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Differentiation of the ant genus *Tapinoma* (Hymenoptera: Formicidae) from the Mediterranean Basin by species-specific cuticular hydrocarbon profiles

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Abstract

Correct species identification is a precondition for biological study, yet despite a long history of morphological investigations, the systematic position of many ant species remains unclear. Here, we compared and identified cuticular hydrocarbon profiles of workers of several species of *Tapinoma* from Algeria, Morocco, Israel, France (mainland and Corsica) and Spain. We used the CHC profiles of workers to identify five *Tapinoma* species: *T. erraticum*, *T. israele*, *T. madeirense*, *T. nigerrimum* and *T. simrothi*. No cryptic species were detected. The species-specific hydrocarbon profiles were found to remain remarkably stable between Morocco and Northern France and between Israel and Algeria. They were not influenced by ecological factors such as vegetation type, soil and climate. In *Tapinoma* genus, cuticular hydrocarbon profiles were found to have a high diversity in CHC composition. These five identified *Tapinoma* species shared only three CHC. Combined with morphological analyses, this result confirms the species status of *T. madeirense*, *T. nigerrimum* and *T. israele*. This study also clarifies the geographical distribution of *T. simrothi* and *T. israele* and gives some indication of the preponderant frequency of *T. nigerrimum*.

Key words: *Tapinoma* complex, cuticular hydrocarbon profiles, gas chromatography-mass spectrometry, sibling species, chemosystematics.

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Introduction

In biology, species are basic units; nevertheless, organisms are sometimes morphologically nearly indistinguishable from each other. Both cryptic and sibling species are species with a very close phenotypic resemblance. "Cryptic species are two or more species which are not separable by primary visual […] of an expert" (SEIFERT 2009). In other words, these species cannot be "safely separable by training of innate pathways of the human cognition system" (SEIFERT 2009). Cryptic species "must not necessarily be the closest of relatives, though a high relatedness is usually the case", whereas, sibling species are "derived from the same common ancestor and are not separable without application of special identification methods" (SEIFERT 2009). Consequently, their reliable identification requires elaborate methods in multi-modal approaches such as high precision morphology analysis, DNA analysis and / or cuticular hydrocarbon compositions (SEIFERT 2009, SEPPÄ & al. 2011). Identifying cryptic or sibling species is essential because morphological similarity may mask great differences in behavior and ecology (SAEZ & LOZANO 2005, GUILLEM & al. 2012). Typical examples in ants are the genera *Tetramorium* MAYR, 1855, *Solenopsis* WESTWOOD, 1840 and *Tapinoma* FÖRSTER, 1850. The impact of misidentification of cryptic or sibling species, for example in pest control, endangered species (GUILLEM & al. 2012) or nature conservation, is increasingly documented and recognized (PATERSON 1991, SAEZ & LOZANO 2005, SEIFERT 2009). Recently, *Tapinoma nigerrimum* (NYLANDER, 1856) (Dolichoderinae) was proposed as a factor limiting the wild spread of the invasive Argentine ant (*Linepithema humile* (MAYR, 1868)) in Corsica (BLIGHT & al. 2009, 2010). However, to date, taxonomists worldwide have not reached a consensus on its taxonomical status, which hinders ecological investigation.

Discrimination and identification are central processes for social insects. Ants employ complex forms of chemical communication to maintain the cohesion and survival of a colony (HÖLLDOBLER & WILSON 1990). They produce numerous chemical signals that encode information about an individual's sex, caste and relatedness, in addition to trail and alarm pheromones (BONAVITA-COUGOURDAN & al. 1987, for review see HOWARD 1993, LENOIR & al. 1999, HOWARD & BLOMQUIST 2005, BLOMQUIST & BAGNÈRES 2010). Colony members learn the recognition cues shortly after hatching, allowing them to form a "template" that serves as a reference (LACY & SHERMAN 1983, BLOMQUIST & BAGNÈRES 2010). Cuticular lipids – particularly hydrocarbons (HCs) – are thought to be involved in species and colony recognition in ants (HOWARD 1993, LUCAS & al. 2005, see also SINGER 1998 or PROVOST & al. 2008). Cuticular hydrocarbon (CHC) profiles are usually colony-specific, with colonies having different relative concentrations of compounds (NIELSEN & al. 1999, FOITZIK & al. 2007, MARTIN & al. 2008). These CHCs consist of a complex mixture of long straight-chain saturated alkanes, which can be modified by addition of methyl groups attached to the chain or introduction of one or more double bonds (MARTIN & DRIJHOUT 2009). All these combinations have led ants to evolve very complex CHC profiles (DAHBI & al. 1996, ELMES & al. 2002). Species-specific hydrocarbon compositions have been characterized for example for *Camponotus vagus* (SCOPOLI, 1763) (see BONAVITA-COUGOURDAN & al. 1987), *Cataglyphis cursor* (FONSCOLOMBE, 1846) (see NOWBAHARI & al. 1990), two *Tetramorium* spp. (STEINER & al. 2002), thirteen species of *Formica* (see MARTIN & al. 2008), seven *Temnothorax* spp. (FOITZIK & al. 2007, PROVOST & al. 2008), and two *Lasius* spp. (MORRISON & WITTE 2011) (reviewed in HEFETZ 2007).

Today, the genus *Tapinoma* is commonly acknowledged to contain 91 valid names and subspecies, and five fossil species (BOLTON & al. 2007, 2012). To illustrate the complexity of the *Tapinoma* European group, since the first description of *T. nigerrimum* by NYLANDER (1856), its status has changed 12 times from species to subspecies of *T. erraticum* (LATREILLE, 1798) or vice versa (ANDRÉ 1882, FOREL 1902, BONDROIT 1918, DLUSSKY & al. 1990, ATANASSOV & DLUSSKY 1992, SHATTUCK 1994, BOLTON 2007, 2012). EMERY (1925) classified *T. nigerrimum* as a species in his revision of the Palearctic members of this genus, but only after changing his mind four times (EMERY 1869, 1916, 1925, EMERY & FOREL 1879). *T. nigerrimum* was considered until recently (SHATTUCK 1994, CASEVITZ-WEULERSSE & GALKOWSKI 2009) as a synonym of *T. erraticum*, in spite of "[…] its very characteristic genital morphology (SEIFERT 1984) and its characteristic formation of large supercolonies containing very large workers in addition to smaller ones […]" (SEIFERT 2012). "A partial explanation of this […]" was given by SEIFERT (2012) when he suggested that "[…] all the synonymizing authors may have based their conclusions on worker material only". In the genus *Tapinoma*, males offer a reliable method of differentiation via the obvious genitalia differences, whereas difficulties arise with workers and even gynes (EMERY 1925, SEIFERT 1984). However, the short lifespan of males makes their capture uncertain and some, like *T. israele* FOREL, 1904, have yet to be formally described. Workers of some species can only be discriminated by using the depth of clypeal incision (SEIFERT 1984, 2012), but variation in the clypeal cleft shape even within a nest makes this character unreliable. SEIFERT (1984) did not include in his statistical study individuals of species from Mediterranean countries like Algeria, Portugal, and Spain because he was not always able to distinguish them from other *Tapinoma* species expected in this area, but he expected seven species of *Tapinoma* for the Mediterranean Basin, including *T. erraticum*, *T. simrothi* KRAUSSE, 1911, *T. nigerrimum*, *T. ambiguum* EMERY, 1925, *T. pygmaeum* (DUFOUR, 1857) and two other as yet unidentified species (SEIFERT 1984). Currently, according to the literature, the *Tapinoma* genus has eleven species with established populations in the Mediterranean Basin (*T. pygmaeum*, *T. melanocephalum* (FABRICIUS, 1793), *T. christophi* EMERY, 1925, *T. festae* EMERY, 1925, *T. simrothi phoeniceum* EMERY, 1925, *T. erraticum*, *T. israele*, *T. madeirense* FOREL, 1895, *T. minor* BERNARD, 1945, *T. simrothi*, and *T. nigerrimum*). The following is a brief account of *Tapinoma* species with established populations in the Mediterranean Basin.

Tapinoma pygmaeum and ***T. melanocephalum*** can easily be distinguished from the other *Tapinoma* species by their smaller size (1.3 - 1.7 mm long) (MENOZZI 1925, CREIGHTON 1950) and extremely reduced polymorphism. The head and thorax of the exotic *T. melanocephalum* are a deep dark brown with gaster and legs opaque or milky white (FABRICIUS 1793). Outside the tropics, this species is a nuisance and has always been found inside buildings (DUBOIS & DANOFF-BURG 1994, DEKONINCK & al. 2006), and greenhouses (HÖGMO 2003, ESPADALER 2007). *Tapinoma pygmaeum* has been recorded only ten times in France (DUFOUR 1857, PÉRU 1999, PARAT 2001, LIVORY 2008), Italy (MENOZZI 1925) and Spain (ESPADALER 1977, 1979, ESPADALER & GARCIA-BERTHOU 1997). Due to their special features and the ease with which these two species can be distinguished from the other *Tapinoma*, they are not included in this study. Three other species belonging to the eastern Mediterranean *Tapinoma* are extremely poorly known: ***T. christophi*** from Lebanon, known exclusively through the male caste, ***T. festae*** from Greece, recently proposed as a valid species (LUSH 2009) and known through its workers and queens, and ***T. simrothi phoeniceum*** from Cyprus, Lebanon and Greece, known through all three castes. They are not included in this work.

