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DOI
10.1007/s10493-010-9413-5

Publication date
2011

Document Version
Submitted manuscript

Published in
Experimental and Applied Acarology

Morphological variation and reproductive incompatibility of three coconut-mite-associated populations of predatory mites identified as *Neoseiulus paspalivorus* (Acari: Phytoseiidae)

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Abstract

Predatory mites identified as *Neoseiulus paspalivorus* DeLeon (Phytoseiidae) have been considered as agents for classical biological control of the coconut mite, *Aceria guerreronis* Keifer (Eriophyidae), in Africa and elsewhere. Preliminary identification of geographically distinct populations as belonging to the same species (*N. paspalivorus*) was based on their morphological similarity. However, laboratory studies recently conducted have shown large differences in feeding behaviors and biological characteristics among individuals collected from three geographic origins: Brazil (South America), Benin and Ghana (West Africa). As morphologically similar specimens do not necessary belong to the same species, we evaluated under laboratory conditions, reproductive compatibility between the specimens from three geographic locations to ascertain their conspecificity. Morphological measurements were also made to determine if there is a means of discriminating between them. Inter-population crosses showed complete reproductive isolation between the three geographic populations, but interpopulation discontinuities in morphometric characters were absent. These results indicate that the tested specimens are distinct biological entities despite morphological similarity. Further molecular genetic studies are therefore proposed, including screening for endosymbionts and assessment of genetic differentiation, to determine the cause of reproductive incompatibility and to clarify the taxonomic relationship between those populations.

Key words: Morphometrics, reproductive isolation, conspecificity, biological species.

Introduction

Coconut, *Cocos nucifera* L., is one of the main crops in the Tropics. The coconut mite, *Aceria guerreronis* Keifer, has become one of the most important arthropod pests of this crop around the world. It causes severe damage to the crop by attacking the meristematic tissues underneath the bracts. According to recent assessments the abundance of the coconut mite and its damage to the crop may be lower, and the predator fauna associated with coconut mite may be richer in certain areas of Brazil than in Africa (Benin, Ghana and Tanzania) and Sri Lanka (Fernando et al. 2003; Lawson-Balagbo et al. 2008; Reis et al. 2008; Negloh et al. 2010; Fernando et al., unpublished). Over the past two decades, attempts were made to control the pest through the use of chemicals and biopesticides. These control measures, however, have proven to be costly, often ineffective and ecologically undesirable (Moore and Alexander 1987; Moore et al. 1989). Because of the exotic nature of the coconut mite in Africa (originated from South America) (Navia et al., 2005), classical biological control, i.e., is the intentional importation and release of natural enemies in the native range of the target pest, where the pest and its natural enemies co-evolved, for permanent establishment and self-sustained control of the target pest, is thought to be a reasonable approach to combat the coconut mites.

Mites in the family Phytoseiidae are mostly predators that are well-known to play a significant role in the biological control of arthropod pests such as mites and small insects (McMurtry et al. 1970; Helle and Sabelis 1985; Lindquist et al. 1996; Sabelis and Van Rijn 1997; Gerson et al. 2003). The predatory mite *Neoseiulus paspalivorus* (DeLeon) (Acari: Phytoseiidae) is the most common natural enemy associated with the coconut mite in Brazil (Lawson-Balagbo et al. 2008; Reis et al. 2008). In Benin and Ghana, West Africa, *N. paspalivorus* was also frequently reported on infested coconuts (Negloh et al. 2010). However, recent laboratory experiments by Famah Sourassou et al. (in prep.) showed large differences in feeding behaviours and biological characteristics among specimens from the three geographic locations: Benin, Ghana (West Africa) and Brazil (Itamaraca, State of Pernambuco). All three geographic populations developed and reproduced on coconut mite prey, but their biological parameters differed. Furthermore, the specimens from Beninese and Brazilian populations were able to develop and reproduce on *Tetranychus urticae* Koch (Acari: Tetranychidae), whereas the Ghanaian ones were able to develop on this prey, yet unable to convert the food obtained from this prey into eggs. Moreover, the specimens from Brazilian and Ghanaian populations were able to develop on coconut pollen, whereas those from Beninese populations were not. All three geographic populations of *N. paspalivorus* did not produce any eggs on coconut pollen. Contrasting results with our data were previously reported by

Lawson-Balagbo et al. (2007), showing that *N. paspalivorus* specimens collected in Acarau (State Cesara, Brazil) could not feed and develop on *T. urticae*, whereas they were able to develop and reproduce on coconut pollen. Taken together, there are clear indications that the three *N. paspalivorus* geographic populations are biologically different despite their morphological similarity. Such biological differences between morphologically similar populations may indicate cryptic species (Muma and Denmark 1969; Monetti and Croft 1997, Tixier et al. 2003, 2004, 2006a, 2008). To test whether the three allopatric populations are 'potentially able to interbreed and produce viable progeny' (Biological species sensu Mayr 1940) is necessary to understand the evident biological differences between the three geographic populations.

The study reported in this article aimed to evaluate reproductive compatibility among three geographic populations of predatory mites (Brazil, Benin, and Ghana) that have been identified as *N. paspalivorus* based morphological similarity only. We also conducted morphological comparison to determine if there are morphological traits other than those used for their identification that may serve to distinguish these populations.

