Proceedings of the Tenth International Conference on Urban Pests
Rubén Bueno-Marí, Tomas Montalvo, and Wm. H Robinson (editors) 2022
CDM Creador de Motius S.L., Mare de Deu de Montserrat 53-59,
08930 Sant Adrià de Besòs, Barcelona, Spain

GENETIC PATTERNS OF *VESPA VELUTINA* IN SPAIN

[1]CAYETANO HERRERA, [2]JOANA F. FERRAGUT, [1]MAR LEZA,
AND [2]JOSE A. JURADO-RIVERA
[1] Department of Biology (Zoology), University of the Balearic Islands, Ctra. Valldemossa km 7.5, Spain
[2] Department of Biology (Genetics), University of the Balearic Islands, Ctra. Valldemossa km 7.5, Spain

Abstract The yellow-legged hornet (*Vespa velutina*) is a social Hymenoptera native from Asia and an invasive species in Europe where was first detected in France in 2004 and have spread across the continent reaching peninsular Spain in 2010 and Mallorca island in 2015. Its predatory habits on a broad diversity of pollinators including *Apis mellifera* are known to have a negative impact on ecosystems. Here we investigate both the origin and phylogenetic relationships of *V. velutina* in peninsular Spain and Mallorca island based on the analysis of DNA sequences from the gene encoding the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and 13 nuclear STR (microsatellite) markers. Our results retrieved the Spanish *V. velutina* population nested in a genetic cluster including other European populations and specimens sampled in Jiangsu and Zhejiang (China). In addition, we provide evidence for an origin of both peninsular Spain and Mallorca *V. velutina* populations derived from the spreading southward of the yellow-legged hornets initially established in France, which have successfully spread an established in much of Europe.

Key words *Vespa velutina*, phylogenetics, *cox1*, STRs

INTRODUCTION

Social insects have high invasion capacities (Beggs et al., 2011; Moller, 1996) mainly due to the ability of a single foundress to found a huge colony and to produce many future queens which in turn offer a large dispersal capacity (Arca et al., 2015; Mikheyev et al., 2009), a phenomenon that is known to increases with global trade and human mobility (Beggs et al., 2011). The yellow-legged hornet (*Vespa velutina*) is the first invasive Vespidae predator accidentally introduced from Asia to Europe (Monceau et al., 2014). It was first detected in France in 2004 and has successfully spread and stablished to neighbouring countries (Laurino et al., 2020) reaching peninsular Spain in 2010 (López et al., 2011). Since then, *V. velutina* populations have colonized all north Iberian peninsula and invaded Mallorca (Balearic Islands, Western Mediterranean) in 2015 (Leza et al., 2021) probably due to an accidental introduction by humans (Robinet et al., 2019). Since the first detection in Mallorca, an intensive protocol to detect and remove nests was implemented (Leza et al., 2021). Thirty-one nests were removed and sixteen queens were captured using spring trapping between 2015 and 2018. No more nests were found in subsequent years and the species was declared officially eradicated until July 2021 when a new secondary nest was detected and removed in the island. Since then, no more individuals have been detected in the island.

The aim of this study is to investigate both the origin and phylogenetic relationships of peninsular Spain and Balearic Islands yellow-legged hornet populations based on the analysis of mitochondrial (cox1) and nuclear (STR) DNA sequences.

MATERIAL AND METHODS

Sampling included 102 individuals of *V. velutina* from six geographic areas of Spain (Figure 1): Mallorca (n = 49), Catalonia (15), Basque Country (14), Asturias (10), Galicia (12) and Extremadura (2). Specimens were collected from nests, traps, and directly from flowers. Since a restricted number of individuals were sampled from each region, individuals were treated as representative samples from each geographic area studied and consequently the term ‘population’ refers to the total number of individuals from each sampled region.

