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RESEARCH ARTICLE

Reflectance-based identification of parasitized host eggs and adult *Trichogramma* specimens

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ABSTRACT

A wide range of imaging and spectroscopy technologies is used in medical diagnostics, quality control in production systems, military applications, stress detection in agriculture, and ecological studies of both terrestrial and aquatic organisms. In this study, we hypothesized that reflectance profiling can be used to successfully classify animals that are otherwise very challenging to classify. We acquired hyperspectral images from adult specimens of the egg parasitoid genus *Trichogramma* (*T. galloi*, *T. pretiosum* and *T. atopovirilia*), which are ~1.0 mm in length. We also acquired hyperspectral images from host eggs containing developing *Trichogramma* instar and pupae. These obligate egg endoparasitoid species are commercially available as natural enemies of lepidopteran pests in food production systems. Because of their minute size and physical resemblance, classification is time consuming and requires a high level of technical experience. The classification of reflectance profiles was based on a combination of average reflectance and variogram parameters (describing the spatial structure of reflectance data) of reflectance values in individual spectral bands. Although variogram parameters (variogram analysis) are commonly used in large-scale spatial research (i.e. geoscience and landscape ecology), they have only recently been used in classification of high-resolution hyperspectral imaging data. The classification model of parasitized host eggs was equally successful for each of the three species and was successfully validated with independent data sets (>90% classification accuracy). The classification model of adult specimens accurately separated *T. atopovirilia* from the other two species, but specimens of *T. galloi* and *T. pretiosum* could not be accurately separated. Interestingly, molecular-based classification (using the DNA sequence of the internally transcribed spacer ITS2) of *Trichogramma* species published elsewhere corroborates the classification, as *T. galloi* and *T. pretiosum* are closely related and comparatively distant from *T. atopovirilia*. Our results emphasize the importance of using high-spectral and high-spatial resolution data in the classification of organism relatedness, and hyperspectral imaging may be of relevance to a wide range of commercial (i.e. producers of biocontrol agents), taxonomic and evolutionary research applications.

KEY WORDS: Hyperspectral imaging, Trichogrammatidae, Biological control, Machine vision, Variogram analysis

INTRODUCTION

A recent review article claims that UAVs (unmanned aerial vehicles) will revolutionize research methods in spatial ecology (Anderson and Gaston, 2013). Progressive expansion of image-based applications in both research and commercial applications is driven by a series of trends, including: progressive improvements in the robustness of vehicles and sensors, lighter and more powerful technologies, lower cost of equipment, and advances in processing and classification of imaging data. Here, we argue that advances in the development of imaging technologies not only improve our ability to acquire, process and interpret spatial data on a large geographical scale but also enable new methods of classifying organisms (animals and growing plants and seeds) and non-destructively assessing their relatedness.

The intensity and composition of photons reflected (here referred to as reflectance profiles) from an object is partially determined by the radiometric energy source (lighting), projection angle, environmental conditions (i.e. temperature and barometric pressure), and the physical structure and/or biochemical composition of the objects' surface (Clark, 1999). Thus, if the lighting source, projection angle and environmental conditions are maintained constant, distinct differences in reflectance profiles between target objects are assumed to be associated with differences in the physical structure and/or biochemical composition of the target objects. Hyperspectral imaging technologies have been used to provide insight into a wide range of biological phenomena, including: (1) how color-blind cuttlefish [*Sepia officinalis* L. (Cep.: Sepiida)] camouflage themselves (Chiao et al., 2011), (2) twilight spectral dynamics and their effects on mass spawning events on coral reefs (Sweeney et al., 2011), (3) vision in honeybees [*Apis mellifera* L. (Hym.: Apidae)] and orb-web spiders [*Nephila pilipes* Fabricius (Ara.: Nephilidae)] (Chiao et al., 2009) and (4) the role of an enlarged claw in mating courtship and territorial displays among fiddler crabs [*Uca vomeris* McNeill (Dec.: Ocypodidae)] (Zeil and Hofmann, 2001). In addition, reflectance-based technologies have been used to differentiate: species of stored grain insects (Singh et al., 2010), tobacco budworm [(*Heliothis virescens* (Fabricius) (Lep.: Noctuidae)] and corn earworm [(*Helicoverpa zea* (Boddie) (Lep.: Noctuidae)] (Jia et al., 2007), and to age-grade biting midges [*Culicoides sonorensis* Wirth and Jones (Dip.: Ceratopogonidae)] (Reeves et al., 2010). Klarica et al. (Klarica et al., 2011) used imaging spectroscopy to discriminate cryptic species of ants [*Tetramorium caespitum* (L.) and *T. impurum* (Foerster) (Hym.: Formicidae)]. Regarding classification based on reflectance profiles acquired from the external surface of insects and other organisms, both physical and biochemical factors are likely involved. It was beyond the scope of this study to identify and characterize the possible mechanisms responsible for species-specific differences in reflectance profiles, but the density and size of body hairs, body shape, and ornamentation and pigmentation of the insect cuticle are believed to be of major importance. As an example, the hydrocarbon composition of insect cuticle is known to be highly dynamic and vary: (1) among closely related species

