Journal of Apicultural Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

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To cite this article: Anna Janczyk , Marina Doris Meixner & Adam Tofilski (2021) Morphometric identification of the endemic Maltese honey bee (*Apis mellifera ruttneri*), Journal of Apicultural Research, 60:1, 157-164, DOI: 10.1080/00218839.2020.1827705

To link to this article: https://doi.org/10.1080/00218839.2020.1827705

Published online: 20 Oct 2020.

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Journal of Apicultural Research, 2021
Vol. 60, No. 1, 157–164, https://doi.org/10.1080/00218839.2020.1827705

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ORIGINAL RESEARCH ARTICLE

Morphometric identification of the endemic Maltese honey bee (*Apis mellifera ruttneri*)

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(Received 25 February 2020; accepted 22 July 2020)

Apis mellifera ruttneri is the endemic honey bee subspecies of the Maltese Islands. This population is endangered by hybridization with other honey bee subspecies that are frequently imported by beekeepers. It deserves conservation because of its unique behavioral traits including adaptation to the local climate. Conservation of honey bee subspecies is not easy, because it requires the identification of unhybridized source colonies by multiple morphological measurements or by molecular analysis. We present here a new method to identify *A. m. ruttneri* that is reliable and easy to use by both scientists and beekeepers. We have tested the new method using 312 workers collected from flowers in 7 locations and 52 managed colonies from Malta. Those bees formed two groups. One of the groups was interpreted as unhybridized *A. m. ruttneri* and the other group was interpreted as hybrids between *A. m. ruttneri* and imported bees. The new method to identify Maltese bees can provide a useful contribution to their conservation.

Keywords: honey bee; subspecies identification; *Apis mellifera ruttneri*; geometric morphometrics; wing morphometry

Introduction

The Western honey bee (*Apis mellifera*) has an unusually wide distribution range with to date about 30 recognized subspecies (Chen et al., 2016; Meixner et al., 2011; Ruttner, 1988; Sheppard & Meixner, 2003). The high number of subspecies results from adaptation to a wide range of environmental conditions including climate, vegetation, and presence of pathogens and predators, maintained by at least partial genetic isolation through geographical barriers such as mountain ranges, large bodies of water, or deserts (Ruttner, 1988). These subspecies were grouped into four evolutionary lineages: lineage A from central and southern Africa, lineage C from southwest Europe, lineage M from Northern and Western Europe, and lineage O from the Middle East (Kandemir et al., 2011; Meixner et al., 2013). From an apicultural and economical point of view beekeepers oftentimes regard native bees as less desirable. Therefore, in many regions, a strong tendency to import foreign subspecies can be observed, with a predominance of *A. m. ligustica*, *A. m. carnica,* or their hybrids (De la Rúa et al., 2009; Meixner et al., 2010). Due to their limited population size, endemic subspecies from small islands are particularly endangered by such importations, since this can lead to hybridization, loss of adaptive features, and extinction of the native gene pool. The native subspecies from the Maltese Islands, *A. m. ruttneri* (Sheppard et al., 1997), is an example of a particularly highly endangered honey bee subspecies.

The Maltese Archipelago is located in the central part of the Mediterranean Sea, approximately 96 km south of Sicily and 290 km north of the African coast. During long periods of the Quaternary, the archipelago was connected to the African mainland (Hunt & Schembri, 1999), as demonstrated by fossil remains of African fauna (Zammit-Maempel, 1985), but became isolated with the rise of sea levels after the last glaciation.

A. m. ruttneri differs both genetically and morphologically from the closest neighboring subspecies, *A. m. intermissa*, *A. m. ligustica* and *A. m. siciliana* (Sheppard et al., 1997; Zammit-Mangion et al., 2017). Despite the proximity to the Italian peninsula, *A. m. ruttneri,* together with *A. m. siciliana,* is more closely related to African rather than European subspecies, and clearly belongs to the evolutionary lineage A (Meixner et al., 2013), as is also confirmed by a high frequency of African mitochondrial *Dra*I haplotypes (Zammit-Mangion et al., 2017). Morphologically, the Maltese bees are characterized by wide abdomen, comparatively dark pigmentation and a characteristic wing venation (Sheppard et al., 1997). The bees are comparatively defensive, with specific behavioral adaptations against predatory hornets (*Vespa orientalis*), and their colony development is well adapted to the local climatic conditions (Meixner et al., 2018).

