignment, i.e. sequences sharing a common haplotype/genotype, a single sequence of each group was included in the alignment to

produce the ML tree for ITSS and 12S rRNA fragments, and the number of times of identical sequences were found in the dataset were indicated into brackets in the phylogenetic tree. The robustness of the trees was assessed by bootstrap analysis, with 1000 bootstrap replicates for all of the analyses.

For the combined analysis, ITSS and 12S rRNA sequences were concatenated according to the mites' population in the output files on ML analyses. The alignments were concatenated in a matrix (21 taxa, 1057 base pairs) using the Mesquite v.3.31 program (Maddison and Maddison 2017). The combined analysis was performed in MrBayes v.3.2.6 (Ronquist et al. 2012). The number of categories used to approximate the gamma distribution was set at four, and four Markov chains were run for 10,000,000 generations; the final average standard deviation of split frequencies was less than 0.01, and the stabilization of model parameters (burn-in=0.25) occurred at approximately 250 generations.

All the new sequences herein obtained have been deposited in GenBank. Available ITSS and 12S rRNA *A. largoensis* sequences in GenBank were added to the dataset (Bowman and Hoy 2012; Navia et al. 2014; Santos and Tixier 2016). *Amblyseius herbicolus* (Chant) sequences retrieved from GenBank were included in the analyses as ingroup and a *Neoseiulus californicus* (McGregor) (Phytoseiidae: Amblyseiniinae) sequence was included in the dataset as the outgroup (Kanouh et al. 2010a; Tsolakis et al. 2012; Navia et al. 2014). The numbers of specimens of each population that were analyzed are shown in Table 2, along with their GenBank accession numbers. The alignments are available upon request.

Results

Morphological characterization

Significant differences are found among the mean values of all characters of the five *A. largoensis* populations (Table 1). Approximately 43% of the total variability was explained by the two first principal components (PC1: 25.8%, PC2: 17.1%). The axes are explained by the distance between setae *St1–St3*, *St2–St2* and *St1–St1*, the length of the *JV5* setae and tibial macrosetae IV (PC1) and the distance between setae *St4–St4* and the length of setae *z5*, *z2*, *z4* and *s4* (PC2). Partial overlap of the populations was observed (Fig. 1), without morphometric discontinuity between them. The first two canonical variables (CV1 and CV2) accounted for 72.9% of the total variance (CV1: 46.6%, CV2: 26.3%) (Fig. 2). The first canonical variable (CV1), explained by the length of setae *z5*, *j6*, *S5*, *j4*, *S4*, and *j5*, macroseta of tarsus IV, *JV5*, *z4*, and *St1–St1*, shows a complete distinction between the population from the Reunion Island and those from the Americas and Asia (Thailand) (Table 3). Comparing those results with the morphometric data (ANOVA) (Table 1), the specimens from Thailand and Roraima differ from those from Reunion because they have longer *z5* setae, shorter macrosetae of tarsus IV, shorter *JV5*, and a smaller distance between the sternal setae *St1–St1*. Specimens from Thailand and Roraima, Brazil distanced themselves from the others along CV2 because of shorter *JV5* setae and *St2–St3*.

The discriminant analysis showed that, on average, 91% of the samples were classified in the population of origin, with 85% being correctly classified in the populations from Roraima and Thailand and 95% in the populations from Reunion, Pernambuco and Trinidad and Tobago.

