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A multidisciplinary approach to discriminating different taxa in the species complex *Pachycondyla villosa* (Formicidae)

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A multidisciplinary approach provides new evidence that strongly supports the differentiation of three taxa previously confounded in a single species, *Pachycondyla villosa*. All specimens studied were collected in the same location at Ilhéus, Bahia, Brazil. Bivariate plots of petiole heights vs. petiole lengths and isozyme patterns clearly differentiate the three taxa. Chain lengths and chemical structures of cuticular hydrocarbons are characteristic of each taxon. This congruent evidence shows that there are three different valid species. Ecological hypotheses are discussed to explain the benefits of these species remaining sympatric. © 2002 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2002, **75**, 249–259.

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INTRODUCTION

Phenotypic variation among sympatric insects may confound the correct identification of some species and further characterization in therefore needed. This is true for the ponerine ant, *Pachycondyla villosa,* described by Fabricius in 1804. *P. villosa* is one of the most common ants of the genus in the Neotropical Region. In recent years, *P. villosa* has been intensively studied in a number of biological fields including: histology and morphology (Camargo-Mathias & Caetano, 1991; Camargo-Mathias *et al.*, 1991; 1992b; 1992b; 1992b; Camargo-Mathias & Caetano, 1992a, 1992b; 1995a; 1995b; 1996; Mariano et al., 2000); regulation of reproduction (Heinze *et al*., 1996; Trunzer *et al.*, 1998); foraging behaviour (Lachaud *et al.*, 1984; Hölldobler, 1985; Lachaud & Fresneau, 1985; Dejean & Corbara, 1990a, 1990b; Dejean *et al.*, 1990; Valenzuela-Gonzalez *et al.*, 1994; Dejean & Corbara, 1998); and ecology (Pérez-Bautista *et al.*, 1985; Dejean, 1990).

Discrepancies in behaviour and ecology between the mentioned studies, suggest some heterogeneity. For example, in Yucatan, Mexico, where *P. villosa* invariably lives with epiphytes, Dejean (1990) observed a strong tendency of young queens to nest in Bromelia epiphytes of the genus *Aechmea*. A number of other papers refer to a similar mode of nesting (Dejean & Corbara, 1990a, 1990b; Dejean *et al*., 1990; Camargo-Mathias & Caetano, 1991; 1992a; 1992b; 1995a; 1995b; 1996). Conversely, other studies refer to hollow branches of living or dead trees, abandoned preexisting nests, or rotten cocoa pods as the most characteristic nesting places (Lachaud *et al*., 1984; Hölldobler, 1985; Pérez-Bautista *et al*., 1985; Valenzuela-Gonzalez *et al*., 1994; Heinze *et al*., 1996; Trunzer *et al*., 1998).

During our current study on colonies identified as *P. villosa*, consistent morphological differences were ob-

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served. This led us to refer to the taxon *Ponera inversa*, that was originally described by Smith in 1858, but was later related to *Pachycondyla villosa* as a subspecies by Forel (1899), Emery (1904, 1911), Kempf (1972) and Bolton (1995). According to Kempf (1972) and Bolton (1995), it is the only valid subspecies of this taxon and has approximately the same geographical range as *Pachycondyla villosa*, i.e. from Texas (Forel, 1899) to Argentina via Mexico (Kempf, 1972).

Biological, as well as taxonomic, data provided by the literature seem to have been intermingled, this suggests that some distinction must be made within the species complex *P. villosa*.

The available species keys are based mainly on morphology and a limited number of specimens. Recent technical developments in chemical analysis allow to distinguish between species using epicuticular hydrocarbon compositions (Lockey, 1991; Howard, 1993; El Messoussi *et al*., 1994). In this study, one individual is sufficient to distinguish species. For example, several taxonomic studies were published on termites (Haverty *et al*., 1988; Bagnères *et al*., 1990; Haverty *et al*., 2000), fruit flies (Bartelt *et al*., 1986; Jallon & David, 1987; Lemeunier *et al*., 1997), tsetse flies (Nelson *et al*., 1988), and ants (Brill *et al*., 1985; Yamaoka, 1990). Furthermore, isozyme electrophoresis and other biochemical techniques have also been used to successfully define taxa in species groups (Ward, 1980; Halliday, 1981; Douwes & Stille, 1987).

In this study, we report here new morphometric, biochemical and chemical data which allow better characterization and differentiation of three taxa, certainly three valid species, within populations recently considered a single species, *P. villosa*.

MATERIAL AND METHODS

Ants

Queen-right colonies were collected from sympatric populations in the experimental fields of the Cocoa Research Center at Ilhéus, Bahia, Brazil. All colonies were reared in the laboratory in artificial nests for at least 6 months before analysis. The nests were maintained at 27 ± 1°C, with approximately 60–80% relative humidity, and a 12L:12D photoperiod. All colonies were provided with an identical diet (honey/apple mixture, *Calliphora* sp. larvae, cockroaches and crickets) twice a week.

