

Near-Infrared Imaging Spectroscopy as a Tool to Discriminate Two Cryptic *Tetramorium* Ant Species

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Abstract Correct species identification is a precondition for many ecological studies. Morphologically highly similar, i.e., cryptic, species are an important component of biodiversity but particularly difficult to discriminate and therefore understudied ecologically. To find new methods for their rapid identification, thus, is important. The cuticle's chemical signature of insects often is unique for species. Near-infrared spectroscopy (NIRS) can capture such signatures. Imaging NIRS facilitates precise positioning of the measurement area on biological objects and high-resolution spatial capturing. Here, we tested the applicability of imaging NIRS to the discrimination of cryptic species by using the ants *Tetramorium caespitum* and *T. impurum*. The classification success of Partial Least Squares Regression was 98.8%. Principal Component Analysis grouped spectra of some *T. impurum* individuals with *T. caespitum*. Combined with molecular-genetic and morphological evidence, this result enabled us to pose testable hypotheses about the biology of these species. We conclude that discrimination of *T. caespitum* and *T.*

impurum with imaging NIRS is possible, promising that imaging NIRS could become a time- and cost-efficient tool for the reliable discrimination of cryptic species. This and the direct facilitation of potential biological insight beyond species identification underscore the value of imaging NIRS to ecology.

Key Words Cuticular hydrocarbons · Ant worker · Infrared spectroscopy · Chemical fingerprinting

Introduction

Species are basic units of ecology. However, species identification can be difficult, especially with morphologically nearly identical, i.e., cryptic, species. This situation hampers the ecological study of cryptic species, now known from many groups of organisms.

Near-infrared spectroscopy (NIRS) can afford an opportunity to make cryptic species accessible to ecology. By using the characteristic reaction of functional groups to infrared light in order to assign peaks to these functional groups, a fast and 'easy-to-use' identification tool has resulted (Workman and Weyer, 2008). It is increasingly used in many disciplines, including in entomology, as the cuticle's chemical signature is important for species discrimination in insects (Steiner et al., 2002; Martin and Drijfhout, 2009; Sikulu et al., 2010; Seppä et al., 2011). However, an exact positioning can be difficult for small specimens. Imaging-NIRS technology allows precise positioning of the measurement area and spectra capturing at a high spatial resolution (up to $6.25 \times 6.25 \mu\text{m}$), and thus has potential advantages over classical, fiber-optic-probe NIRS, where each spectrum represents an area of several square millimeters.

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Imaging NIRS has not been used for cryptic-species discrimination so far. Here, we investigated whether the cryptic ant species *Tetramorium caespitum* and *T. impurum*, which otherwise can be identified only via molecular genetics or high-precision morphometrics (Schlick-Steiner et al., 2006), can be discriminated by their NIR spectra.

Methods and Materials

We selected 15 nest samples for each species from various European countries, collected in –2007 and already species-identified using mitochondrial (mt) DNA and morphometrics (Schlick-Steiner et al., 2006; Steiner et al., 2010). Individuals had been killed with and stored in 99% ethanol p.a. A plane measurement area that could be identically positioned in all individuals was chosen, i.e., a 400×400 μm frons area. Thus, the heads of three workers per nest were stuck on a microscope slide with wallpaper paste in such a way that head width and length were maximum. We excluded incompletely colored, i.e., very young workers.

Spectra were recorded using a Spectrum 400 (Perkin Elmer) spectrometer and a Spotlight 400 microscope with a nitrogen-cooled MCT detector array (16×2). At a resolution of 25×25 μm, we collected 256 spectra per individual from the 400×400 μm area, for wavelengths 7,800–2,000 cm⁻¹. The 256 spectra were reduced to one spectrum representing the measurement area. Data were analyzed using The Unscrambler 10.0.1 (Camo).

Spectra obtained from a smaller region (150×150 μm) for the individuals of nests TM45, TM308, i62, TM235, TM290, and i36 were used in a pre-test (data unpublished). As spectra of the TM45 and TM308 individuals could not be separated from *T. caespitum* spectra by Principal Component Analysis (PCA; see results), DNA was extracted from these six decapitated individuals, and a cytochrome-C-oxidase subunit one fragment of mtDNA was reverse sequenced as in Schlick-Steiner et al. (2006), with slight modifications.