Workers of ***Tapinoma erraticum*** were initially found in France near Brive and described by LATREILLE (1798). Males have subgenital plates with strong broad lobes truncated terminally (WETTERER & al. 2007). This species has a Palearctic distribution, and is recorded for example, in several central and southern European countries (EMERY 1925, BERNARD 1967, SEIFERT 1984, 2012, BOER 2010, CZECHOWSKI & al. 2012) and also from Algeria, Egypt, and Israel (EMERY 1925, SHATTUCK 1994) although samples named as *T. erraticum* from those last three countries are probably misidentifications. The biology of this species was investigated by MEUDEC (1973).

Tapinoma israele: Workers of *Tapinoma erraticum israele* were found for the first time in Jerusalem and described by FOREL (1904). Subsequently, Emery described queens and workers from Palestine, Syria and Crete, thereby raising *T. israelis* to species status (EMERY 1925). Workers of *T. israele*, hereafter named following the latest version

of BOLTON's Catalogue (2007), show longer antennae and a smaller depth of clypeal incision than *T. erraticum* (see FOREL 1904, EMERY 1925). Emery reported that he was unable to distinguish *T. israele* from *T. simrothi* based on queens alone (1925). Since then, there has been no description of males, nor any genetic or chemical investigations of this species. In this study, *Tapinoma israele* was named based on FOREL's description (1904) and EMERY's revision (1925), many samples from Israel having been made available to one author (X.E.) by Israeli entomologists. They included both samples preserved in alcohol and samples prepared dry. Workers and worker-associated males – and a few queens – were available from two species: *Tapinoma israele* and *Tapinoma simrothi phoeniceum*. The former definitely differs from *Tapinoma erraticum* based on the much more shallow clypeal incision and male genitalia (X. Espadaler, unpubl.). Factors indicating male genitalia are noted by EMERY (1925). Forthcoming work will present the evidence and propose minor taxonomic rearrangements and synonymies in Eastern Mediterranean *Tapinoma*.

Tapinoma madeirense: In 1895, FOREL described workers from Madeira (Portugal), distinguishing them from the other *Tapinoma: T. erraticum* var. *madeirense*. The queen was described by EMERY (1925). The female caste differs in the degree of clypeal incision, which is shorter in *T. madeirense* (see also SEIFERT 1984). The males of *T. madeirense*, described as *T. ambiguum* by EMERY (1925), have genitalia with thin elongated lobes, directed somewhat externally (WETTERER & al. 2007).

First described by BERNARD (1945), ***Tapinoma minor*** has been cited only from Morocco by Henri Cagniant as a junior synonym of *T. simrothi* (see CAGNIANT 2006). Workers are black with a head longer than its width (BERNARD 1982). No further investigations have been undertaken.

KRAUSSE-HELDRUNGEN (1911) described workers from Sardinia as *Tapinoma erraticum var. simrothi*; then EMERY (1925) described queens and males, and raised ***T. simrothi*** to species status. Small workers are very similar to *T. erraticum* but male genitalia are easily distinguishable (EMERY 1925). *T. simrothi* are currently recorded in several countries like France (Corsica: EMERY 1925, CASEVITZ-WEULERSSE 1989), Italy (Sardinia and Sicily: EMERY 1925), Spain (EMERY 1925, CARPINTERO & al. 2000), Morocco (CAGNIANT 1964, 2006) and Algeria (CAGNIANT 1968, 1970, BERNARD 1976). *T. simrothi* was characterized as a tramp species (BERNARD 1976) owing to its massive exploitation of aphids (BERNARD 1982).

Oddly enough, while ***Tapinoma nigerrimum*** was listed as a junior synonym of *T. erraticum* by BOLTON & al. (2007), this polygynic "species" is one of the best known European *Tapinoma*. Large workers of *T. nigerrimum* differ from *T. erraticum* not only in absolute size (EMERY 1925), but also in their proportionally shorter scape and a much more elongated second funiculus segment. Although equally-sized workers of both species have almost the same scape length, clypeal notch is deeper in *T. nigerrimum*. Other statistical differences are shown in table 1 of SEIFERT (2012). *Tapinoma nigerrimum* is an originally Ibero-Mauritanian ant with a Mediterranean distribution; it has shallow extensive nests and is particularly abundant in man-influenced areas (PASSERA 1977, COMIN & DE HARO 1980, ESPADALER 1986). Colonies are composed of several nests, with many entrances inter-connected with trails (CERDÁ & al. 1989). The species builds nests preferentially in spaces with scarce arboreal strata (BERNARD 1980); their entrances are characteristically domed (CASTELLÓ & ARIAS DE REYNA 1982) or delimited by accumulated sand (BONARIC 1971). Its capacity to withstand floods (BERNARD 1983), together with its biological features and the ability to colonize degraded areas (ACOSTA 1980), enable this species to colonize coastal areas, where it is particularly abundant (PASSERA 1977, FERNANDEZ & RODRIGUEZ 1982).

The impact of misidentification of cryptic or sibling species, for example in pest control or nature conservation, is increasingly documented and recognized (e.g., PATERSON 1991, SAEZ & LOZANO 2005, SEIFERT 2009). *Tapinoma nigerrimum* has been proposed as a factor limiting the wild spread of the invasive Argentine ant (BLIGHT & al. 2009, 2010), but in the Mediterranean Basin, several *Tapinoma* species may also occupy the same type of habitat. We thus decided to study the Mediterranean *Tapinoma* complex, which is widespread and ecologically important in many ecosystems, using both morphological and chemical analyses. Our principal aims were (1) to facilitate reliable determination of the Mediterranean *Tapinoma* species, (2) to confirm the species status of *T. israele*, *T. madeirense* and *T. nigerrimum*, and (3) to clarify the complex Mediterranean *Tapinoma* distribution.

Materials and methods

Field collection

Workers of *Tapinoma* sp. were collected from 51 nesting sites in Algeria, France, Israel, Morocco, Madeira Island and Spain (Fig. 1 and Tab. 1). The nesting sites sampled ranged over 1900 km from the northern to the southern part and over 4800 km from the eastern to the western part of the study area. Four colony fragments were collected in Algeria: two in Tlemcen [Tlemcen University and Tlemcen Lalla Setti], one in Tizi-Ouzou and one in Itma. 31 colony fragments were collected in France: eight along the Corsican coast [Ajaccio, Bastia, Favona, Lac Palo, Ostriconi, Porto-Vecchio, Propriano, Santa Giulia], and 23 in continental France [Aubagne, Auriol, Sainte Baume, Azay-sur-Cher, six close to Bléré, four near Fos-sur-Mer, Fréjus, Frioul island, Gardanne, Istres, Mimet, Plage de Piemanson, Saint Aygulf, Salin de Giraud, and Velaux]. Two nesting sites were sampled in Morocco [Ijjoukak and Marrakech]. Three colony fragments were collected in Israel [Lower Galilee]. One colony fragment was sampled in Madeira island [Funchal]. The remaining ten colony fragments were collected in Spain [Algéciras, Bahia de Cadiz, Dragados, Línea de la Concepción, San Pedro de Alcántara, El Pedroso, Puerto Santa María, Sotogrande, Tadeo, and Tarifa].

Fields for ant collection were chosen following the literature. Algeria, South of Spain, Morocco, and Corsica were chosen for *Tapinoma simrothi* (from CAGNIANT 1968, 1970, BERNARD 1976, 1982, CASEVITZ-WEULERSSE 1989, CARPINTERO & al. 2000), Israel for *T. israele* (from EMERY 1925), southern France and Spain for *T. nigerrimum* (from EMERY 1925, BERNARD 1983, CARPINTERO & al. 2000), Morocco for *T. minor* (from BERNARD 1982, CAGNIANT 1964, 2006), Madeira island for *T. madeirense* (from WETTERER & al. 2007), and central France for *T. erraticum* (from MEUDEC 1973).

Tab. 1: Collection sites for *Tapinoma* ants. Latitude (X) and longitude (Y) are given in decimal World Geodetic System (WGS 84). ♂ represents nesting sites morphologically determined via the male genitalia.