Material and methods

Source populations and rearing techniques

Specimens of *N. paspalivorus* were collected from coconuts in October 2005 in Ouidah (06^{o}21N; 02^{o}097E), Southern Benin, in September 2006 in Itamaraca (07^{o}46S; 34^{o}52W), State of Pernambuco, Northeastern Brazil and in December 2008 near Winneba (05^{o}22'907N; 00^{o}38'685W), Southern Ghana. These three samples were used for propagation in a rearing unit consisting of a black PVC tile (4 x 4 x 0.1 cm) placed on top of a foam pad (4 x 4 x 1 cm) resting in a Petri dish (14.5 cm in diameter and 1 cm in height). The edges of the tile were covered with a band of tissue paper that also contacted the foam pad. To prevent mites from escaping, distilled water was supplied to the Petri dish on a daily basis to keep wet the foam pad and the tissue paper. A tuft of hydrophobic cotton wool covered by a piece of transparent plastic was placed in the center of each rearing unit to serve as an oviposition site for the predators. Colonies of all populations were maintained in a climate-controlled room at 25-27^{o}C, 70-90% relative humidity and a 12-12h light-dark cycle. The colonies were provided with a new supply of immature stages of *T. urticae* at three day intervals.

Crossing experiments

Each experiment started with a cohort of 1-day old eggs obtained from gravid females of each of the three populations. To obtain them, one hundred females were confined to rearing units similar to that described above and offered eggs of *T. urticae* as prey. After 24h, each egg laid by the predator was transferred to a new unit, again similar to that previously described above, except that the PVC was 2.5 cm diameter black PVC disk and had a hole of 2 mm diameter in the center to serve as an oviposition site. The nymphal stages of the three populations were reared on all stages of *A. guerreronis* supplied ad libitum. When reaching adulthood, predators were sexed and females and males of each population were kept apart for the subsequent crosses.

Mating of all possible combinations of recently moulted females and males of the three populations were considered to determine reproductive compatibilities. All crosses were set up as a single pair mating between virgin females and males. Additionally, 10 virgin females of each population were kept in isolation, to ascertain that mating is necessary for oviposition to take place. Each pair was observed daily and the number of eggs laid was recorded for a period of 10 days. Eggs found in the arenas of a given male-female combination of geographic strains were pooled in a unit similar to that previously described (8.5 cm in diameter and 1.5 cm in height), and reared to adulthood to determine egg viability, post-embryonic survivorship and sex-ratio. Offsprings of each cross were backcrossed to assess hybrid fertility. All crossing experiments were carried out simultaneously under the same environmental conditions as described previously (25-27^{o}C, 70-90% relative humidity and a light-dark cycle of 12h-12h). At the end of the observation period, females and males of each population were preserved in 70% alcohol for morphological analysis.

Morphological analysis

Twenty adult females and ten adult males of each population were slide-mounted in Hoyer's medium for examination under a phase-contrast microscope. Measurements were done with an ocular micrometer, at 400x magnification to measure body size and shield dimensions and at 1000x magnification to measure the length of setae, spermathecal calyx (spermatodactyl for male) and cheliceral digits. Females and males were characterized based on 32 morphological traits (Tables 2, 3) that are used commonly for the identification of phytoseiid mites (i.e., Chant and McMurtry 1994, Moraes et al. 2004; Chant and McMurtry 2005; Zannou et al. 2007). Setal nomenclature follows that of Lindquist and Evans (1965), as applied to the phytoseiids by Rowell et al. (1978) and Chant and Yoshida-Shaul (1991). For measurement of cheliceral digits and spermatodactyl, another set of at

least 20 females and 20 males of each population were slide-mounted separately. The gnathosoma was cut from the rest of the body and a slight pressure was applied to the mount by touching the slide coverslip with the rear end of a small brush to help in distinguishing the two cheliceral digits. Holotype measurements, taken from the original description (DeLeon, 1957), were also included in the results for comparison.

Data analysis

Crossing data

A single-factor ANOVA was used to test the effects of crossing types on fecundity and duration of the pre-oviposition period. Data of the latter were log-transformed [ln(x+1)] for ANOVA. Means were compared using the Student-Newman-Keuls multiple range test (SNK) at $P < 0.05$.

Morphological data

Morphological characters of females and males were analyzed separately with SAS software (SAS Institute 2003). For each population and each character examined, descriptive statistics (mean, standard error, maximum, minimum) were calculated using the MEANS procedure (PROC MEANS). Differences among geographic populations were tested by one-way analysis of variance (PROC ANOVA) followed by a Newman-Keuls multiple comparison test at $P < 0.05$. Next, a multifactorial analysis (Principal Component Analysis) and a Canonical Discriminant Analysis were performed to assess patterns of morphological variation and to identify morphological characters that contribute most to morphological differentiation among the three geographic populations. Prior to multifactorial analysis, a discriminant analysis was carried out to examine how many specimens were correctly classified into their original populations.

Results

Cross-breeding experiments

All mated females of intra-population crosses laid eggs, but the proportion of ovipositing females from inter-population crosses ranged between 80 and 100%. Unmated females did not oviposit (Table 1). The pre-

oviposition period of offspring from each of the inter-population crosses (ranging between 2.9 and 5.4 days) was longer than that of offspring from the intra-population crosses (< 2.0 days) ($P < 0.001$; Table 1).