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Received 11 August 2013; Accepted 27 November 2013

The Journal of Experimental Biology (2014) doi:10.1242/jeb.095661

(Dowell et al., 1999; Roux et al., 2008), (2) in relative composition (Geiselhardt et al., 2009) or in actual composition (Howard and Pérez-Lachaud, 2002) among males and females within a species, (3) during the lifespan of insect individuals (Butler et al., 2009; Howard and Baker, 2003; Lapointe et al., 2004; Roux et al., 2008; Zhu et al., 2006), (4) among eusocial individuals with different tasks (Ferreira-Caliman et al., 2010; Nunes et al., 2009), (5) according to mating behavior and status (Howard and Baker, 2003; Howard and Pérez-Lachaud, 2002; Steiner et al., 2007), and (6) in response to environmental conditions (Howard and Baker, 2003; Howard and Pérez-Lachaud, 2002; De Loof et al., 2010). Thus, there is ample evidence of the composition of the insect cuticula being highly dynamic and tightly associated with complex internal physiological processes in insects. And differences in the biochemical composition of the cuticula would imply corresponding differences in reflectance profiles and therefore the possibility of classifying even closely related species.

Trichogramma species are obligate egg endoparasitoids; about 210 *Trichogramma* species have been identified (Pinto, 2006) and they are among the mostly commercialized natural enemies for control of Lepidopteran pests (Li, 1994). Commercial mass-rearing of *Trichogramma* species occurs in at least 16 countries, including: (1) Russia, (2) Mexico, (3) China (Lenteren, 2008), (4) Cuba and (5) Brazil (Parra, 2010). *Trichogramma* species are released into a wide range of cropping systems, including horticultural crops, cereals, cotton (*Gossypium* spp. L.), corn (*Zea mays* L.), sugarcane (*Saccharum officinarum* L.), tobacco (*Nicotiana tabacum* L.) and pastures (Lenteren, 2008; Li, 1994; Parra, 2010). The success of *Trichogramma* spp. as biocontrol agents is partially linked to the possibility of mass rearing them on factitious host eggs from stored grain moth species, such as the Mediterranean flour moth [*Ephestia kuehniella* Zeller (Lep.: Pyralidae)] (Hassan, 1997; Lewis, 1976; Parra, 2010). However, Danks (Danks, 1988) and Smith (Smith, 1996) reported on several examples of costly biocontrol releases with limited effect on target pest populations, and the lack of success was partially attributed to misclassification and/or cross-contamination of species during the mass-rearing process. Thus, numerous studies have investigated the variability in quality of mass-reared *Trichogramma* species (Bigler, 1989; Cerutti and Bigler, 1995; Kuhlmann and Mills, 1999; Losey and Calvin, 1995; Lundgren and Heimpel, 2003). Zucchi and Querino (Zucchi and Querino, 2010) argued that continuous taxonomic monitoring of *Trichogramma* populations is important both in commercial mass-rearing facilities and under field conditions after mass releases. However, species identification of micro-hymenoptera requires a high level of technical experience and is also time consuming (Pinto and Stouthamer, 1994). Furthermore, as discussed by Almeida (Almeida, 2004), identification of *Trichogramma* species has in the past been widely based on male genitalic characteristics, which is a particular challenge as many *Trichogramma* species have partial or completely parthenogenic forms. Furthermore, identification based on male genitalic characteristics involves considerable practical experience, and it may also require fairly elaborate chemical procedures, including chemical treatments and correct mounting of genitalia on microscope slides (Querino and Zucchi, 2011). As part of a drive to improve methods used to classify *Trichogramma* species, Almeida (Almeida, 2004) discussed the potential of a range of molecular-based methods and developed a classification key for 17 South American species based on the DNA sequence of the internally transcribed spacer ITS2 (Stouthamer et al., 1999). The classification key was based on the size of the nuclear ribosomal gene ITS2 PCR product and three endonucleases (*Eco*RI, *Mse*I and *Mae*I). Recently, Querino and Zucchi (Querino and Zucchi, 2011) published a new key to *Trichogramma* species, but it is still important to emphasize that identification of minute adult *Trichogramma* specimens (1.0 mm in length) is challenging and requires considerable technical experience.