No.	Nesting-sites and abbreviations		Countries	X	Y	Morphological determination	Chemical determination
1	Itma	Itm	Algeria	36.74733333	3.0754	*T. nigerrimum*	*T. nigerrimum*
2	Tizi-Ouzou	Tiz	Algeria	36.71666667	4.05	*T. nigerrimum*	*T. nigerrimum*
3	Tlemcen	Tle	Algeria	34.87855	-1.313116667		*T. nigerrimum*
4	Tlemcen : Lalla setti	Lal	Algeria	34.87875	-1.275466667		*T. israele*
5	Ajaccio	Aja	France	41.91923333	8.738616667	♂ *T. nigerrimum*	*T. nigerrimum*
6	Aubagne	Aub	France	43.28065	5.579866667	*T. nigerrimum*	*T. nigerrimum*
7	Auriol	Aur	France	43.35691667	5.6408	*T. nigerrimum*	*T. nigerrimum*
8	Auriol : Sainte Baume	Bau	France	43.38835	5.6537		*T. erraticum*
9	Azay-sur-Cher	Aza	France	47.32741667	0.809666667	*T. erraticum*	*T. erraticum*
10	Bastia	Bas	France	42.70213333	9.45075	♂ *T. nigerrimum*	*T. nigerrimum*
11	Bléré	Blé	France	47.30955	0.952916667	*T. erraticum*	*T. erraticum*
12	Bléré	Blr	France	47.30941667	0.953616667	*T. erraticum*	*T. erraticum*
13	Bléré	Bré	France	47.30925	0.9532	*T. erraticum*	*T. erraticum*
14	Bléré	Tou	France	47.3094	0.953583333	*T. erraticum*	*T. erraticum*
15	Bléré	Cen	France	47.30918333	0.953016667	*T. erraticum*	*T. erraticum*
16	Bléré	Ind	France	47.30921667	0.952816667	*T. erraticum*	*T. erraticum*
17	Favona	Fav	France	41.7729	9.396816667	♂ *T. nigerrimum*	*T. nigerrimum*
18	Fréjus	Fre	France	43.45233333	6.734833333	*T. madeirense*	*T. madeirense*
19	Fréjus : Saint Aygulf	Sai	France	43.43246667	6.735183333	*T. nigerrimum*	*T. nigerrimum*
20	Frioul island	Fri	France	43.28	5.297666667	*T. nigerrimum*	*T. nigerrimum*
21	Istres	Ist	France	43.51013333	4.9994	*T. nigerrimum*	*T. nigerrimum*
22	Lac Palo	Lac	France	41.94758333	9.40755	*T. nigerrimum*	*T. nigerrimum*
23	Plage de l'Ostriconi	Ost	France	42.66136667	9.063016667	*T. nigerrimum*	*T. nigerrimum*
24	Plage de Piémanson	Pla	France	4.347166667	4.78475	♂ *T. nigerrimum*	*T. nigerrimum*
25	Porto-Vecchio	Por	France	41.59098333	9.279483333	♂ *T. nigerrimum*	*T. nigerrimum*
26	Propriano	Pro	France	41.67585	8.9032	♂ *T. nigerrimum*	*T. nigerrimum*
27	Salin de Giraud	Sal	France	43.37545	4.741333333	♂ *T. nigerrimum*	*T. nigerrimum*
28	Santa Giulia	San	France	41.51973333	9.271666667	♂ *T. nigerrimum*	*T. nigerrimum*
29	Velaux	Vel	France	43.53098333	5.250166667	*T. madeirense*	*T. madeirense*
30	Fos-sur-Mer	Fme	France	43.47751667	4.874733333	*T. madeirense*	*T. madeirense*
31	Fos-sur-Mer	FoM	France	43.47865	4.8744	*T. madeirense*	*T. madeirense*
32	Fos-sur-Mer	Fos	France	43.47798333	4.874883333	*T. nigerrimum*	*T. nigerrimum*
33	Fos-sur-Mer	FSM	France	43.4729	4.876083333	*T. madeirense*	*T. madeirense*
34	Gardanne	Gar	France	43.44311667	5.483016667	*T. nigerrimum*	*T. nigerrimum*
35	Mimet	Mim	France	43.44021667	5.472583333	*T. nigerrimum*	*T. nigerrimum*
36	Lower Galilee	Ira	Israel	32.72973333	35.44328333	*T. israele*	*T. israele*
37	Lower Galilee	Isr	Israel	32.75086667	35.43805	*T. israele*	*T. israele*
38	Lower Galilee	Iae	Israel	32.74455	35.48606667	*T. israele*	*T. israele*
39	Ijjoukak	Ijo	Morocco	30.9967	-8.159883333	*T. nigerrimum*	*T. nigerrimum*
40	Marrakech	Mar	Morocco	31.63333333	-8.00	*T. nigerrimum*	*T. nigerrimum*
41	Funchal	Fun	Portugal	32.664159	-16.87515	*T. madeirense*	*T. madeirense*
42	Algéciras	Alg	Spain	36.14578333	-5.448633333	*T. simrothi*	*T. simrothi*
43	Cadiz : Bahia de Cadiz	Cad	Spain	36.5004	-6.273566667	*T. nigerrimum*	*T. nigerrimum*
44	El Pedroso	Ped	Spain	37.84103333	-5.768416667	*T. nigerrimum*	*T. nigerrimum*
45	El puerto de Santa María	PSM	Spain	36.57793333	-6.214166667	*T. nigerrimum*	*T. nigerrimum*
46	El puerto de Santa María : Dragado	Dra	Spain	36.56831667	-6.2223	*T. nigerrimum*	*T. nigerrimum*
47	El puerto de Santa María : Tadeo	Tad	Spain	36.58045	-6.226816667	*T. nigerrimum*	*T. nigerrimum*
48	La Línea de la Concepción	Lin	Spain	36.16758333	-5.36665	*T. simrothi*	*T. simrothi*
49	San Pedro de Alcántara	PDA	Spain	36.48356667	-4.980633333	*T. nigerrimum*	*T. nigerrimum*
50	Sotogrande	Sot	Spain	36.28703333	-5.301483333	*T. simrothi*	*T. simrothi*
51	Tarifa	Tar	Spain	36.02183333	-5.615283333	*T. simrothi*	*T. simrothi*

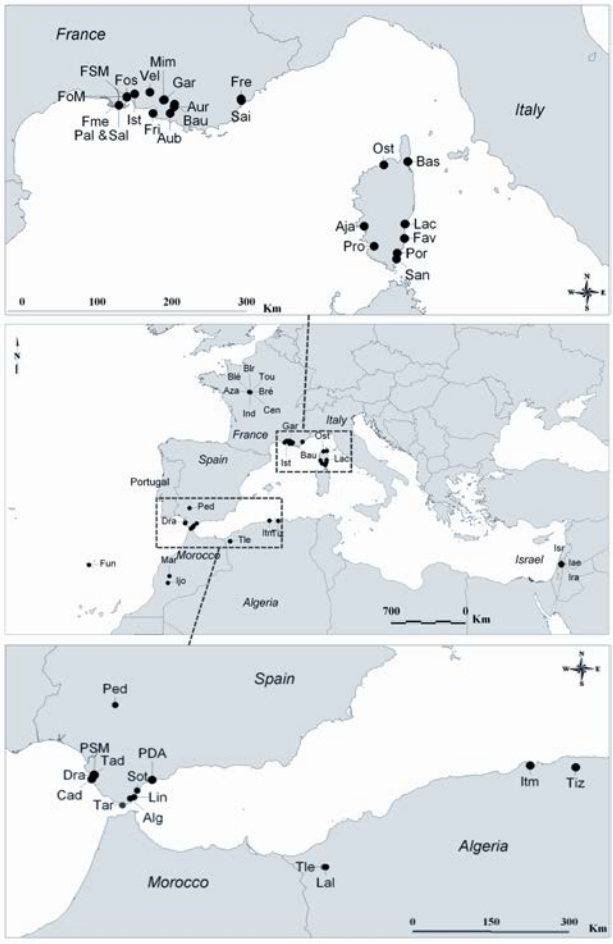

Fig. 1: Location of the 51 studied nesting sites of *Tapinoma* in the Mediterranean Basin.

Colony fragments (about 50 workers) were collected in 2009, 2010 and 2011, between April and October from a variety of habitats including landscaped residential lots or sand dunes, but only three workers from Madeira Island arrived alive at the laboratory, so we did not include them in the statistical analyses. Workers were killed by freezing and maintained at -18°C, in separate glass tubes.

Chemical analyses of cuticular hydrocarbons

Seven samples from each nesting site (7 × 50 = 350), consisting of a single worker, were immersed in 5 µl of hexane for 15 minutes in order to extract and estimate cuticular hydrocarbons. 3 µl of this extract were used for capillary gas chromatography (GC), carried out using a Varian 3 900 gas chromatograph equipped with a flame ionization detector (FID) and a Chrompack CPSil5WCOT apolar capillary column (fused silica, 25 m × 250 µm; thickness of stationary phase, 0.12 µm) and interfaced with Star 5.5 (Varian) software. Oven temperature was held at 100°C, then increased to 220°C at 10°C / min, then to 320°C at 3°C / min and finally held at constant temperature for 10 min. The injector and flame-ionization detector were at 280 and 250°C respectively, with helium the carrier gas. Quantitative data were obtained by integrating peaks.

Gas chromatography analysis of the 350 worker profiles yielded five different types of chromatograms. Cuticular compounds of workers from two nesting sites of each of the five profiles were determined by coupled Gas Chromatography / Mass Spectrometry (GC-MS) [Aza and Blé for *group 1*; Isr and Ira for *group 2*; Fre and FoM for *group 3*; Fri and Sai for *group 4*; Sot and Tar for *group 5*] as reference nests. Total body washes of 20 pooled workers from each reference nest were used for extraction. Workers were immersed in 100 µl of hexane for 20 min. Four micro-liters of each extract were run into an Agilent 6890N GC equipped with Chrompack CPSil5WCOT apolar capillary column (25 m × 0.25 mm, 0.12 µm). The GC was coupled with a 5375 Agilent Technologies Mass Spectrometer. Electron impact mass spectra were obtained with an ionization voltage of 70 eV. The carrier gas was helium at 1 ml / min with the injector in splitless mode. Oven temperature was isothermal at 70°C for 1 min, followed by 30°C / min to 180°C, then increased at a rate of 5°C / min to 320°C, and finally held for 15 min. Specific peak identity was determined with hydrocarbon standards and by matching diagnostic peaks with those from published spectra.

