

## Article

# Compound Eye Morphology Revealed by SEM Enhances Species Discrimination in *Temnothorax* Ants (*tuberum* Group)

Joaquín L. Reyes-López 

Department of Botany, Ecology and Plant Physiology, Campus of Rabanales, University of Córdoba,  
14071 Córdoba, Spain; cc0reloj@uco.es

## Abstract

The delimitation of cryptic species represents one of the main challenges, particularly in groups with low external morphological differentiation. In this context, scanning electron microscopy (SEM) enables the detection of diagnostic characters at the microscale. This study evaluates the potential of ocular morphology for interspecific discrimination within a group of species of the genus *Temnothorax*. A total of 246 workers from 52 nests, 13 populations, and 8 species from the Iberian Peninsula and North Africa were analyzed. Morphometric variables related to compound eyes were quantified from SEM images (ommatidia number, interommatidial setae, eye diameter). Data were analyzed using ANOVA, effect size estimation ( $\eta^2$ ), Linear Discriminant Analysis (LDA), and Random Forest models. All variables showed significant differences among species ( $p < 0.001$ ), with varying discriminative power. Variables related to ommatidial organization exhibited the highest classification performance, achieving high rates of correct species assignment in multivariate analyses. The use of nest-level means values substantially improved classification accuracy. These results demonstrate that ocular morphology analyzed through SEM represents a promising complementary tool for species discrimination in *Temnothorax*, complementing traditional and molecular approaches.

**Keywords:** ocular morphology; setae; species delimitation; SEM; Formicidae

## 1. Introduction

Taxonomy is a fundamental discipline for the study, management, and conservation of biodiversity, as it provides the conceptual and methodological framework to identify, describe, delimit, and classify species. Without this reference system, it is not possible to reliably estimate species richness, understand distribution patterns, interpret ecological interactions, or design effective conservation strategies. It has currently evolved toward integrative taxonomy [1,2], a methodological approach that delimits species through the explicit integration of multiple independent lines of evidence (morphological, digital imaging, molecular, ecological, and ethological data). In this way, integrative taxonomy enhances the rigor of species delimitation. This approach has led to more accurate biodiversity inventories, as it can result in both increases and decreases in the number of recognized species. Notably, increases are often associated with the discovery of cryptic species [3].

At its core, alpha taxonomy addresses the fundamental process of recognizing, describing, and naming species. Traditionally, this process has relied on observable morphological features; however, more recent approaches have expanded its scope through techniques such as X-ray-based analyses. In particular, soft X-ray tomography (SXT) allows the ex-



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amination of internal cellular and tissue structures, facilitating species identification and characterization when conventional methods are insufficient [4,5].

Furthermore, recent advances in computational technology and microscopy have opened new possibilities for generating three-dimensional and interactive imaging [6]. Among these techniques are scanning electron microscopy (SEM), micro-computed tomography (micro-CT) [6], and synchrotron X-ray tomography [7]. These tools have the potential to revolutionize studies based on biological collections by enabling detailed analyses of both external and internal morphology in valuable or rare specimens without causing damage.

Scanning electron microscopy (SEM) is a highly valuable tool in alpha taxonomy, particularly for the detailed examination of surface features and structures that are difficult to resolve using light microscopy. Owing to its superior magnification and resolution, SEM enables precise characterization of traits such as surface sculpturing, fine appendage morphology, and other microstructural features of taxonomic relevance.

In the study of ants, SEM is especially useful for investigating external morphology, providing high-resolution images of exoskeleton. This includes detailed observation of structures such as setae, scales, and other surface ornamentations, as well as specialized appendages including antennae, legs, and mouthparts. Furthermore, SEM facilitates the analysis of ultrastructural features, such as cuticular layering and sensilla, which are critical for species delimitation and functional interpretation. SEM has been widely applied in myrmecological research, including the characterization of cuticular microstructures in *Strumigenys* [8], the analysis of mandibular cuticle morphology in *Atta* [9], and the description of spongiform bodies in *Strumigenys* [10].

Compound eyes are key sensory organs in insects, enabling visual perception finely tuned to their ecological and behavioral requirements. They are composed of multiple photoreceptive units known as ommatidia, each contributing a portion of the overall visual field and collectively providing panoramic vision with high sensitivity to motion and changes in light intensity [11]. This visual system is essential for critical behaviors such as foraging, predator avoidance, navigation in complex environments, and intraspecific visual communication [11,12]. Additionally, morphological variation in compound eyes may reflect ecological differences among species and can, in some cases, serve as a diagnostic character in taxonomic delimitation. For example, between *Lasius flavus* (Fabricius, 1782) and *L. myops* Forel, 1894 [13], or between *Solenopsis invicta* Buren, 1972 vs. *S. richteri* Forel, 1909 [14], or between *Temnothorax cokendolpheri* (Mackay, 2000) vs. *T. carinatus* (Cole, 1957) [15].

Taxonomic studies rarely involve counting all ommatidia, particularly in species with large eyes. Instead, standardized metrics are typically employed, such as the number of ommatidia in the longest row or the maximum number of ommatidia across the eye, usually assessed in lateral or oblique view [15]. These measurements are widely used in modern descriptions due to their repeatability using microscopy and digital imaging. In taxonomic revisions, they are commonly reported as “eyes with ~X ommatidia in the longest row.” Clear examples can be found in revisions of several genera and species complexes, such as *Tetramorium* (*T. shiloense* species group) [16,17], as well as in comparative diagnoses of hyperdiverse groups [18].

In other cases, ocular indices derived from standard morphometric measurements (e.g., eye length relative to head width) are employed, often in combination with ommatidial counts to provide an estimate of both eye size and functional “resolution.” In ant taxonomic literature, abbreviations such as EL (eye length) and OI (ocular index) are routinely used, frequently alongside ommatidial counts, particularly in species with reduced eyes [16]. As noted above, these characters are especially valuable in the diagnosis of cryptic species:

when other morphological traits exhibit limited variation or overlap, eye size and structure—including ommatidial number—can provide reliable diagnostic features.

Moreover, these traits carry important ecological signal. Strongly reduced eyes or those with few ommatidia are typically associated with hypogean lifestyles or low-light microhabitats, whereas more developed eyes reflect a greater reliance on vision. Comparative studies on eye structure and ecology in ants have revealed consistent patterns across lineages and life-history strategies, which helps explain why these characters are effective not only for taxonomic diagnosis but also for ecological interpretation [19].

In *Temnothorax* (Crematogastrini: Myrmicinae), ommatidial counts are a commonly employed character in regional revisions and clade-level studies, typically expressed as “X ommatidia in the longest row” and often accompanied by indices such as OI and EL. This approach is particularly informative in this genus, which comprises small-bodied species with relatively reduced eyes [20,21], where even slight differences in ommatidial number may carry diagnostic value.

Although not always strictly focused on systematics, studies in *Temnothorax* have also incorporated ommatidial counts in behavioral and biological contexts (e.g., asymmetry and lateralization [22]). Such work provides valuable insight into the actual range of intraspecific variation, including differences among castes and sexes, thereby offering an important contextual framework for taxonomic studies.

The primary aim of this study is to assess the value of compound eye structures and their immediate surrounding features as complementary characters for the delimitation of morphologically similar or cryptic species. We hypothesize that ocular morphology—particularly traits related to ommatidial organization and periocular structures—encodes a species-specific morphological signal that is partially independent of overall body size and provides additional discriminatory power beyond traditional morphometric characters. To this end, both the individual discriminatory power of these variables and their combined contribution in a multivariate framework are evaluated and compared with a set of traditional morphological variables commonly used in such studies. As a secondary objective, their applicability is explored across different species groups of *Temnothorax* within the same taxonomic complex.