In this study, we hypothesized that classification of closely related animals can be conducted successfully on the basis of the spatial structure of high spatial (45 pixels mm^{-2}) and spectral (160 spectral bands from 405–907 nm) resolution imaging data. As a challenging model system of reflectance-based classification of insects and possibly other living organisms (other animals, growing plants and seeds), we acquired hyperspectral images of three *Trichogramma* species, which are obligate egg endoparasitoids. We acquired hyperspectral images of adult specimens (*T. galloi* Zucchi; *T. pretiosum* Riley and *T. atopovirilia* Oatman and Platner) and of parasitized host eggs. The classification of reflectance profiles was based on a combination of average reflectance and variogram parameters (describing the spatial structure of reflectance data) of reflectance values in individual spectral bands. Our data suggest that classification of hyperspectral imaging data may be used both to identify species and also to characterize their relatedness.

RESULTS

Identification of parasitized host eggs

Fig. 1A shows representative photos of non-parasitized (grayish) and parasitized (dark) host eggs, and Fig. 1C clearly indicates that parasitism of host eggs was detectable for the entire spectral range between 405 and 907 nm. Fig. 1B shows representative photos of host eggs parasitized by the three *Trichogramma* species, and it can be seen that they were virtually indistinguishable based on visual inspection. However, average reflectance profiles in Fig. 1C

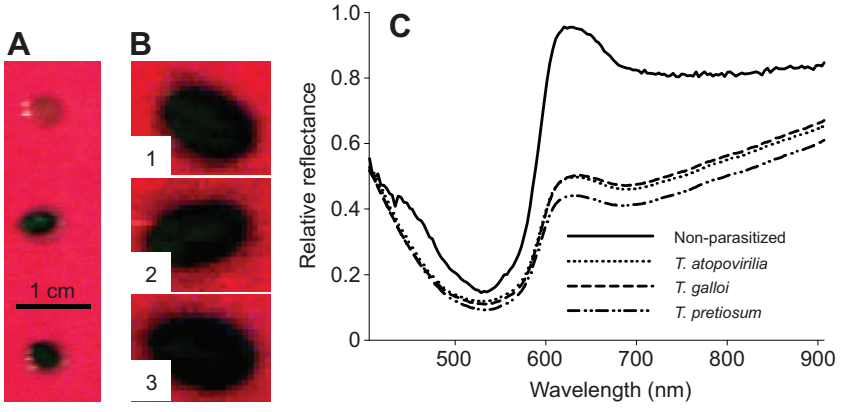

Fig. 1. Host eggs parasitized by *Trichogramma*. (A) Images of host eggs with (dark) or without (grayish) parasitism. (B) Host eggs parasitized by (1) *T. atopovirilia*, (2) *T. galloi* and (3) *T. pretiosum*. (C) Relative reflectance from host eggs in 160 spectral bands from 405 to 905 nm.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.095661