Beekeeping has been present on the Maltese Islands since ancient times (Brincat, 1995; Crane, 1999). Traditionally, the bees were kept in bottle-shaped horizontal hives made of fired clay ("Qolla", Ball, 2006). In 1970, about 3000 such traditional hives existed on the

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Maltese Islands (Crane, 1999). Nowadays, especially since the arrival of *Varroa destructor* on the islands in 1992, beekeepers use modern hives. Most apiaries are relatively small (Jansen, 2018) with an average number of 15 colonies per beekeeper (Jones, 2004). In 2018, 222 registered beekeepers in Malta managed a total of 4109 colonies (Jansen, 2018). While many Maltese beekeepers oppose the introduction of non-native bees (Jansen, 2018), some of them frequently import both queens and whole colonies from Europe, mainly the productive *A. m. ligustica*, for economic reasons. Despite the frequent and on-going importation of foreign stock, some colonies on the Maltese Islands still show the typical morphological and genetic traits of *A. m. ruttneri* (Zammit-Mangion et al., 2017), and this heritage deserves conservation.

Conservation of a native honey bee subspecies can, for instance, be achieved by identification of colonies showing traits specific for the subspecies in question, and using such colonies to produce queens to requeen colonies showing non-native traits. However, an important prerequisite for the success of such activities is an easy and reliable method to discriminate between colonies of native and non-native origin. Originally, the identification of honey bee subspecies was based on the measurement of multiple morphological traits including various body parts, pilosity and pigmentation, followed by statistical analysis (Ruttner et al., 1978). However, this method is not easily available and not practicable for beekeepers, whose involvement is essential for the conservation of local subspecies.

More recently, the use of genetic analyses to characterize subspecies has become more common, but a rapid and cost-effective method for molecular subspecies diagnosis is not yet widely available (Meixner et al., 2013). While morphometric reference data of *A. m. ruttneri* have been published (Sheppard et al., 1997), the only genetic reference data for this subspecies that exist to date are mitochondrial DNA haplotypes (Zammit-Mangion et al., 2017) that can identify the maternal lineage, but not the nuclear genetic makeup nor any hybridization of different subspecies.

A simplified morphometric method that is based on forewing measurements only, was introduced to overcome the difficulties associated with standard morphometry and to facilitate morphometric characterization of honey bee subspecies (Francoy et al., 2008; Tofilski, 2008). Recently, the computer software "IdentiFly" was developed (Nawrocka et al., 2017) that is based on geometric morphometric wing measurements and can be used to identify unknown samples via inbuilt reference data of a number of different honey bee subspecies, including *A. m. ruttneri*. However, due to the still limited number of inbuilt reference samples for each subspecies, the level of confidence that can be achieved for subspecies identification is comparatively low, while identification of an unknown sample to its evolutionary lineage is possible with a high level of confidence. For instance, the reference sample set for *A. m. ruttneri* that was available for the current version of the IdentyFly software consisted of only five colonies. However, as the reference sample size correlates with the estimation of variation and covariation of the variables used for identification, a substantial size of the reference sample set appears essential for a reliable identification of subspecies with higher confidence.

In this study we aimed, firstly, to increase the reference samples of *A. m. ruttneri*, and secondly, to test the improved reference sample set with field data. In order to increase the reference sample size we used colonies that previously had been confirmed as *A. m. ruttneri* using both molecular and morphometric methods. The new, larger reference sample set was implemented into the IdentiFly software, which can be used by both scientists and beekeepers. In order to achieve the second objective, we investigated the prevalence of native *A. m. ruttneri* or hybridized bees by analyzing a collection of worker bees from both apiaries and flowers from random locations on the island.

Materials and methods

Preparation of reference sample set

In the first step, we increased the reference sample set. Five of the colony samples belonged to the original data set used in the description of this subspecies and were collected from two locations in Malta (Dingli and San Julian's) (Sheppard et al., 1997). These data were obtained from the Morphometric Bee Data Bank in Oberursel, Germany. The remaining 19 reference samples were collected in 2015 and 2016, in the context of the SMARTBEES project (www.smartbees.org), from selected beekeepers who never imported foreign stock. These colonies were confirmed to be pure *A. m. ruttneri* by standard morphometry and genetic analyses (Parejo et al., 2018).