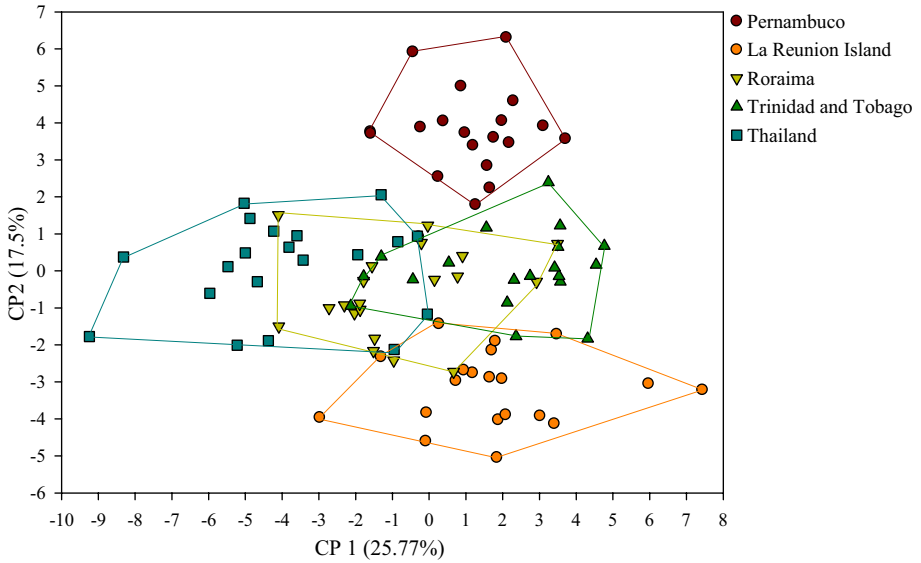


Fig. 1 Principal component analysis of 36 morphological characters of females from five populations of *Amblyseius largoensis*. Polygons were formed based on the projection of individuals from each population to PC 1 and 2 (variation explained by the two main components is shown in parentheses)

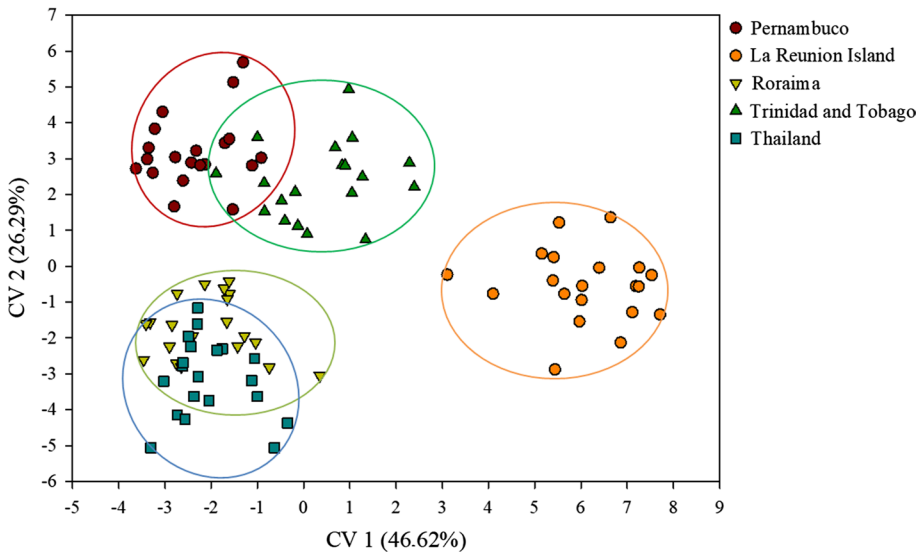


Fig. 2 Canonical variable analysis of 36 morphological characters of females from five populations of *Amblyseius largoensis*. Ovals were formed based on the projection of individuals from each population to CV 1 and 2 (variation explained by the two canonical variables is shown in parentheses)

Table 3 Analysis of canonical variables for the females of five populations of *Amblyseius largoensis*

Morphological characters	CV1 weight	Morphological characters	CV2 weight
<i>z5</i>	-0.3923	<i>S2</i>	0.5448
<i>j6</i>	-0.3195	<i>z2</i>	-0.4670
<i>S5</i>	-0.2230	<i>Z1</i>	0.4009
<i>j4</i>	0.2205	<i>z4</i>	-0.4001
<i>S4</i>	-0.2063	<i>z5</i>	-0.3452
<i>j5</i>	0.2009	<i>r3</i>	0.3493
Macrosetae of tarsu IV	0.1959	<i>J5</i>	0.3038
<i>JV5</i>	0.1814	<i>JV5</i>	0.2044
<i>z4</i>	-0.1612	<i>St2–St3</i>	0.1947
<i>St1–St1</i>	0.1592	<i>S4</i>	0.1915

The first 10 morphological (eigenvector) characters with higher weights (eigenvalues) are shown for two canonical variables (CV1 and CV2), in descending order of absolute values

Molecular characterization

A BLAST search in GenBank showed that the ITSS and 12S rRNA sequences aligned with those of Phytoseiidae.