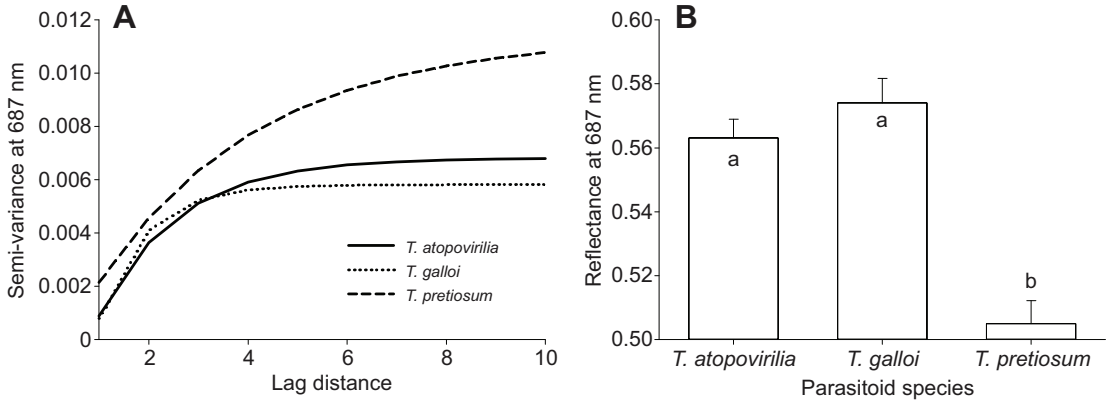

Fig. 2. Classification of parasitized host eggs. (A) Mean *c* parameter (derived from Eqn 3 and (B) mean (±s.e.) reflectance data at 687 nm of parasitized host eggs. Different letters represent a significant difference at the 0.05 level.

suggested that eggs parasitized by *T. pretiosum* were consistently darker than those parasitized by *T. atopovirilia* or *T. galloi* in all spectral bands beyond 600 nm. Thus, based on qualitative/visual assessment of average reflectance profiles of parasitized host eggs (Fig. 1C), there was substantial justification for pursuing a classification approach in which reflectance data in selected spectral bands were used to differentiate host eggs parasitized by different *Trichogramma* species. A total of 6500 pixels (mean ± s.e. of 108.13±4.45 pixels per host egg) were included after deploying a radiometric filter (Eqn 1) to hyperspectral images of parasitized host eggs. Based on derived variogram parameters (Eqn 3 and Fig. 2), we found that the spectral structure (variogram regression fit) of reflectance data at 687 nm (Fig. 2A) could be used to accurately separate host eggs parasitized by the three *Trichogramma* species with 86.67% accuracy (Table 1). All three *Trichogramma* species were classified with similar accuracy. Fig. 2B shows mean reflectance at 687 nm, which also varied significantly among *Trichogramma* species (d.f.=2,59, F=27.76, P<0.001), but there was no significant difference between *T. atopovirilia* and *T. galloi*. Thus, simple comparisons of reflectance averages at 687 nm could not be considered a reliable indicator of all three classes of parasitized host eggs. The linear discriminant classification model based on variogram parameters was validated with reflectance data from 25 independent host eggs parasitized by *T. atopivirilia* or *T. pretiosum*, which were classified with 91.17% accuracy (Table 1).