Identification of unknown samples

In the second step, we investigated the current status of the hybridization of bees from Malta. In 2018, workers were collected from 52 colonies in 5 randomly selected apiaries located in the Northern District of Malta. Each apiary was represented by 9-12 colonies, and each colony was represented by 20 workers. In total, 1040 workers were analyzed. Additionally, a total of 312 worker bees were collected from flowers in 7 locations (in the Northern, Western and Southern Harbour District of Malta) in March 2015. The number of workers per location varied between 19 and 90. Bees from apiaries and field samples were preserved in 70% ethanol. The forewings of the bees were dissected and their images were obtained using a CMOS camera attached to a 25-mm lens or a stereomicroscope. The resolution of the images was 768 × 576 or higher.

Table 1. Procrustes distances between reference colonies of *A. m. ruttneri* and evolutionary lineages (lower triangle) and statistical significance of pairwise comparisons between the groups (upper triangle).

group	*A. m. ruttneri*	lineage A	lineage C	lineage M	lineage O
A. m. ruttneri	–	***	***	***	***
lineage A	0.0168	–	***	***	***
lineage C	0.0214	0.0281	–	***	***
lineage M	0.0200	0.0138	0.0279	–	***
lineage O	0.0150	0.0129	0.0256	0.0176	–

***$P < 0.0001$.

Wing measurements and analysis

All wing images were measured by indicating 19 characteristic points called landmarks as described in a previous study (Nawrocka et al., 2017). The landmark coordinates were aligned using the Procrustes fit method in the MorphoJ 1.04 software (Klingenberg, 2011). The aligned coordinates were averaged across colonies (in case of bees from apiaries) or locations (in case of bees from flowers). All further analyses were performed on the average values. Procrustes distances were used to describe differences between groups. The significance of differences between groups was based on permutation tests for the Procrustes distances (Klingenberg, 2011).

In order to simplify the identification of an unknown sample, the subspecies were grouped into lineages C, M and O. Using lineages instead of subspecies reduced the number of groups which makes interpretation and presentation of the identification results easier. Lineage A was split into *A. m. ruttneri* and the remaining subspecies from this lineage. Although *A. m. ruttneri* belongs to lineage A, for the purpose of identification only, it was treated as a separate group. For brevity, we use "lineage A" when we refer to the remaining subspecies from lineage A. Reference samples for the evolutionary lineages consisted of 187 colonies representing 25 subspecies. The same data were used by the IdentiFly software to discriminate lineages in an earlier study (Nawrocka et al., 2017) and were originally obtained from the Morphometric Bee Data Bank in Oberursel. The identification was based on canonical variate analysis. First, the reference samples were used to calculate coefficients for canonical variables, means of canonical variables, and variance covariance matrices. These data were used to project the unknown landmark configuration into the canonical space. The classification of reference samples was verified with leave-one-out cross-validation using PAST 3.11 software (Hammer et al., 2001). In the case of colonies from beekeepers and field samples cross-validation was not used because those data were not used to calculate the classification model. All statistical analyses were performed with STATISTICA v.13 software (TIBCO Software Inc, 2017). The identification of *A. m. ruttneri* presented in this study can be replicated using the IdentiFly software (Nawrocka et al., 2017). The identification data were saved in file "apis-mellifera-ruttneri-classification.dw.xml" which can be downloaded from http://drawwing.org/identifly.

Results

Reference samples

The *A. m. ruttneri* reference samples collected in 1995 were more similar to lineage A (squared Mahalanobis distance between lineage A and 1995 and 2015/16 reference samples was 0.0105 and 0.0181, respectively) and less similar to lineage C (squared Mahalanobis distance between lineage C and 1995 and 2015/16 reference samples was 0.0233, and 0.0215, respectively) than the reference samples from 2015/16. However, the differences in wing shape between the 1995 and 2015/16 reference samples were not statistically significant (MANOVA: $F = 4.01$; $P = 0.3774$); therefore, the two groups were combined for further analysis.