Statistical analysis

Bivariate plots of morphometric measurements were performed by Statistica 5.0 software (Statsoft Inc., Tulsa, OK, USA). To compare biometric data, we used a non-parametric test (Mann–Whitney *U-test*) with the sequential Bonferroni correction of the *P*-values. Principal component analysis (PCA) was performed, on normalized variables for a better comparison, with SPAD 3.0 software (Cisia-Ceresta, Cedex, France).

Sample preparation for scanning electron microscopy

Three colonies per species were studied. From each colony, three were taken and killed by freezing, cleaned in an ultrasonic-wave bath, and dehydrated using increasing concentrations of ethanol. The individuals were air-dried and then placed on aluminium stubs. They were coated with a mixture of 80% gold / 20% palladium and examined with a Leica Stereoscan 440 scanning electron microscope (Cedex, France) at 10 kV.

Biometric study

A total of 86 workers taken from nine colonies (three for each species) were measured with an ocular micrometer on a Leica MZFL3 binocular microscope. The seven recorded measurements were taken where the studied part was the largest: length of the right scape (SL, magnification × 32); dorsal width of head, including eyes (HW, magnification × 32); dorsal petiolar width (PW, magnification × 63); petiolar length (PL, magnification × 63) in lateral view from the lateral flanges of the anterior peduncle to the posterior margin of the petiole (Ward, 1985); diagonal length of thorax (TL, magnification × 20) in lateral view; dorsal width of thorax (TW, magnification × 50);and finally height of the anterior face of petiole in lateral view (PHaf, magnification × 63) from the anterior margin of petiole to the maximum height of the node (Fig. 1).

Extraction procedure and characterization of cuticular hydrocarbon*s*

Five to 10 workers per colony were selected from nine colonies (three for each species) with clean forceps, placed individually into chilled vials and killed by freezing at –20°C. Cuticular hydrocarbons were extracted from each whole individual by immersion for 5 min in 200 µL of hexane with three-dimensional agitation. The samples were dried under nitrogen and dissolved in 50 µL of hexane.

Each individual sample was analysed by gas chromatography-mass spectrometry (GC-MS) carried out with a Fisons mass spectrometer MD 800 (electron impact at 70 eV) coupled directly with a Carlo Erba gas chromatograph GC 8065MS (Thermo-Finnigan, Cedex, France). The GC-MS was fitted with a 25QC2 BP1 methylsilicone capillary column (25 m × 0.22 mm, ID × 0.1 µm). 5 µL of sample. The oven temperature

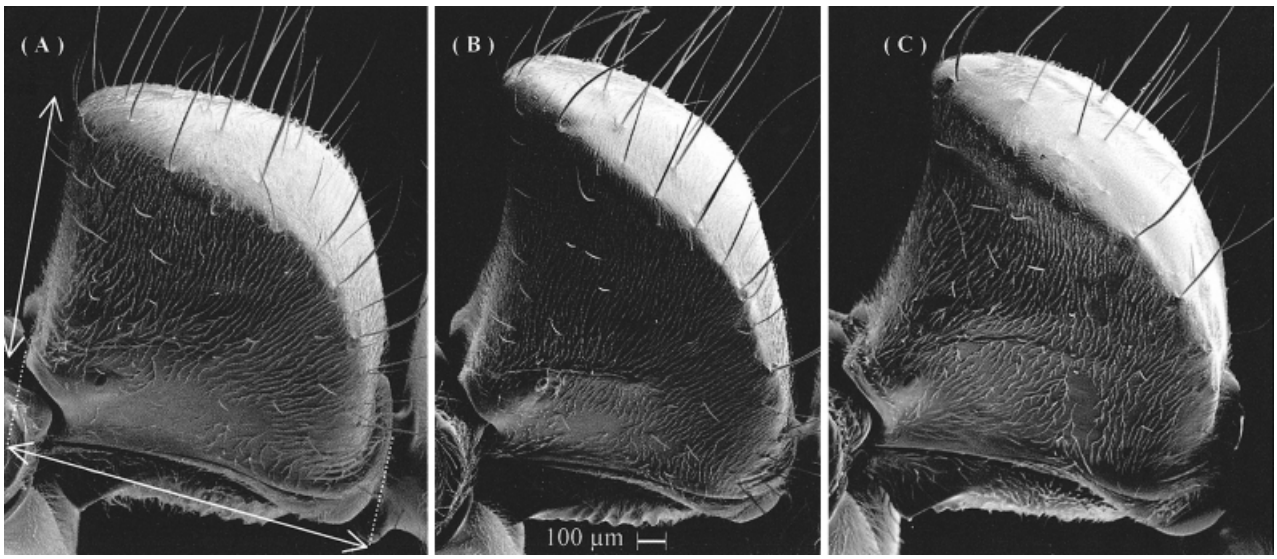

Figure 1. Scanning electron microscopy photographs of the left side of workers' petioles of Pvv (A), Pvi1 (B) and Pvi2 (C).Magnification × 152. Vertical white arrow: PHaf, horizontal white arrow: PL.

was increased 1 min after injection from 60 to 225°C at 20°C min^{-1} and from 225 to 325°C at 3°C min^{-1} (isotherm 10 min). Data were integrated using an IBM-PC with MassLab 1.27 , data acquisition, plotting and analysis software. Masses were scanned between 40 and 700 amu at 0.45 scan s^{-1}. The mass spectra of alkanes were interpreted according to the criteria proposed by McCarthy *et al*. (1968), Nelson *et al*. (1972), Nelson (1978), and Pomonis *et al*. (1978, 1980).