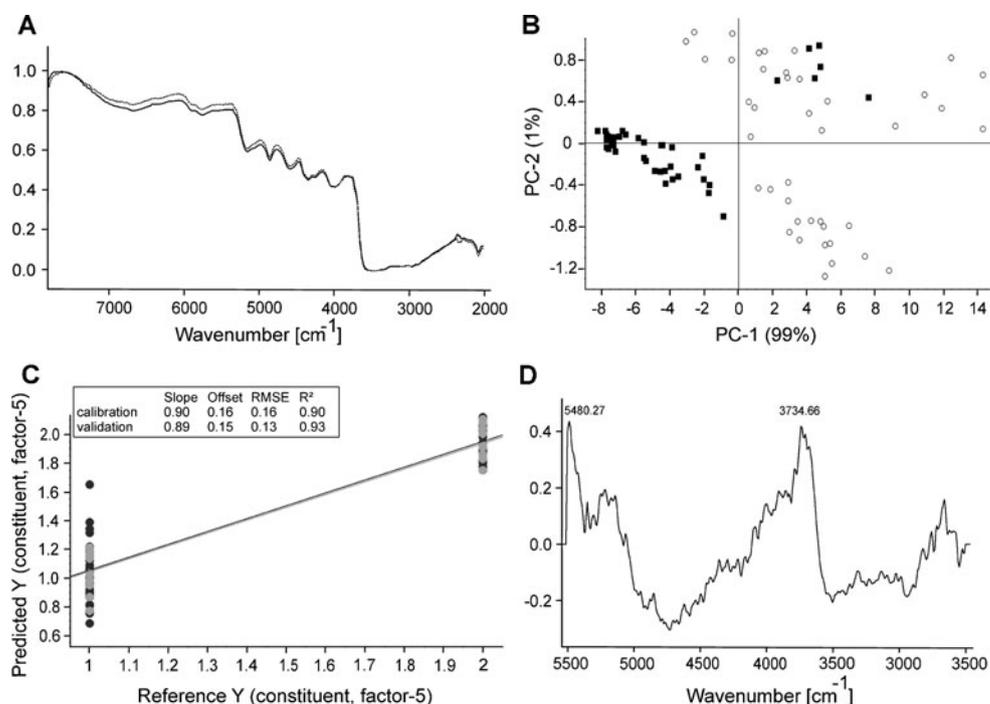
For statistical analysis, an unsupervised method, PCA, and a supervised method, Partial Least Squares (PLS) Regression (NIPALS algorithm) were used. We randomly chose 14 *T. caespitum* and 15 *T. impurum* spectra as a validation set. For PCA, data were mean-centred; the first and second PC were plotted. For PLS Regression, data were mean-centred and converted to first Savitzky-Golay derivative. We set the constituent to 1 and 2 for *T. caespitum* and *T. impurum* individuals, respectively. Calibration quality was evaluated using R^2 , root mean standard error of calibration (RMSEC), root mean standard error of prediction (RMSEP), and validation BIAS.

Regression coefficient plots were noisier in regions 7,800–5,500 and 2,500–2,000 cm⁻¹; therefore, we compared the regression coefficient plots of region 5,500–2,500 cm⁻¹.

Results

Heads of four individuals were destroyed during the measurement procedure. After checking spectra manually

Fig. 1 Differences between species. **a** Example mean spectra (normalized 0–1) of a *Tetramorium caespitum* (dotted line) and a *T. impurum* (solid line) individual. **b** PCA of 84 individuals' mean spectra. Circle: *T. caespitum*; square: *T. impurum*; the six squares in the range of *T. caespitum* represent the individuals of *T. impurum* nests TM45 and TM308. **c** PLS Regression, predicted vs. reference plot using five factors of 84 individuals for the calibration set (black) and 29 individuals for the validation set (grey). **d** Regression coefficient plot of region 5,500–2,500 cm⁻¹ for five factors



and via PCA, two outlier mean spectra were excluded. The remaining 84 spectra (for examples, see Fig. 1a) were used for the analyses.