Average fecundity was about two- to three-fold higher in intra-population than in inter-population crosses ($P < 0.001$; Table 1). All eggs obtained from intra-population crosses were viable, with 71% producing females. All eggs produced by inter-population crosses were malformed and non-viable, similar to crosses between females from the Beninese population and males from the Brazilian population. In the crossings between females from Brazil and males from Benin, for which nearly 14% of the eggs produced (13 eggs out of 92) had a normal shape, only 7.6% of the eggs (7 eggs) hatched giving rise to male progeny only. Eggs were not produced by females of any of the backcrosses involving hybrid males from Brazilian females and Beninese males or Beninese males and Brazilian females (n= 3 for each combination), although mating was observed. In the control backcrosses (n= 3 for each combination), average of 11.3 and 11.0 eggs per female were recorded for Brazilian and Beninese populations respectively.

Morphological analysis

Mean, standard errors and range of the 32 characters are shown in the Tables 2 and 3 respectively for female and male populations. Significant differences were observed for 22 and 15 out of the 32 characters measured, respectively among female and male populations. However, these differences were very small, except for the width of the ventrianal shield at anus level (POST-WVS), which was smaller for the Ghanaian females and the length of seta S5, which was slightly shorter for Beninese females. The standard errors within populations were low and the differences between the minimal and the maximal values for each of the measured morphological variables (except for POST-WVS of the Ghanaian females) were very small.

Predicted classification of specimens based on discriminant analysis is shown in Table 4. Overall, 86.7% of female specimens and 63.3% of male specimens were classified in the population of origin. The majority of misclassifications for males concerned the Ghanaian and Brazilian specimens (7 of 11, or 63.6%), whereas for females 50% of the misclassifications concerned the Beninese and Brazilian populations, and the Ghanaian and the Brazilian populations. The results of discriminant analysis were corroborated by the principal component analysis (Figure 1); substantial overlaps of measurements of females occurred between the Brazilian and Beninese specimens, and between the Brazilian and the Ghanaian specimens. Overlaps were observed between the Ghanaian and Beninese female specimens (Figure 1a). In relation to male specimens, considerable level of overlap was found between the measurements on the Brazilian and Beninese specimens (Figure 1b). The

canonical discrimination analysis allowed a clear distinction between the females of the three populations (Figure 2a). The Brazilian and Beninese specimens are the most distant, while the Ghanaian specimens were intermediate between latter two. However, in males, only the Ghanaian specimens could be clearly distinguished from the two others (Figure 2b). The posterior width of the ventrianal shield (VSW_POST) and the lengths of j1, Z5, S5 and r3 contributed the most to the morphological differentiation between females of the three geographic populations, whereas the width of the dorsal and genital shields and length of seta S5 contributed the most to the morphological differentiation of males from the three geographic populations.

N. paspalivorus females from Ghana had a slightly narrower dorsal shield and relatively longer ventrianal shield with a reduced width at the level of the anal shield (posterior width of ventrianal shield) and slightly shorter seta Z5, while males of the same population had slightly narrower dorsal and genital shields. The two sexes of Beninese specimens differed from those of Brazil and Ghana by having slightly shorter seta S5. Specimens from Brazil appeared to be intermediate, but much closer to Beninese specimens by having a similar size of ventrianal shield (for female) and genital shield (for male) with the Beninese specimens and similar length of setae S5 with the Ghanaian specimens.

Discussion

The results of univariate and multivariate analyses are consistent with the previous identification of all three populations– from Benin, Ghana and Brazil– as belonging to the same species. Overlapping morphometric values indicate great morphological similarity among the geographic populations tested. The measurements of the specimens examined were similar to the holotype measurements of *N. paspalivorus* (De Leon 1957) and measurements of females for the same species from Sri Lanka (Moraes et al. 2004). Differences in setal measurements were small, and the discrimination between the geographic populations by multifactorial analyses was based on only some characters that have low weight in species differentiation. McMurtry (1980) indicated that caution should be exercised in using relatively small differences in setal length to differentiate species. Tixier et al. (2006b) reported large differences in setal lengths between *Kampimodromus hmiminai* (McMurtry and Bounfour) from France and Morocco and *K. adrianae* (Ferragut and Pena-Estévez) from the Canary Islands, but molecular analysis indicated that they are synonyms. However, the results of cross-breeding indicate reproductive isolation between the populations investigated, which, should therefore be considered as separate species (Mayr 1940). The absence of oviposition in unmated *N. paspalivorus* females observed in this study was

in agreement with what is known to date for the majority of phytoseiids (Croft 1970; McMurtry et al. 1976; McMurtry 1980; Moraes and McMurtry 1981; Noronha and Moraes 2002, 2004). Thus, the high proportion of ovipositing females observed in inter-population crosses imply that mating had occurred, therefore indicating the absence of pre-mating reproductive barriers between the populations under test. The complete bidirectional post-mating reproductive incompatibility observed between the geographic populations was expressed in the form of reduced fecundity, zygotic mortality and a male-biased sex ratio among the few sterile offspring.

Bidirectional incompatibility is a rare phenomenon, as unidirectional incompatibilities are the most common in phytoseiid and tetranychid mite populations (Hoy and Cave 1988; Gotoh and Noguchi 1989; Gotoh et al. 1995; Breeuwer 1997; Johanowicz and Hoy 1998; Vala et al. 2000, 2002; Noronha and Moraes 2002, 2004). Our results of bidirectional incompatibility among geographic populations of *N. paspalivorus* are therefore quiet exceptional, but are similar to those reported by Monetti and Croft (1996) with respect to crosses between the morphologically similar phytoseiid species *Neoseiulus californicus* (McGregor) and *N. fallacis* (Garman). Moreover, Klimov et al. (2004) observed postzygotic reproductive isolation between two cryptic species of *Sancassania* mites, *Sancassania salasi* and *S. ochoai* (Klimov, Lekveishvili & OConnor), and molecular analysis of these populations showed that they represent distinct species.