Identification of *Trichogramma* adults

Fig. 3A shows representative *Trichogramma* adults. The micro-hymenoptera are about 1 mm in length and are virtually indistinguishable based on visual inspection. However, average reflectance profiles from the six classes (three species and males/females) suggested that beyond 700 nm, males and females of *T. atopivirilia* were slightly darker than reflectance profiles from the other two species (Fig. 3B). However, there was no spectral region with a clear distinction of reflectance profiles between *T. pretiosum* and *T. galloi*. Thus, separation of adult specimens based on differences in average reflectance in one or more spectral bands appeared to be considerably more challenging than separation of parasitized host eggs. In total, 6318 pixels were included in the classification of *Trichogramma* adults with no significant difference in the number of pixels among species (d.f.=2,119, F=0.48, P=0.620) but with males (55.93±2.28 pixels) being significantly larger (more pixels acquired per specimen) than females (49.37±1.56 pixels) (d.f.=1,119, F=5.57, P=0.019). Testing variogram parameters in individual spectral bands in 10-band intervals between 405 and 907 nm, we found that a combination of variogram parameters and average reflectance acquired from six spectral bands (465, 497, 529, 560, 718 and 813 nm) could be used to accurately classify adult specimens of the three *Trichogramma* species with 85.0% accuracy (Table 2). Using only average reflectance in these six spectral bands (not using variogram parameters) enabled classification of adult *Trichogramma* specimens with 74.17% accuracy. In other words, including variogram parameters in the linear discriminant analysis added considerable accuracy to the classification. The linear discriminant classification model of adult *Trichogramma* specimens was validated with reflectance data from nine hyperspectral images of 10 (*T. galloi* only) or 12 adult (10 *T. galloi* and two *T. atopivirilia* or *T. pretiosum*) *Trichogramma* specimens. In validation analyses of hyperspectral images of 10 *T. galloi* specimens, none of the specimens were identified as *T. atopivirilia*, but 53% of them were incorrectly classified as *T. pretiosum*. In validation analyses of hyperspectral images of 10 *T. galloi* specimens mixed with two *T. pretiosum* specimens, none of the specimens were identified as *T. atopivirilia*, but 32% of them were incorrectly classified as *T. pretiosum* (higher proportion than two out of 12 individuals). In validation analyses of hyperspectral images of 10 *T. galloi* specimens mixed with two *T. atopivirilia* specimens, two *T. atopivirilia* specimens were correctly identified in all validation samples, but 36% of the adult specimens were incorrectly classified as *T. pretiosum* (although they were *T. galloi* specimens). Thus, the classification model accurately detected whether *T. atopivirilia* individuals were present or absent in the mixtures (classification accuracy=100%). However, the classification model was not able to accurately differentiate between adult specimens of *T. galloi* and *T. pretiosum* (classification accuracy <65.0%).

Table 1. Linear discriminant classification of parasitized host eggs

	Assigned class by training model		
Actual class	*T. atopovirilia*	*T. galloi*	*T. pretiosum*
T. atopovirilia	**85.00** (83.33)	5.00	10.00 (16.67)
T. galloi	20.00	**80.00**	0.00
T. pretiosum	10.00 (0.00)	0.00	**90.00** (100.00)

Hyperspectral image samples (N=60) of parasitized host eggs were classified with 85% accuracy (mean of numbers is in bold). For independent validation, 25 image samples (in parentheses) were used and classified with 91.17% accuracy.

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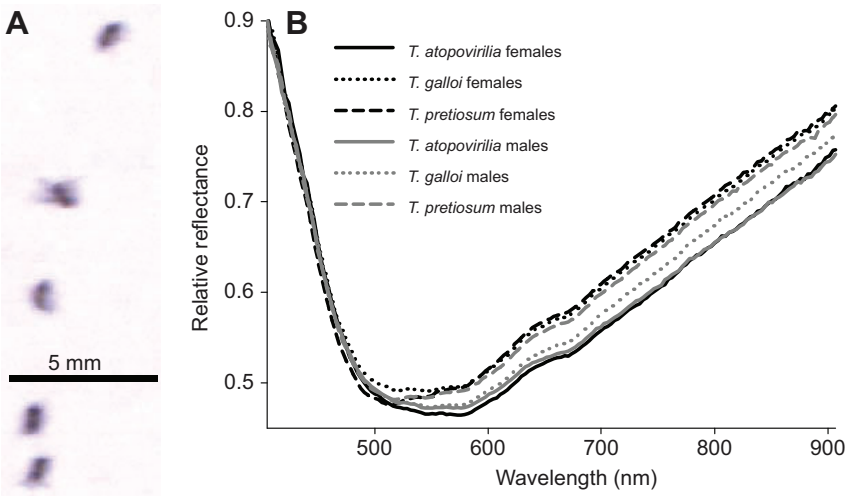

Fig. 3. Adult *Trichogramma* individuals. (A) Images of adult *Trichogramma* specimens. (B) Relative reflectance from host eggs in 160 spectral bands from 405 to 905 nm.