The wing shape of *A. m. ruttneri* reference samples differed significantly from all lineages (Table 1). The differences in wing shape between *A. m. ruttneri* and evolutionary lineages were located in various parts of the wing (Figure 1) including landmarks 3, 10, 13, and 18. The reference samples of *A. m. ruttneri* formed a separate cluster in the graphs of the first three canonical variates (Figure 2A, B), particularly in the graph depicting the third canonical variate (Figure 2B) there is a visible lack of overlap with lineage A. Canonical Variate Analysis allowed to correctly classify (with cross-validation) all the reference colonies of *A. m. ruttneri*. However, one colony of *A. m. intermissa* (from lineage A) was misclassified as *A. m. ruttneri*. Moreover, one colony of *A. m. capensis* (from lineage A) was misclassified as lineage O, one colony of *A. m. anatoliaca* and one colony of *A. m. syriaca* (both from lineage O) were misclassified as lineage A. Overall, the correct classification rate was 98.06%.

Identification of unknown samples

The bees collected from flowers at all locations of Malta were most similar to *A. m. ruttneri* and they did not differ from them significantly (Table 2). The probabilities of classification as these subspecies were larger than 0.01, which indicates a relatively high similarity of those bees to the reference samples. In comparison to bees collected from flowers, the bees collected from apiaries were less similar to the reference colonies and they differed from them significantly (Table 2). Nonetheless, almost all (98.08%) colonies from apiaries were most similar to *A. m. ruttneri*. Only one colony was most similar to lineage C. However, some of the colonies that were most similar to *A. m. ruttneri* still differed markedly from the reference of this subspecies. In the graphs of canonical variates (Figure 2A, B) the colonies from apiaries formed two clusters. Both of the