The causes of reproductive incompatibility are poorly studied in mites, particularly in phytoseiids. Only in the phytoseiid *Galendromus occidentalis* (Nesbitt) and in some spider mites, the endosymbionts *Wolbachia* and more recently *Cardinium* have been demonstrated to mediate unidirectional reproductive incompatibility (Hess and Hoy 1982; Gotoh et al. 1995; Johanowicz and Hoy 1996; Breeuwer 1997; Vala et al. 2000, 2002, 2003; Gotoh et al. 2006; Ros and Breeuwer 2009). Bidirectional incompatibility is assumed to be caused by either negative nuclear-nuclear genes interactions, as has been reported in the spider mite *Panonychus mori* Yokoyama (Gotoh et al. 2005), or infection by different strains of *Wolbachia,* as is well documented for insects (Laven 1959, 1967; Mercot et al. 1995; O'Neill and Karr 1990; Clancy and Hoffmann 1996). Although males and females from different geographic origins were able to mate, we did not obtain F1 hybrids (except in the cross involving Beninese females and Brazilian males where very few sterile males were produced). This apparent lack of gene exchange among the three geographically isolated populations revealed a pattern of reproductive isolation among them. Moreover, the crossing data appeared to corroborate the previously known biological differences observed among these three geographic populations (Famah et al. in prep).

Taken together, we expect the reproductive incompatibility observed in this study, to be the result of genetic divergence due to allopatric differentiation among the populations investigated (Hurt and Hedrick 2003;

Dettman et al. 2008) rather than to be caused by *Wolbachia*. Even if endosymbionts would be present, there is no guarantee that they were the cause of reproductive isolation because some *Wolbachia* strains are incapable of inducing reproductive incompatibility in their host (Giordano et al. 1995; Turrelli and Hoffmann 1995; Gotoh et al. 2005). For example, Gotoh et al. (2005) observed bidirectional reproductive incompatibility between two Japanese populations of *P. mori* (from Hanayama and Toyama) although harbouring the same *Wolbachia* strain. In this case, the procedure suggested by Breeuwer (1997) i.e. crossing experiments in combination with antibiotic treatment should be used to demonstrate whether they are involved in the reproductive incompatibility. Reproductive isolation is generally thought to develop by the gradual accumulation of genetic differences between populations as a by-product of other adaptive or neutral genetic changes that take place in allopatry (Mayr 1963; Charlesworth et al. 1987; Coyne 1992, 1993; Wu and Davis 1993; Drès and Mallet 2002; Gavrilets 2003; Coyne and Orr 2005; Dettman et al., 2008). Moreover, reproductive isolation has been shown to represent an initial step in the speciation process by preventing or greatly reducing gene flow between populations (Laven 1959, 1967; Conner and Saul 1986; Thompson 1987; 1995; Dettman et al. 2008), and our data are therefore consistent with incipient allopatric speciation (Drès and Mallet 2002; Gavrilets 2003; Coyne and Orr 2005) among these three geographically isolated specimens identified as *N. paspalivorus*.

In conclusion, we found that three geographically isolated populations of predatory mites preliminarily identified as *N. paspalivorus*, show reproductive isolation indicating that they are distinct biological species despite morphological similarity. Based on the crossing data reported in this study, we propose that there is every reason to screen for the presence of endosymbionts and to perform molecular characterization in order to test the hypothesis on allopatric speciation. It would be also informative to use molecular methods for determining the origin and invasion route of these predatory mites in comparison with what is already known for *A. guerreronis* (Navia et al. 2005), i.e the prey of these predatory mites and the pest of coconut palms. By including more geographic populations, our work may provide a basis for inferring the intercontinental and between-country invasion of these predatory mites and creates a solid basis for finding the best strains for developing biological control of coconut mites.

Acknowledgements

We thank R. Houndafoche, P. Sovimi, C. Kededji and B. Bovis for their assistance in maintaining the predator colonies and conducting experiments. We also thank A. Onzo, D. Gnanvossou and M. Toko for their encouragements and advices. Many thanks go to Sam Korie for providing assistance on the statistical analysis of the data, and to Fabien Hountondji for his comments on the manuscript. The authors also thank M.G.C. Gondim Jr, for helping to locate and for supplying the specimens of *N. paspalivorus* from Brazil. This research was supported by the International Institute of Tropical Agriculture (IITA) and the University of Amsterdam, the Netherlands, through a grant from the Board of the Netherlands Foundation for the advancement of Tropical Research (WOTRO). This study is part of a PhD thesis of the senior author.

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Figure captions

Fig.1 Principal component analysis based on 32 morphometric characters measured on adult females (**a**) and (**b**) males from three geographic specimens identified as *Neoseiulus paspalivorus*: Polygons formed based on the projection of the individuals of each population onto the principal components 1and 2

Fig.2 Canonical discrimination analysis of 32 morphometric characters measured on adult females (**a**) and (**b**) males from three geographic populations of *Neoseiulus paspalivorus*: Polygons formed based on the projection of the individuals of each population onto the canonical variable 1 and 2.