ITSS rDNA Eight *A. largoensis* partial ITSS DNA sequences from Thailand were obtained (approximately 620 bp). For *A. cinctus*, six sequences (approximately 610 bp) were obtained (Table 2). Half of these specimens had their exoskeletons recovered and mounted on microscope slides in Hoyer's medium.

Three groups of identical sequences were identified within the 28 ITSS sequences of *A. largoensis* (Table 2; Figures S1 and S4). Genotype G1 included specimens (16) from Asia (Thailand), the Americas (Brazil, Trinidad and Tobago) and Oceania (New Caledonia). Genotype G2, represented by the sequence KF219649, consisted only of specimens (7) from Indian Ocean islands (Reunion) as reported by Navia et al. (2014), and G3 was exclusively composed of a specimen collected in Oceania (New Caledonia) (Figure S2). A single nucleotide located at position 121 bp of the alignment separated the G1 (C) genotype from G2 (T). This variable site corresponds to that described by Navia et al. (2014). The New Caledonia specimen (G3) differed only at position 222, where the A base appears in place of G. The average nucleotide composition in the ITSS sequences was equal to T=30.7%, A=27.0%, C=20.0% and G=22.3% (G+C content=42.3%, A+T content=57.7%).

The topologies of the main branches for ML and BIC trees were similar, and therefore, only the ML phylogeny is presented in the Supplementary Material (Figure S1). The *A. largoensis* populations formed a clade strongly supported by the bootstrap value (99.7%). This clade showed three internal clusters consisting, respectively, of genotype G1 (the Americas, Thailand and New Caledonia), G2 (Indian Ocean islands) and G3 (New Caledonia) specimens.

The mean intraspecific variability within *A. largoensis* populations was 0.05% (Table 4, A1). The mean distances between *A. largoensis* and *A. herbicolus* and between *A. largoensis* and *A. cinctus* were 4.2 and 12.1%, respectively (Table 4, A1). The mean intraspecific variation between sequences of *A. largoensis* from the Americas (Brazil and Trinidad and

Table 4 Mean genetic Kimura 2-parameter distances (%) below the diagonal (minimum and maximum values in parentheses), with standard error estimates (above diagonal), between and within the ITSS region of *Amblyseius* species and the outgroup *Neoseiulus californicus*