Isozyme electrophoresis

Head, thorax and gaster of individual workers were ground separately in 50 µL of distilled water. The homogenate (6–14 µL) was subsequently applied onto presoaked cellulose acetate plates. Protein separation was carried out in one of three buffer systems (0.1 M Tris-citrate pH 8.2, Tris-maleate-EDTA pH 7.4 and pH 8.3) with 1–7 mA per gel for 5–35 min, depending on the enzyme system. Using slightly modified protocols from Murphy *et al*. (1990), 29 enzymes were stained, six of which appeared to be of diagnostic value: alkaline phosphatase (ALP), esterases (EST), glucose-6-phosphate isomerase (GPI), isocitrate dehydrogenase (IDH), trehalase (TRE) and xanthine dehydrogenase (XDH). One to six workers were investigated in each of the 58 colonies except for ALP (five colonies) and XDH (15 colonies).

RESULTS

All results allow us to distinguish three taxa in the species complex *Pachycondyla villosa*: the *Pachycondyla villosa villosa* (Pvv), the taxa provisionally called *Pachycondyla villosa inversa* phenotype 1 (Pvi1), and *Pachycondyla villosa inversa* phenotype 2 (Pvi2).

Morphology

Workers in the species complex *P. villosa* are morphologically very similar, with a dark colouration except for mandibles, legs and other ventral parts of the ants. A dark reddish colouration is most frequently observed among individuals of Pvv and also in a few cases of Pvi2, where the red is less intense. Taxa may be differentiated more precisely by the morphology of the petiole. In lateral view, the petiole of Pvv is globally as long as it is high, its anterior face is straight or weakly concave and forms a right angle (sometimes a little rounded) with the dorsal face (Fig. 1A). In Pvi1, the petiole is higher than its length, the anterior face is highly concave (Smith, 1858) and forms an acute angle (±60°) with the dorsal face (Fig. 1B). For Pvi2, it is as high as, or slightly higher than its length, the anterior face is concave and forms a weak acute angle with the dorsal face (Fig. 1C). This lack of clearly defined morphological and colour differences, especially between Pvv and Pvi2, may have been responsible for the current confusion regarding the taxonomy of the species complex *Pachycondyla villosa*. This confusion highlights the requirement of quantitative data in order to obtain a definitive characterization of each taxon.

BIOMETRY

All measures (Fig. 2) were statistically significantly different ($P < 0.05$) between any two taxa with two exceptions: TL and PL were not significantly different between Pvv and Pvi2. All other measurements, except PHaf, were larger for Pvv. As for Pvi1, all measures were significantly the smallest with two exceptions: PW is the smallest in Pvi2 and PHaf in Pvv.

Bivariate plots of morphometric measurements revealed that only the plot of PHaf vs. PL clearly differentiated the three taxa (Fig. 3).

CHEMICAL DATA

The analysis of cuticular hydrocarbons was very useful for the final distinction between Pvv, Pvi1 and Pvi2. Their hydrocarbons (HCs) have even- or odd-numbered chain lengths, are saturated or unsaturated, linear or branched with branching occurring indifferently on even- or odd-numbered carbon atoms. Internal monomethylalkanes (starting from 7-methyl) appear as mixtures of unresolved isomers among which the 11- to 13- isomers are dominant. Both types make up homologous series in each species. Dimethylalkanes are also observed as mixtures, but there is more diversity in branchings. Unsaturated HCs are linear or internally methylbranched monoenes separated from the corresponding alkanes by approximately –0.3 equivalent chain length (ECL). *n*-alkenes are easily identifiable, whereas the mass spectra of methylalkenes are more complex with clusters of diagnostic fragments 1–3 amu lower than the corresponding doublets of methylalkanes, as well as additional clusters resulting from β-cleavage at the branching points. Furthermore, with the presence of isomers under the same peak, the interpretation of the spectra may be more complex. Nevertheless, their general patterns and molecular masses are suggestive of the same methyl branching, as it has been found in the immediate following saturated methylalkanes. A more precise structural determination, as well as double bond location, would need their isolation and chemical modification, they are therefore included as Xi-MeCn:1 in Table 1.

The range of the prevalent HCs' chain lengths (mean percentage >2) differs from one subspecies to another: 25–27 C for Pvi2, 27–30 C for Pvi1 and 29–41 C for Pvv. Indeed, only 3-MeC29 (peak 53) is common to Pvv and Pvi1. The chromatographic profile of each taxon is therefore characteristic (Fig. 4).

Table 1 gathers the percentages of the individual HCs present in any of the three subspecies. In Pvi2, n-C25 and a series of monomethyl- and dimethylpentacosanes represent 90% of the total HCs. The bulk of monomethylalkanes are end-branched whereas the dimethylalkanes comprise almost exclusively one series of isomers with one external and one internal branch. If we also consider the minor components, the same scheme is discernible for different chain lengths. A trimethylalkane (5,13,17-triMeC25) is present in small quantities only in this subspecies, whereas no unsaturated HCs are observed.