Table 1: Outcome of crosses involving three geographic populations identified as *Neoseiulus paspalivorus*

Cross *Female* x *male*	N	% ovipositing females	Pre-oviposition period (±SE)	No. eggs /female (±SE)	% deformed eggs/female	% egg hatchability	Immature survivorship (±SE)	Sex ratio (% female) (±SE)
Intra-population crosses								
Brazil x Brazil	20	100	1.6 ± 0.19c	12.8 ± 0.64a	0	100	99.0±0.65	71.9±3.92
Benin x Benin	20	100	1.8 ± 0.26c	12.2 ± 0.48a	0	100	99.4±0.51	71.8±1.11
Ghana x Ghana	15	100	1.9 ± 0.19c	10.1 ± 0.55b	0	100	100	74.1±4.01
Inter-population crosses								
Brazil x Benin	30	83.3	3.8 ± 0.31b	5.2 ± 0.33c	86	7.6	100	0.0*
Benin x Brazil	30	93.3	2.9 ± 0.25b	5.7 ± 0.55c	100	0.0	-	-
Brazil x Ghana	15	100	5.4 ± 0.13a	3.8 ± 0.29c	100	0.0	-	-
Ghana x Brazil	15	93.3	5.2 ± 0.22a	4.2 ± 0.41c	100	0.0	-	-
Benin x Ghana	10	100	3.1 ± 0.10b	5.8 ± 0.66c	100	0.0	-	-
Ghana x Benin	10	80	5.1 ± 0.54a	3.9 ± 0.84c	100	0.0	-	-

Means followed by the same letter in a column are not significantly different (SNK test $p < 0.05$)
N= number of pair crosses
*All progeny are males

Table 2: Mean, standard error and range of 32 morphometric variables measured on adult females of three geographic populations identified as *N. paspalivorus.* All measurements in micrometers

	Benin		Ghana		Brazil		Holotype (De Leon
Morphological characters	Mean ± SE	Min – Max	Mean ± SE	Min – Max	Mean±SE	Min – Max	1957)
Length of dorsal shield	343.1 ± 0.99 a	336 – 352	338.2 ± 0.73 c	333 – 342	340.6 ± 0.74 b	336 – 349	340
Width of dorsal shield	137.9 ± 0.96 a	130 – 146	138.8 ± 0.78 a	130 – 146	138.3 ± 0.98 a	127 – 143	140
j1	11.4 ± 0.13 a	10 – 12	11.5 ± 0.15 a	10 – 12	10.3 ± 0.12 b	10 – 11	11
j3	10.5 ± 0.14 b	10 – 12	11.4 ± 0.18 a	10 – 12	10.1 ± 0.22 b	8 – 11	11
j4	9.3 ± 0.16 a	8 – 10	9.4 ± 0.23 a	8 – 11	9.0 ± 0.17 a	10 – 12	9
j5	9.0 ± 0.13 a	8 – 10	9.5 ± 0.24 a	8 – 11	9.2 ± 0.16 a	8 – 10	10
j6	9.8 ± 0.12 b	9 – 11	10.4 ± 0.18 a	9 – 11	9.6 ± 0.17 b	8 – 10	11
J2	10.9 ± 0.17 a	9 – 12	11.1 ± 0.16 a	10 – 12	10.4 ± 0.13 b	10 – 11	11
J5	9.0 ± 0.12 b	8 – 10	9.9 ± 0.23 a	8 – 11	9.3 ± 0.14 b	9 – 10	9
z2	9.3 ± 0.19 b	8 – 10	10.7 ± 0.19 a	9 – 12	9.6 ± 0.13 b	9 – 10	11
z4	10.3 ± 0.17 b	9 – 11	11.0 ± 0.17 a	9 – 12	10.8 ± 0.14 b	10 – 11	11
z5	9.2 ± 0.13 b	9 – 10	10.0 ± 0.12 a	9 – 11	8.8 ± 0.17 b	7 – 10	9
Z1	11.1 ± 0.20 a	10 – 14	11.4 ± 0.20 a	9 – 12	10.1 ± 0.21 a	9 – 12	11
Z4	17.0 ± 0.21 a	15 – 18	17.0 ± 0.19 a	15 – 19	16.0 ± 0.17 b	15 – 17	17
Z5	54.6 ± 0.36 a	51 – 56	50.2 ± 0.40 a	48 – 52	52.5 ± 0.27 b	51 – 55	52
s4	12.5 ± 0.15 a	11 – 14	12.4 ± 0.16 a	11 – 15	11.8 ± 0.19 b	10 – 12	12
S2	13.6 ± 0.16 a	12 – 15	13.9 ± 0.20 a	12 – 15	12.8 ± 0.17 b	11 – 14	14
S4	14.5 ± 0.19 b	14 – 16	15.3 ± 0.15 a	14 – 16	14.6 ± 0.18 b	14 – 16	15
S5	15.8 ± 0.18 b	15 – 18	18.7 ± 0.16 a	17 – 20	19.0 ± 0.17 a	18 – 20	19
r3	11.7 ± 0.22 b	10 – 14	13.8 ± 0.14 a	12 – 15	11.8 ± 0.19 b	11 – 14	11
R	11.0 ± 0.23 a	9 – 12	11.4 ± 0.16 a	10 – 12	10.1 ± 0.12 b	9 – 11	10
St IV	17.6 ± 0.20 b	16 – 19	18.4 ± 0.22 a	16 – 20	17.5 ± 0.19 b	16 – 19	-
ST1-ST3	80.8 ± 0.43 a	76 – 82	80.5 ± 0.42 a	79 – 86	80.7 ± 0.36 a	79 – 82	-
ST2-ST2	51.9 ± 0.42 ab	47 – 54	50.8 ± 0.48 b	47 – 54	52.6 ± 0.42 a	51 – 57	-
ST5-ST5	59.6 ± 0.37 a	57 – 63	58.3 ± 0.35 b	57 – 60	57.4 ± 0.39 b	54 – 60	-
VSW-ANT	81.4 ± 0.33 a	79 – 82	81.1 ± 0.48 a	79 – 85	82.0 ± 0.47 a	79 – 86	-
VSW-POST	73.5 ± 0.49 a	70 – 76	60.8 ± 1.11 b	54 – 73	73.8 ± 0.40 a	70 – 76	-
Length of ventrianal shield	103.6 ± 0.56 a	98 – 108	107.8 ± 0.57 a	101 – 111	102.2 ± 0.75 b	95 – 108	-
Length of fixed digit	17.8 ± 0.19 a	17 – 19	18.0 ± 0.27 a	16 – 19	18.3 ± 0.19 a	17 – 19	-
Length of movable digit	23.7 ± 0.19 a	23 – 25	23.1 ± 0.38 a	22 – 25	23.3 ± 0.27 a	21 – 25	-
Length of calyx	7.8 ± 0.18 a	6 – 9	8.2 ± 0.17 a	6 – 9	8.0 ± 0.16 a	6 – 9	-
Diameter of calyx	6.8 ± 0.14a	6 – 7	6.6 ± 0.18 a	5 – 7	6.3 ± 0.10 a	6 – 7	-