Genomic DNA was extracted and purified from single specimens using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions and RNA was removed using 15 µl of RNAse A solution (Promega, Madison, WI, USA). For each specimen, the mitochondrial cytochrome c oxidase subunit 1 gene (cox1) was sequenced using the primer-pair LCO/HCO. PCR conditions used 0.2 µM of each primer and 3.5 mM MgCl2 (50mM) on a standard protocol of 40 cycles with annealing temperature of 48ºC (30s), denaturation at 94 °C (30s) and elongation at 72°C (60s). PCR products were visualized by 1% agarose gel electrophoresis and subsequently purified using the MSB Spin PCRapace kit (Invitek, Berlin, Germany). Sanger sequencing was performed with the same primers using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing process was carried out in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Electropherograms were assembled and edited with CodonCode aligner 9.0.1 (CodonCode Corporation, Dedham, MA, USA).

To determine the phylogenetic relationships and the origin of *V. velutina* specimens in Spain, sequences obtained in this study were aligned with those available in GenBank (Release 2022.01.19) using MAFFT 7 online version (Katoh et al., 2018). A median joining haplotype network was generated using the popART 1.7 software and a maximum likelihood (ML) phylogenetic tree was inferred using IQTREE 1.6.12 (Nguyen et al., 2015). Individuals were genotyped using a total of 18 microsatellites (Arca et al., 2012; Daly et al., 2002; Hasegawa & Takahashi, 2002), where forward primers were labelled with 6-FAM fluorescence (Blue), VIC (Green), NED (Yellow) and PET (Red). PCR amplifications were performed in two separate multiplex reactions using a final volume of 10 µl: 1.0 µl of template DNA, 5.0 µl of Multiplex PCR Kit (Qiagen), 3.6 µl of primer mix and 0.4 µl of ddH2O. It was carried out in a thermal cycler using a protocol of 40 cycles with annealing temperature of 50ºC (30s), denaturation at 94°C (30s) and elongation at 72°C (60s). The amplification product was separated by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems) and fragments sizes and alleles were determined using GeneMapper 3.2 ID software (Applied Biosystems).

Genetic differentiation among individuals from different populations was estimated by computing pairwise FST values in Arlequin 3.5.2.2 (Excoffier et al. 2005), specifying 10.000 random permutations to assess significance (p-value < 0.05). To explore the genetic relationships among individuals and whether there is genetic structure in Europe and in Spain, a model-free principal coordinate analysis (PCoA) was run in GENALEX 6.5 (Peakall & Smouse, 2006).

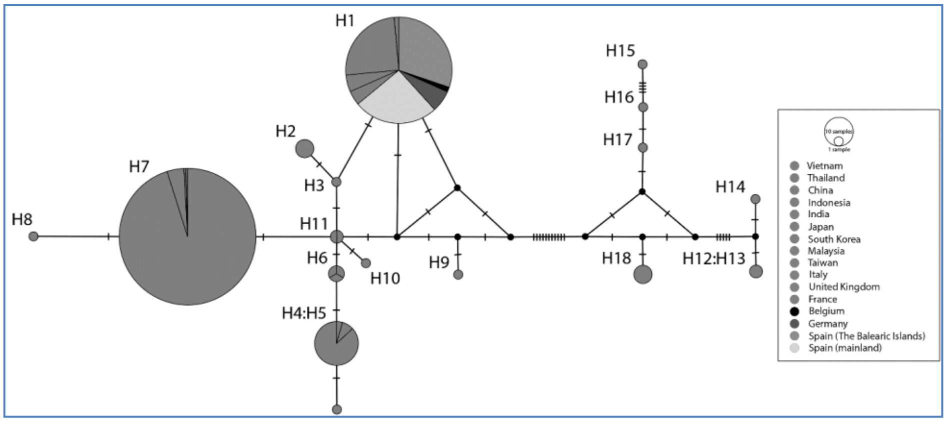

Figure 1. Median joining network of *V. velutina* haplotypes constructed in PopART. The pie charts display haplotype frequency for the mitochondrial coxI marker in each sampled region and their size is proportional to the sampling effort. Missing haplotypes are indicated by small black circles and each mutational step is indicated by hatch marks.