Subsequently, quantitative analyses obtained by gas chromatography were performed with only the hydrocarbons as variables. CHC peaks areas were standardized to 100% by calculating the percentage contribution of each compound to the cuticular hydrocarbon blend. Comparison of cuticular profiles was based on this proportion. A correction coefficient (K) was applied in order to adjust the integrated values to the sensitivity of the detector according to the number of carbon atoms of molecules. We determined this coefficient after injection of the same quantity of 10 n-alkanes (18, 20, 22, 24, 28, 32, 34, 36, 38, and 40 carbon atoms, respectively) where $K = 0.0038x^2 - 0.1738x + 2.9683$ (x is the number of carbon atoms per molecule of the substance).

Morphological analyses

Immediately following field collection and during chemical analyses, several workers from each nesting site were morphologically identified (Tab. 1). Morphological identification and chemical analyses were performed in blind tests in different laboratories (respectively Spain and France).

Over the fifty nesting sites, two kinds of morphological analyses were used, according to whether or not males were present in the nest. During field collection, males were found in eight nesting sites, two in continental France (Pla and Sal) and six in Corsica (Aja; Bas; Fav; Por; Pro; and San: Tab. 1). The morphological identification of workers from these eight nesting sites was based on male genitalia.

Identifications of the species from the other nesting sites were based on worker cephalic characteristics: clypeal notch, clypeal seta insertion and antennal segments. Two to five workers per sample were examined. The following characteristics and measurements were used: (a) depth of clypeal notch / distance from deepest point of clypeal notch to most posterior point of clypeus, which is easily seen on clean, dry specimens and using tangential illumination; (b) relative length of second and third antennal segments (Fig. 2, Tab. 2). We measured mounted, dried specimens. A stereomicroscope Nikon SMZ-U with magnification (up to 112.5×) and dual arm fiber optic illuminator was used to measure the two described variables and get the corresponding indices. Measurements and indices used have not been validated as a formal statistical and revisionary tool for *Tapinoma*; however, they have been

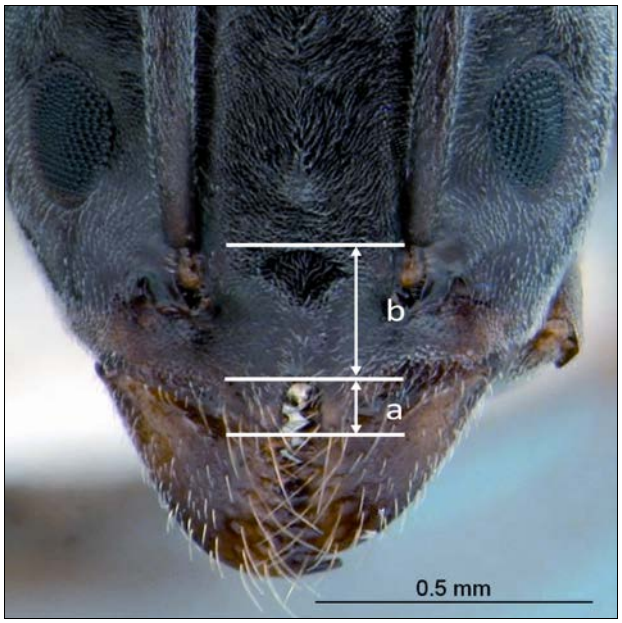

Fig. 2: Metric characters measured in *Tapinoma* species: (a) Maximum depth of clypeal notch as it appears in frontodorsal view. (b) Distance from deepest point of clypeal notch to most posterior point of clypeus. Example of a head of *Tapinoma nigerrimum*.

routinely used over the past years and have been confirmed whenever worker-associated males have been available.

Statistical analyses

Statistical analyses were performed using hydrocarbons as sole variables, ignoring fatty acids and all other compounds. 168 cuticular compounds were separated and identified by GC-MS (Tab. 3). Multivariate data analyses were performed using R language and environment (R 2.15.1 software). The standardized peak areas were used to perform multivariate principal component analyses (PCA). The PCA were performed on the principal factors of overall variance. We also conducted a separate *K*-means cluster analysis to group together data points (factor coordinates of the variables) showing similar cuticular hydrocarbon profiles.

A pairwise matrix of CHC differences was constructed by measuring the binary squared Euclidean distance (the total number of CHCs that are absent in one species but present in the other, and vice versa), in order to look at the relationship between species using Ward's linkage method (Euclidean distance) (WARD 1963) with Statistica 6.0 software.

Results

Morphological analyses

Workers from 39 nesting sites were morphologically analyzed on the basis of several workers each using the morphological indices (Tab. 2, Fig. 2). These analyses revealed the presence of five species: *Tapinoma erraticum*, *T. israele*, *T. madeirense*, *T. nigerrimum*, and *T. simrothi*. Workers from 21 nesting sites were identified as *T. nigerrimum*. Workers from eight nesting sites, all in France, were identified as *T. erraticum*. Workers from four nesting sites, all in Spain, were identified as *T. simrothi*. Workers from three nesting sites, all in Israel, were identified as *T. israele*, and

Tab. 2: Indices and key for measurements of *Tapinoma* species.

Tapinoma	**b / a**	**Funicle segments**
israele	> 4	2 ≥ 3
madeirense	> 4	2 < 3
erraticum	~ 3.2	2 = 3
nigerrimum	2 - 2.3	2 > 3
simrothi	1.6 - 2	2 ≥ 3

workers from five nesting sites, all in France, as *T. madeirense*. The three workers from Madeira Island were also morphologically identified as *T. madeirense*. Workers from Tle, Lal and Bau nesting sites could not be identified morphologically due to their deterioration in transit from France to Spain (Tab. 2).

Field collection yielded eight nesting sites with males (Aja, Bas, Fav, Pla, Por, Pro, Sal, and San). For these sites, therefore, identification was based on male genitalia; all were morphologically identified as *Tapinoma nigerrimum* (Tab. 1).

Cuticular hydrocarbon analysis

Chemical analyses of all 350 worker-profiles revealed clearly five qualitative and quantitative CHC profiles (Fig. 3), leading us to expect five *Tapinoma* species over the 50 nesting sites. In order to visualize the different chemical signatures, multivariate principal component analysis (PCA) was performed (Fig. 4) on the cuticular hydrocarbons found in the seven workers from each of the 50 nesting sites (Tab. 3). The PCA was performed on 53 factors which accounted for 95.1% of overall variance. The PCA (Fig. 4) revealed a marked discrimination on axis 1 between on one side, workers from group one [dark grey triangles] and, on the other side, workers from group two [light grey circles]. The first axis accounted for 33.29% of total variance. The second axis, which accounted for 26.41% of cumulative variance, revealed a marked separation between both sides of the axis, with group 4 on the negative side [black circles] and both groups 1 and 2 on the positive side. Group 3 was located near the axis intersection, in the middle of the three other groups. All GC-MS control individuals from groups 1, 2 and 4 from a given location were assigned to the same group. The *K*-means analysis identified four chemical groups. Group 1 (grey triangles, Fig. 4) was composed of all the workers from Alg, Lin, Sot, and Tar (GC-MS controls), all from Spain. Group 2 (light grey circles) was composed of workers from Algeria (Itm, Tiz, and Tle), France (Aub, Aur, Cad, Fos, Fri, Gar, Ist, Lac, Mim, Ost, and Sai), Morocco (Ijo and Mar) and Spain (Dra, PDA, Ped, PSM, and Tad). Group 3 (dark grey rectangle) was composed of all the workers from Aza, Bau, Blé, Blr, Bré, Cen, Fre, FSM, FMe, FoM, Ind, Tou, and Vel (France). Group 4 (black circles) was composed of all the remaining workers [Israel: Iae, Isr, Ira; and Algeria: Lal]. Given the perfect concordance between the morphological and chemical discriminations, groups 1, 2 and 4 were identified as *T. simrothi*, *T. nigerrimum*, and *T. israele* respectively.