In Pvi1, apart from the preponderance of *n*-alkanes and monomethylalkanes (3- and internally branched), two features are to be noted: the presence in small abundance of dimethylalkanes (2.67%), and the significant percentage of some isomeric internal monomethylalkenes (10.11%), particularly methylnonacosenes (6.44%).

In Pvv, cuticular HCs occurred in two distinct groups: early eluting external monomethylalkanes (42, 52, 53, 58 and 63) representing 15.37% and late eluting compounds amounting to 81.29% (Fig. 4). This latter group may be divided into two characteristic

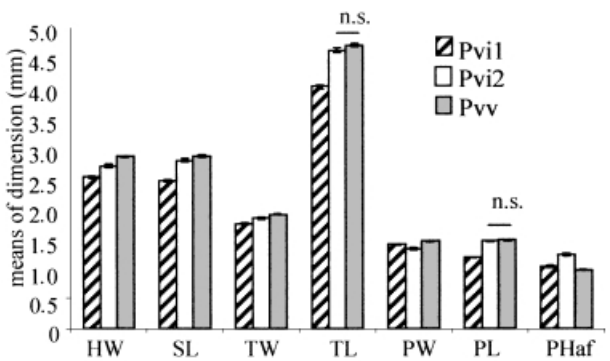

Figure 2. Mean (± SD) of the biometric measurements of Pvi1, Pvi2 and Pvv ($n = 26$, 30 and 30, respectively). There are significant differences ($P < 0.05$) between measures of each group except for the two groups marked ns.

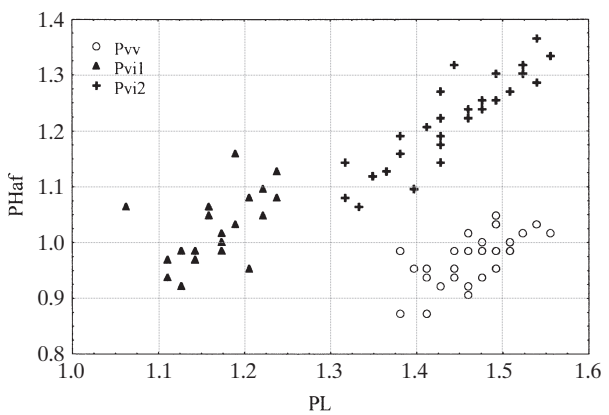

Figure 3. Bivariate plots of PHaf by PL measurements (mm) of Pvv, Pvi1 and Pvi2.

Table 1. Percentages of cuticular hydrocarbons of workers of Pvv, Pvi1 and Pvi2 in order of increasing retention times. Mean values ± SD, n = 22, 24 and 19, respectively; **bold type** > 2%; trace (tr) < 0.1%