A1	Interspecific values				Intraspecific values		
	1	2	3	4			
1 <i>A. largoensis</i> AM, NC, TH, IOI		0.00904	0.01697	0.01987	0.05 ± 0.0004% (0.00–0.22)		
2 <i>A. herbicolus</i>	4.16% (4.13–4.37)		0.01529	0.01802	0.00 ± 0.00%		
3 <i>A. cinctus</i>	12.14% (12.11–13.38)	10.02% (10.02–10.02)		0.015977	0.00 ± 0.00%		
4 <i>N. californicus</i>	14.81% (14.78–15.06)	12.10% (12.10–12.10)	10.81% (10.81–10.81)		n/c		
A2	Interspecific values					Intraspecific values	
	1	2	3	4	5		
1 <i>A. largoensis</i> AM, NC, TH		0.00025	0.00897	0.01648	0.01832	0.05 ± 0.00047% (0.00–0.22)	
2 <i>A. largoensis</i> IOI	0.03% (0.00–0.22)		0.00896	0.01648	0.01830	n/c	
3 <i>A. herbicolus</i>	4.16% (4.13–4.37)	4.13% (4.13–4.13)		0.01523	0.01679	0.00 ± 0.00%	
4 <i>A. cinctus</i>	12.14% (12.11–12.38)	12.11% (12.11–12.11)	10.02% (10.02–10.02)		0.01559	0.00 ± 0.00%	
5 <i>N. californicus</i>	14.81% (14.78–15.06)	14.78% (14.78–14.78)	12.10% (12.10–12.10)	10.81% (10.81–10.81)		n/c	
A3	Interspecific values						Intraspecific values
	1	2	3	4	5	6	
1 <i>A. largoensis</i> AM, NC		0.00046	0.00046	0.00887	0.01647	0.01856	0.087 ± 0.0008% (0.00–0.22)
2 <i>A. largoensis</i> TH	0.05% (0.00–0.22)		0.00000	0.00885	0.01645	0.01854	0.00 ± 0.00%
3 <i>A. largoensis</i> IOI	0.05% (0.00–0.22)	0.00%		0.00885	0.01645	0.01854	n/c
4 <i>A. herbicolus</i>	4.18% (4.13–4.37)	4.13% (4.13–4.13)	4.13% (4.13–4.13)		0.01483	0.01713	0.00 ± 0.00%
5 <i>A. cinctus</i>	12.17% (12.11–12.38)	12.11% (12.11–12.11)	12.11% (12.11–12.11)	10.02% (10.02–10.02)		0.01545	0.00 ± 0.00%
6 <i>N. californicus</i>	14.84% (14.78–15.06)	14.78% (14.78–14.78)	14.78% (14.78–14.78)	12.10% (12.10–12.10)	10.81% (10.81–10.81)		n/c

For information on population data see Table 1. *Amblyseius largoensis* populations from the Americas (AM) and New Caledonia (NC), Thailand (TH) and Indian Ocean islands (IOI) were grouped as a single taxon (A1) and as separate taxa (A2 and A3)

Tobago), Oceania (New Caledonia) and Asia (Thailand) was 0.05% (Table 4, A2). The mean distance between the populations of *A. largoensis* from the Americas, Oceania and Asia and the population of the islands of the Indian Ocean (Reunion) was 0.03% (Table 4, A2). Figure 3 presents the four intra-population distances and the three inter-population distances of *A. largoensis*. The ITSS maximum intraspecific distance observed was 0.22% and overlap was observed between all intra- and inter-population distances. Similar low intraspecific values were reported for *A. largoensis* (0.16%; Navia et al. 2014), *Euseius nicholsi* (0.2%; Yang et al. 2012) and usually do not exceed 3%, as pointed by Tixier et al. (2017).

12S rRNA Seven sequences of the 12S rRNA of *A. largoensis* from Thailand (410 bp) (five exoskeletons recovered) and five *A. cinctus* sequences were obtained (four exoskeleton recovered). The lengths were approximately 400 bp. Among the 12S rRNA sequences of *A. largoensis*, 14 haplotypes were identified (Table 2; Figure S4). Haplotype H6, including the specimens (7) from Thailand, differed from haplotypes including the populations of