In order to discriminate workers of the remaining species, a new PCA (Fig.5) was performed on worker pro-

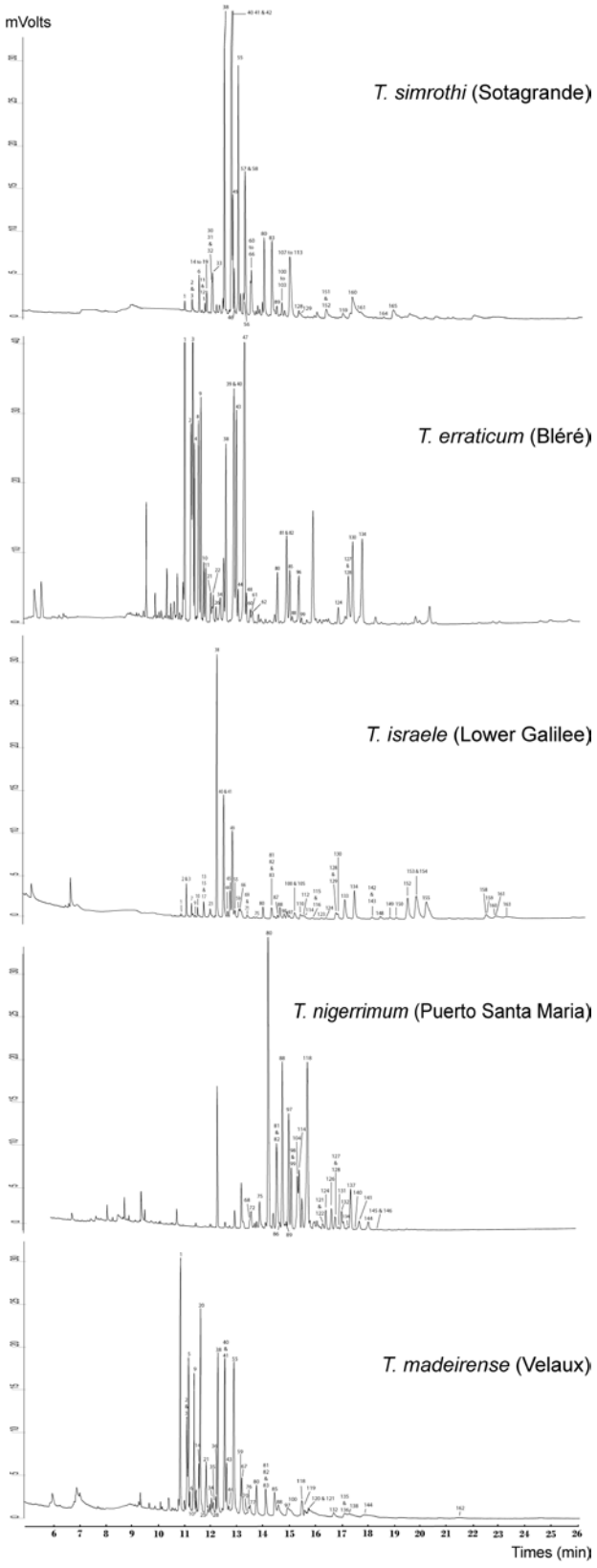

Fig. 3: Chromatograms obtained by injection of total body wash of workers of *Tapinoma simrothi* (Sot), *T. erraticum* (Ind), *T. israele* (Iae), *T. nigerrimum* (Tad) and *T. madeirense* (Vel).

files of all the nesting sites of group 3 from the first PCA. This PCA was based on their 77 CHCs and was performed

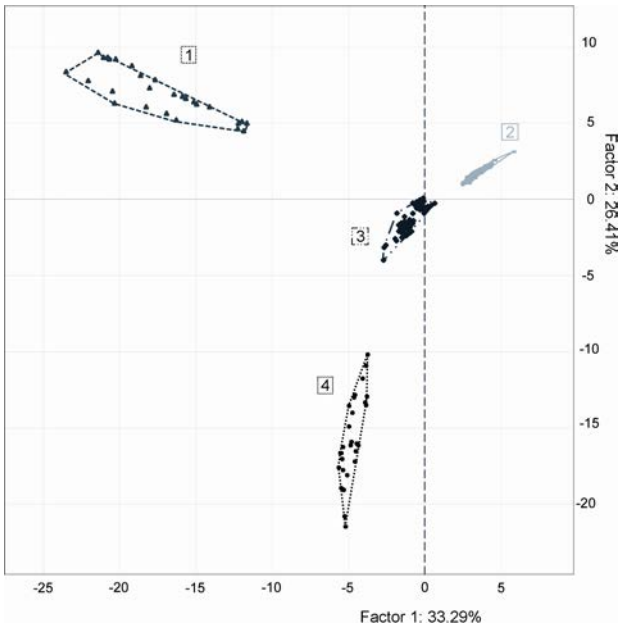

Fig. 4: Principal Component Analysis was based on the proportion of worker cuticular hydrocarbons from the 51 nesting sites. The projection was performed on two dimensions, where the first axis accounts for 33.29% of the total variance and the second for 26.41%. *K*-means cluster analysis distinguishes four groups. Morphological analysis allows us to identify these groups as *Tapinoma simrothi* [1-grey triangles], *T. nigerrimum* [2-light grey circles], indistinguishable *T. madeirense* and *T. erraticum* [3-black rectangles], and *T. israele* [4-black circles]. The areas correspond to the 95% confidence limit of the data.

on the 37 major factors which accounted for 95.2% of the overall variance. This second PCA (Fig. 5) revealed a marked discrimination between the four Fréjus worker profiles and the other nesting sites. The first and the second axes, which accounted respectively for 29.37% and for 13.52% of total variance, discriminated between, on one side, workers from group 3a (grey circles) and on the other side, workers from group 3b (black circles).The *K*-means analysis distinguished three chemical groups. The first group [3a] was composed of workers from southern France [Fre, FSM, FMe, FoM, and Vel (grey circles, Fig. 5)]. The second group [3b] was composed of workers from southern and central France [Aza, Bau, Blé, Blr, Bré, Cen, Ind, and Tou (black circles, Fig. 5)]. In the first two groups, all the individuals from a given location were assigned to the same group, except for four workers from Fréjus which formed a separate group [3c, dark grey triangles]. Morphological and chemical discriminations led to groups 3a and 3b being identified *Tapinoma madeirense* and *T. erraticum* respectively (Tab. 1). Given their chemical composition and their morphological discrimination, workers from Fréjus were identified as *T. madeirense*.

Across the five species, 168 distinct CHCs were found, belonging to five distinct hydrocarbon groups (Tab. 3). These 168 CHCs were identified as ranging from 25 to 39 carbon atoms. Among the five species, the sequence of occurrence is as follows: monomethylalkanes > dimethylalkanes > *n*-alkanes > trimethylalkanes > *n*-alkenes (Tab. 3). Cuticular profiles of all *Tapinoma* species present methyl-

Tab. 3: List of the 168 compounds identified by GC-MS and their percentage contribution (Mean), in the cuticular extract sample of workers from field populations of the five *Tapinoma* species. All nests were summed per site ($N_{T.erraticum}$ = 56; $N_{T.israele}$ = 28; $N_{T.\ madeirense}$ = 35; $N_{T.\ nigerrimum}$= 203; $N_{T.\ simrothi}$ = 28).

No.	Compounds		*T. madeirense*	*T. israele*	*T. nigerrimum*	*T. erraticum*	*T. simrothi*
1	n-Pentacosane	n-C25	7.27	0.9		12.9	8.45
2	13-Methylpentacosane	13-Me C25	4.02	1.08		4.69	12.8
3	11-Methylpentacosane	11-Me C25	2.5	1.06		4.44	12.8
4	7-Methylpentacosane	7-Me C25				5.62	
5	5-Methylpentacosane	5-Me C25	8.24			2.22	
6	3-Methylpentacosane	3-Me C25	4.04	0.62		5.23	8.27
7	11,15-Dimethylpentacosane	11,15-Dime C25		0.78			
8	7,9-Dimethylpentacosane	7,9-Dime 25				2.06	
9	5,13-Dimethylpentacosane	5,13-Dime C25	12.7	0.22		7.49	
10	n-Hexacosane	n-C26	1.3	0.87		2.25	
11	4,11,13-Trimethylpentacosane	4, 11, 13-Trime C25				1.57	1.6
12	4,9,13-Trimethylpentacosane	4,9,13-Trime C25					1.6
13	14-Methylhexacosane	14-Me C26	2.44	0.46			
14	13-Methylhexacosane	13-Me C26					0.99
15	12-Methylhexacosane	12-Me C26		0.31			0.99
16	11-Methylhexacosane	11-Me C26					0.99
17	10-Methylhexacosane	10-Me C26		0.26			0.99
18	9-Methylhexacosane	9-MeC26					0.99
19	8-Methylhexacosane	8-Me C26				1.59	0.99
20	5,9,11-Trimethylpentacosane	5,9,11-Trime C25	3.56				
21	12,14-Dimethylhexacosane	12,14-Dime C26	1.67			0.57	
22	12,13-Dimethylhexacosane	12,13-Dime C26				0.59	
23	n-Heptacosene	C27:1		0.83			
24	X,Y-Dimethylhexacosane	X,Y-Dime C26				0.65	
25	8,12-Dimethylhexacosane	8,12-Dime C26	1.2				0.99
26	6-Methylhexacosane	6-Me C26				0.63	
27	5-Methylhexacosane	5-Me C26	0.57				
28	4-Methylhexacosane	4-Me C26	0.32				
29	3-Methylhexacosane	3-Me C26					0.99
30	10,16-Dimethylhexacosane	10,16-Dime C26					0.8
31	10,14-Dimethylhexacosane	10,14-Dime C26					0.8
32	10,12-Dimethylhexacosane	10,12-Dime C26					0.8
33	2-Methylhexacosane	2-Me C26	0.38				1.76
34	6,12-Dimethylhexacosane	6,12-Dime C26	0.68			0.68	
35	5,12-Dimethylhexacosane	5,12-Dime C26	0.53				
36	4,12-Dimethylhexacosane	4,12-Dime C26	0.63				
37	4,6-Dimethylhexacosane	4,6-Dime C26				0.42	
38	n-Heptacosane	n-C27	4.52	16.2	1.12	6.44	6.21
39	15-Methylheptacosane	15-Me C27				3.65	
40	13-Methylheptacosane	13-Me C27	3.95	6.05		3.65	3.01
41	11-Methylheptacosane	11-Me C27	3.59	4.33			3.01
42	9-Methylheptacosane	9-Me C27					3.01