DISCUSSION

We are unaware of any published studies on the cuticular hydrocarbon composition of *Trichogramma* species. However, several studies have described how cuticular hydrocarbon composition of adult individuals of other minute parasitoids is influenced by age, mating status and diet, and how it also may vary among males and females (Howard and Baker, 2003; Howard and Pérez-Lachaud, 2002). Thus, our reflectance-based profiling of parasitoid species is likely associated with species-specific variation in cuticular composition. Compared with classical taxonomy (under the microscope) or molecular-based classification of minute and closely related animals, plant seeds and growing plants, a reflectance-based method may be of considerable relevance to a wide range of biological studies. There are numerous approaches to classification of hyperspectral imaging data, and only recently has the approach (a combination of variogram analysis and linear discriminant analysis) used in this study been described and successfully applied (Nansen, 2012; Nansen et al., 2010a; Nansen et al., 2009; Nansen et al., 2014). However, this analysis represents the first application of this classification method to the identification of animals. There appear to be two main advantages of the proposed method. (1) The classification model only requires reflectance values in a few narrow spectral bands. This means less computer-processing requirements than when support vector machine (Vapnik, 1995) or other computer-intensive classification methods are deployed. (2) Incorporation of information about the spatial data structure (variogram parameters derived from variogram analysis) appears to provide substantial accuracy and robustness to the classification model. The latter point implies that the proposed classification method relies on reflectance data being acquired at a fairly high spatial resolution. The current study was based on data acquired at a spatial resolution of 45 pixels mm^{-2}. We found clear spectral trends, with reflectance data acquired from certain spectral ranges providing better separation than in other spectral ranges. We also demonstrated that classification of parasitized host eggs and adult *Trichogramma* specimens required reflectance data in different spectral bands. Parasitized host eggs were classified with high accuracy (>90% classification accuracy). Regarding adult specimens, we were able to accurately separate adult *T. atopivirilia* specimens from *T. galloi* and *T. pretiosum*, but the latter two species could not be accurately classified. Interestingly, Almeida (Almeida, 2004) developed a dichotomous key of 17 South American *Trichogramma* species based on the DNA sequence of the internally transcribed spacer ITS2 and (Fig. 4). Almeida (Almeida, 2004) found that the ITS2 product size varied from 379 to 632 bp among species, and its size successfully grouped the species into two groups (this was the first and therefore most important identification trait). This key suggested that *T. galloi* and *T. pretiosum* are closely related and comparatively distant from *T. atopovirilia*, as the latter species is separated from the other two species at the first dichotomous level (whether the size of the PCR product is above or below 620 bp). This may indicate that the difficulty associated with the separation of *T. galloi* and *T. pretiosum* may be linked to their phylogenetic proximity. Our results emphasize the importance of using high-spectral and high-spatial resolution data in the classification of closely related organisms, and hyperspectral imaging may be of relevance to a wide range of commercial (i.e. producers of biocontrol agents), taxonomic and evolutionary research applications.

Table 2. Linear discriminant classification of adult *Trichogramma* specimens

	Assigned class by training model		
Actual class	*T. atopovirilia*	*T. galloi*	*T. pretiosum*
T. atopovirilia	**93.75**	6.25	0.00
T. galloi	7.14	**82.14**	10.71
T. pretiosum	4.55	9.09	**86.36**

Hyperspectral image samples (*N*=120) of adult *Trichogramma* specimens were classified with 87.42% accuracy (mean of numbers is in bold).

MATERIALS AND METHODS

Insects

Newly oviposited Mediterranean flour moth eggs (0–24 h) are here referred to as host eggs, and they were glued (Arabic gum, Goyana) individually onto a piece of pink paper (4×0.5 cm), referred to as egg cards (Fig. 1A). Gluing host eggs to cards does not affect developing *Trichogramma* parasitoids, and most commercial shipments of *Trichogramma* parasitoids consist of parasitized host eggs glued onto egg cards. Each egg card was exposed for 12 h to parasitism by a single *Trichogramma* female inside a 1.5 cm (diameter) by 7 cm (length) glass vial maintained at 25±1°C, relative humidity 70±10% and 14 h:10 h light:dark light program. One droplet of honey was placed inside the vial as a food source for each *Trichogramma* female. As a control, we also prepared egg cards without parasitism. Hyperspectral images were acquired from egg cards at 24 h intervals after parasitism during six consecutive days. Hyperspectral images acquired