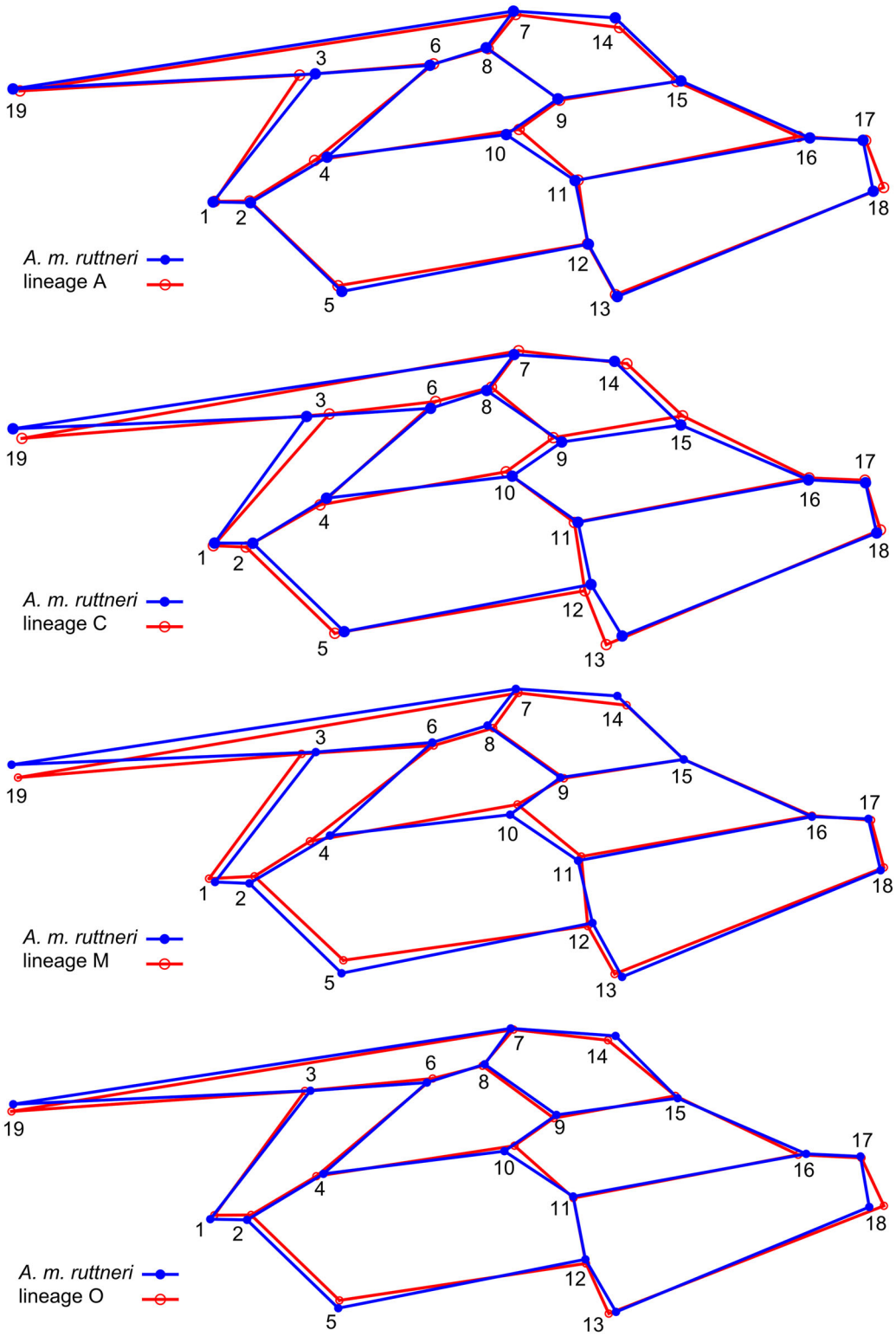

Figure 1. Differences in wing shape between *A. m. ruttneri* and evolutionary lineages A, C, M and O (from top to bottom, respectively). The differences in shape were magnified twofold for better visualization.

clusters differed significantly from *A. m. ruttneri* (for both clusters $P < 0.0001$). In comparison to reference samples of *A. m. ruttneri* the bees collected both from flowers and apiaries were on average further away from lineage A and closer to lineage C (Figure 2A, B).

Discussion

In this paper, we present geometric morphometric reference data which can be used for the identification of *A. m. ruttneri*. The identification is based on a wing