43	7-Methylheptacosane	7-Me C27	1.35			3.3	
44	5-Methylheptacosane	5-Me C27	1.25	0.78		1.23	
45	11,15-Dimethylheptacosane	11,15-Dime C27		2.41			0.4
46	11,13-Dimethylheptacosane	11,13-Dime C27					0.4
47	7,15-Dimethylheptacosane	7,15-Dime C27				7.5	
48	5,15-Dimethylheptacosane	5,15-Dime C27				1.93	
49	5,13-Dimethylheptacosane	5,13-Dime C27	5.97	0.59			0.18
50	5,12-Dimethylheptacosane	5,12-Dime C27					0.18
51	5,11-Dimethylheptacosane	5,11-Dime C27					0.18
52	5,9-Dimethylheptacosane	5,9-Dime C27					0.18
53	4,16-Dimethylheptacosane	4,16-Dime C27			0.15		
54	4,12-Dimethylheptacosane	4,12-Dime C27			0.15		
55	3-Methylheptacosane	3-Me C27	4.23	6.53			2.86
56	3,14-Dimethylheptacosane	3,14-Dime C27					2.4
57	3,12-Dimethylheptacosane	3,12-Dime C27					2.4
58	3,7-Dimethylheptacosane	3,7-Dime C27					2.4
59	n-octacosane	n-C28	1.19	1.19			
60	14-Methyloctacosane	14-Me C28	1.27	0.27		1.23	0.18
61	13-Methyloctacosane	13-Me C28				0.74	0.18
62	12-Methyloctacosane	12-Me C28		0.27		0.67	0.18
63	11-Methyloctacosane	11-Me C28					0.18
64	10-Methyloctacosane	10-Me C28					0.18
65	9-Methyloctacosane	9-Me C28					0.18
66	8-Methyloctacosane	8-Me C28		0.35			0.18
67	5,9,13-Trimethylheptacosane	5,9,13-Trime C27	1.11				
68	14,15-Dimethyloctacosane	14,15-Dime C28			0.63		
69	12,16-Dimethyloctacosane	12,16-Dime C28		0.43			
70	12,14-Dimethyloctacosane	12,14-Dime C28	0.67				
71	10,14-Dimethyloctacosane	10,14-Dime C28		0.41			
72	8,12-Dimethyloctacosane	8,12-Dimethyl C28			0.71		
73	6-Methyloctacosane	6-Me C28	0.6				
74	4-Methyloctacosane	4-Me C28	0.34				
75	n-Nonacosene	C29:1		2.39	0.1		
76	6,14-Dimethyloctacosane	6,14-Dime C28	0.35				
77	5,13-Dimethyloctacosane	5,13-Dime C28	0.42				
78	4,14-Dimethyloctacosane	4,14-Dime C28	0.22				
79	4,12-Dimethyloctacosane	4,12-Dime C28	0.22				
80	n-Nonacosane	n-C29	1.16	3.28	12.3	2.2	1.34
81	15-Methylnonacosane	15-Me C29	0.64	0.29	2.58	1.4	
82	13-Methylnonacosane	13-Me C29	0.69	0.28	3.98	1.4	
83	11-Methylnonacosane	11-Me C29	0.62	0.28			3.73
84	4,8,12-Trimethyloctacosane	4,8,12-Trime C28			2.52		
85	7-Methylnonacosane	7-Me C29	0.66			1.47	
86	2,6,12-Trimethyloctacosane	2,6,12-Trime C28			0.34		
87	13,15-Dimethylnonacosane	13,15-Dime C29		1.05			
88	5-Methylnonacosane	5-Me C29	0.62	0.76	9.92	0.59	
89	11,17-Dimethylnonacosane	11,17-Dime C29			0.49		0.15
90	11,15-Dimethylnonacosane	11,15-Dime C29		0.77			0.15
91	11,13-Dimethylnonacosane	11,13-Dime C29					0.15
92	9,17-Dimethylnonacosane	9,17-Dime C29					0.05

No.	Compounds		*T. madeirense*	*T. israele*	*T. nigerrimum*	*T. erraticum*	*T. simrothi*
93	9,15-Dimethylnonacosane	9,15-Dime C29					0.05
94	9,13-Dimethylnonacosane	9,13-Dime C29					0.05
95	7,19-Dimethylnonacosane	5,19-Dime C29			1.32		
96	7,15-Dimethylnonacosane	7,15-Dime C29				1.25	
97	3-Methylnonacosane	3-Me C29	0.46	0.39	5.12		
98	5,17-Dimethylnonacosane	5,17-Dime C29			1.39		
99	5,15-Dimethylnonacosane	5,15-Dime C29			1.28	0.61	
100	5,13-Dimethylnonacosane	5,13-Dime C29	1.17	0.8			0.07
101	5,12-Methylnonacosane	5,12-Dime C29					0.07
102	5,11-Dimethylnonacosane	5,11-Dime C29					0.07
103	5,9-Dimethylnonacosane	5,9-Dime C29					0.07
104	3,17-Dimethylnonacosane	3,17-Dime C29			4.89		
105	11,15,17-Trimethylnonacosane	11,15,17-Trime C29		3.62			
106	16-Methyltriacontane	16-Me C30	0.33				
107	15-Methyltriacontane	15 Me C30					1.12
108	14-Methyltriacontane	14-Me C30	0.32				1.12
109	13-Methyltriacontane	13-Me C30					1.12
110	12-Methyltriacontane	12-Me C30		0.26			1.12
111	11-Methyltriacontane	11-Me C30					1.12
112	10-Methyltriacontane	10-Me C30		0.25			1.12
113	9-Methyltriacontane	9-Me C30					1.12
114	8-Methyltriacontane	8-Me C30		0.25	6.32		
115	14,20-Dimethyltriacontane	14,20-Dime C30		0.09			
116	12,18-Dimethyltriacontane	12,18-Dime C30		0.09			
117	10,14-Dimethyltriacontane	10,14-Dime C30	0.4				
118	8,12-Dimethyltriacontane	8,12-Dime C30	4.91		24.3		
119	6,14-Dimethyltriacontane	6,14-Dime C30	0.4				
120	4,14-Dimethyltriacontane	4,14-Dime C30	0.31				
121	4,12-Dimethyltriacontane	4,12-Dime C30	0.3		0.44		
122	4,8-Dimethyltriacontane	4,8-Dime C30			0.43		
123	n-Untriacontene	C31:1		0.08			
124	n-Untriacontane	n-C31	0.21	0.19	1.47	0.84	
125	2,8,12-Trimethyltriacontane	2,8,12-Trime C30	0.07				
126	4,8,12-Trimethyltriacontane	4,8,12-Trime C30			3.03		
127	15-Methyluntriacontane	15-Me C31	0.07		0.83	1.19	
128	13-Methyluntriacontane	13-Me C31	0.07	0.7	0.81	1.21	0.17
129	11-Methyluntriacontane	11-Me C31	0.07	0.7			0.17
130	7-Methyluntriacontane	7-Me C31	> 0.01	0.89		2.08	
131	13,24-Dimethyluntriacontane	13,24-Dime C31			0.43		
132	5-Methyluntriacontane	5-Me C31	0.42		1.89		
133	13,15-Dimethyluntriacontane	13,15-Dime C31		4.3			
134	7,15-Dimethyluntriacontane	7,15-Dime C31		0.25	0.61	1.86	
135	7,13-Dimethyluntriacontane	7,13-Dime C31	0.63				
136	7,11-Dimethyluntriacontane	7,11-Dime C31	0.51				
137	5,17-Dimethyluntriacontane	5,17-Dime C31			3.91		
138	5,13-Dimethyluntriacontane	5,13-Dime C31	0.45				

