



## Delineating species boundaries using an iterative taxonomic approach: The case of soldierless termites (Isoptera, Termitidae, Apicotermitinae)

Thomas Bourguignon<sup>a,b</sup>, Jan Šobotník<sup>c,d</sup>, Robert Hanus<sup>a,c</sup>, Jana Krasulová<sup>c,e,\*</sup>, Vladimír Vrkoslav<sup>c</sup>, Josef Cvačka<sup>c</sup>, Yves Roisin<sup>a</sup>

<sup>a</sup> Evolutionary Biology and Ecology, CP 160/12, Université Libre de Bruxelles, Avenue F.D. Roosevelt 50, 1050 Brussels, Belgium

<sup>b</sup> Department of Biological Sciences, National University of Singapore, 117543 Singapore, Singapore

<sup>c</sup> Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, 166 10 Prague, Czech Republic

<sup>d</sup> Czech University of Life Sciences, Faculty of Forestry and Wood Sciences, Kamýcká 129, 165 21 Prague 6 – Suchbát, Czech Republic

<sup>e</sup> Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Albertov 6, 128 43 Prague 2, Czech Republic

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### ABSTRACT

Species boundaries are traditionally inferred using morphological characters, although morphology sometimes fails to correctly delineate species. To overcome this limitation, researchers have widely taken advantage of alternative methods such as DNA barcoding or analysis of cuticular hydrocarbons (CHs) profiles, but rarely use them simultaneously in an iterative taxonomic approach. Here, we follow such an approach using morphology, DNA barcoding and CHs profiles to precisely discriminate species of soldierless termites, a diversified clade constituting about one-third of the Neotropical termite species richness, but poorly resolved taxonomically due to the paucity of useful characters. We sampled soldierless termites in various forest types of the Nouragues Nature Reserve, French Guiana. Our results show that morphological species determination generally matches DNA barcoding, which only suggests the existence of three cryptic species in the 31 morphological species. Among them, *Longustitermes manni* is the only species whose splitting is corroborated by ecological data, other widely distributed species being supported by DNA barcoding. On the contrary, although CHs profiles provide a certain taxonomic signal, they often suggest inconsistent groupings which are not supported by other methods. Overall, our data support DNA barcoding and morphology as two efficient methods to distinguish soldierless termite species.

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### 1. Introduction

The concept of species has been largely debated and more than 20 definitions, sometimes incompatible, have been proposed (Mayden 1997). Recently, de Queiroz (2007) attempted to reconcile those concepts emphasizing that they all share a common element, and proposed to define species as separately evolving metapopulation lineages. This unifying concept clearly separates the conceptual problem of defining the species category from the methodological problem of species delimitation, i.e. inferring species boundaries (de Queiroz 2007).

Traditional species delimitation is often based on morphological characters, but in many cases, morphology fails to discriminate among closely related species. This is especially true for species in which individuals do not recognize each other based on visual cues. In those taxa, differentiation between species may involve

chemical or acoustic cues rather than visual ones, making species morphologically indistinguishable (Bickford et al., 2007; Henry 1994). Such “cryptic” species have now been revealed in many animal taxa, generally by molecular data (Bickford et al., 2007), such as DNA barcoding.

DNA barcoding was proposed as an easy and cheap method to distinguish between species (Hebert et al., 2003). Species identification relies upon a COI tag sequence and a cut-off dissimilarity value, generally set at 2% for vertebrates and 3% for invertebrates, below which individuals are considered as conspecific and above which they are deemed to represent distinct species (Hebert et al., 2003). The simplicity of the concept is appealing and has attracted many researchers, quickly gathering a huge quantity of DNA barcodes (Hajibabaei et al., 2007). However, there are cases where DNA barcodes fail to identify species well characterized by other methods, e.g. where gene trees do not match species trees due to introgression or retention of ancestral polymorphism (Funk and Omland, 2003). For this reason and some other drawbacks, DNA barcoding has been heavily criticized by its detractors (e.g. Wheeler 2005; Will et al., 2005), while its proponents underline

\* Corresponding author at: Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, 166 10 Prague, Czech Republic

E-mail address: [krasulova@uochb.cas.cz](mailto:krasulova@uochb.cas.cz) (J. Krasulová).

its ease and advance that the method overcomes morphologically based taxonomy (e.g. Hebert et al., 2004; Packer et al., 2009).

Another commonly used non-morphological taxonomic characters are profiles of cuticular hydrocarbons (CHs), which have been proved useful to distinguish insect species, especially among social insects including termites (e.g. Haverty et al., 1992, 2000, 2005; Bagine et al., 1994). CHs, albeit in small amounts, form a substantial part of insect cuticle and are responsible for its waterproof properties. Insect hydrocarbons are in general synthesized *de novo*, often by modified epidermal secretory cells, the oenocytes (Chapman 1998; Howard and Blomquist 2005). At the same time, they are exploited by social insects as important identification tags allowing caste-, age-, or task-related recognition, as well as intra- and inter-specific discrimination (Blomquist et al., 1998; Singer 1998; Martin et al., 2008). Using CHs profiles for species recognition thus allows comparing directly the chemical characters through which social insects recognize each other. A few studies focusing on termites have also shown the species-specificity of soldier frontal gland secretion (for review see Šobotník et al., 2010) or isoenzymatic composition (Lopes and Ruvolo-Takasusuki 2010) and their usefulness to infer species identity, but the DNA sequences and to a lesser extent the CHs profiles remain the two most common alternatives to morphology-based taxonomy.