The Journal of Experimental Biology (2014) doi:10.1242/jeb.095661

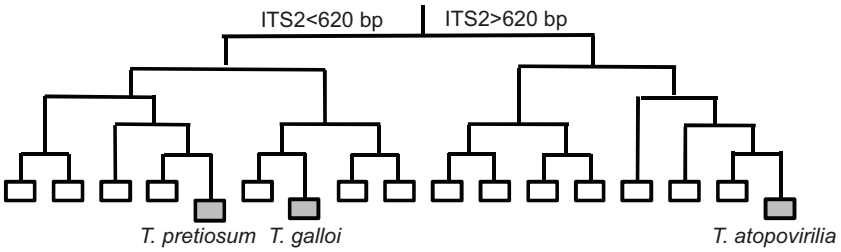

Fig. 4. Phylogenetic tree of *Trichogramma* species. The tree (Almeida, 2004) of 17 South American *Trichogramma* species is based on the size (bp) and three endonucleases (*Eco*RI, MseI and MaeI) of the DNA sequence entitled internally transcribed spacer ITS2 (Stouthamer et al., 1999). The three species included in this study are highlighted (*T. atopovirilia*, *T. galloi* and *T. pretiosum*).

from non-parasitized eggs were only used to develop a spectral filter to exclude non-parasitized eggs from the classification (see Eqn 1 below). We also acquired hyperspectral images of samples of male and female adult parasitoids of all three *Trichogramma* species, which had been killed in 80% ethanol, dried and placed on a white piece of paper. *Trichogramma* species identification was based on Querino and Zucchi (Querino and Zucchi, 2011).

Hyperspectral imaging

Similar to previously published studies (Nansen et al., 2010a; Nansen et al., 2010b; Nansen et al., 2008), we used a hyperspectral push broom spectral camera (PIKA II, Resonon Inc., Bozeman, MT, USA), which collects 160 bands in the range from 405 to 907 nm. The objective lens had a 35 mm focal length (maximum aperture of F1.4) and was optimized for the visible and NIR spectra. The main specifications of the spectral camera are as follows: interface, Firewire (IEEE 1394b); output, digital (12 bit); 160 bands (spectral) by 640 pixels (spatial); angular field of view, 7 deg; and spectral resolution, <3 nm. All hyperspectral images were collected with artificial lighting from 15 W, 12 V LED light bulbs mounted in two angled rows, one on either side of the lens, with three bulbs in each row. A voltage stabilizer (Tripp-Lite, PR-7b, www.radioreference.com) powered the lighting. Ambient climate conditions were between 19 and 22°C and between 30% and 40% relative humidity. A piece of white Teflon (K-Mac Plastics, MI, USA) was used for white calibration, and 'relative reflectance' refers to proportional reflectance compared with that obtained from Teflon. Consequently, relative reflectance values ranged from 0 to 1. Hyperspectral images of host eggs and adult *Trichogramma* specimens were acquired at a spatial resolution of 45 pixels mm^{-2}.

Reflectance data processing and analysis

A customized software package was used to convert hyperspectral image files in BIL-format into txt-files, and these were subsequently imported into PC-SAS 9.2 (SAS Institute, NC, USA) for processing and analysis. As part of omitting the pink background, non-parasitized eggs and stochastic noise from all hyperspectral images of host eggs (Fig. 1A), we applied a radiometric filter (Eqn 1), so that reflectance profiles were only included if the relative reflectance in the spectral band at 700 nm (R_{700}) was:

$$0.30 < R_{700} < 0.70 . \quad (1)$$

A different radiometric filter was applied to hyperspectral images of *Trichogramma* adults (Fig. 3A, Eqn 2), so that reflectance profiles were only included if the relative reflectance in the spectral band at 544 m (R_{544}) was:

$$0.25 < R_{544} < 0.65 . \quad (2)$$

With a spatial resolution of 45 pixels mm^{-2} and after radiometric filtering, we obtained, on average, 108.13±4.45 pixels per host egg and 52.65±1.62 pixels per adult *Trichogramma* specimen. Analysis of variance (PROC MIXED) was used to compare averages of pixels acquired from adult male and female specimens.