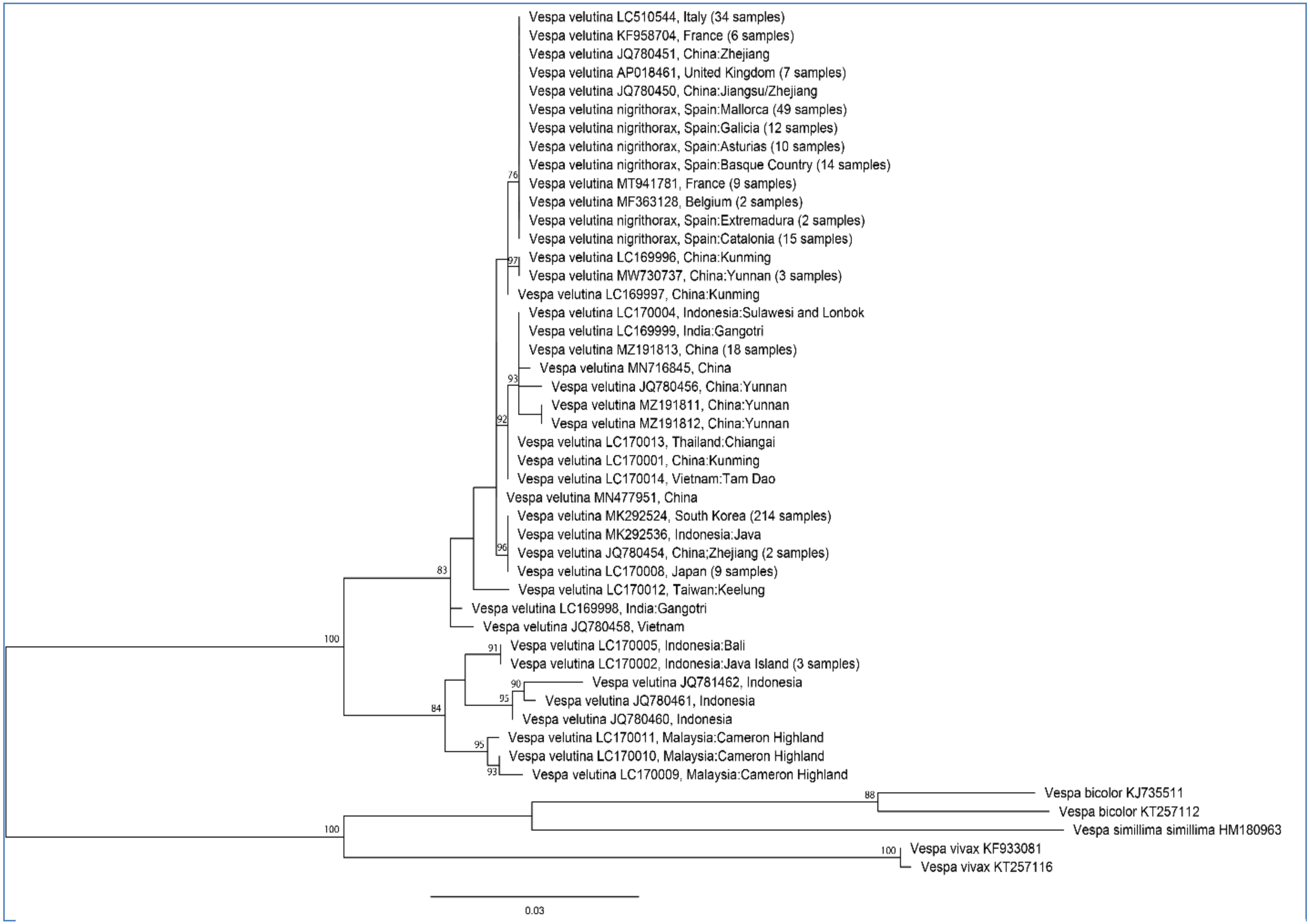

Figure 2. Maximum Likelihood tree (log-likelihood = 1942.5559, AIC = 4035.1118) computed using the TN - F - G4 model selected by the ModelFinder algorithm (Kalyaanamoorthy et al., 2017) implemented in the IQ-TREE software. Numbers represent ultrafast bootstrap support (only >75% are shown).