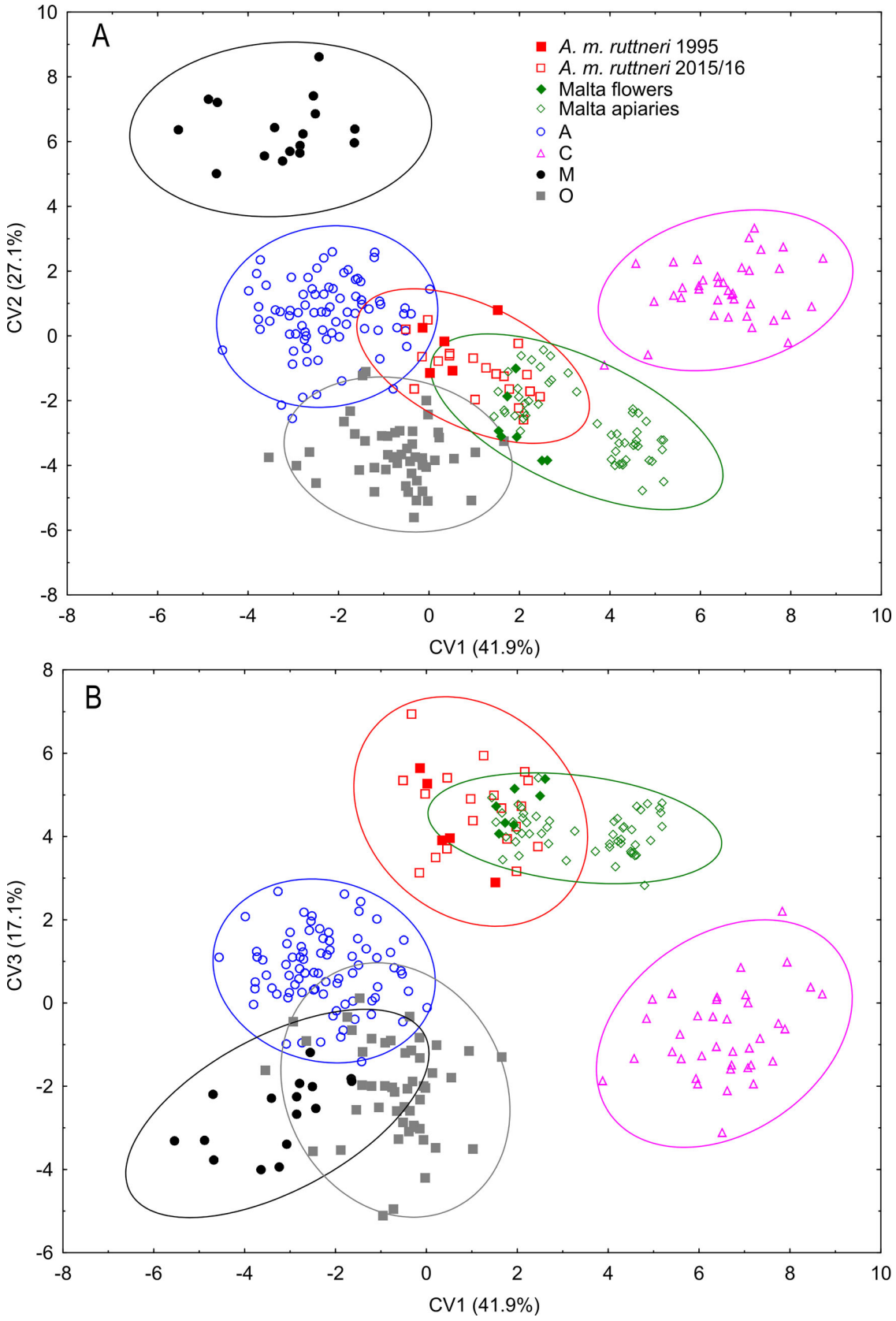

Figure 2. Discrimination of *A. m. ruttneri* from four honey bee evolutionary lineages based on canonical variate analysis of forewing shape. Samples of bees collected from Malta were projected into the space of canonical variates obtained from the analysis of reference samples of lineages and subspecies.

Table 2. Procrustes distances between reference colonies of *A. m. ruttneri* and bees collected in Malta from apiaries or flowers (lower triangle) and statistical significance of pairwise comparisons between the groups (upper triangle).

group	*A. m. ruttneri*	apiaries	flowers
A. m. ruttneri	–	***	NS
apiaries	0.0174	–	***
flowers	0.0073	0.0129	–

***$P < 0.0001$; NS: $P > 0.05$.

measurement method that does not require any sophisticated equipment and is available both to scientists and beekeepers. By identifying the similarity of unknown samples to reference data of *A. m. ruttneri*, this effective and relatively easy method can contribute to the conservation of this endangered honey bee subspecies native to Malta. In comparison to the earlier version, where all subspecies were identified (Nawrocka et al., 2017) the new method is easier because it is specifically designed for the identification of one particular subspecies. To make the analysis results easier to interpret, the number of reference groups was reduced to the different lineages instead of numerous individual subspecies. By comparing *A. m. ruttneri* with lineages we do not suggest that this subspecies forms another lineage; the purpose of this treatment is to make the interpretation of the identification results easier. To increase the resolution, it would be useful to also compare *A. m. ruttneri* with its closest relatives *A. m. intermissa* and *A. m. siciliana* (Sheppard et al., 1997); however, to perform this analysis, we do not have adequate numbers of reference data from those two subspecies.

In addition, as the analysis is now based on an extended reference dataset including *A. m. ruttneri* samples that were identified as pure based on standard morphometry and genomic sequence analysis (Parejo et al., 2018), the accuracy of the identification has also been considerably improved. Nonetheless, there is still space for further improvement of the reference set, especially by the inclusion of historic samples and more samples verified by molecular methods.

Hybridization between honey bee subspecies leads to a continuous range of hybrids. In this situation, it is often an arbitrary decision to pronounce an investigated colony as pure or not. It should be stressed that the IdentiFly software can indicate that the most similar subspecies is *A. m. ruttneri*, even if it is markedly hybridized with other subspecies. Therefore, attention should be paid to the probability of classification. If the probability of classification as *A. m. ruttneri* is smaller than 10^{-4}, the colony under analysis should not be considered as belonging to this subspecies. In practice, for conservation purposes, colonies that are most similar to the reference colonies should be chosen. In some cases, the colony can be markedly hybridized, but it is selected for the next generation because there is no better alternative.