The classification of reflectance data was based on a combination of variogram analysis (Nansen, 2012; Nansen et al., 2014) and linear discriminant analysis (Fisher, 1936). In brief, the analytical approach consists of conducting variogram analysis of reflectance values in individual spectral bands from each hyperspectral image. We conducted variogram analysis (PROC VARIOGRAM) using the following settings for host eggs: lagdistance=1, maxlags=10 and outpdistance=10, and lagdistance=1, maxlags=5 and outpdistance=5 for adult *Trichogramma* specimens. The difference in the lagdistance number was based on host eggs being considerably larger than adult specimens. Then, a non-linear regression (Eqn 3) is fitted to each of the output variogram data:

$$F(D) = a + b\,[1 - e^{(-cD)}] , \quad (3)$$

in which a, b and c are fitted parameters, and $F(D)$ is the semi-variance at each lag distance interval, D. As illustrated in Fig. 5: (1) the a parameter denotes the intercept, (2) the b parameter denotes the asymptote and (3) the c parameter denotes the slope of the increase towards the asymptote. Thus, the proposed classification approach is based on the assumption that the three variogram parameters (a, b and c) can be used to describe the spatial structure of the reflectance data in the single reflectance band. The model training data sets consisted of 60 parasitized host eggs (20 of each of the three *Trichogramma* species) and 120 adult *Trichogramma* specimens (20 of each combination of *Trichogramma* species and sex). Variogram analysis was conducted in individual spectral bands in 10-band intervals (16 spectral bands), so a total of 2880 variogram analyses were conducted to develop the classification models of parasitized host eggs and for adult specimens [(60 hyperspectral images of host eggs + 120 hyperspectral images of adult specimens)×16 spectral bands]. Subsequently, the derived variogram parameters (a, b and c) and average reflectance values in the same spectral band were used as input variables in a linear stepwise discriminant analysis (PROC STEPWISE) of host eggs or adult specimens selecting the combination of variogram parameters and spectral band with the best separation of classification classes (species of parasitoids). For each of the two classifications (host eggs and adult specimens), linear discriminant analysis (PROC DISCRIM option=crossvalidate) with jack-knife cross-validation was used to assess the classification accuracy of each of the two data sets. Jack-knife cross-validation implies removal of one sample and its use for independent validation, repeating this procedure with all samples. As a second validation approach, the classification models were used to classify independent hyperspectral imaging data from 25 parasitized host eggs (12 host eggs parasitized by *T. atopovirilia* and 13 host eggs parasitized by *T. pretiosum*) and from nine mixtures of adult *Trichogramma* specimens. These mixtures of adult *Trichogramma* specimens consisted of 10 *T. galloi* only or

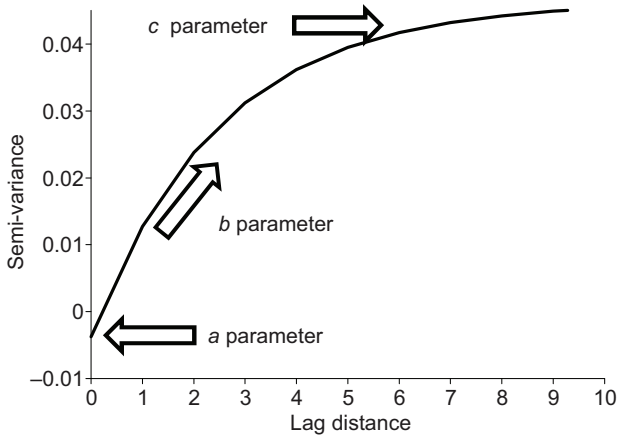

Fig. 5. Variogram analysis. The fit describes the spatial structure (variogram analysis) of reflectance data with: (1) the *a* parameter denoting the intercept, (2) the *b* parameter denoting the asymptote and (3) the *c* parameter denoting the slope of the increase towards the asymptote.

10 *T. galloi* individuals mixed with two specimens of *T. atopovirilia* or *T. pretiosum*, and there were three replicated hyperspectral images for each combination.

Author contributions

C.N., A.C. and J.M.V. designed and carried out the experiments; C.N. and J.R.P.P. wrote and revised the manuscript.

Funding

We thank the National Council of Technological and Scientific Development (CNPq) and São Paulo research foundation (Fapesp) for part of financial support.

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