RESULTS AND DISCUSSION

All specimens sampled from Mallorca and the Spanish mainland (Catalonia, Basque Country, Asturias, Galicia, and Extremadura) shared the same cox1 haplotype (H1), which was identical to the haplotype shared by all European invasion of *V. velutina* reported to date and by the specimens sampled from Jiangsu and Zhejiang (China) (Figure 1). H1 haplotype found in Spain was the second most frequently detected, after H7 haplotype detected in South Korea (Figure 1). Everything seems to indicate that only one mitochondrial lineage has entered Europe, originally from China, results corroborated by other studies (Arca et al., 2015; Granato et al., 2019; Quaresma et al., 2022). Phylogenetic analyses based on cox1 sequences divided *V. velutina* into two main clades (Figure 2) in agreement with previous analyses (Granato et al., 2019; Perrard et al., 2014; Takeuchi et al., 2017). The inferred tree supported the results derived from the network analysis and showed the Spanish samples nested within the European invasion clade.

A total of 18 microsatellite loci were screened in the individuals from different regions from Spain, of which five were excluded from the study due to inconsistent amplification, and 6 out of the remaining 13 matched confidentially with data from other studies and therefore were used for subsequent analyses (Arca et al., 2015; Quaresma et al., 2022). Model-free principal coordinate analysis (PCoA) retrieved individuals from Asia and France conforming a tight cluster (Figure 3), suggesting that *V. velutina* invasion started in France as was reported by Arca et al. (2015). Meanwhile, the other European populations formed two groups within the PCoA (Figure 3) defined by coordinate 1 axis which explains 36.23% of the total variation. This could be indicative of a possible genetic divergence within the native populations and the original population of the invasion in Europe. This hypothesis is reinforced by the FST distances between native and invasive populations (Table 1) where we can appreciate that among the invasive populations, the higher geographical distance from the original invasion (France), the higher genetic distance. For example, the FST distance between France and Spain is 0.25064, while between France and Portugal it is 0.37784 (Table 1). Likewise, we can observe that many sampled regions have a lower FST value with individuals genotyped from Zheijang/Jiangsu and Yunnan. Indicating that they would be the populations from which the invasion in Europe originated, as other studies conclude (Arca et al., 2015; Quaresma et al., 2022).

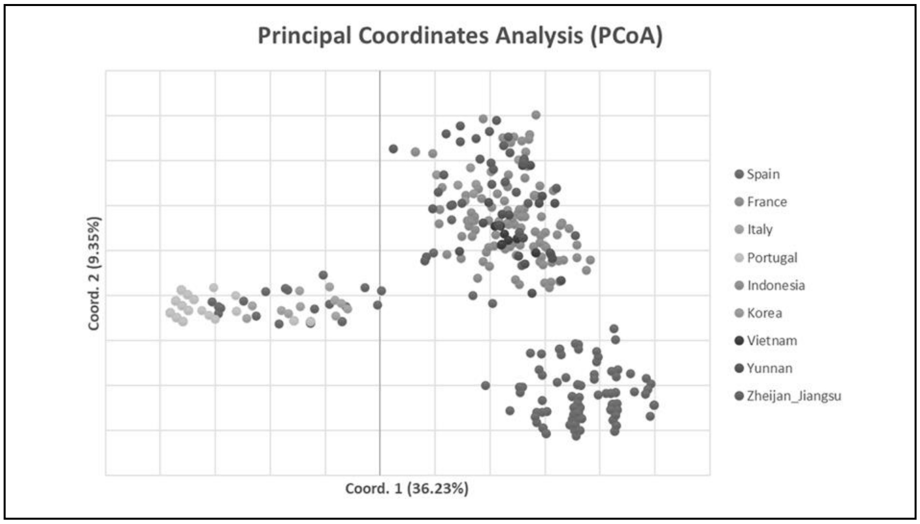

Figure 3. Principal Coordinates Analysis (PCoA) based on genetic distances of the 6 microsatellites used in *Vespa velutina* shared between studies. Dot colours refer to the regions where *V. velutina* was sampled