The improved identification method was tested on a relatively large sample set of "unknown" bees. The results show that some of these samples were similar to the reference data of *A. m. ruttneri*, thereby confirming that native bees are still present in Malta as indicated by an earlier report (Zammit-Mangion et al., 2017). While this study was based on a collection from selected beekeepers that were known to keep the native bee and not to import foreign genetic stock, we have instead collected from randomly chosen apiaries. In addition, we also included foragers collected from flowers that probably represent numerous colonies within the range of a few kilometers. Our results showed that bees collected from flowers were more similar to *A. m. ruttneri* in comparison to bees collected from apiaries. This difference should be interpreted with care because the bees from apiaries and flowers were collected in different years. It is also possible that *A. m. ruttneri* colonies are stronger or more active in March when the bees were collected from flowers.

While the native Maltese bee is recently regaining popularity among beekeepers (Uzunov et al., 2018), the results might also point towards the existence of some feral colonies. At first glance, the environment on Malta does not appear suitable for maintaining a feral population of honey bees, because it is highly anthropogenically influenced and very few large trees exist that would offer nesting sites. However, we have observed two feral colonies in piles of stones that had been removed from cultivated land. Such potential nesting sites are relatively common in Malta and could be utilized by a feral population of honey bees. The persistence of a relatively unhybridized population of *A. m. ruttneri* could be supported by partial reproductive isolation between *A. m. ruttneri* and other subspecies (Zammit-Mangion et al., 2017) as was observed in *A. m. mellifera* (Oleksa et al., 2013) and Africanized bees (Kraus et al., 2007). This mechanism alone can contribute to the conservation of Maltese bees, as recently *A. m. ruttneri* is benefiting from coordinated breeding efforts with an increasing number of beekeepers (Meixner et al., 2018; Uzunov et al., 2018).

Although native honey bees are still present in Malta, signs of hybridization with imported bees can be observed. Our results show that although comparatively pure *A. m. ruttneri* prevail, many samples probably represent hybrids between *A. m. ruttneri* and non-native subspecies. It can also be demonstrated that the wing venation of reference colonies collected in the nineties is more similar to lineage A references than that of the bees collected twenty years later, although the difference is not statistically significant. Nonetheless, the set of reference samples that was collected later had been confirmed to be pure *A. m. ruttneri* by standard morphometry and genomic sequencing (Parejo et al., 2018). Clearly, the bees collected from Malta in this study appear less similar to lineage A reference bees and the

reference bees from Malta, which may be interpreted as a result of partial hybridization of the local population with non-native bees.

From our results, it is difficult to precisely identify the origin of the imported bees that contributed to this hybridization. In an earlier study, it was shown that the wing venation of hybrids is intermediate between those of parental colonies (Węgrzynowicz et al., 2019). The hybrids detected in this study appear between *A. m. ruttneri* and lineage C, however, not as expected close to an imaginary line between the centers of the two groups (Figure 2A, B). The observed morphology of hybrids can be affected both by genetic drift and specific phenotype of imported bees (founder effect). The importation of foreign genetic stock into Malta has been common since at least the 1990s, after the parasitic *V. destructor* mite reached the archipelago (Sheppard et al., 1997). While there is no documentation available on the number and source of imported queens or colonies, the results of Zammit-Mangion et al. (2017) indicate a predominance of Italian (*A. m. ligustica*) and Carniolan (*A. m. carnica*) stock. The two subclusters of bees from apiaries could have various reasons which are difficult to explain; they can reflect two incidents of importation of a large number of colonies or consecutive generations of hybrids between imported and local bees.

In this paper, we present an easy and effective method of identification that can make a valuable contribution to future monitoring and conservation of the native *A. m. ruttneri* in Malta. Due to its small population size and frequent and on-going importations of foreign stock, this honey bee subspecies is one of the most endangered ones and has been threatened by extinction for the past decades. Nonetheless, our results confirm previous observations that numerous *A. m. ruttneri* colonies still exist on the islands and the population could be stabilized and increased by breeding from suitable native stock. Our improved method provides an easy and rapid method of identification of suitable source colonies that can be utilized in conservation efforts.

Acknowledgements

We would like to thank Carmel Psaila and Bartłomiej Piotrowski for helping to obtain honey bee samples and two anonymous reviewers for the helpful comments on an earlier version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Science Centre under Grant DEC-2013/10/E/NZ9/00682.

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