Peak	Compound	Pvv	Pvi1	Pvi2	Peak	Compound	Pvv	Pvi1	Pvi2
1	nC23	0	0	tr	42	**4-,2-MeC28**	**2.23 (0.35)**	1.25 (0.16)	0
2	11-,9-,7-MeC23	0	0	tr	43	3-MeC28	0	0.41 (0.04)	0
3	5-MeC23	0	0	tr	44	nC29 : 1	0	0.87 (0.22)	0
4	3-MeC23	0	0	tr	45	**nC29**	1.65 (0.31)	**8.61 (1.41)**	0.10 (0.02)
5	5,15-; 5,17-; 7,15-diMeC23	0	0	tr	46	**Xi-MeC29 : 1**	0	**6.44 (1.38)**	0
6	nC24	0	0	0.17 (0.04)	47	**15-,13-,11-,9-,7-MeC29**	0.29 (0.07)	**14.45 (1.53)**	0.42 (0.17)
7	12-,11-,10-,9-,8-,7-MeC24	0	0	tr	48	5-MeC29	0	tr	tr
8	6-,5-MeC24	0	0	tr	49	diMeC29 : 1	0	0.61 (0.29)	0
9	4-,2-MeC24	0	0	tr	50	9,13-; 11,15-diMeC29	0	1.14 (0.31)	0
10	3-MeC24	0	0	0.13 (0.07)	51	5,17-diMeC29	0	0	0.17 (0.14)
11	**nC25**	0	0.32 (0.05)	**16.48 (1.97)**	52	2-MeC29	tr	0	0
12	**13-,11-,9-MeC25**	0	tr	**5.88 (0.60)**	53	**3-MeC29**	**7.73 (0.79)**	**5.80 (0.67)**	tr
13	**7-MeC25**	0	tr	**3.48 (0.29)**	54	nC30	0.74 (0.12)	tr	0
14	**5-MeC25**	0	0	**11.05 (0.56)**	55	3,X-diMeC29	0	0.20 (0.05)	0
15	**3-MeC25**	0	tr	**21.49 (1.38)**	56	Xi-MeC30 : 1	0	tr	0
16	**5,19-; 5,17-; 5,15-; 5,13-diMeC25**	0	0	**31.65 (1.84)**	57	15-,14-,13-,12-,11-,10-MeC30	0	0.31 (0.06)	0
17	nC26	0	0.54 (0.09)	0.92 (0.34)	58	**2-MeC30**	**3.99 (0.48)**	0	0
18	3,17-; 3,15-; 3,13-diMeC25	0	0	1.27 (0.16)	59	nC31	0.36 (0.07)	0	0
19	5,13,17-triMeC25	0	0	0.41 (0.08)	60	Xi-MeC31 : 1	0	0.43 (0.13)	0
20	13-,12-,11-,10-,9-,8-,7-MeC26	0	0.18 (0.05)	0.42 (0.06)	61	15-,13-,11-MeC31	0.17 (0.05)	0.86 (0.11)	0
21	6-,5-MeC26	0	tr	0.22 (0.03)	62	11,15-diMeC31	0	tr	0
22	4-,2-MeC26	0	0.35 (0.09)	tr	63	3-MeC31	1.40 (0.19)	0	0
23	3-MeC26	0	0.15 (0.05)	tr	64	11-,13-MeC33	tr	0	0
24	6,18-; 6,14-; 5,17-diMeC26	0	0	0.21 (0.02)	65	13,17-diMeC33	tr	0	0
25	4,18-; 4,16-; 4,14-diMeC26	0	0	tr	66	Xi-MeC35 : 1	0.17 (0.05)	0	0
26	nC27 : 1	0	0.97 (0.20)	0	67	17-,15-,13-MeC35	0.52 (0.11)	0	0
27	**nC27**	0	**21.87 (1.56)**	1.31 (0.29)	68	diMeC35 : 1	tr	0	0
28	Xi-MeC27 : 1	0	0.44 (0.12)	0	69	**13,19-; 13,17-diMeC35**	**6.42 (0.64)**	0	0
29	3,13-; 3,15-diMeC26	0	0	tr	70	13,23-diMeC35	0	0	0.13 (0.05)
30	**13-,11-,9-,7-MeC27**	0	**13.78 (2.35)**	1.20 (0.14)	71	14,18-; 12,16-diMeC36	0.59 (0.12)	0	0
31	5-MeC27	0	tr	0.40 (0.18)	72	Xi-MeC37 : 1	1.18 (0.33)	0	0
32	9,13-; 11,15-diMeC27	0	0.33 (0.09)	0	73	**19-,17-,15-,13-MeC37**	**5.42 (0.30)**	0	0
33	5,19-; 5,17-; 5,15-; 5,13-diMeC27	0	0.13 (0.05)	1.13 (0.24)	74	**15,19-; 13,17-diMeC37**	**54.69 (3.37)**	0	0
34	**3-MeC27**	0	**13.58 (1.41)**	1.03 (0.27)	75	13,25-; 15,23-diMeC37	0	0	0.12 (0.04)
35	nC28 : 1	0	0.11 (0.03)	0	76	14,18-; 13,17-diMeC38	0.44 (0.12)	0	0
36	**nC28**	tr	**3.12 (0.30)**	tr	77	Xi-MeC39 : 1	0.90 (0.30)	0	0
37	3,11-; 3,9-; 3,7-diMeC27	0	0.85 (0.18)	0	78	**19-,17-,15-,14-,13-MeC39**	**3.03 (0.42)**	0	0
38	3,19-; 3,17-; 3,15-diMeC27	0	0	tr	79	**13,19-diMeC39**	**7.47 (0.76)**	0	0
39	Xi-MeC28 : 1	0	0.14 (0.04)	0	80	21-,19-,15-,13-MeC41	0.13 (0.06)	0	0
40	14-,13-,12-,11-,10-MeC28	0	1.58 (0.12)	0	81	15,21-; 13,19-diMeC41	0.20 (0.09)	0	0
41	6-MeC28	0	0.12 (0.02)	0	82	13-,19-diMeC43	tr	0	0