## 2. Materials and Methods

### 2.1. Species Sampling

This study included several species of the genus *Temnothorax* belonging to the *tuberum* group (sensu [23]): *T. bejaraniensis* Reyes-López & Carpintero-Ortega, 2013 (=BEJ), *T. curtulus* (Santschi, 1929) (=CUR), *T. luteus* (Forel, 1874)(=LUT), *T. pardoii* (Tinaut, 1987) (=PAR), *T. racovitzaei* (Bondroit, 1918) (=RAC), *T. tuberum* (Fabricius, 1775) (=TUB), and *T. unifasciatus* (Latreille, 1798) (=UNI).

To account for intraspecific geographic variation (Supplementary Materials, Figure S1), most species were sampled from two or three geographically distant localities (in all cases separated by more than 50 km), except for *T. tuberum* and *T. unifasciatus*, for which only a single locality was available.

For *T. luteus*, two populations were compared: one from the Natural Park of the Sierras de Cazorla, Segura y Las Villas and another from the Sierra Mágina Natural Park, separated by approximately 60 km in a straight line. In *T. pardoii*, populations from the Sierras de Cazorla, Segura y Las Villas Natural Park and the Sierra de Guadarrama National Park were analyzed, with an approximate separation of 330 km. In the case of *T. curtulus*, three populations were included: two from Spain (Strait of Gibraltar region and Subbetic area), separated by approximately 200 km, and one from North Africa (Morocco). For

*T. racovitzae*, populations from Sierra Morena and Sierra de Cardena (central Andalusia, southern Spain) were compared, with a separation of approximately 60 km.

*Temnothorax bejaraniensis* was compared with a morphologically similar, yet undescribed species from the autonomous city of Ceuta (North Africa). Based on their morphological affinities, both forms can be assigned to the so-called “*tebesae* superspecies” [19], here treated as *T. cfr tebesae* (in *tebesae* complex) within the *tuborum* group. The distance between these populations is approximately 230 km, including a marine barrier. For *T. unifasciatus*, colonies were sampled from the Sierra de Guadarrama National Park, whereas *T. tuborum* specimens were collected in the Sierra de Albarracin (Teruel, Spain). Detailed information on the sampled material is provided in Table S1 of the Supplementary Materials.

## 2.2. Preparation for Scanning Electron Microscopy (SEM)

Samples intended for scanning electron microscopy (SEM) require specific preparation procedures, including dehydration and coating with a conductive material (e.g., gold or carbon), to optimize imaging conditions. In this study, specimens were oven-dried at 60 °C for 48 h. Subsequently, the head of each worker was dissected and mounted on a metallic stub using a conductive adhesive (double-sided copper tape). Samples were then sputter-coated with a thin layer of gold to provide surface conductivity and enhance image quality. Only the head of each worker was prepared and mounted. For imaging and analysis, one side of the head was selected based on optimal orientation and preservation state (i.e., absence of debris, structural damage, etc.). When possible, measurements were also taken from the second eye of the same specimen.

Images were acquired using a JEOL JSM-6300 scanning electron microscope (JEOL Ltd., Tokyo, Japan). The instrument provides a high depth of field compared to optical microscopy, allowing detailed visualization of three-dimensional structures. Observations were conducted at Accel Volt of 10 kV and the magnifications ranging from 100× to 400× (wd = 39 mm). At 400×, the field of view was approximately 255 × 195 µm, allowing the entire compound eye to be imaged along with the surrounding setae. The microscope is housed at the Central Research Support Services (SCAI), University of Córdoba.

## 2.3. Morphometric Variables

The morphology of insect compound eyes is strongly influenced by their habitat and lifestyle, allowing adaptation to different environmental conditions such as light availability and ecological niche. (see e.g., recent reviews on insect vision [24]). From the possible variables, the following have been chosen:

SM: Mean length of the setae surrounding the compound eye (µm, 400×). Five setae were selected from the pericocular region, distributed between the anterior and posterior areas (see Figure 1), and their mean length was calculated. Each seta was measured as a straight-line distance from its base to the distal tip (Figure 1). Preference was given to setae that were as straight as possible. In SEM images, these setae typically appear closely appressed to the cuticle, facilitating measurement (Figure 2).

EL: Maximum diameter of the compound eye (µm, 400×), including all structurally visible ommatidia (Figure 3).

OMAT: Total number of ommatidia (400×). A key advantage of SEM is that specimens are coated with a conductive material, making visualization independent of ommatidial pigmentation. This is particularly relevant because, under light microscopy, some ommatidia may lack pigmentation and thus be difficult to discern.

NEIS: Number of interommatidial setae (400×) (Figure 1). Many insect groups possess simple external setae located between ommatidial lenses [25], with diverse functional roles. These structures are also referred to as “interommatidial bristles” or “ommatrichia”.

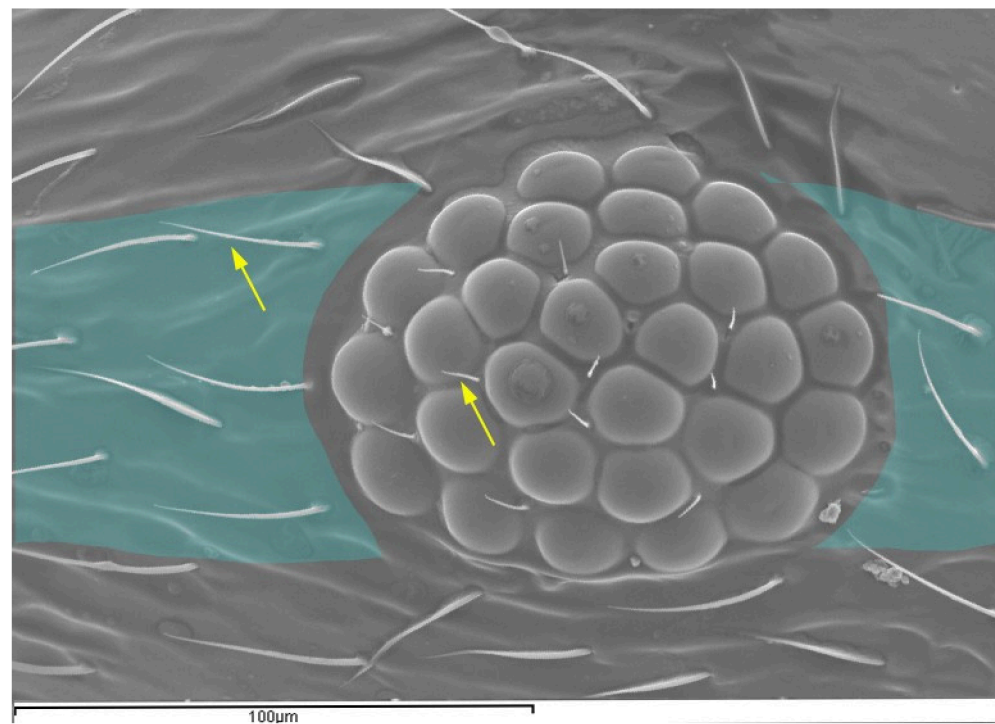


**Figure 1.** Setae surrounding and interommatidial setae of compound eye of *T. bejaraniensis*. Note: The colored arrows indicate different types of setae. The blue area indicates the location where the setae to be measured are selected.