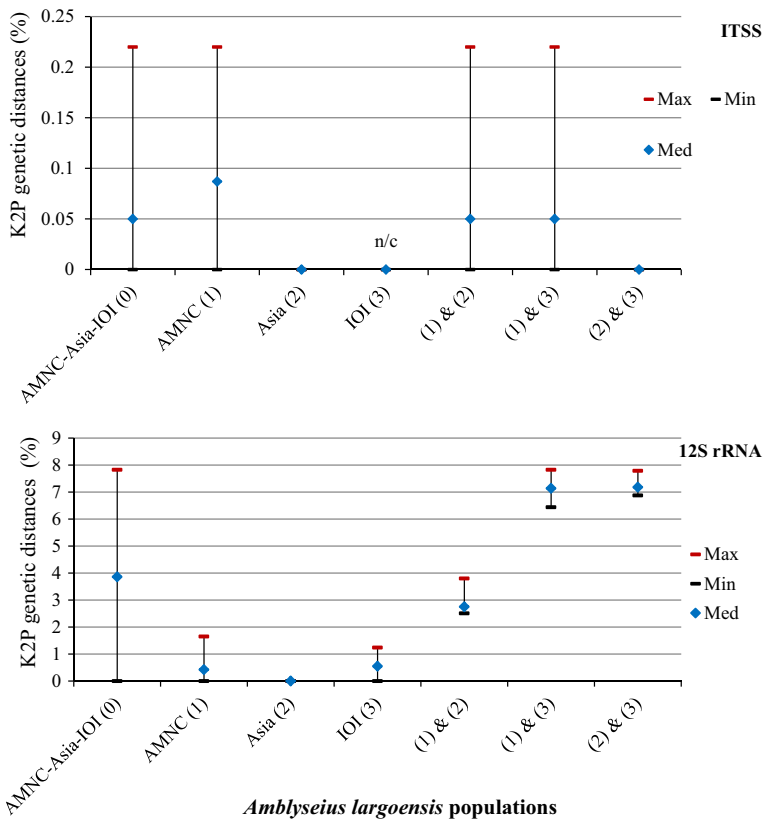


Fig. 3 K2P genetic distances within (0, 1, 2 and 3) and between three populations (1 vs. 2, 1 vs. 3, 2 vs. 3) of *Amblyseius largoensis* for the two molecular markers, ITSS and 12S rRNA. (0) *A. largoensis* as a single group; (1) *A. largoensis* from AMNC=America/New Caledonia; (2) *A. largoensis* from Asia; (3) *A. largoensis* from IOI=Indian Ocean islands

the Americas (Brazil, Trinidad and Tobago, and the USA) and New Caledonia (H1–H3, H7–H10) in 13 nucleotides, and from haplotypes from Indian Ocean islands (Reunion and Mauritius) (H4, H5, H11–H14) in 26 nucleotides (Figure S3). The average nucleotide composition in the 12S rRNA sequences was T=41.9%, A=33.8%, C=9.3% and G=15.1% (G+C content=24.3%, A+T content=75.7%).

The phylogenetic tree was constructed with the maximum likelihood (ML) criterion with the HKY+G model, Ti/Tv=1.2737 and a gamma distribution shape parameter (G) of 0.4040 (Figure S2). The topologies of the NJ, ML and BIC trees were similar, and only the ML phylogeny is shown (Figure S2). All specimens of *A. largoensis* are included in a well-supported monophyletic group (bootstrap score of 100%). This group is divided in two clades. Populations of the Americas (Brazil, USA and Trinidad and Tobago), Oceania (New Caledonia), and Asia (Thailand) clustered in the first clade (bootstrap value: 93.1%). Within this clade, the population of Asia (Thailand) is separated from the populations of the Americas and of Oceania (New Caledonia) (Figure S2). The second clade contains *A. largoensis* specimens from Indian Ocean islands (Mauritius and Reunion) (bootstrap value: 99.8%).

The mean intraspecific variability for the *A. largoensis* specimens considered was 3.9% (min–max=0.0–7.8%) (Table 5, B1; Fig. 3). The mean distances between *A. largoensis* and *A. herbicolus* and between *A. largoensis* and *A. cinctus* were 24.2 and 26.1%, respectively (Table 5, B1). Considering the populations of *A. largoensis* from Indian Ocean islands (IOI) as an isolated group apart from the Americas/New Caledonia (AMNC) and Asia (as observed in the 12S rRNA ML tree; Figure S2), the intra-group mean distances were 1.5% (IOI) and 0.55% (AMNC), respectively, and the mean distance between these two groups was 7.2% (6.4–7.8%) (Table 5, B2). More accurately, the mean genetic distance between specimens from the Americas was 0.42% (0.0–1.7%) (Table 5, B3; Fig. 3) and that between specimens from Asia was 0.0%. The mean genetic distance between specimens from Asia and the Americas was 2.8% (2.5–3.8%) (Table 5, B3; Fig. 3).