The genetic relationship between individuals sampled in Spain during this study were also inspected using a PCoA. In this case, two clades were observed on the 1 axis (Fig 4), which explains the 26.79% of the total variance. The first one includes all the individuals from the mainland Spain, slightly dispersed between them, while the individuals from Mallorca form a more consistent clade with some individuals related to regions of northern Spain (Figure 4). These results suggest an origin of the invasion in the Mediterranean island of Mallorca from either Catalonia or the Basque Country, a pattern that is also supported by the relatively low FST genetic distance values between Mallorca population and those from Catalonia and the Basque Country (Table 2).

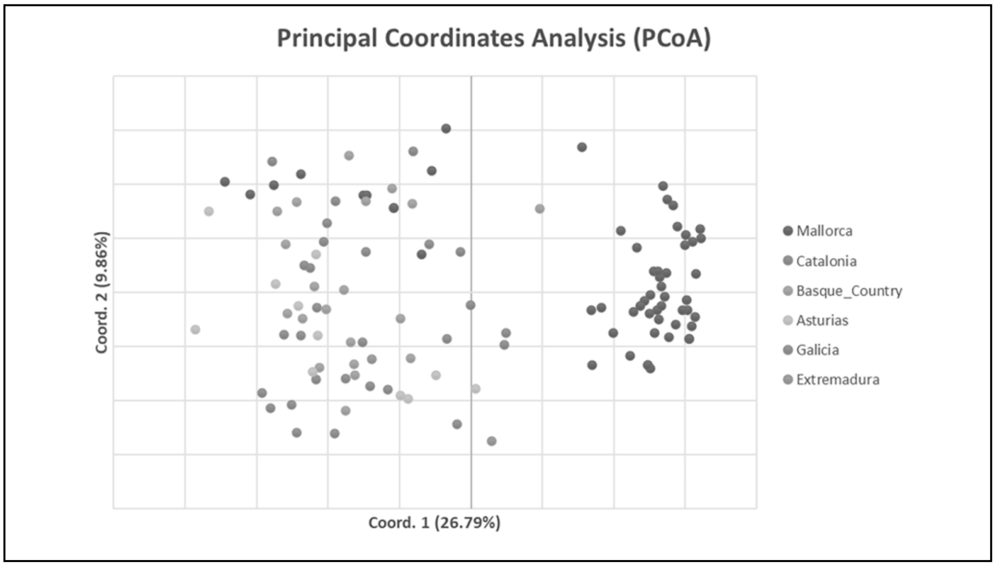

Figure 4. Principal Coordinates Analysis (PCoA) based on genetic distances of the 13 microsatellites used in *Vespa velutina* for this study. Dot colours refer to the regions where *V. velutina* was sampled.

The mitochondrial and nuclear markers concurrently employed in this study suggest that the invasions of *V. velutina* into Spain (mainland and Mallorca) stemmed from the particularly successful population of France, rather than from multiple independent introductions from the native range. Convincing evidence comes from the unique mitochondrial haplotype identified in Europe (Arca et al., 2015; Budge et al., 2017; Granato et al., 2019; Jones et al., 2020; Quaresma et al., 2022), congruent with other mitochondrial surveys undertaken in Europe and with the hypothesis of a single mated-queen propagule introduced into Europe.

Table 1. Multilocus estimates of F_{ST} (below diagonal) between populations of the invaded and native ranges. Bold numbers represent statistically significant differences (p.value < 0.05).