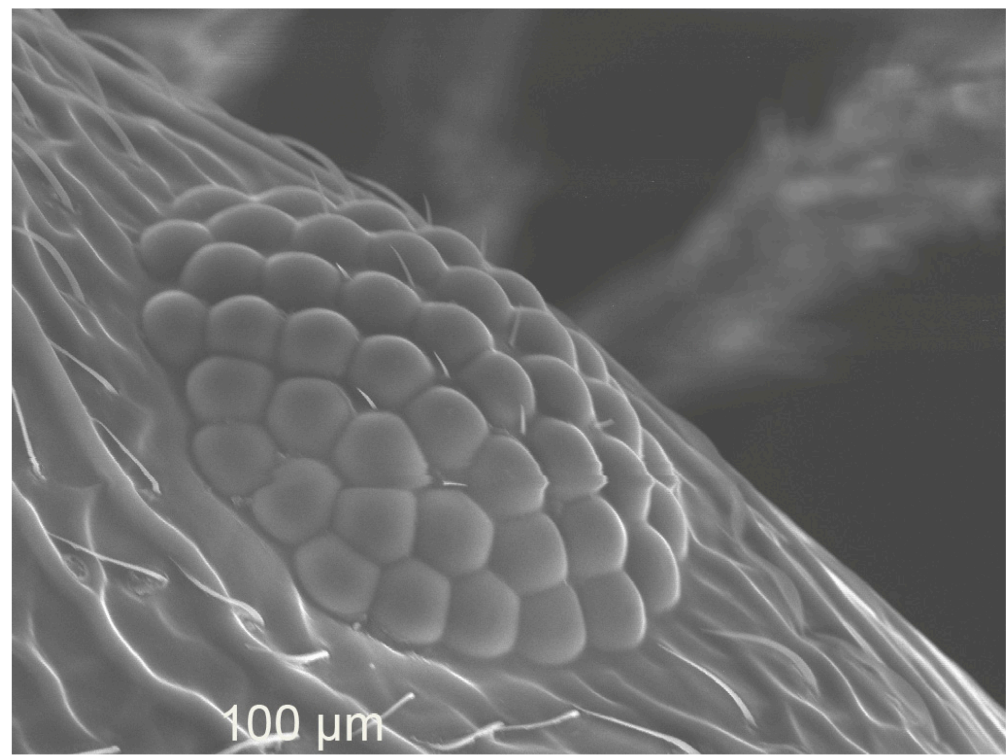


**Figure 2.** Tangential view of the compound eye where the setae are best seen.

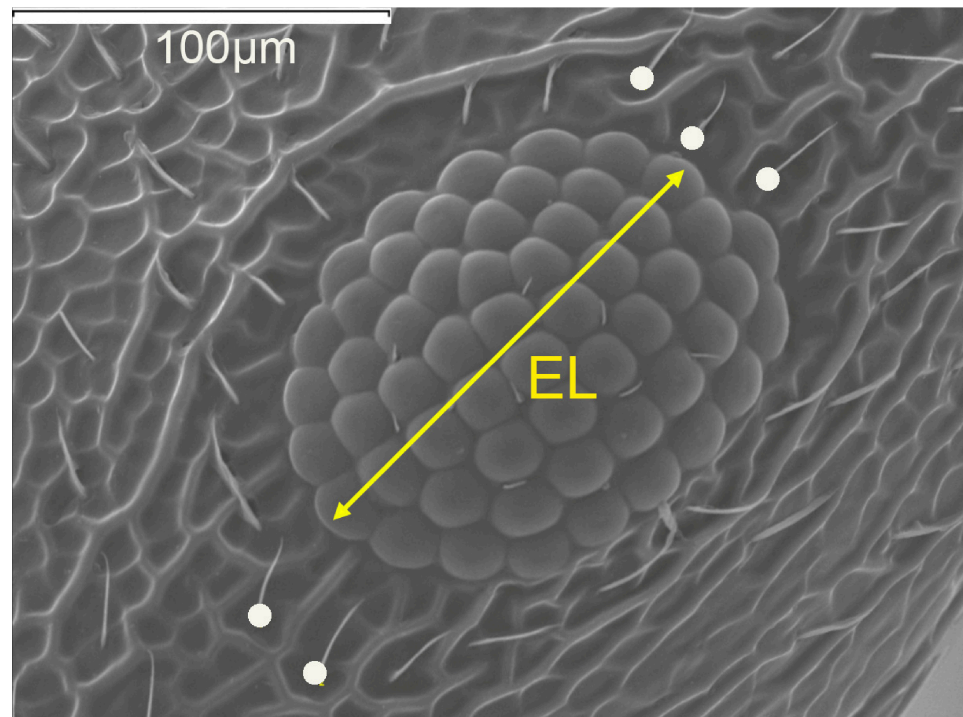


**Figure 3.** Maximum diameter of the compound eye of *T. curtulus*. The white circles indicate the base of the setae chosen for measurement in that photograph.

Traditional morphometric variables ( $\mu\text{m}$ , all measured at  $100\times$ ).

HW: Head width, measured in frontal view just above the compound eyes (Figure 4).

HL: Head length. Length of the head capsule excluding mandibles, measured in full-face view as the straight-line distance from the midpoint of the anterior clypeal margin to the midpoint of the posterior head margin.

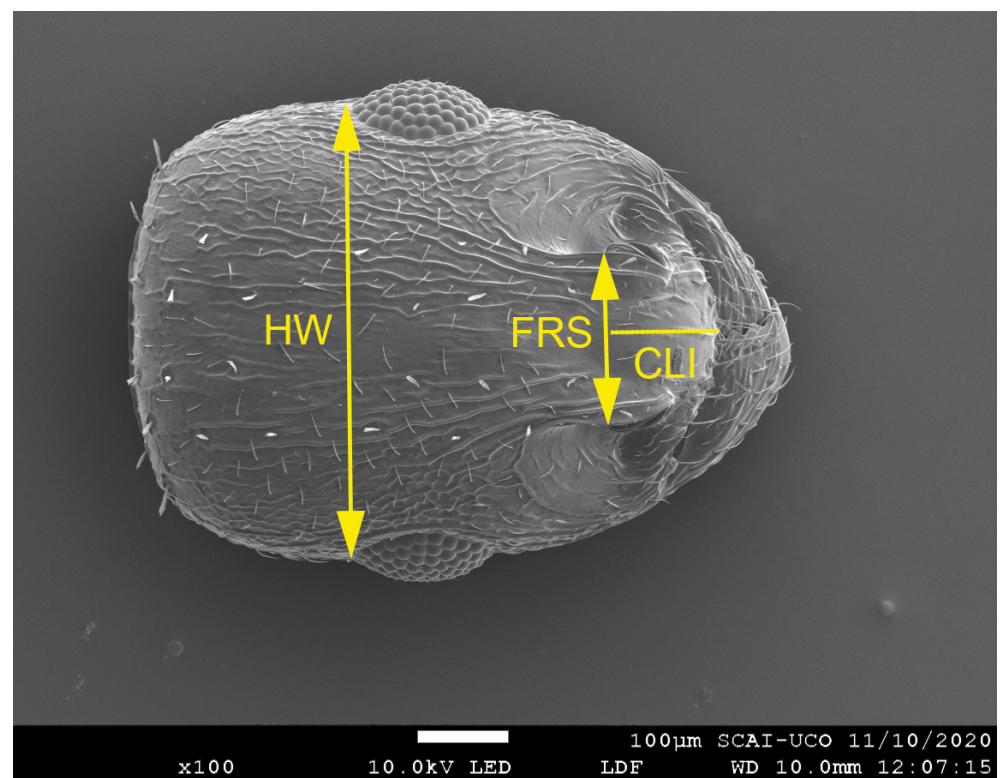


**Figure 4.** Front view of the head of *T. luteus* with some of the measured variables.

FRS: Distance between the frontal carinae (Figure 4), measured immediately caudal to the posterior intersection points between the frontal carinae and the lamellae dorsal to the torulus [26].