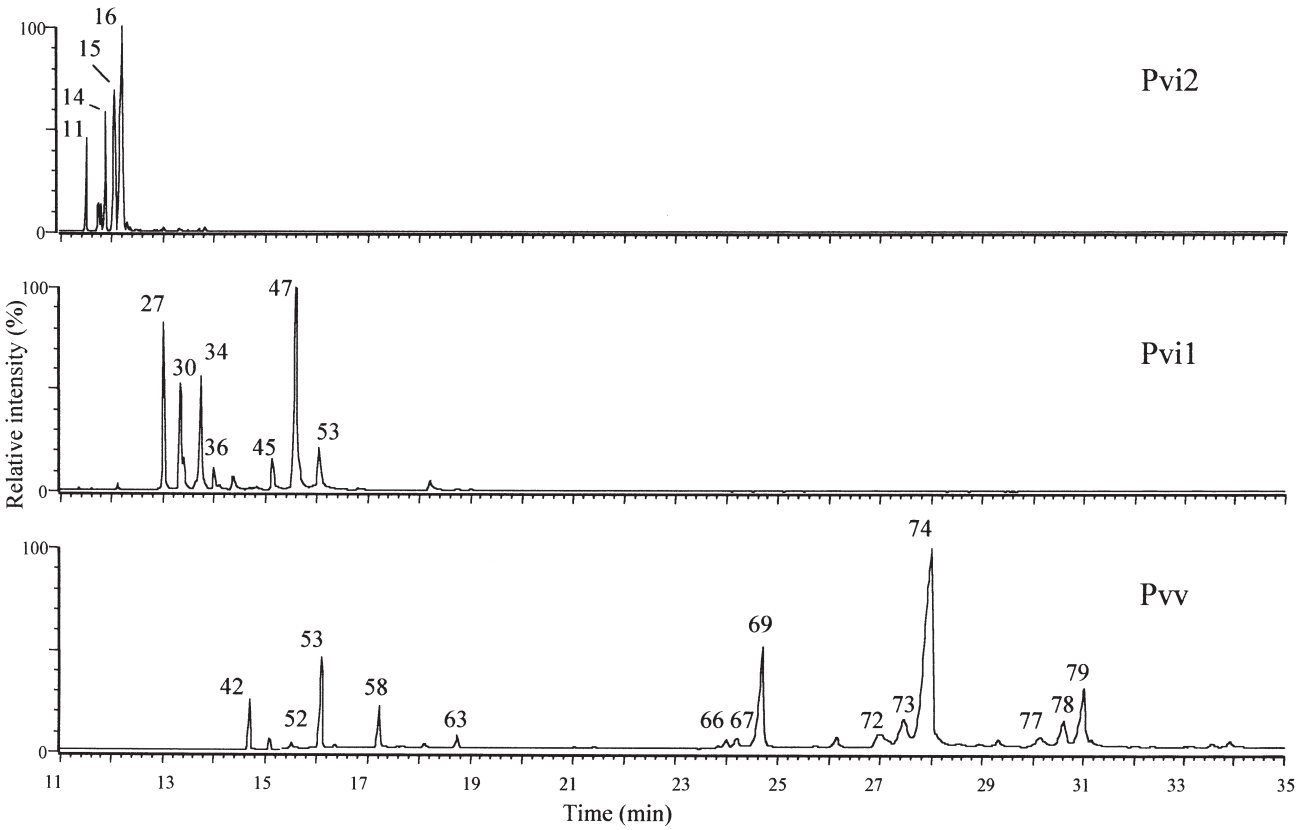

Figure 4. Total ion chromatograms of the HCs from Pvv, Pvi1 and Pvi2. Numbers refer to the peaks listed in Table 1.

subgroups, each of which associating a Xi-methylalkene (66, 72 and 77), a 13-methylalkane (67, 73 and 78) and a 13,17- and/or 13,19-dimethylalkane (69, 74 and 79), in order of increasing abundance (2.25, 8.97 and 68.58%, respectively). On the whole, beside monomethylalkanes (25%), the most important compounds in Pvv are dimethylalkanes (70%) with two internal branches, whereas those of Pvi2 (35.36%) have both external and internal branches. On the contrary, *n*-alkanes and methylalkenes are only minor ones.

The PCA of the percentages of the cuticular HCs supports the distinction between the three taxa (Fig. 5). The variables were normalized during the analysis in order to obtain a better comparison. The first two factors, F1 and F2, represent, respectively, 29 and 23% of the total variance. This analysis involving 82 compounds, the cumulative percentage of these two factors (52%), is a good index of reliability. The plot of the coordinates of all the individuals against the F1 and F2 axes shows three clearly separated and rather elongated clusters, each of which corresponding to only one subspecies. Pvi2 is positively correlated to axis 1, whereas Pvv and Pvi1 are negatively correlated

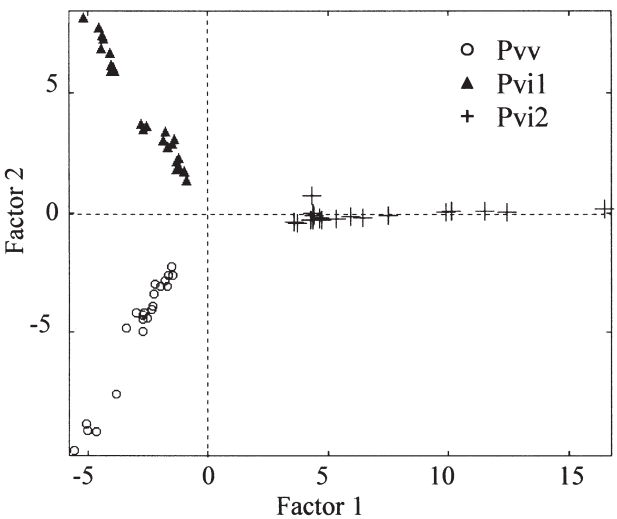

Figure 5. PCA of HC percentages of Pvv, Pvi1 and Pvi2.The first two factors F1 and F2 represent, respectively, 29 and 23% of the total variance.

Table 2. Electromorph patterns of six enzymes in the species complex *P. villosa.* Migration velocity of different electromorphs in the gel is indicated by f (fast), m (medium), and s (slow). Buffer 1: 0.1M Tris-citrate pH 8.2; buffer 2: Tris-maleate-EDTA pH 7.4; buffer 3: Tris-maleate-EDTA pH 8.3, current: 3–6 mA, voltage: 180–230V.