In the PCA, the two species were separated, except for two nests (TM45, TM308; Fig. 1b) genetically and morphologically assigned to *T. impurum* by Schlick-Steiner et al. (2006). Our mtDNA analysis here of exactly those TM45 and TM308 individuals analyzed by NIRS also resulted in *T. impurum*. However, the independent imaging-NIRS pre-test had led to the same discrimination problem in PCA (data unpublished). When not considering TM45 and TM308, *Tetramorium impurum* showed less variability in its spectra than *T. caespitum*.

The PLS calibration was performed using five factors, and resulted in a correct classification of 98.8%, an R^2 of 0.90 for calibration and of 0.93 for validation. RMSEC and RMSEP were 0.16 and 0.13, respectively, and validation BIAS was -0.01 , meaning that the spectra used for calibration supported the separation and prediction of the two species (Fig. 1c).

The regression coefficients had two distinct peaks in the five-factor plot at 3,730 and 5,481 cm^{-1} (Fig. 1d).

Discussion

Our results suggest that cryptic ant species can be separated by their near-infrared spectra. The two distinct peaks in the regression-coefficient plots correspond to hydrocarbon overtone vibration (Workman and Weyer, 2008), suggesting that these peaks represent species-diagnostic characters.

Concerning the two *T. impurum* nests not assigned to this species via PCA, we exclude sample confusion and misidentification, because the six scanned individuals were assigned to *T. impurum* by mtDNA, and spectra of these individuals also clustered with *T. caespitum* in the NIRS pre-test. An influence of the preservation method over time (cf. Perez-Mendoza et al., 2002) is also unlikely to be causal to the situation because samples did not group according to age in the PCA, and other samples collected in the same years as TM45 (2000) and TM308 (2002) were discriminated correctly. Instead, we are aware of three possible biological explanations, now available for future testing: (i) Interspecific hybridization was inferred for the *T. caespitum/impurum* complex (Steiner et al., 2010). Mitochondria are passed uniparentally rendering hybridization undetectable by mtDNA alone. If, for example, TM45 and TM308 were older hybrids, i.e., not F1 hybrids, different identification results from morphometrics and NIRS (*T. caespitum*) could reflect different selection pressures on the morphological and the chemical phenotype. (ii) We had excluded very young workers, but the age of the included individuals could still influence CHC profiles as

known from other insects (Sikulu et al., 2010). (iii) TM45 and TM308 could have rare CHC profiles, and our *T. impurum* sample might under-represent the true variability of that species. Possibly in line with this, *T. impurum* was less variable in its spectra than *T. caespitum* when the two ‘problematic’ nests were excluded. However, two *Formica* ant species also differed strongly in CHC variability (Seppä et al., 2011), and such pattern could, alternatively, be common for closely related ant species.

With imaging NIRS, hands-on time for sample preparation and measurement was 25 min per individual, at 0.01 Euro costs. Imaging NIRS, thus, is faster than COI sequencing and morphometrics (85 and 75 min, respectively) and cheaper than COI sequencing (10.20 Euro); morphometrics causes no costs. Fiber-optic NIRS is even faster (Sikulu et al., 2010) and, like morphometrics, incurs no costs, but our imaging-NIRS-based PLS-classification success was higher when compared to that in fiber-optic-probe NIRS studies (e.g., Jia et al., 2007; Sikulu et al., 2010). Not included in the scanning area, the surface a specimen is placed on cannot influence measurements with imaging NIRS, and this might be one reason for the particularly good discrimination result. Biological insight beyond species identification may be facilitated by capitalizing on the incongruence for two nests across the results of the NIRS-based PCA and mtDNA- and morphometrics-based species identification. Finally, by allowing exact positioning of the measurement area and image capturing at a high resolution, investigating whether the chemical signature differs across body parts will be possible. This could open up additional possibilities for, e.g., NIRS-based behavioral-ecological studies (e.g., Newey et al., 2008). Thus, imaging NIRS gives a time- and cost-efficient opportunity to make cryptic species accessible to ecology and to further investigate their ecological significance.

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