CLI: Distance from the most distal point of the clypeus to the midpoint of the line defined by the FRS measurement (Figure 4).

ML: Mesosoma length (Weber's length). Distance from the caudalmost point of the propodeal lobe to the transition point between the anterior pronotal slope and the anterior propodeal shield, measured in lateral view.

SPST: Distance between the center of the propodeal stigma and the tip of the propodeal spine, measured in lateral view (Figure 5).

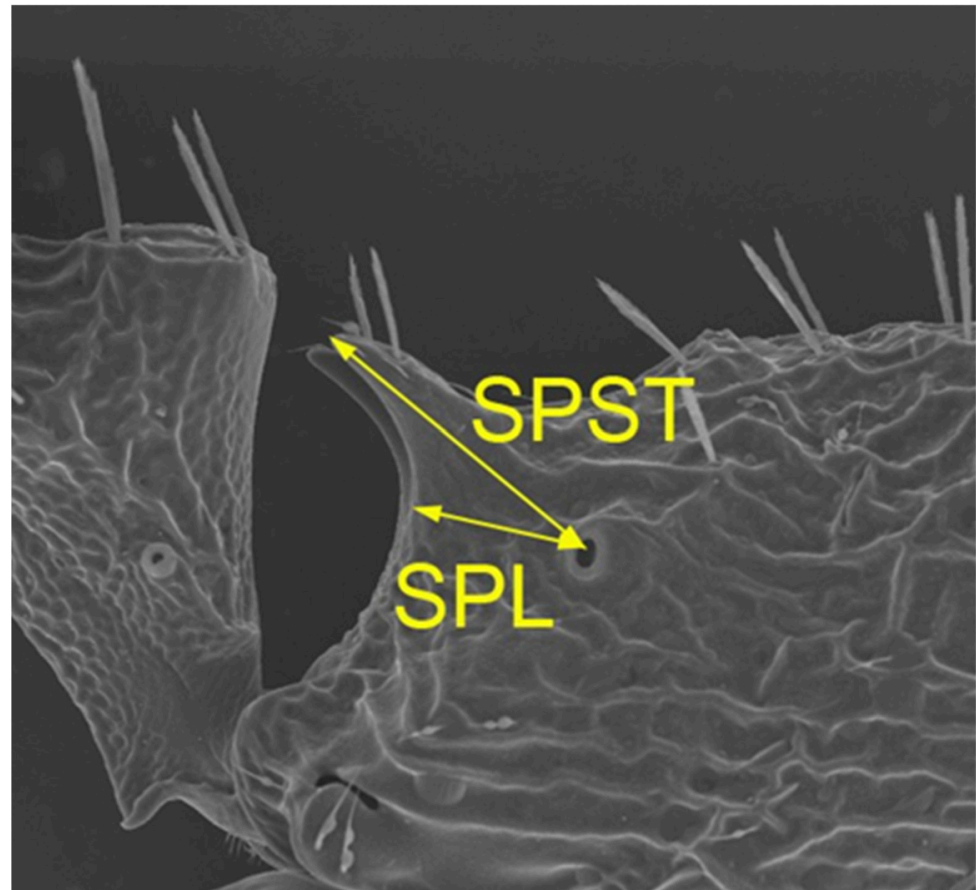


**Figure 5.** Side view of the spine, with the measured variables.

SPL: Minimum distance between the center of the propodeal spiracle and the subspinal excavation, measured in lateral view (Figure 5).

#### 2.4. Statistical Analysis

To keep this section from becoming overly complex, only a brief outline is provided here; it will be expanded upon in each of the Results sections, where the procedures are described in greater detail.

The discriminative power of the morphometric variables was assessed using multiple analytical approaches. First, a correlation matrix was examined to identify potential redundancy among variables. This was followed by an evaluation of the individual discriminatory capacity of each variable through univariate comparisons. Subsequently, multivariate analyses were performed, including Linear Discriminant Analysis (LDA) and Random Forest (RF), with species identity (SPP) treated as the response variable.

Linear Discriminant Analysis (LDA) is a parametric method that assumes multivariate normality and equal covariance matrices across groups, and seeks linear combinations of variables that maximize class separation. In contrast, Random Forest (RF) is a non-parametric ensemble method that builds multiple decision trees using bootstrap sampling and random subsets of variables and aggregates their predictions. While LDA is more interpretable and efficient under its assumptions, RF is more flexible, can capture non-linear relationships, and is generally more robust to violations of distributional assumptions.

All species included in this study are currently regarded as valid (“good”) species; therefore, the objective of the analyses was not to detect new taxonomic entities, but rather to assess whether the selected variables allow for a reliable separation among them. Additional methodological details are provided in the corresponding sections.

All analyses were conducted in the R statistical environment [27].

### 3. Results

The analysis was based on a dataset comprising 246 workers, representing 52 nests, 13 populations, and 8 species.

#### 3.1. Correlation Matrix Among Variables

Table 1 shows the Pearson linear correlation coefficients between the variables proposed in this study and the traditional morphometric variables.

**Table 1.** Pearson correlations between SM, OMAT, NEIS, and EL and the remaining variables. Asterisks indicate significance levels:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*).  $n = 246$ .

| Variable | SM         | OMAT      | NEIS      | EL        |
|----------|------------|-----------|-----------|-----------|
| CS       | −0.165 **  | 0.854 *** | 0.163 *   | 0.915 *** |
| FRS      | −0.083 ns  | 0.858 *** | 0.097 ns  | 0.899 *** |
| CLI      | −0.331 *** | 0.131 *   | 0.042 ns  | 0.185 **  |
| ML       | −0.005 ns  | 0.878 *** | 0.180 **  | 0.895 *** |
| SPST     | −0.158 *   | 0.473 *** | 0.499 *** | 0.576 *** |
| SPL      | 0.008 ns   | 0.516 *** | 0.450 *** | 0.594 *** |

OMAT and EL show the highest correlation values, predominantly positive and mostly statistically significant. In contrast, SM tends to be negatively correlated with several variables (CLI, SPST, CS), although with small to moderate effect sizes and, in some cases, lacking statistical significance. NEIS also exhibits moderate correlations, mainly with SPST and SPL, and weaker associations with ML and CS. A particularly noteworthy finding is the linear relationship between OMAT (number of ommatidia) and CS (head size), indicating that larger head size is associated with a greater number of facets in the compound eyes. Consequently, techniques aimed at minimizing this effect will be applied subsequently.

#### 3.2. Variable-by-Variable Basis

The following sections examine, on a variable-by-variable basis, the potential interspecific differences among the measured traits.

The approach followed in the analysis was to begin with comparisons between species variable by variable, using univariate ANOVAs. After checking the theoretical assumptions (normality of residuals and homogeneity of variances), heteroscedasticity was detected. Therefore, Welch’s test was chosen. The R function ‘welch\_anova\_test()’ from the rstatix package (version 0.7.3) was used. For the post hoc tests, the function ‘games\_howell\_test()’, also from rstatix, was applied [28].

### 3.2.1. Mean Length of the Setae Surrounding the Compound Eye (SM)

Differences in SM among species were assessed using one-way ANOVA. Due to heterogeneity of variances (Levene's test), a Welch's ANOVA was applied, followed by post hoc comparisons using the Games–Howell test ( $\alpha = 0.05$ ). Significant differences among species were detected (Welch  $F(7, 91.57) = 45.43, p < 0.001$ ). The Games–Howell post hoc analysis revealed multiple significant differences (Table 2). Species grouping showed a clear gradient in mean SM values: BEJ exhibited the highest values; TUB and TEB formed a high-intermediate group; UNI and LUT an intermediate group; RAC showed moderate values; and PAR and CUR exhibited the lowest values.