Bayesian combined analysis

A combined analysis (Fig. 4), which included unique variants of nucleotide sequences of both the internal transcribed spacer (ITSS) region and 12S rRNA, supported the results of individual analyses using ITSS and 12S rRNA fragments separately (Figures S1 and S2). Strong support (posterior probability=0.93) was confirmed for the clade containing the specimens from the Americas, New Caledonia and Thailand, although the specimens from Thailand remain internally separated in this clade. The Indian Ocean islands populations formed a distinct and well-supported clade (0.99).

Discussion

Morphological and molecular differences between the *A. largoensis* populations considered have been observed. The first question concerns thus the taxonomical meaning of these differences. Morphological variation was observed for all the characters considered, setae and spermatheca lengths, body and ventrianal shield dimensions and distance between the sternal setae. However, even if significant differences were observed, the mean values were very close and some of these characters—especially distances between sternal

Table 5 Mean genetic Kimura 2-parameter distances (%) below the diagonal (minimum and maximum values in parentheses), with standard error estimates (above diagonal), between and within 12S rRNA *Amblyseius* species and the outgroup *Neoseiulus californicus*

B1	Interspecific values				Intraspecific values
	1	2	3	4	
1 <i>A. largoensis</i> AM, NC, TH, IOI		0.03300	0.03523	0.03900	3.86 ± 0.00809% (0.00–7.83)
2 <i>A. herbicolus</i>	24.20% (23.27–25.53)		0.03472	0.04053	0.00 ± 0.00%
3 <i>A. cinctus</i>	26.08% (25.60–27.42)	25.02% (25.02–25.02)		0.03860	0.00 ± 0.00%
4 <i>N. californicus</i>	32.15% (30.26–33.40)	30.88% (30.88–30.88)	27.85% (27.85–27.85)		n/c

B2	Interspecific values					Intraspecific values
	1	2	3	4	5	
1 <i>A. largoensis</i> AM, NC, TH		0.01591	0.03345	0.03527	0.04260	1.47 ± 0.00504% (0.00–3.80)
2 <i>A. largoensis</i> IOI	7.15% (6.44–7.83)		0.03374	0.03628	0.03938	0.55 ± 0.00219% (0.00–1.24)
3 <i>A. herbicolus</i>	23.99% (23.27–24.39)	24.71% (24.39–25.53)		0.03400	0.04028	0.00 ± 0.00%
4 <i>A. cinctus</i>	25.69% (25.60–26.19)	27.01% (26.82–27.42)	25.02% (25.02–25.02)		0.03858	0.00 ± 0.00%
5 <i>N. californicus</i>	32.82% (32.75–33.40)	30.60% (30.26–31.49)	30.88% (30.88–30.88)	27.85% (27.85–27.85)		n/c

B3	Interspecific values						Intraspecific values
	1	2	3	4	5	6	
1 <i>A. largoensis</i> AM, NC		0.01001	0.01658	0.03386	0.03582	0.04238	0.42 ± 0.00202% (0.00–1.65)
2 <i>A. largoensis</i> TH	2.75% (2.51–3.80)		0.01713	0.03464	0.03581	0.04203	0.00 ± 0.00%
3 <i>A. largoensis</i> IOI	7.14% (6.44–7.83)	7.18% (6.88–7.79)		0.03469	0.03715	0.03991	0.55 ± 0.00214% (0.00–1.24)
4 <i>A. herbicolus</i>	23.79% (23.27–24.39)	24.39% (24.39–24.39)	24.71% (24.39–25.53)		0.03561	0.03999	0.00 ± 0.00%
5 <i>A. cinctus</i>	25.73% (25.60–26.19)	25.60% (25.60–25.60)	27.01% (26.82–27.42)	25.02% (25.02–25.02)		0.03779	0.00 ± 0.00%
6 <i>N. californicus</i>	32.85% (32.75–33.40)	32.76% (32.76–32.76)	30.60% (30.26–31.49)	30.88% (30.88–30.88)	27.85% (27.85–27.85)		n/c

Table 5 (continued)