Enzyme	Pvv	Pvi1	Pvi2	No. of colonies	Buffer	Time (min)	No. of applications
ALP	*mm*	*ff*	*ss*	5	1	10–20	4
EST	*ss*	*ff*	*mm*	58	1,2,3	30–35	3–4
GPI	*ff*	*mm*	*ss*	58	1	30	1
IDH	*mm*	*ff*	*ss*	58	2	25	5
TRE	*mm*	*ss*	–	58	1	20	3–5
XDH	*ss*	*mm*	*mm*	15	1	20	5

to this axis. Conversely, the correlation to axis 2 is positive for Pvi1 and negative for Pvv.

Isozyme electrophoresis

Twenty of a total 29 examined enzyme systems could be reliably stained. The banding patterns of five enzymes (ALP, EST, GPI, IDH, and TRE) showed clear differences between the taxa, and heterozygote individuals were never found (Table 2). It appears that the three taxa are fixed for different electromorphs at these five diagnostic loci. Another enzyme, XDH, differed consistently between Pvv and Pvi1/2, but had identical electromorphs in Pvi1 and Pvi2.

Any intraspecific variation at the respective loci was not found. Banding patterns of four additional enzymes (malate dehydrogenase, phosphate-dependent malate dehydrogenase, phosphoglucomutase, phosphogluconate dehydrogenase) could not be sufficiently resolved using cellulose acetate plates, but we propose that they might also differ between species.

DISCUSSION

Discrepancies regarding the identification of *P. villosa* in recent studies, and consequently in results obtained, e.g. the number of ovarioles (Camargo-Mathias & Caetano, 1992b), have led us to look at further means of characterization. It soon became obvious that morphological data provided by electron scanning microscopy, biometric measurements and statistical analysis allowed the distinction of three clearly different taxa. Nevertheless, additional and less tedious techniques were desirable and both enzyme electrophoresis and cuticular HCs analysis are commendable ones.

Cuticular HCs play a number of roles in insects, from water-proofing, thermal insulation, protection from predators, to chemical communication, reproduction isolation, and kin and species recognition. They are stable end-products of biosynthetic pathways endogenous to insects (Blomquist & Dillwith, 1985; Chang Yong & Jallon, 1986), thus reflecting their genotypes and are expected to be species-specific (Lockey, 1991; Howard, 1993). Cuticular HCs have been shown to be largely genetically determined (Ferveur, 1991; Kaib *et al.*, 1991; Page *et al*., 1991; Coyne *et al.*, 1994; Ferveur & Jallon, 1996; Coyne *et al.*, 1999).

A growing body of experimental evidence suggests that insect HC compositions are species-, colony- and caste-specific (Singer, 1998). Taxonomic studies, however, of insects based on chemical analysis are often confronted with similar qualitative profiles with only quantitative differences (Lockey, 1991; El Messoussi *et al*., 1994), some of which may be attributed to non-specific factors such as age, diet, habitat and other environmental factors (Toolson, 1982; Vander Meer *et al.*, 1989; Dahbi *et al*., 1996). In this study, chromatographic profiles reveal important differences in chain lengths and types of components (linear, unsaturated, monomethyl- or dimethyl-, end- or internally branched). We have chosen sympatric colonies reared under identical conditions, ruling out the possibility that differences in cuticular composition might result from differences in diet or other environmental effects.

As the species specificity of these HCs has been demonstrated, the next step, naturally, is to determine which of the various potential roles cited in the literature is played in the three species we have studied. One of the most well known roles is the regulation of cuticular permeability. Gibbs & Pomonis (1995) and Gibbs (1998a) have shown that the structures of HCs clearly affect the melting point (Tm) of HC model mixtures or arthropod's cuticles (Gibbs, 1998b). Grasshoppers (*Melanoplus sanguinipes*) exhibit geographical variation of their cuticular HC compositions with latitude, their Tm being higher with warmer temperature (Gibbs & Mousseau, 1994). Studies on the coackroach *Blatella germanica* (Young *et al*., 2000) have shown that the HCs predicted to have Tm increasing effects are more abundant in

tissues that require greater waterproofing as the oothecal exterior or the epicuticle. It will be interesting to study the correlation, if any, between cuticular HC compositions and nesting habits of the three *Pachycondyla* species.

Numerous studies have also evidenced the role of cuticular HCs in chemical communication and nestmate recognition (Singer, 1998). Solitary insects of various genera identify their potential mates using species- and sex-specific HCs. Social insects also need HC signals to recognize their nestmates from non-nestmates in order to preserve their resources and maintain their social structure. Whole cuticular lipid extracts have been shown to elicit aggressive reactions between non-nestmate workers in such ant species as *Camponotus vagus* (Bonavita-Cougourdan *et al.*, 1987) and *Cataglyphis cursor* (Nowbahari *et al*., 1990). More recently, HC fractions of the extract have been tested and their role in nestmate recognition confirmed for *Iridomyrmex purpureus* (Thomas *et al*., 1999), *Cataglyphis niger* (Lahav *et al*., 1999) and *Pogonomyrmex barbatus* (Wagner *et al*., 2000). We have already set up a new ethological test allowing the determination of characteristic behavioural items when two species are present. Work is under way to assess the role of cuticular extracts and HCs in interactions between the three species.