**Table 2.** Differences among species in SM, OMAT, NEIS, and EL. Values are presented as mean,  $\pm$ , st, ard, deviation. Identical letters within each column indicate no significant differences among species according to Games–Howell post hoc comparisons ( $\alpha = 0.05$ ) following a Welch's ANOVA.

| SPP | n  | SM ( $\mu\text{m}$ ) | OMAT               | NEIS               | EL ( $\mu\text{m}$ ) |
|-----|----|----------------------|--------------------|--------------------|----------------------|
| BEJ | 28 | 29.07 $\pm$ 2.73 a   | 35.32 $\pm$ 3.97 f | 8.18 $\pm$ 1.68 c  | 114.96 $\pm$ 6.70 e  |
| CUR | 38 | 21.69 $\pm$ 2.32 c   | 40.92 $\pm$ 5.15 e | 6.76 $\pm$ 1.15 d  | 122.95 $\pm$ 7.42 d  |
| LUT | 27 | 25.51 $\pm$ 1.77 b   | 55.04 $\pm$ 5.10 d | 10.19 $\pm$ 1.47 b | 141.11 $\pm$ 8.08 b  |
| PAR | 38 | 22.36 $\pm$ 1.18 c   | 62.87 $\pm$ 4.65 c | 9.68 $\pm$ 1.45 b  | 145.87 $\pm$ 6.89 b  |
| RAC | 44 | 24.38 $\pm$ 1.76 b   | 55.36 $\pm$ 8.24 d | 11.75 $\pm$ 2.06 a | 138.36 $\pm$ 11.43 c |
| TEB | 21 | 26.86 $\pm$ 2.64 a   | 43.76 $\pm$ 4.84 e | 10.62 $\pm$ 1.32 a | 124.00 $\pm$ 6.16 d  |
| TUB | 29 | 27.61 $\pm$ 2.60 a   | 76.10 $\pm$ 8.60 b | 8.90 $\pm$ 1.61 b  | 155.72 $\pm$ 8.73 a  |
| UNI | 21 | 25.55 $\pm$ 1.41 b   | 89.86 $\pm$ 9.03 a | 8.90 $\pm$ 1.37 b  | 160.76 $\pm$ 9.44 a  |

### 3.2.2. Number of Interommatidial Setae (NEIS)

Differences in NEIS among species were assessed using a one-way ANOVA. Due to heterogeneity of variances (Levene's test), a Welch's ANOVA was applied, followed by post hoc comparisons using the Games–Howell test ( $\alpha = 0.05$ ). Significant differences among species were detected (Welch  $F(7, 93.51) = 39.30, p < 0.001$ ). The Games–Howell post hoc analysis identified 23 significant pairwise comparisons. Species grouping revealed a clear interspecific gradient: RAC exhibited the highest NEIS values; TEB and LUT formed a high-intermediate group; PAR showed intermediate values; UNI, TUB, and BEJ formed a low-intermediate group; and CUR exhibited the lowest values.

### 3.2.3. Number of Ommatidia

Differences in the number of ommatidia per compound eye (OMAT) among species (SPP) were assessed using a one-way Welch's ANOVA. Highly significant differences in OMAT were detected among species. The Welch's ANOVA was significant ( $F(7, 92.99) = 211.20, p < 0.001$ ). The Games–Howell post hoc analysis identified 27 significant pairwise comparisons, revealing a pronounced interspecific gradient: UNI exhibited the highest OMAT values, followed by TUB, PAR, RAC, and LUT (the latter two not significantly different from each other), whereas TEB, CUR, and BEJ showed progressively lower values. Overall, these results demonstrate a very strong interspecific differentiation in the number of ommatidia per compound eye.

## 3.3. Interocular Correlation

### 3.3.1. Ommatidia

Measuring both eyes of the same specimen under SEM is technically challenging; therefore, only a limited subset of individuals with both eyes measured was available ( $n = 37$  workers). To assess bilateral symmetry in the number of ommatidia per compound eye (OMAT), the two measurements obtained per individual (OMAT and OMAT2) were analyzed.

As laterality (right vs. left eye) was not recorded, directional asymmetry could not be evaluated. Instead, bilateral asymmetry was estimated using the absolute difference between eyes ( $|OMAT - OMAT2|$ ). In addition, Pearson correlation coefficient was calculated between both measurements as indicators of overall bilateral symmetry. Measurements from both eyes showed an extremely high correlation (Pearson  $r = 0.996$ ,  $p < 0.001$ ), indicating strong bilateral symmetry in ommatidial number. The mean absolute difference between eyes was low ( $1.32 \pm 1.27$  ommatidia; maximum = 5), representing minimal variation relative to the total number of ommatidia per individual. Overall, these results demonstrate very high bilateral symmetry, although directional asymmetry could not be assessed due to the lack of laterality information.

3.3.2. NEIS

Asymmetry in the number of interommatidial setae (NEIS) was evaluated in individuals with measurements available for both eyes (NEIS and NEIS2; both eyes of the same worker;  $n = 37$ ). As laterality (right vs. left eye) was not recorded, directional asymmetry could not be assessed. Instead, bilateral asymmetry was estimated using the absolute difference between eyes ( $|NEIS - NEIS2|$ ). To test whether the magnitude of asymmetry differed among species, a one-way Welch’s ANOVA was performed. Mean difference in bilateral asymmetry in NEIS was low ( $1.49 \pm 1.37$  setae), with a maximum observed difference of 5 setae. No significant differences in asymmetry magnitude were detected among species (Welch ANOVA:  $F(5, 7.49) = 0.95$ ,  $p = 0.504$ ). Overall, these results indicate that, although NEIS exhibits moderate intra-individual variation between eyes, this variation is relatively consistent across species and does not display a significant interspecific pattern.

3.4. Univariate Discriminative Power of the Variables

The discriminative power of the variables was assessed using Linear Discriminant Analysis (LDA) and Random Forest (RF) with 5-fold cross-validation. Random Forest classifiers were implemented using the default settings of the “randomForest” package in R (for example, tree = 500).

As shown in Table 3, OMAT and FRS exhibited the highest classification performance, whereas SM, NEIS, SPL, and CLI showed considerably lower discriminative power.

**Table 3.** Univariate discriminative power of the variables ( $n = 246$ ). The ranking is based on the average of  $\eta^2$ , LDA accuracy, and Random Forest accuracy.

| Variable | $\eta^2$ (Welch ANOVA) | LDA Accuracy | RF Accuracy | Ranking |
|----------|------------------------|--------------|-------------|---------|
| OMAT     | 0.860                  | 0.505        | 0.415       | 1       |
| FRS      | 0.855                  | 0.511        | 0.476       | 2       |
| SPST     | 0.847                  | 0.435        | 0.39        | 3       |
| EL       | 0.747                  | 0.403        | 0.325       | 4       |
| ML       | 0.728                  | 0.407        | 0.354       | 5       |
| SM       | 0.583                  | 0.374        | 0.309       | 6       |
| SPL      | 0.542                  | 0.309        | 0.284       | 7       |
| NEIS     | 0.511                  | 0.366        | 0.342       | 8       |
| CLI      | 0.191                  | 0.195        | 0.325       | 9       |

To remove the effect of body size, each variable was regressed against CS, and the resulting residuals were used in subsequent analyses (Table 4). Thus, the variables primarily reflect variation in shape and proportions. Discriminative power was assessed using ANOVA effect size ( $\eta^2$ ), as well as univariate LDA and Random Forest analyses (RF). After this correction, SPST exhibited the highest discriminative power among species, followed by OMAT and SM, whereas CLI showed very low discriminative ability.