139	11,15,17-Trimethyluntriacontane	11,15,17-Trime C31		3.81			
140	7,11,15-Trimethyluntriacontane	7,11,15-Trime C31			0.21		
141	5,9,13-Trimethyluntriacontane	5,9,13-Trime C31	0.25		2.79		
142	12-Methyldotriacontane	12-Me C32		0.1			
143	11-Methyldotriacontane	11-Me C32		0.1			
144	8,12-Dimethyldotriacontane	8,12-Dime C32	0.56		3.36		
145	8,12,16-Trimethyldotriacontane	8,12,16-Trime C32			0.11		
146	6,10,14-Trimethyldotriacontane	6,10,14-TrimeC32			0.11		
147	X,Y-Dimethyldotriacontane	x,y-Dime C32		0.41			
148	n-Tritriacontene	C33:1		0.63			
149	14,18,22-Trimethyldotriacontane	14,18,22-Trime C32		0.34			
150	n-Tritriacontane	n-C33		0.34			
151	13-Methyltritriacontane	13-Me C33					0.09
152	11-Methyltritriacontane	11-Me C33		4.55			0.09
153	13,19-Dimethyltritriacontane	13,19-Dime C33		4.03			
154	13,17-Dimethyltritriacontane	13,17-Dime C33		4.26			
155	11,15,17-Trimethyltritriacontane	11,15,17-Trime C33		6.24			
156	12-Methyltétratriacontane	12-Me C34		0.13			
157	11-Methyltétratriacontane	11-Me C34		0.13			
158	13-Methylpentatriacontane	13-Me C35		1.04			
159	11-Methylpentatriacontane	11-Me C35		1.02			0.06
160	13,19-Dimethylpentatriacontane	13,19-Dime C35		1.11			> 0.01
161	13,17-Dimethylpentatriacontane	13,17-Dime C35		1.17			> 0.01
162	5,13-Dimethylpentatriacontane	5,13-Dime C35	0.44				
163	11,15,17-Trimethylpentatriacontane	11,15,17-Trime C35		1.45			
164	11-Methylheptatricontane	11-Me C37					> 0.01
165	15,19-Dimethylheptatriacontane	15, 19-Dime C37					> 0.01
166	15,17-Dimethylheptatriacontane	15, 17-Dime C37					> 0.01
167	13,17-Dimethylheptatriacontane	13,19-Dime C37					> 0.01
168	13,17-Dimethylheptatriacontane	13,17-Dime C37					> 0.01

branched alkanes (mono-, di- and trimethyl) and some straight-chain *n*-alkanes, but only *T. israele* and *T. nigerrimum* present *n*-alkenes.

Cuticular profiles of *Tapinoma erraticum*, *T. israele*, *T. madeirense* and *T. simrothi* (Fig. 3) were dominated by monomethylalkanes (respectively 44%, 30.5%, 42% and 68%), while the cuticular profile of *T. nigerrimum* workers was dominated by dimethylalkanes (43%) (Tab. 3, Fig. 3). The profile of the *T. simrothi* worker was more diverse and wide-ranging (70 CHCs ranging from C25 to C39) than that of the four other species. The profile of the *T. israele* worker ranged from C25 to C38 (65 CHCs), while that of the *T. erraticum* worker presented 35 CHCs ranging from C25 to C33 carbon atoms. *T. nigerrimum* worker profile ranged from C27 to C35 (35 CHCs) and that of the *T. madeirense* worker was composed of 62 CHCs ranging from C25 to C35 carbon chain lengths (Tab. 3).

Only *n*-C27, *n*-C29 and 13-MeC31 were common to all five species (1.9%). Ten cuticular hydrocarbons were common to four species (5.9%), 14 were common to three species (8.3%), 33 were common to two species (19.6%) and 108 were found in the profile of workers from one species only (64.3%). On average, workers from two different species had 16 CHCs in common; however *Tapinoma israele* and *T. madeirense* workers shared 26 compounds (respectively 40% and 42% of their entire profile), whereas *T. simrothi* and *T. nigerrimum* shared only four compounds (C27, C29, 11, 17 dimethyl-C29 and 13me-C31), respectively 5.7% and 11% of the entire profile of each species. *T. erraticum* shared 48.7% of its profile with *T. israele* (19 CHCs), while *T. israele* shared with this species only 29% of its own profile (Tab. 3). It is possible, using putative species recognition cues, to correctly identify each *Tapinoma* species through compounds which greatly contribute to their discrimination. *T. israele* can be identified easily by the presence of C27, C29 and C33 alkene, dimethyl-C33, methyl-C34, and a suite of 11, 15, 17 trimethyl-C29 / C31 / C33 / C35. The *T. nigerrimum* CHC profile is dominated by compounds greatly contributing to the discrimination of this species (8, 12 dimethyl-C30 (24.3%); 7, 9- & 3, 17- dimethyl-C29 and 4, 8, 12 trimethyl-C28 / C31). *T. simrothi* can be easily discriminated from the other *Tapinoma* species by the presence of suites like 9 to 13 methyl-C26 / C30; 5, 13- 5, 12- & 5,9- dimethyl-C27 and 3, 12- 3, 7- & 3,4- dimethyl-C27. Four dimethyl-alkanes (7, 5 dimethyl-C25; 12, 13- & 4, 6- dimethyl-C26 and 7, 15

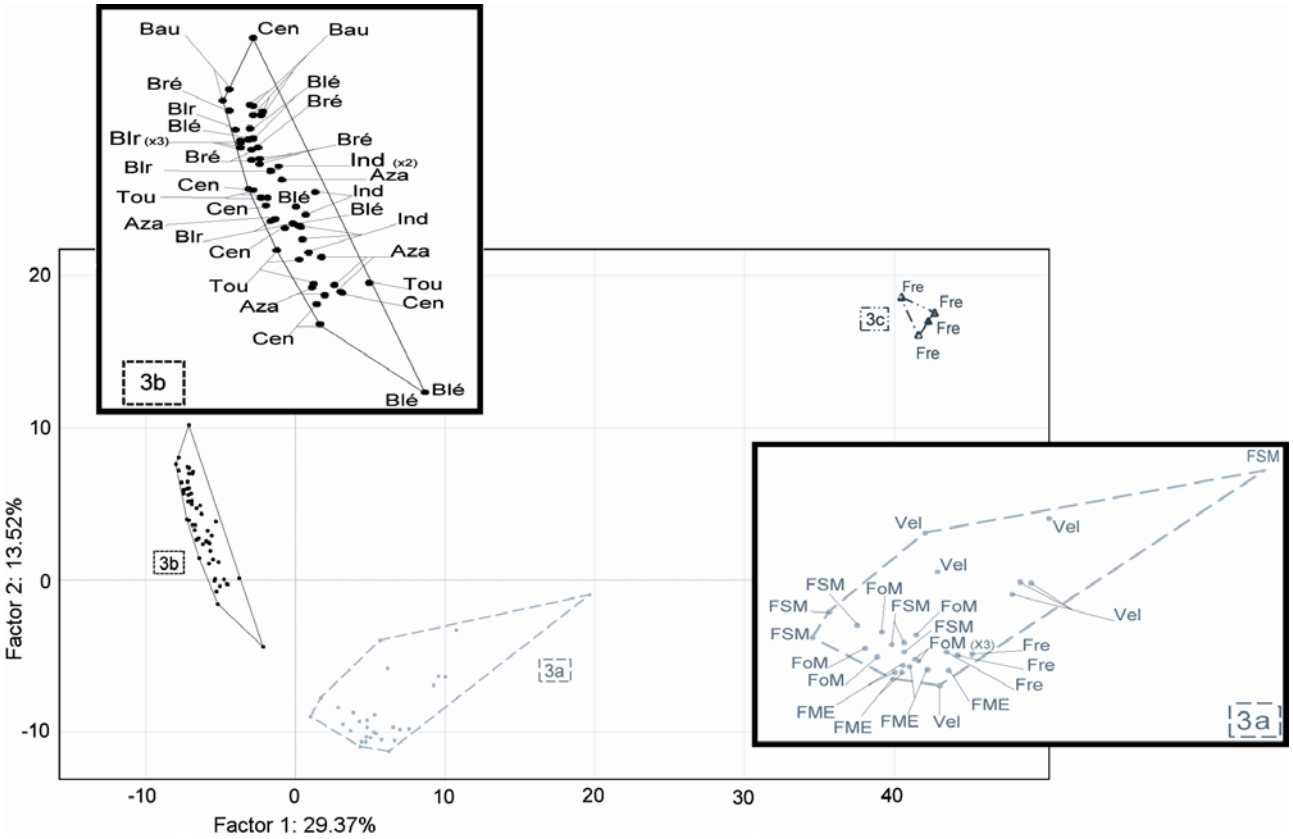

Fig. 5: Principal Component Analysis based on the 77 CHCs of worker profiles of the third group of the first PCA. The projection was performed on factorial plane 1 - 2. Axis 1 accounts for 29.37% of the total variance and axis 2 for 13.52%. *K*-means cluster analysis distinguishes three groups: *Tapinoma madeirense* [3a-grey circles], *T. erraticum* [3b-black circles] and four workers from Fréjus [3c-dark grey triangles]. The areas correspond to the 95% confidence limit of the data.

dimethyl-C27) greatly contributed to the discrimination of *T. erraticum. T. madeirense* can be identified by using both methyl-alkane (5 & 4 methyl-C26 and 6 & 4 methyl-C28) and dimethyl- alkane (5, 12- & 4, 12 dimethyl-C26; 6, 14- & 5, 13- dimethyl-C28; 4, 14- & 4, 12 dimethyl-C28; 7, 13- 7, 11- dimethyl-C31 and 5, 13 dimethyl-C31) (Tab. 3).

In order to better understand the contribution of the five hydrocarbon groups in the species determination, a PCA based only on dimethyl- and trimethyl- alkanes was performed. The same discrimination (PCA not shown) between the five species was obtained irrespective of the presence or not of the alkenes, alkanes and methyl-alkanes. The *K*-means analysis identified the same four chemical groups. Moreover, the exclusion of the dimethyl- and / or trimethyl-alkanes significantly decreases the capacity to distinguish species.