	Spain	**France**	**Italy**	**Portugal**	**Indonesia**	**Korea**	**Vietnam**	**Yunnan**
France	**0.28064**							
Italy	**0.27690**	**0.33055**						
Portugal	**0.37784**	**0.47890**	**0.44027**					
Indonesia	**0.35050**	**0.38820**	**0.51621**	**0.66213**				
Korea	**0.24187**	**0.28471**	**0.44239**	**0.52062**	**0.28217**			
Vietnam	**0.31420**	**0.34844**	**0.45161**	**0.64007**	**0.37447**	**0.27793**		
Yunnan	**0.23047**	**0.20443**	**0.32994**	**0.54866**	**0.25091**	**0.12987**	**0.18380**	
Zheij/Jiangsu	**0.19146**	**0.21278**	**0.33144**	**0.50120**	**0.25945**	**0.06904**	**0.20511**	**0.07332**

Table 2. Multilocus estimates of F_{ST} (below diagonal) between populations of Spain. Bold numbers represent statistically significant differences (p.value < 0.05).

	Mallorca	Catalonia	Basque Country	Asturias	Galicia
Catalonia	**0,21307**	-	-	-	-
Basque Country	**0,24576**	**0,05601**	-	-	-
Asturias	**0,36160**	**0,03454**	**0,09103**	-	-
Galicia	**0,29290**	**0,13149**	**0,16242**	**0,13575**	-
Extremadura	0,24359	**0,20187**	**0,21389**	**0,22397**	0,00816

CONCLUSIONS

This study builds upon the seminal analysis of the founding propagule in France to greatly expand current understanding on the population genetics aspects underlying the phenomenal invasion of *V. velutina* in Europe. Our findings further stress the importance of surveillance and control measures to halt gene flow and call for more stringent policies within the European Union preventing circulation of propagules, which may lead to secondary founder events favouring establishment and expansion of alien organisms.

ACKNOWLEDGMENTS

This work has been possible thanks to a FPI grant (FPI_014_2020) from the *Conselleria d'Innovació, Recerca i Turisme del Govern de les Illes Balears*. This study has been supported by *Fundación Biodiversidad* with Spanish MITECO (*Ministerio para la Transición Ecológica y el Reto Demográfico*) with a project titled "*STOP* Vespa velutina*: descifrando de dónde ha llegado y cómo se dispersa para establecer mecanismos de gestión de esta especie exótica invasora que amenaza a las abejas*".

REFERENCES CITED

Arca, M., Capdevielle-Dulac, C., Villemant, C., Mougel, F., Arnold, G. and Silvain, J. F. 2012. Development of microsatellite markers for the yellow-legged Asian hornet, *Vespa velutina*, a major threat for European bees. *Conservation Genetics Resources*, 4(2), 283–286.

Arca, M., Mougel, F., Guillemaud, T., Dupas, S., Rome, Q., Perrard, A., Muller, F., Fossoud, A., Capdevielle-Dulac, C., Torres-Leguizamon, M., Chen, X. X., Tan, J. L., Jung, C., Villemant, C., Arnold, G. and Silvain, J. F. 2015. Reconstructing the invasion and the demographic history of the yellow-legged hornet, *Vespa velutina*, in Europe. *Biological Invasions*, 17(8), 2357–2371.

Beggs, J. R., Brockerhoff, E. G., Corley, J. C., Kenis, M., Masciocchi, M., Muller, F., Rome, Q., & Villemant, C. 2011. Ecological effects and management of invasive alien Vespidae. *BioControl*, 56(4), 505–526.

Budge, G. E., Hodgetts, J., Jones, E. P., Ostojá-Starzewski, J. C., Hall, J., Tomkies, V., Semmence, N., Brown, M., Wakefield, M. and Stainton, K. 2017. The invasion, provenance and diversity of *Vespa velutina* Lepeletier (Hymenoptera: Vespidae) in Great Britain. *PLoS ONE*, 12(9), 1–12.