**Table 4.** Values represent the discriminative power among species after removing the effect of body size (CS) through linear regression. Residuals from these regressions were used in the analyses. The ranking is based on the average of  $\eta^2$ , LDA accuracy, and Random Forest accuracy (n = 246).

| Variable (Residual) | $\eta^2$ | LDA Accuracy | RF Accuracy | Ranking |
|---------------------|----------|--------------|-------------|---------|
| SPST                | 0.875    | 0.545        | 0.443       | 1       |
| OMAT                | 0.678    | 0.371        | 0.284       | 2       |
| SM                  | 0.561    | 0.317        | 0.273       | 3       |
| FRS                 | 0.546    | 0.306        | 0.212       | 4       |
| NEIS                | 0.520    | 0.350        | 0.366       | 5       |
| SPL                 | 0.506    | 0.289        | 0.227       | 6       |
| EL                  | 0.490    | 0.305        | 0.223       | 7       |
| ML                  | 0.307    | 0.321        | 0.317       | 8       |
| CLI                 | 0.191    | 0.118        | 0.318       | 9       |

### 3.5. Comparison of Interspecific Discrimination at the Individual vs. Nest Level

Morphometric variables were size corrected by linear regression against head size (CS), used as a proxy for body size. For each variable, a linear model was fitted against CS, and the residuals were used as size-independent variables.

Species discrimination was assessed using LDA. Two independent multivariate analyses were performed: one based on individual worker measurements and another on nest-level mean values. In both cases, a forward stepwise procedure was applied, whereby variables were sequentially included if they improved classification accuracy as estimated by 5-fold cross-validation.

For each final model, cross-validated classification accuracy, apparent accuracy, confusion matrix, proportion of discriminant variance explained by LD1 and LD2, and Wilks'  $\lambda$  (with  $\chi^2$  approximation) were calculated to assess overall model significance (See more details in the R script).

To specifically evaluate the discriminative power of the variables proposed in this study (SM, OMAT, NEIS, and EL), univariate LDAs were performed using each variable separately as a predictor of species identity. Classification accuracy was estimated using 4-fold cross-validation and compared with that of traditional morphometric variables.

The stepwise procedure applied to individual workers selected seven variables: SPST, OMAT, FRS, SM, SPL, NEIS, and CLI. This model achieved a cross-validated accuracy of 0.871 and an apparent accuracy of 0.894. The first two discriminant axes explained 84.2% of the total discriminant variance (LD1 = 60.8%, LD2 = 23.4%; Figure 6). The model was highly significant (Wilks'  $\lambda \approx 0$ ;  $\chi^2 = 4891$ ; df = 49;  $p < 0.001$ ).

In the nest-level analysis, the stepwise procedure selected four variables: SPST, OMAT, NEIS, and CLI. This model showed slightly higher classification performance (CV accuracy = 0.901; apparent accuracy = 0.923). In this case, LD1 and LD2 explained 76.7% and 18.6% of the discriminant variance, respectively (95.3% cumulative; Figure 7). The model was also highly significant (Wilks'  $\lambda \approx 0$ ;  $\chi^2 = 470.3$ ; df = 28;  $p < 0.001$ ).

Confusion matrices indicated that most species were correctly classified. In the individual-based analysis, the main misclassifications occurred among *T. luteus*, *T. pardoii* and *T. racovitzai*, as well as among *T. bejaraniensis*, *T. cfr. tebesae*, and *T. unifasciatus*, suggesting higher morphological similarity within these groups. In contrast, the nest-based analysis substantially reduced classification errors, with several species classified without error (BEJ, CUR, TEB, and UNI).

Univariate LDA analyses showed that SPST had the highest individual discriminative power (accuracy = 0.62), followed by CS (0.54) and SPL (0.45). Among the variables proposed in this study, SM and NEIS exhibited moderate discriminative ability comparable to several traditional morphometric traits, whereas OMAT and EL showed lower performance

when considered in isolation. However, the substantially higher accuracy of multivariate models indicates that species discrimination primarily depends on the combined effect of multiple morphological traits, suggesting that these variables provide complementary information in a multivariate framework.

Overall, these results indicate that using nest-level means results in simpler and slightly more accurate models, suggesting that part of the variation observed at the individual level reflects within-nest variability rather than true interspecific morphological differences.

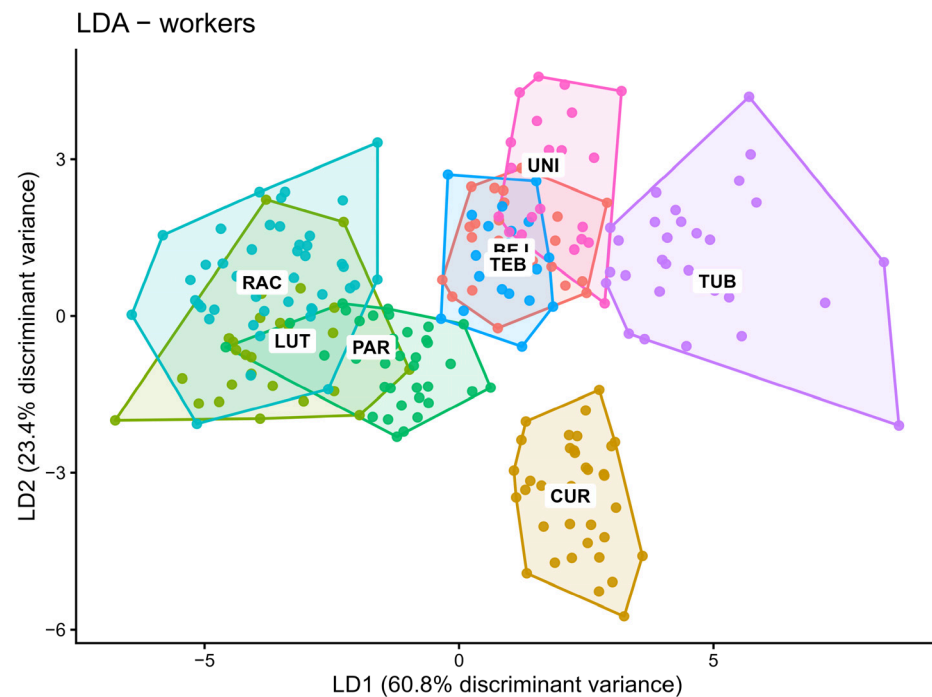


**Figure 6.** Relative position of the species on the first two axes of the LDA. Each point represents a worker. Note: Species are indicated by their abbreviated code. In addition, they have been assigned an arbitrary color.