For information on population data see Table 1. *Amblyseius largoensis* populations from the Americas (AM) and New Caledonia (NC), Thailand (TH) and Indian Ocean islands (IOI) were grouped as a single taxon (B1) and as separate taxa (B2 and B3)

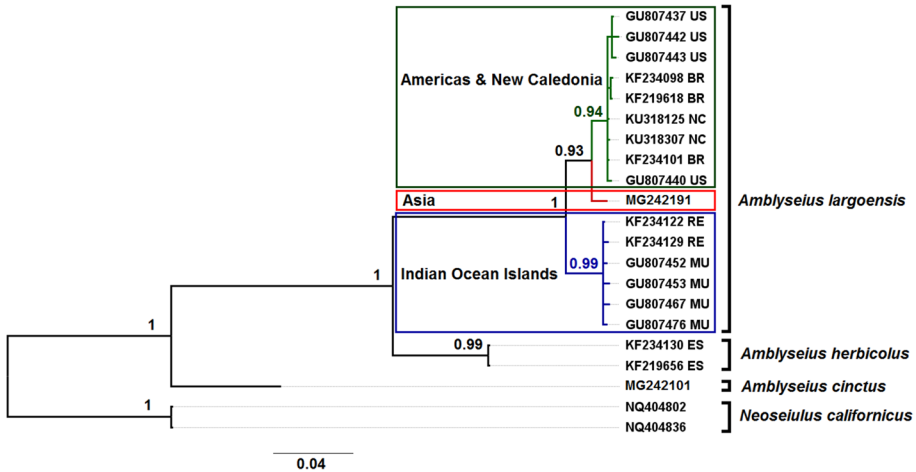


Fig. 4 Combined Bayesian inference (BI) analysis tree for *Amblyseius* species on coconut plants calculated from the ribosomal region ITSS and 12S rRNA sequences. Statistical support indicates Bayesian posterior probabilities; only probabilities > 0.6 are indicated above branches. *Amblyseius largoensis* populations from different geographic locations are highlighted in colored squares. The species names based on morphological identification are to the right of the tree

setae—are rarely used for diagnosis issues. Furthermore, Tixier (2013) and Tixier et al. (2013) proposed statistical approaches to establish limits to distinguish between intra- and interspecific variability based on the lengths of phytoseiid setae on the dorsal shield. The minimum difference between the mean values of samples belonging to two distinct species should be 10.58 μm (for setae < 65 μm) and 33.99 μm (for setae > 65 μm) (Tixier 2013). According to these values, the variability found among the population of *A. largoensis* of Thailand and the other populations studied reflects intraspecific variability.

Molecular analyses confirm this conclusion. The ITSS genetic distances observed between all specimens of *A. largoensis* herein considered (0.05%) are lower than the intraspecific distances determined for other phytoseiid species of the genera *Neoseiulus* (0–0.4%) (Okassa et al. 2011), *Neoseiulella* (0–1.00%) (Kanouh et al. 2010b), and *Typhlodromus* (0–1.2%) (Tixier et al. 2006b, 2012b). Furthermore, the ITSS mean interspecific distance between *A. largoensis* and *A. herbcicolus* (4.2%) was significantly greater than the distance between *A. largoensis* populations. The 12S rRNA mean genetic distance between populations of *A. largoensis* in Asia and the populations of the Americas plus Oceania (2.8%) and islands in the Indian Ocean (7.1%) are comparable with the intraspecific distances observed for other phytoseiid species of the genera *Euseius* (0–3%) (Okassa et al. 2009) and *Phytoseius* (maximum value = 9%) (Tixier et al. 2017), *Typhlodromus* (0–4.7%) (Tixier et al. 2012a), *Neoseiulus* (0–5.2%) and *Neoseiulella* (0–8.0%) (Kanouh et al. 2010b). In addition, the 12S rRNA distance values for *A. largoensis* populations are lower than

the interspecific distances for species of the same genus: distance found in this study for *A. largoensis* and *A. herbicolus* (26.1%), and among species of the genera *Typhlodromus* (25–26.7%) (Tixier et al. 2012a), *Euseius* (14–22%) (Okassa et al. 2009) and *Neoseiulus* (9–12.5%) (Jeyaprakash and Hoy 2002; Okassa et al. 2011).