A hierarchical cluster analysis was performed on all the qualitative cuticular hydrocarbon dissimilarities (Fig. 6). This cluster was constructed by measuring the binary squared Euclidean distance, in order to look at the relationship between species using Ward's linkage method. This cluster shows a first separation between, on the one hand *Tapinoma simrothi* and *T. israele* and, on the other hand, *T. madeirense*, *T. erraticum* and *T. nigerrimum*. A second separation distinguishes *T. nigerrimum* from *T. erraticum* and *T. madeirense.*

Cuticular hydrocarbons from the three workers from Madeira Island which arrived alive at the laboratory were analyzed by gas chromatography. Their profiles were qualitatively identical to the profile of *Tapinoma madeirense*.

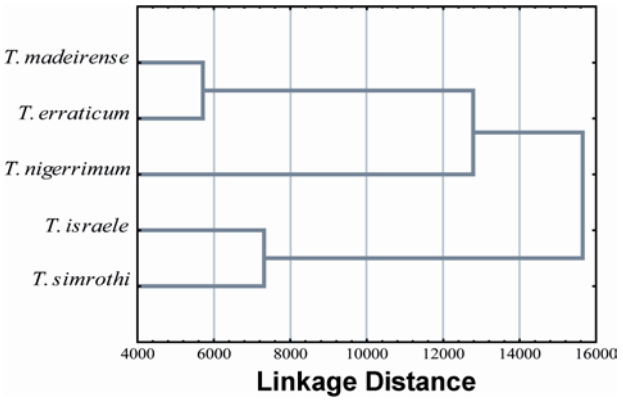

Fig. 6: Cluster analysis of cuticular hydrocarbon compositions of workers of *Tapinoma erraticum*, *T. madeirense*, *T. israele*, *T. nigerrimum*, and *T. simrothi* using Ward's linkage method (Euclidean distance) (WARD 1963).

Discussion

High correlation and reliability between CHCs and morphological analyses for species identification has previously been shown, for example, with the cryptic *Pachycondyla villosa* complex (LUCAS & al. 2002) or the European

Tetramorium sibling species (SCHLICK-STEINER & al. 2006). CHCs can discriminate sibling species (CREMER & al. 2008), cryptic species (LUCAS & al. 2002), or even hybrids (VANDER MEER 1986), like any phenotypic character that is an expression of DNA (for a review see SEIFERT 2009). Here, based on multiple comparisons, both chemical and morphological, we concluded that all the groups studied are distinct *Tapinoma* species. Chemical analyses support and corroborate the morphological separation of the total nesting sites into five groups and allow us to identify them as *T. israele*, *T. erraticum*, *T. nigerrimum*, *T. simrothi*, and *T. madeirense*. These differences lend support to the species status of the five studied taxa.

Our study shows that the CHCs are extremely different among all five species. The species can be reliably separated by principal component analysis of their cuticular hydrocarbons, since considerable interspecific dissimilarities were found in the composition of the five *Tapinoma* CHC profiles. Recently, MORRISON & WITTE (2011) showed strong differences in chemical recognition cues between two species of *Lasius*. *Lasius niger* and *L. platythorax* shared 17 compounds (i.e., 26.6% of the *L. niger* CHCs and 34% of those of *L. platythorax*). Here, we found that *T. israele* shared with *T. nigerrimum* only ten compounds (i.e., 15.4% of the *T. israele* CHCs and 28.6% of those of *T. nigerrimum*) and *T. erraticum* shared with *T. simrothi* 13 compounds (i.e., 33.33% of the *T. erraticum* CHCs and 18.6% of those of *T. simrothi*). Maximum interspecific dissimilarities were found between *T. simrothi* sharing 5.7% of its profile and *T. nigerrimum* sharing 11.4% of its profile, with only four compounds in common. All five *Tapinoma* species share only three CHCs and only *T. nigerrimum* and *T. israele* have *n*-alkenes. Of the three shared CHCs, two are odd-numbered *n*-alkanes (C27 and C29) which are typically dominant in ant CHC profiles (MARTIN & DRIJFHOUT 2009). They may be used as chemical communication signals, but their ubiquity means that they could be general indicators only (MARTIN & DRIJFHOUT 2009). As previously shown by MARTIN & al. (2008), the exclusion of the methyl-alkane, alkane and alkene did not significantly reduce the capacity of distinguish species. However, the exclusion of the dimethyl and / or trimethyl-alkanes in the PCA significantly decreases the discrimination, suggesting that it is these compounds that contain the maximum information.

Moreover, VAN WILGENBURG & al. (2011) showed that ant species have on average 18.7 ± 9.84 different homologous alkenes and methyl branched alkane series within their profile; *Tapinoma* species have on average 53.6 ± 16.02 different CHCs. These closely-related *Tapinoma* species appear to differ strongly in their CHCs, as with *Formica fusca* and *F. lemani* (see MARTIN & al. 2008) or others (AKINO & al. 2002, LUCAS & al. 2005), suggesting that major changes in their composition may have occurred during speciation events (VAN WILGENBURG & al. 2011).

In addition to being very different chemically, these closely-related *Tapinoma* species seem to produce a wide range of CHCs, leading to diverse biophysical properties. Across the 78 ant species included in their review, MARTIN & DRIJFHOUT (2009) found 993 different compounds; these five *Tapinoma* species presented more than 16% of the total of these compounds. The image of the ant as a chemical factory is fully reinforced by the *Tapinoma* genus.

Because of their species-specificity, CHCs were first considered for chemotaxonomy in 1970 (JACKSON & BAKER 1970). They may help to delimit a number of different species and can be used as an additional taxonomic tool in combination with morphological methods. Cluster analysis was based here on the presence / absence of hydrocarbons rather than on their proportions, because conspecific colony profiles differ in CHC ratios. In the light of the chemical cluster analysis, *T. simrothi* seems to be closer to *T. israele* and *T. erraticum* to *T. madeirense*, with *T. nigerrimum* in between. This result converges with morphological characteristics. However, a cluster tree based on the presence or absence of compounds must be taken with precaution. Using CHC profile information is potentially problematic when major changes in species composition have occurred at speciation events, as with several *Formica* (see MARTIN & al. 2008) or with our *Tapinoma* species. For example, these five *Tapinoma* species belonging to the same genus would not be clustered together in the dendrogram of MARTIN & DRIJFHOUT (2009) because they do not all produce *n*-alkenes. When such speciation events are involved, it is preferable to use CHC profiles only for intra-genus phylogeny.

Chemical cuticular profiles are known to be influenced by genetic and environmental factors (e.g., CROSLAND 1989, WAGNER & al. 2001, VAN ZWEDEN & al. 2008). If adaptations to abiotic parameters such as humidity or temperature (WAGNER & al. 2001) were the driving force for chemical differentiation between species, we would expect longer-chained hydrocarbons with the more xero- and thermophilic species (*Tapinoma simrothi* and *T. israele*), as they offer better protection against desiccation (GIBBS 1998). *Tapinoma erraticum* presented shorter-chained hydrocarbons than *T. simrothi or T. israele* (Tab. 3).

Chemical profiles of species can be altered by a close contact with the prey profiles (LIANG & SILVERMAN 2000) or by environmental factors. In most cases, the changes remain small or moderate, mainly affecting the proportions of some of the compounds (MORRISON & WITTE 2011). But in our study, the chemical similarity within *Tapinoma* species is remarkable. The effect of environmental factors and diet seems negligible in spite of the obvious environmental gradient induced by the fact that some of these populations are separated by 900 kilometers (*T. erraticum*), 1300 km (*T. nigerrimum*) and even more than 3000 km (*T. israele*). At shorter distances, the same stability of CHCs has been shown, for example, in *Lasius niger* in western Europe [France and Belgium (LENOIR & al. 2009); Germany (DINTER & al. 2002) and Denmark (DREIER & D'ETTORRE 2009)], in *Formica lemani* [England, Finland and Ireland (MARTIN & al. 2009, SEPPÄ & al. 2011)], and in *Formica exsecta* (see MARTIN & al. 2008). Although the pronounced interspecific dissimilarities found here may well result from ecological differences, this cannot explain the intraspecific similarities. In addition to passive mechanisms, adaptations to specific niches might have been expected to lead to chemical differences between *T. israele* from Algeria and from Israel, or between *T. nigerrimum* from Morocco and from France. If the parameters principally influencing CHCs were environmental, species would consequently have been more similar within sites, where they share the same environment. If CHCs serve mainly protective functions, selection should drive the species' pro-

files apart, but we did not find intermediate chemical phenotypes.

Among all the nesting sites, 58% of the nesting sites were identified as *Tapinoma nigerrimum.* This species seems to be well spread around the Mediterranean Basin. Despite the major search made, *T. simrothi* nesting-sites were not found in southern mainland France and Corsica. This absence of *T. simrothi* in southern mainland France and Corsica could be explained by misidentification (in precedent works), insufficient field collection (in this work) or the disappearance of the species. The presence of the nesting site from Lalla Setti (Lal) permits us to demonstrate the existence of *T. israele* in Algeria. Despite the major search made in France and Spain, no *T. israele* nesting-sites were found.

The findings from our work clearly show how using chemical and morphological techniques together facilitates the study of these species, by allowing precise differentiation and identification. The chemistry of the CHC bouquet is shown to be an excellent tool for taxonomists, being species-stable over thousands of kilometers.

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