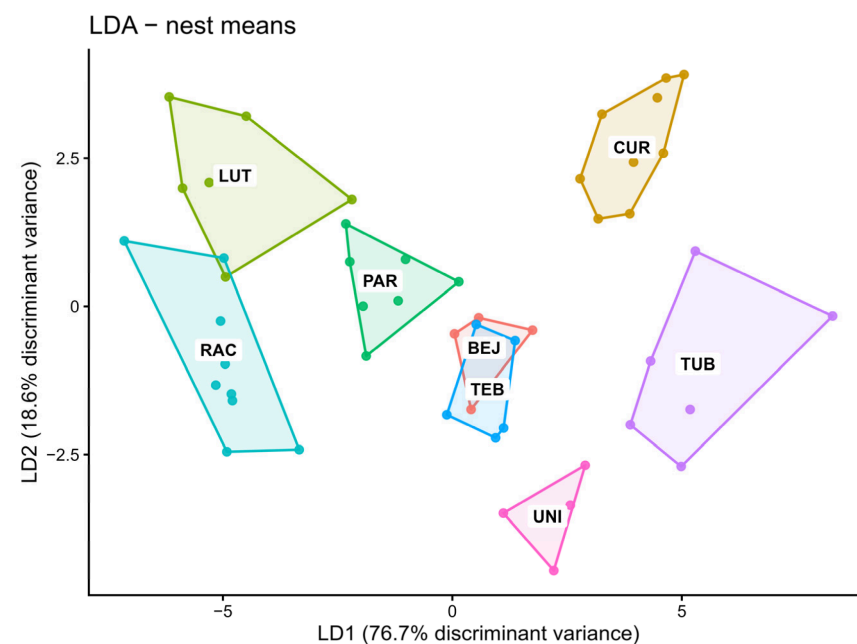


**Figure 7.** Relative position of the nests on the first two axes of the LDA. Note: Species are indicated by their abbreviated code. In addition, they have been assigned an arbitrary color.

### 3.6. Application to the Tebesae Complex

In this case, only *T. bejaraniensis*, *T. cfr. tebesae*, and *T. curtulus* were compared. To reduce within-nest variation, measurements were averaged at the nest level, retaining species and geographic location as grouping factors.

A head size metric (CS) was calculated from head length and width. Subsequently, nest-level means were computed for all morphometric variables. The structure of morphological variation was explored using Principal Component Analysis (PCA), based exclusively on SM, OMAT, EL, NEIS, and CS. Prior to analysis, all variables were centered and standardized. Geographic differentiation was visualized in space defined by the first two principal components. Convex hulls were computed for each geographic group to illustrate the occupied morphospace. In addition, centroids were calculated for each group in the PC1–PC2 plane, and Euclidean distances between centroids were used to quantify morphological divergence. The PCA revealed a strong dimensionality reduction of the dataset. The first principal component (PC1) explained 60.1% of the total variance, while the second component (PC2) explained 31.6%, together accounting for 91.7% of the total variation.

Morphological variables showed a clear correlation structure: EL and OMAT were strongly positively correlated, whereas SM was negatively correlated with head size (CS), indicating that variation in eye morphology and head size contributes substantially to the main morphological gradients. In the PC1–PC2 space, geographic groups formed distinct clusters. BEJ-SCO was located at the negative extreme of PC1, whereas CEUTA was primarily separated along PC2. CUR populations clustered in the positive region of PC1 (Figure 8). Centroid distances indicated that CUR-BOU and CUR-SRI were the most similar populations, whereas CUR-TAR showed greater differentiation, particularly relative to CUR-BOU. The mean distance among CUR populations in PC1–PC2 space was 1.27, compared to 3.01 between BEJ-SCO and TEB.

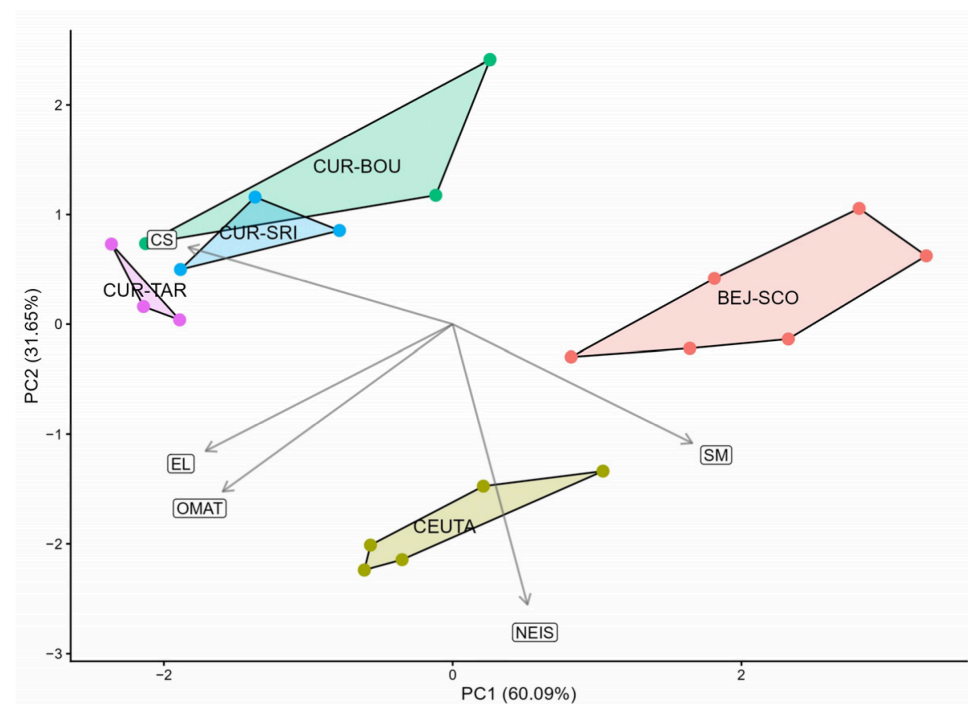


**Figure 8.** Nests of *T. tebesae* complex on the first two axes of the PCA. Note: An arbitrary color has been assigned to each of the populations. The arrows indicate the variables used.

Multivariate differences in morphospace among species were assessed using permutational multivariate analysis of variance (PERMANOVA), implemented in the adonis2

function (vegan package). Euclidean distances were calculated from the first two PCA axes. Statistical significance was assessed using 9999 permutations. Homogeneity of group dispersions was tested using a multivariate analogue of Levene's test (betadisper).

PERMANOVA revealed strong differences in morphospace among species ( $F = 30.20$ ,  $R^2 = 0.78$ ,  $p < 0.001$ ). Tests for homogeneity of dispersion were non-significant ( $F = 0.40$ ,  $p = 0.68$ ), indicating that these differences are driven by centroid separation rather than dispersion effects.

## 4. Discussion

### 4.1. Eyes in Ants

The eyes of insects exhibit remarkable structural and functional diversity, enabling vision across a wide range of light environments. Compound eyes, composed of multiple ommatidia, are evolutionarily optimized to balance spatial resolution, motion sensitivity, and photon capture according to ecological demands. Diurnal insects generally possess apposition eyes with numerous small ommatidia that maximize visual acuity under bright conditions, whereas nocturnal species often exhibit superposition optics, enlarged facets, and neural summation mechanisms that enhance sensitivity under dim light. Many insects are also capable of detecting ultraviolet and polarized light, supporting behaviors such as navigation, prey detection, and pollinator–plant interactions [29,30].

In ants, the eyes play a fundamental role in orientation and navigation [31], as well as in prey and predator detection. When ecological specialization promotes a smaller worker size and a more cryptic or hypogaeic lifestyle, a reduction of energetically expensive visual structures may occur [32]. This pattern is evident in subterranean and leaf-litter foraging ants, which generally possess relatively smaller eyes than surface-foraging species.

Johnson & Rutowski [19] identified a consistent nocturnal ecomorph in four distantly related genera (Formicinae: *Myrmecocystus*; Myrmicinae: *Aphaenogaster*, *Temnothorax*, and *Veromessor*). This syndrome is characterized by reduced cuticular pigmentation together with increased eye size, ommatidial number, and facet diameter. In contrast, extreme reduction of compound eyes, ranging from only a few ommatidia to complete blindness, is even more widespread and constitutes part of the subterranean ecomorph syndrome [30]. Arboreal ants, including both lignicolous and carton-nesting species, typically possess much larger eyes than subterranean, leaf-litter, or ground-nesting ants [33]. Eye size is also correlated with both habitat and trophic level: ants inhabiting subterranean or litter environments tend to have smaller eyes than surface-foraging species, while predatory ants generally exhibit smaller eyes than omnivorous species [33,34].