The integrative approach herein proposed, combining morphological and molecular analyses, shows that the population identified as *A. largoensis* collected in Thailand, as well as that from New Caledonia, are conspecific to the populations previously studied in the Americas and Indian Ocean islands by Navia et al. (2014). Regarding the ITS polymorphism observed among genotypes G1 (Americas, Asia, New Caledonia) and G2 (Indian Ocean islands), the C–T transition (alignment position 121) could be used as a trait to differentiate these geographic populations as reported by Navia et al. (2014), just as the A–C transition (alignment position 222) could be used to discriminate genotype G1 from G3 (New Caledonia). The data herein obtained will be helpful for the development of molecular diagnosis in establishing a cutting threshold for the decision between intra- and interspecific variation. Indeed, various aspects may affect the genetic delimitation values for diagnosis purposes such as the number of sequences analyzed, the sensibility of the clustering methods, the taxonomic entity and details about biological features, making the decision difficult (Tixier et al. 2017). Thus, even though 7.8% is higher compared to values obtained in some other studies (Okassa et al. 2009, 2011; Tixier et al. 2012a, b; Navia et al. 2014) it is a reliable value for *Amblyseius* species based on biological observations.

As all populations considered belong to the same species, the second question concerns the meaning of the intraspecific differences herein observed. Morphological and molecular analyses (with both markers) indicate consistent results and the existence of three clear groups, one including populations from the Americas, one including specimens from Asia and one including specimens of Indian Ocean islands. The two former clades are more related to each other than to the one including specimens from Indian Ocean islands. Several hypotheses can be proposed for explaining this clustering. The first hypothesis is related to the geographical proximity of the populations. Populations from Thailand are geographically closer to populations from Indian Ocean islands, than from populations of the Americas, but included in the same clade as the latter. Specimens from New Caledonia are also geographically remote from those from the Americas, yet included in the same clade. Thus the geographical hypothesis to explain the molecular and morphological clustering of the populations of *A. largoensis* cannot be retained. The second hypothesis is related to dispersal between the various places and to the ‘history’ of the species, via human transport and/or vicariance effects. We can hypothesize a late dispersal to Ocean islands from the continent and subsequently a differentiation of these populations. It is also possible that specimens from Thailand or other Asian countries with the same genetic lineage were introduced into the Americas via human transport. However, to really conclude on this point, clearly more samples of *A. largoensis* are needed, especially from Africa. Finally, a third hypothesis is related to ecological isolation. Although at present no clear factor has been identified for *A. largoensis* (such as, e.g., plant or prey), recent studies have associated genetic differences with variation in the biological performance of populations of single species within the family Phytoseiidae (Ferrero et al. 2007; Furtado et al. 2007; Tixier et al. 2010). Navia et al. (2014) hypothesized that the genetic differences observed between populations of *A. largoensis* from Brazil and Reunion Island may be related to the biological differences reported by Domingos et al. (2013). Variation in the period of oviposition, prey consumption and net reproduction rate were observed between Reunion Island

and Brazilian populations, and these parameters were significantly higher for the Reunion population (Domingos et al. 2013). Because of the proximity between the Thailand and the Americas populations, it is likely that they are also more biologically similar when compared to the populations from the islands of the Indian Ocean. However, it is essential to test this hypothesis with further studies to elucidate the biological performance of the Asian population under laboratory and field conditions in comparison to populations from the Americas and Indian Ocean islands.

Finally, because of the molecular and morphological variations observed, this study indicates that these tools might be helpful for monitoring these populations when released in the field. Clearly, if these phenotypic and genetic differences could be associated with biological features, such integrated information could be used for modeling and forecasting population dynamics, in case of releases. However, further experiments are needed to better characterize the biological parameters of these populations, as well as their interaction (e.g., competition, intraguild predation) in case of co-occurrence.

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