Beside the debate surrounding DNA barcoding and its oversimplified species concept, several authors have argued about the necessity of switching over an integrative taxonomy approach. The term “integrative taxonomy” was coined simultaneously by Dayrat (2005) and Will et al. (2005) and refers to the use of independent sets of characters to precisely assess species boundaries. In this study, we followed a multisource approach to distinguish species, combining the results of three disciplines, i.e. morphology, DNA barcoding, and CHs profiling, analyzed separately and compared for concordance (Schlick-Steiner et al., 2010). This comparative approach, testing species boundaries against several lines of evidence, was termed “iterative taxonomy” by Yeates et al. (2011), who defined “integrative taxonomy” as the use of different data contributing together to the delimitation of species boundaries. Here, we will follow Yeates et al. (2011) nomenclature and relate to the term “iterative taxonomy”.

Termites are abundant and diversified in tropical and subtropical ecosystems. Several studies have already pointed out the presence of cryptic species in termites, highlighting that as in many other animal taxa, distinct termite species are sometimes morphologically indistinguishable (e.g. Bagine et al., 1994; Cheng et al., 2011; Davison et al., 2001; Roy et al., 2006; Watson et al., 1989). Additionally, genetic characters have also been used as evidence to synonymize introduced species characterized by a disjunct distribution (Austin et al., 2005; Scheffrahn et al., 2005a,b). Here, we focus on soldierless termites, mostly represented by soil-dwellers, feeding on degraded organic matter (e.g. Bourguignon et al., 2011a). Soldierless termites constitute about one-third of the termite diversity in African and South American rainforests (Eggleton 2000). Because they lack the soldier caste, species identification must be based on alate imagoes, when collected, and on workers, and requires the dissection and close examination of their digestive tube (Noirot 2001). Such a complicated way to discriminate species, coupled with the cryptic life-style of most species, explains the paucity of studies devoted to their taxonomy (Sands 1972, 1999; Fontes 1986; Bourguignon et al., 2010), leaving a very large gap in termite knowledge.

This study aims at two goals:

- (1) We applied an iterative taxonomic approach, taking advantage of the multitude of methods currently available, to provide an accurate image of species boundaries (Schlick-Steiner et al., 2010; Yeates et al., 2011). We

combined morphological examination, DNA barcoding, and CHs profiling using two analytical approaches in order to understand the species diversity and boundaries of the soldierless termite fauna in the rainforest of the Nouragues Nature Reserve in French Guiana. Our first aim was to compare the effectiveness of morphological, molecular and chemical characters for species delimitation.

- (2) The Nouragues reserve is composed of a wide variety of habitats from which we sampled a continuum from the forest growing on poorly drained soil beside a creek, to the low forest growing on the foothills of the Nouragues inselberg (Poncy et al., 2001). We previously showed that different forest habitats differ in their termite fauna, although some species are ubiquitously found throughout a wide range of habitats (Bourguignon et al., 2011b). A recent study demonstrated that generalist springtail soil species often form complexes of cryptic species, more specialized than previously expected (Emerson et al., 2011). Our second aim was therefore to find out whether the ubiquitously distributed soil-feeding termite species are true species, or if they actually are clusters of ecologically specialized cryptic species.

## 2. Material and methods

### 2.1. Study site

Termite sampling took place in the Nouragues Nature Reserve (04°05'N, 52°41'W). The site is covered by primary tropical rainforest characteristic of the Guyana Shield. Although the Nouragues research station is dominated by the inselberg, culminating at 430 m, the altitude of sampling sites was between 80 m and 200 m. We sampled termites in 10 parcels around the station to cover the diversity of available habitats (Fig. 1). All the sites but three (H, I and J) consisted in squares of 100 × 100 m delimited by paths. The remaining three sites were not as strictly delimited but covered a comparable area. Sites A–C and E–G were in mature lowland rainforest growing on well-drained soil with an average canopy height reaching 30–40 m. Site D was very similar to the former six, but differed in the high density of lianas (Schnitzer and Bongers, 2002). Site H consisted of a forest growing beside a creek on poorly drained soil. Site J was a low forest growing on the foothills of the inselberg, transitional between lowland rainforest and the rock savanna growing on the granitic outcrop. This short-stature forest was dominated by *Clusia minor* (Clusiaceae) intermingled with other low trees not more than 10 m tall. Finally, site I was located at the boundary between a low forest and a typical primary lowland rainforest, representing an intermediate between the two types. All forests experience about 3000 mm of rainfall per year, mostly distributed between January and June. The mean annual temperature is 26 °C. The region is uninhabited and thereby almost free of human disturbance.

### 2.2. Sampling

We inspected all places suitable for soldierless termites, i.e. rotten wood, abandoned nests and soil. Specimens were collected with forceps and/or aspirator. For all groups of termites we encountered, three subsamples were collected: one in 80% alcohol for morphological species identification; one in pure ethanol, subsequently stored at –20 °C, for DNA extraction; and one, consisting of termite workers only, stored in vials partially filled with anhydrous magnesium sulfate (Sigma–Aldrich, St. Louis, MO, USA) plugged at the bottom with clean cotton wool, for CHs analysis.

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