Eye size and ommatidial number are also strongly correlated with body size [14,35]. After accounting for mesosoma length, significant interspecific variation in eye structure was found among *Temnothorax* species [19], including differences in both ommatidial number and facet diameter. Also, the paler species tended to exhibit larger ommatidial diameters [19]. One common correlate of reduced integument pigmentation is low environmental light availability. Pale animals with little or no melanin are commonly associated with habitats lacking ambient light, such as caves, deep-sea environments, soils, or parasitic lifestyles inside host bodies. Similarly, species active under dim-light conditions, including nocturnal insects, are often paler than their diurnal congeners. However, the adaptive significance of reduced pigmentation in low-light environments remains unclear, particularly because melanin is relatively inexpensive to produce and may provide several physiological advantages.

Among the eight species considered in the present study, four (*Temnothorax bejaranien-sis*, *T. curtulus*, *T. pardoii*, and *T. cfr. tebesae*) are clearly forest-associated [23,36], whereas the remaining four occur in more open habitats. Nevertheless, all species are small ants that

forage within the leaf litter. The forest-associated species exhibit pale yellow coloration and comparatively smaller eyes, possibly as an adaptation to habitats with reduced ambient light [33]. In the less forest-dependent species, the eyes are larger and darker pigmentation may occur in parts of the body, particularly in the head or gaster, although none of the species exhibit uniformly dark coloration. All species examined can be considered diurnal in their activity patterns and therefore do not display the extremely enlarged eyes and very pale coloration typical of the *laurae* group described by [19] a syndrome characteristic of strongly nocturnal species.

#### 4.2. Setae

Interommatidial setae (often referred to as ommatrichia) in Formicidae are microscopic sensory hairs located between the ommatidia of the compound eye. These structures are common in many Hymenoptera, and their presence, absence, density, and length vary considerably among species according to their ecological niche, such as diurnal versus nocturnal activity or aerial versus terrestrial lifestyles [29]. Consequently, interommatidial setae are frequently used as diagnostic characters in taxonomic descriptions, with some species characterized as having “eyes bare” and others as possessing “compound eyes bearing interommatidial setae”.

The function of the appressed setae surrounding the compound eyes in the species studied remains unknown, although they may have a tactile or mechanosensory role.

#### 4.3. Morphometric Traits

The presence of morphologically cryptic species represents a major challenge in insect systematics, including ant taxonomy. In many groups, evolutionarily distinct lineages may exhibit highly similar external morphology, complicating species delimitation when relying exclusively on traditional characters observable under optical microscopy. This limitation has stimulated the development of new methodological approaches, particularly the use of high-resolution imaging techniques and the exploration of novel quantitative morphological traits.

In this context, scanning electron microscopy (SEM) has become an especially valuable tool because it allows the detailed examination of microscopic exoskeletal structures that often remain undetectable using conventional methods. SEM enables precise characterization of traits such as cuticular microsculpture, setal arrangement, compound eye structure, and sensory morphology.

At the same time, the incorporation of new morphometric variables represents a key strategy for addressing cryptic diversity. Although ant taxonomy has traditionally relied on a relatively limited set of linear measurements, the inclusion of additional traits—such as eye morphology and cephalic geometry—can provide substantial discriminatory information, particularly when analyzed within a multivariate framework. These findings suggest that ocular morphology represents a largely underexplored but evolutionarily relevant axis of morphological differentiation in ants.

An important advantage of the *Temnothorax* species examined in this study is their relatively low number of ommatidia, in contrast to species with highly developed compound eyes such as *Melophorus bagoti* (421–590 ommatidia [35]), *Cataglyphis bicolor* (400–1200; [37]), or *Myrmecia pyriformis* (2724–3593; [38]). The relatively low number of ommatidia facilitates accurate quantification and comparative analyses of eye morphology.

Multivariate approaches allow the detection of variation patterns that are not evident when characters are analyzed individually. Traits with limited discriminatory power on their own may become informative when combined with others. Accordingly, the

integration of high-resolution SEM imaging and novel quantitative variables represents a promising strategy for species delimitation in morphologically conserved groups.

The results demonstrate that morphometric approaches effectively discriminate among the studied species. Multivariate Linear Discriminant Analysis (LDA) models achieved high classification accuracy (approximately 0.87–0.90), indicating a strong taxonomic signal. Extremely low Wilks' lambda values further confirm the robustness of interspecific differentiation. In other studies on *Temnothorax* using the usual morphological variables, classification success typically ranges between 0.95 and 1 [39,40].

Analyses performed at the nest level produced simpler and slightly more accurate models than those based on individual workers, suggesting that part of the observed individual-level variation reflects intracolony variability rather than true interspecific differences. Averaging measurements at the colony level therefore appears to reduce intracolony "noise" and enhance the discriminatory signal.

Confusion matrices indicate that most species are clearly separated, although some overlap exists among certain groups (e.g., *T. luteus*, *T. pardoii*, and *T. racovitzaei*), suggesting morphological proximity or possible evolutionary affinities. In contrast, species such as *T. curtulus* and *T. tuberosum* exhibit much stronger differentiation.

A key aspect highlighted by this study is the influence of body size on morphometric variables. Size correction revealed that a substantial proportion of the discriminatory power is driven by allometric effects. After removing this component through regression against CS, interspecific differences primarily reflected variation in shape and proportions, which are more directly associated with evolutionary and functional divergence. Thus, the observed interspecific differences primarily reflect variation in shape and proportions relative to body size rather than absolute size differences.

In this context, the high discriminatory power of SPST suggests that propodeal spine morphology contains a strong taxonomic signal, potentially associated with mechanical or defensive adaptations. Similarly, OMAT (n° ommatidia) retained high discriminatory capacity even after size correction, reinforcing its value as a functional and ecological trait. The contribution of SM (setae surrounding the compound eye) indicates that pericocular pilosity also provides relevant information, although to a lesser extent. In contrast, the low discriminatory power of CLI (clypeus) suggests that this character is relatively conserved or strongly influenced by overall body size, thereby limiting its taxonomic utility. These results highlight the importance of disentangling size-related and shape-related effects in morphometric analyses.

Taken together, these findings demonstrate that the incorporation of novel morphometric traits—particularly those related to ocular morphology—improves the quantitative characterization of interspecific variation and contributes to a more robust species delimitation framework within multivariate analyses.

## 5. Conclusions

The results of this study demonstrate that the combination of scanning electron microscopy (SEM) and the use of novel morphometric variables significantly improves species delimitation in morphologically conserved groups such as *Temnothorax*. Variables related to ocular morphology and cephalic structures provide relevant discriminatory information, particularly when integrated within multivariate frameworks. Correcting for body size is essential to disentangle allometric effects from true shape variation, revealing morphological patterns more directly associated with evolutionary divergence. In this context, traits such as SPST and OMAT exhibit high discriminative power, while other variables contribute complementary information in multivariate models. Moreover, the use of nest-level means enhances model accuracy and reduces the influence of intra-colony variability, represent-

ing a recommended methodological approach in morphometric studies. Overall, these findings highlight the value of integrating new quantitative morphological approaches with high-resolution imaging techniques to improve the delimitation of cryptic species, providing a robust framework complementary to molecular methods. This approach may be broadly applicable to other morphologically conserved insect groups facing similar taxonomic challenges.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/taxonomy6020035/s1>, Table S1. Different populations with their geographical data; Figure S1. Location of the different populations of *Temnothorax* used.

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