VSW-ANT, Width of ventrianal shield at level of ZV2; VSW-POST, Width of ventrianal shield at anus

Means with same letter in a row are not significantly different (SNK; $P < 0.05$

Table 3: Mean, standard error and range of 32 morphometric variables measured on adult males of three geographic populations identified as *N. paspalivorus.* All measurements in micrometers

	Benin		Ghana		Brazil		Holotype (De Leon
Morphological characters	Mean ± SE	Min – Max	Mean ± SE	Min – Max	Mean ± SE	Min – Max	1957)
Length of dorsal shield	257.1 ± 1.73 a	250 – 269	257.7 ± 1.16 a	253 – 263	256.8 ± 2.11 a	241 – 263	260
Width of dorsal shield	124.5 ± 0.67 a	120 – 127	117.6 ± 0.87 b	114 –120	124.0 ± 0.56 a	120 – 127	120
j1	9.6 ± 0.19 a	8 – 10	9.8 ± 0.25 a	9 – 11	10.0 ± 0.12 a	9 – 10	-
j3	10.0 ± 0.26 a	8 – 11	10.2 ± 0.12 a	9 – 11	10.3 ± 0.16 a	9 – 11	-
j4	7.8 ± 0.25 b	6 – 9	7.6 ± 0.22 b	6 – 9	8.5 ± 0.16 a	7 – 9	-
j5	7.8 ± 0.26 a	6 – 9	8.1 ± 0.21 a	7 – 9	8.4 ± 0.19 a	7 – 9	-
j6	8.6 ± 0.22 a	7 – 10	9.4 ± 0.27 a	8- 11	9.0 ± 0.36 a	7 – 10	-
J2	9.2 ± 0.33 a	8 – 11	9.6 ± 0.19 a	8 – 10	10.0 ± 0.26 a	9 – 11	-
J5	7.0 ± 0.20 b	6 – 8	6.6 ± 0.19 b	6 – 8	7.6 ± 0.29 a	6 – 9	-
z2	8.2 ± 0.27 b	6 – 9	9.4 ± 0.20 a	8 – 10	8.6 ± 0.22 b	7 – 10	-
z4	9.1 ± 0.26 b	8 – 10	10.0 ± 0.18 a	9 – 11	9.8 ± 0.22 a	9 – 11	-
z5	7.3 ± 0.22 a	6 – 9	8.0 ± 0.20 a	7 – 9	7.8 ± 0.19 a	7 – 9	-
Z1	9.6 ± 0.19 a	8 – 10	9.2 ± 0.42 a	7 – 11	9.5 ± 0.33 a	9 – 10	-
Z4	14.4 ± 0.27 ab	12 – 15	14.0 ± 0.36 b	13 – 16	15.2 ± 0.31 a	14 – 16	-
Z5	40.4 ± 0.64 a	36 – 44	41.1 ± 0.65 a	38 – 44	40.0 ± 0.69 a	37 – 45	-
s4	10.5 ± 0.27 a	9 – 11	11.2 ± 0.18 a	10 – 12	10.8 ± 0.19 a	10 – 11	-
S2	11.2 ± 0.18 b	10 – 12	12.1 ± 0.19 a	11 – 13	11.4 ± 0.22 b	10 – 12	-
S4	12.2 ± 0.31 b	11 – 14	13.1 ± 0.33 a	11 – 15	11.8 ± 0.20 a	11 – 13	-
S5	12.7 ± 0.29 b	11 – 14	14.8 ± 0.34 a	13 – 16	15.0 ± 0.32 a	13 – 16	-
r3	9.6 ± 0.19 b	9 – 10	10.2 ± 0.25 ab	9 – 11	10.8 ± 0.26 a	10 – 12	-
R	9.1± 0.32 b	8 – 10	9.5 ± 0.20 ab	8 – 10	10.2 ± 0.25 a	9 – 11	-
St IV	15.0 ± 0.18 b	14 – 16	16.1 ± 0.22 a	15 – 17	15.3 ± 0.25 b	14 – 16	-
ST1-ST3	67.2 ± 0.63 a	63 – 70	66.2 ± 0.56 a	63 – 70	65.9 ± 0.42 a	63 – 66	-
ST2-ST2	40.2 ± 0.48 ab	38 – 41	40.0 ± 0.51 b	38 – 41	41.5 ± 0.31 a	41 – 44	-
ST5-ST5	33.6 ± 0.51 a	32 – 35	30.7 ± 0.67 b	28 – 35	33.0 ± 0.51 a	31 – 35	-
VSW-ANT	118.0 ± 1.62 a	111 – 127	117.0 ± 1.31 a	111 – 123	116.6 ± 1.31 a	108 – 120	-
VSW-POST	76.7 ± 1.55 a	70 – 82	75.1 ± 1.06 a	70 – 79	78.6 ± 0.92 a	73 – 82	-
Length of ventrianal shield	92.5 ± 0.92 a	89 – 98	94.4 ± 0.63 a	92 – 98	92.0 ± 1.05 a	85 – 95	-
Length of fixed digit	12.0 ± 0.20 b	11 – 12	12.8 ± 0.26 a	11 – 14	12.3 ± 0.25 ab	11 – 14	-
Length of movable digit	17.6 ± 0.30 a	16 – 19	17.2 ± 0.25 a	16 – 19	17.7 ± 0.22 a	16 – 19	-
Length of spermatod. shaft	11.7 ± 0.20 a	11 – 12	12.0 ± 0.20 a	11 – 12	11.8 ± 0.21 a	11 – 12	-
Length of spermatod. foot	4.6 ± 0.32 a	4 – 6	4.8 ± 0.25 a	4 – 6	4.5 ± 0.27 a	4 – 6	-