Another issue must be raised regarding *Pachycondyla* HCs. A recent publication (Hefetz *et al*., 2001) has shown that in *Pachycondyla apicalis* workers, long chain alkadienes, particularly 5,9-C27:2, are clearly dominant in cuticular and postpharyngeal gland HC profiles, whereas methyl-branched alkanes are present only in trace amounts. The authors have raised the question as to whether the presence of alkadienes as major constituents is specific for the *Pachycondyla* genus. Our data show no detectable alkadiene in the three species studied and comply with most of the results on ants' chemistry published up until now. In *Harpegnathos saltator*, Liebig *et al*. (2000) have found C35:2 and C37:2 in dispersing and callow queens, but these dienes are almost absent in outside workers. More data on various ant species in general and particularly on *Pachycondyla* species are needed before the species specificity of this class of cuticular HCs can be assessed.

Isozyme studies confirmed the results of chemical analysis. At least five diagnostic enzyme loci distinguish the three taxa and a sixth locus allows differentiation between Pvv on one side and Pvi1 and Pvi2 on the other. The absence of heterozygotes in the sympatric populations at Ilhéus suggests that the three taxa are reproductively isolated. This is supported by the preliminary observation that mating between Pvv/Pvv and Pvv/Pvi1 produced workers only in the first case.

Because of the ease of isozyme electrophoresis on cellulose acetate plates and the clarity of the results, this technique could be used in the future to determine whether gene flow is also restricted in other populations and to which taxon particular individuals belong. Preliminary data suggest that certain loci consistently differ between the *P. villosa* group and related species, such as *P. obscuricornis*.

Biometric studies also afford diagnostic elements, although intermediacies in morphology/biometry and in chromatographic profiles do not coincide. A hierarchical analysis (using Ward's method for clustering) based on biometric and chemical data links together Pvv/Pvi2 and Pvi1/Pvi2, respectively (data not shown).

The congruence between our data from biometric, genetic and chemical analyses clearly demonstrates that three subspecies or, more probably, three species, are to be distinguished in the species complex considered until now as *P. villosa*.

According to descriptions in the literature, Pvv appears to be *P. villosa* (see, e.g. Wheeler, 1908; Gallardo, 1918). The taxonomic status of Pvi1 and Pvi2 is not completely clear. Pvi1 is presumably *P. inversa*, that can be considered to be a valid species as originally described by Smith (1858). Pvi2 is a new species that we provisionally call *Pachycondyla subversa,* until complete taxonomic description is performed.

Our study provides a new insight into *P. villosa* taxonomy and suggests that some of the data recently obtained may have to be reinvestigated as far as species identification is concerned. The present situation of the genus *Pachycondyla* remains very confuse as Brown (1973) has put in synonymy several genera under that name, including *Neoponera* that formerly included *P. villosa* (Kempf, 1972). Well accepted by most taxonomists (Kempf & Lenko, 1976; Bolton, 1995), this synonymy has never been clearly justified and the genus in its modern form needs, as the whole taxa under it, an exhaustive revision. Current investigations in progress aim to obtain more information regarding the status of the *P. villosa* species complex, including biogeographical, behavioural and karyological observations. Only after these have been completed, will it be possible to make a large and complete redescription of the species included in the complex. This situation is not an isolated case, as recent (unpublished) taxonomical, biological and ecological observations strongly suggest that several other Neotropical *Pachycondyla*, until now considered as good species, would be actually species complexes as in the case presented here. Chemistry and genetics have also proven to be crucial for the taxonomy of other insects as evidenced, e.g. data obtained by Nelson *et al*. (2001) on termites.

An interesting issue is the maintenance of a '*villosa* morph' formed by these three species, that remain

similar in general morphology, and by at least two spider species, their Batesian mimics. These spiders belong to two distinct families: *Sphecotypus niger* (Clubionidae) and *Zuniga* sp. (Salticidae). The myrmecomorphy is not so rare in the Neotropics, ant models are generally found in dominant arboreal genera such as *Camponotus*, *Crematogaster*, *Dolichoderus*, *Ectatomma* and *Pseudomyrmex*. In the *Pachycondyla* genus, only the '*villosa* morph' is an evident model for spiders (Delabie, 2000). One could hypothesize that the '*villosa* morph' allows this group of species, individually weakly competitive and not dominant, to appear all together, omnipresent on trees, numerically abundant, and disuasive towards different larger predators, thus forming a potential Müllerian mimicry ring (McIver & Stonedahl, 1993).

Further studies should be carried out in order to understand better the structure and ecology of the '*villosa* morph' not only in Ilhéus, but everywhere where its different taxa live together. As at least two of them are largely sympatric, according to the literature the phenomenon described herein may not be the product of a local situation, but is probably distributed throughout the Neotropical region.

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