Daly, D., Archer, M. E., Watts, P. C., Speed, M. P., Hughes, M. R., Barker, F. S., Jones, J., Odgaard, K. and Kemp, J. 2002. Polymorphic microsatellite loci for eusocial wasps (Hymenoptera: Vespidae). *Molecular Ecology Notes*, 2, 273–275.

Granato, A., Negrisolo, E., Bonomi, J., Zulian, L., Cappa, F., Bortolotti, L. and Mutinelli, F. 2019. Recent confirmation of a single haplotype in the Italian population of *Vespa velutina*. Biolog. Invasions 21 2811–2817.

Hasegawa, E. and Takahashi, J. 2002. Microsatellite loci for genetic research in the hornet *Vespa mandarinia* and related species EISUKE. *Molecular Ecology Notes*, 2, 306–308.

Jones, E. P., Conyers, C., Tomkies, V., Semmence, N., Fouracre, D., Wakefield, M. and Stainton, K. 2020. Managing incursions of *Vespa velutina nigrithorax* in the UK: an emerging threat to apiculture. *Scientific Reports*, 10(1), 1–8.

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., Von Haeseler, A. and Jermiin, L. S. 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods,* 14(6), 587–589.

Katoh, K., Rozewicki, J. and Yamada, K. D. **2018**. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, 20(4), 1160–1166.

Laurino, D., Lioy, S., Carisio, L., Manino, A. and Porporato, M. 2020. *Vespa velutina*: An alien driver of honey bee colony losses. *Diversity*, 12(1).

Leza, M., Herrera, C., Picó, G., Morro, T. and Colomar, V. 2021. Six years of controlling the invasive species *Vespa velutina* in a Mediterranean island: The promising results of an eradication plan. Pest Management Science, 77(5), 2375–2384.

López, S., González, M. and Goldarazena, A. 2011. *Vespa velutina* Lepeletier, 1836 (Hymenoptera: Vespidae): First records in Iberian Peninsula. *EPPO Bulletin*, 41(3), 439–441.

Mikheyev, A. S., Bresson, S. and Conant, P. 2009. Single-queen introductions characterize regional and local invasions by the facultatively clonal little fire ant *Wasmannia auropunctata*. *Molecular Ecology* 18: 2937–2944.

Moller, H. 1996. Lessons for invasion theory from social insects. *Biological Conservation,* 78(1–2), 125–142.

Monceau, K., Bonnard, O. and Thiéry, D. 2014. *Vespa velutina*: A new invasive predator of honeybees in Europe. *Journal of Pest Science,* 87(1), 1–16.

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A. and Minh, B. Q. 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, 32(1), 268–274.

Peakall, R. and Smouse, P. E. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6(1), 288–295.

Perrard, A., Arca, M., Rome, Q., Muller, F., Tan, J., Bista, S., Nugroho, H., Baudoin, R., Baylac, M., Silvain, J. F., Carpenter, J. M. and Villemant, C. 2014. Geographic variation of melanisation patterns in a hornet species: Genetic differences, climatic pressures or aposematic constraints? *PLoS ONE*, 9(4).

Quaresma, A., Henriques, D., Godinho, J., Gmaside, X., Bortolotti, L. and Pinto, M. A. 2022. Invasion genetics of the Asian hornet *Vespa velutina nigrithorax* in Southern Europe. *Biological Invasions*, 9.

Robinet, C., Darrouzet, E. and Suppo, C. 2019. Spread modelling: a suitable tool to explore the role of human-mediated dispersal in the range expansion of the yellow-legged hornet in Europe. *International Journal of Pest Management*, 65(3), 258–267.

Takeuchi, T., Takahashi, R., Kiyoshi, T., Nakamura, M., Minoshima, Y. N. and Takahashi, J. 2017. The origin and genetic diversity of the yellow-legged hornet, *Vespa velutina* introduced in Japan. *Insectes Sociaux*, 64(3), 313–320.