Spermatod, Spermatodactyl

Means with same letter in a row are not significantly different (SNK; $P < 0.05$

Table 4: Classification based on discriminant analysis of 32 morphological characters of three geographic populations of *N. paspalivorus* females and males

	Female				Male			
	% well-classified*	Benin	Ghana	Brazil	% well-classified*	Benin	Ghana	Brazil
Benin	90	18	0	2	60	6	2	2
Ghana	85	0	17	3	80	0	8	2
Brazil	85	2	1	17	50	0	5	5
Total	86.7	20	18	22	63.33	6	15	9

*Percentage of well-classified individuals in their original populations

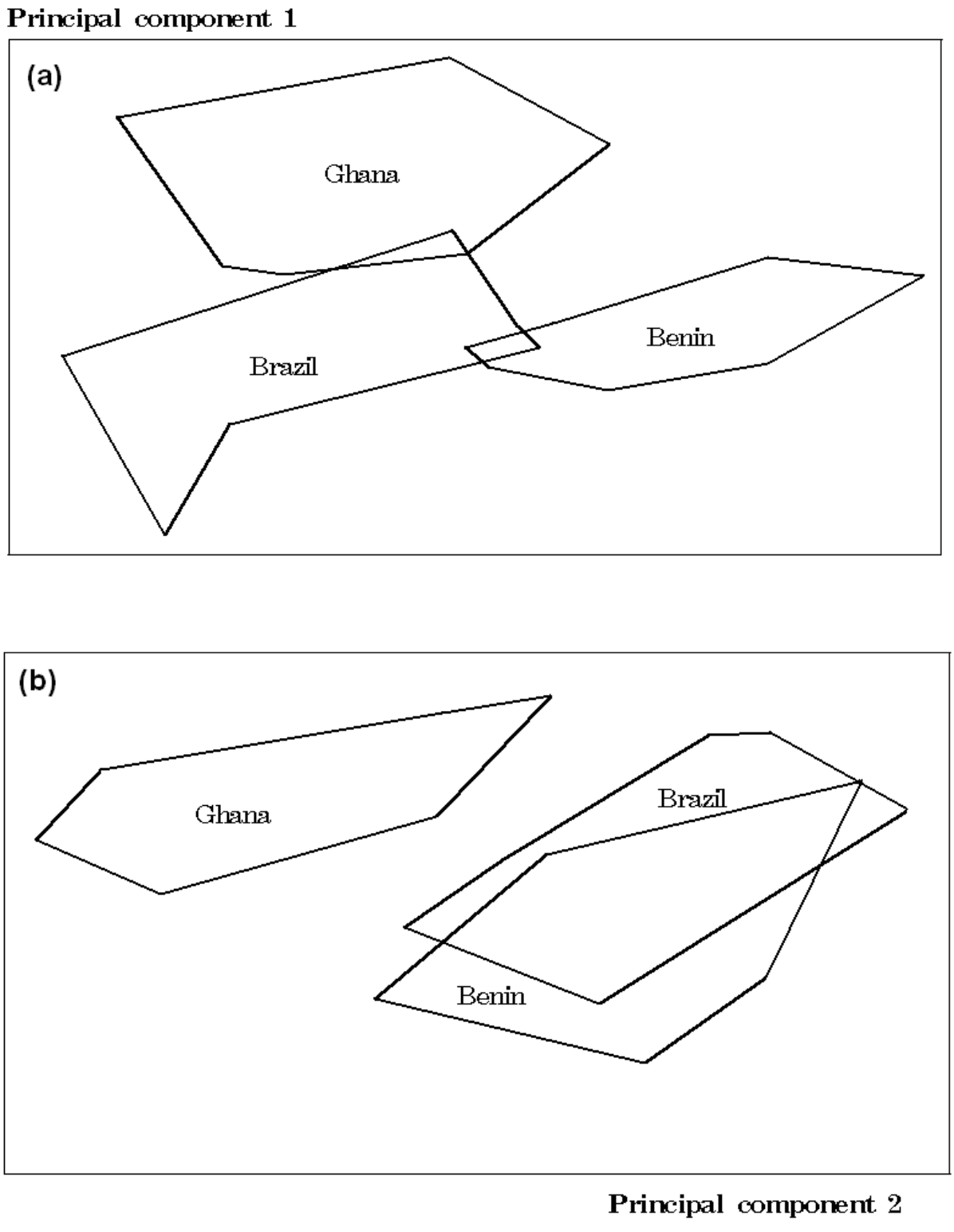
Principal component 1
(a)
Ghana
Brazil
Benin
(b)
Ghana
Brazil
Benin
Principal component 2

Figure 1

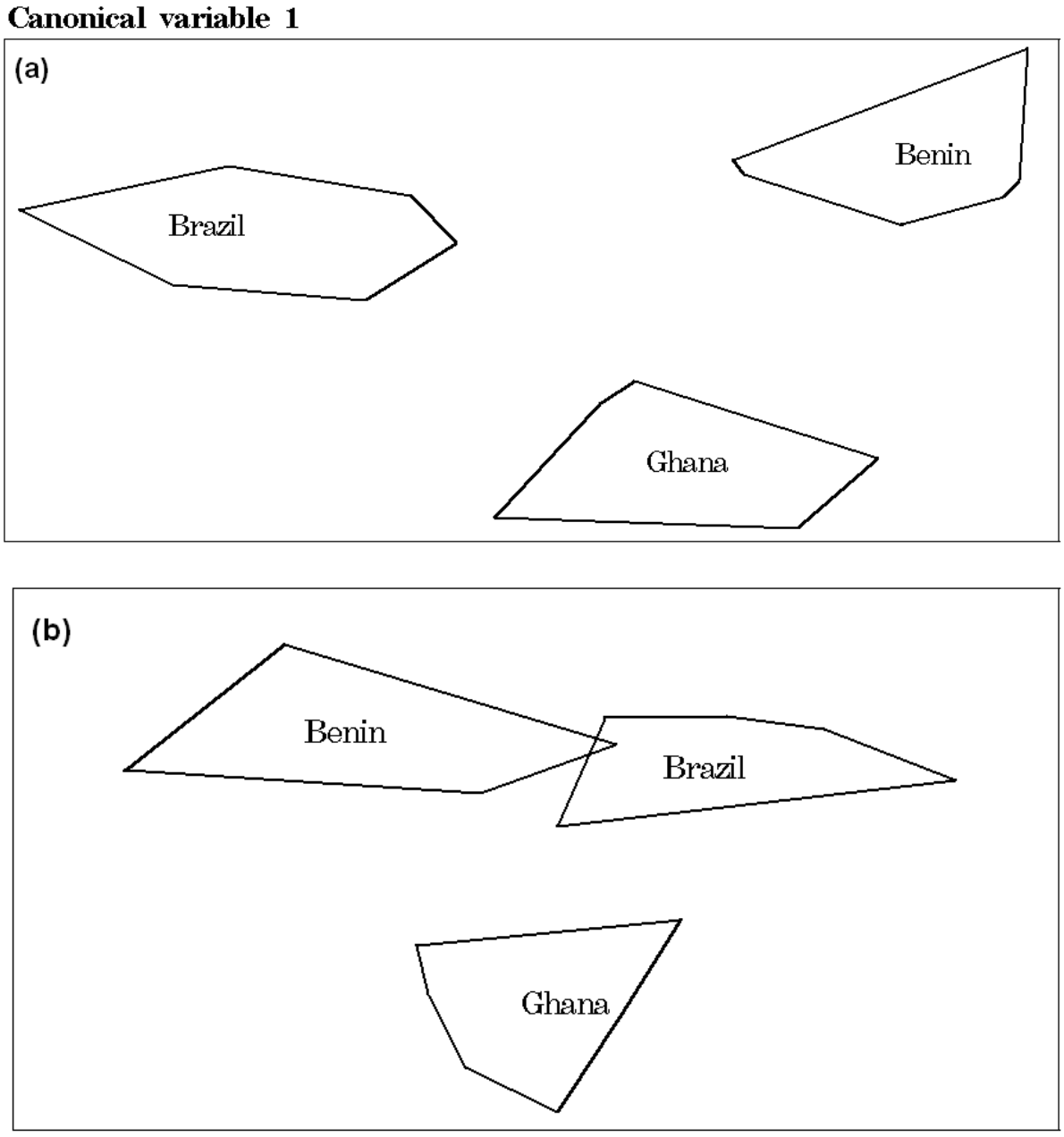
Canonical variable 1
(a)
Benin
Brazil
Ghana
(b)
Benin
Brazil
Ghana
Canonical variable 2

Figure 2