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# Comparison of intra-colonial genetic diversity of the founder and leading edge populations of *Vespa velutina*

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"We are all connected.

To each other, biologically.

To the earth, chemically.

To the rest of the universe, atomically."

Neil deGrasse Tyson

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**SRPV:** Société Régionale de la Protection des Végétaux

**SNP:** Single Nucleotide Polymorphism

**SSR:** Simple Sequence Repeat

**STR:** Short Tandem Repeat

**SSLP:** Simple Sequence Length Polymorphism

**IAM:** Infinite Allele Model

**GSMM:** General Stepwise Mutation Model

**MCMC:** Markov Chain Monte Carlo

**PCoA:** Principal Coordinates Analysis

**Ho:** Observed Heterozygosity

**He:** Expected Heterozygosity

**uHe:** Unbiased Expected Heterozygosity

**Na:** Observed Number of Alleles

**Ne:** Effective Number of Alleles

**HWE:** Hardy-Weinberg Equilibrium

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**ٱلْحَمْدُ لِلَّهِ**

# ABSTRACT

*Vespa velutina nigrithorax*, commonly known as the yellow-legged hornet is a species of Vespidae native to Southwestern Asia. It was first observed in 2004 in Southwestern France (Lot-et-Garonne) and rapidly spread to neighboring countries; reaching Spain in 2010 and Portugal in 2011 in Viana do Castelo. Since 2011 it has been spreading through Portugal, with Bragança representing the most marginal distribution area of *V. velutina* in the northern region. Its spread rate is explained by its great capacity for climate adaptation and its polyandry. The invasion of *V. velutina* in Europe has caused significant ecological and economic damages. In fact, this hornet is a major threat to beekeeping since *Apis mellifera* consists in a third of its diet.

Genetic studies have provided insights into the colonization patterns of this invasive predator, revealing a genetic bottleneck upon its arrival in Europe, which led to reduced genetic diversity. In this study, we used 16 microsatellites to genotype a total of 120 female hornets from two nests of Viana do Castelo (Vila de Punhe) and two from Bragança (Macedo de Cavaleiros and Mirandela).

This study aims to assess and compare the genetic diversity of *V. velutina* population in the districts of Bragança and Viana do Castelo in Portugal.

Bragança represents the leading edge of the northeastern expansion in the Portuguese territory. Surprisingly, the genetic diversity observed in the two colonies from Bragança was higher than that in the colonies from Viana do Castelo. This discrepancy suggests that the population in Bragança is a result of expansion from multiple sources, introducing new alleles to the local population.

**Keywords:** *Vespa velutina*; Yellow-legged hornet; invasive species; microsatellites; population genetics; molecular markers

# RÉSUMÉ

*Vespa velutina nigrithorax* communément appelée frelon à pattes jaunes est une espèce de Vespidae originaire de l'Asie du Sud-ouest. Elle a été observée pour la première fois en 2004 dans le Sud-ouest de la France (Lot-et-Garonne) et s'est rapidement répandue dans les pays voisins ; atteignant l'Espagne en 2010 et le Portugal en 2011 à Viana do Castelo. Depuis 2011 elle se propage à travers le Portugal, avec Bragança représentant la zone de distribution la plus marginale de *V. velutina* dans la région nord. Sa vitesse de propagation s'explique par sa grande capacité d'adaptation au climat et sa polyandrie  L'invasion de *V. velutina* en Europe à provoqué d'importants dégâts écologique et économique. En effet, ce frelon est une grande menace pour l'apiculture, car *Apis mellifera* représente un tiers de son régime alimentaire.

Les études génétiques ont fourni des perspectives sur les schémas de colonisation de ce prédateur invasif, révélant un goulot d'étranglement lors de son arrivée en Europe, entraînant une réduction de la diversité génétique. Dans cette étude, nous avons utilisé 16 microsatellites pour génotyper un total de 120 frelons femelles de deux nids de Viana do Castelo (Vila de Punhe) et de deux nids de Bragança (Macedo de Cavaleiros et Mirandela).

Cette étude vise à évaluer et comparer la diversité génétique de la population de *V. velutina* dans les districts de Bragança et Viana do Castelo au Portugal.

Bragança représente la pointe de l'expansion Nord-est sur le territoire portugais. De manière surprenante la diversité génétique observée dans les deux colonies de Bragança était plus élevée que celle dans les colonies de Viana do Castelo. Cette disparité suggère que la population de Bragança résulte d'une expansion à partir de sources multiples, introduisant de nouveaux allèles dans la population locale.

**Mots-clés :** *Vespa velutina* ; Frelon à pattes jaunes ; espèces invasives ; microsatellites ; génétique des populations ; marqueurs moléculaires

# RESUMO

*Vespa velutina* nigrithorax, conhecida como vespa-asiática, é uma espécie da família Vespidae nativa do Sudoeste Asiático. Foi observada pela primeira vez em 2004 no Sudoeste Francês (Lot-et-Garonne) e tem-se expandido rapidamente para países vizinhos, tendo chegado a Espanha em 2010 e a Portugal em 2011 (Viana do Castelo). Desde 2011, tem-se expandido por Portugal, com Bragança a representar a área de distribuição mais marginal na região norte de Portugal. A taxa de expansão da *V.velutina* é explicada pela sua grande capacidade de adaptação a diferentes climas e poliandria. A invasão de *V. velutina* na Europa tem causado da nos significativos ecológicos e económicos. Em particular esta vespa é uma ameaça para a apicultura, pois a *Apis mellifera* representa um terço da sua dieta.

Estudos genéticos têm fornecido informações sobre os padrões de colonização deste predador invasor. Neste estudo, foram utilizados 16 microssatélites para genotipar um total de 120 fêmeas provenientes de dois ninhos em Viana do Castelo (Vila de Punhe) e dois em Bragança (Macedo de Cavaleiros e Mirandela). Este estudo tem como objetivo avaliar e comparar a diversidade genética das populações de *V. velutina* nos distritos de Bragança e Viana do Castelo.

Bragança representa o limite da expansão nordeste no território português, no entanto, surpreendentemente, a diversidade genética observada nas duas colónias de Bragança foi superior à observada nas colónias de Viana do Castelo. Este resultado sugere que a população de Bragança é o resultado da expansão de vespas de múltiplas origens que vão introduzindo novos alelos na população local.

**Palavras-chave:***Vespa velutina*; espécies invasoras; microssatélites; genética populacional; marcadores moleculares

# I. Introduction

## 1. Framework

The term "invasive" refers to an alien species that is introduced to a new area and quickly spreads, causing social and economic damage and also impacting the local biodiversity (Beggs et al. 2011).

*Vespa velutina nigrithorax* or commonly named the yellow-legged hornet is native to the temperate zones of Southern Asia (Archer 1994) but it is an invasive species in Europe. In 2004, *V. velutina* was observed for the first time in Southwestern France (Lot-et-Garonne). Even with a completely different climate in Europe, *V. velutina* has colonized almost 70% of France and already spread to the bordering countries (Spain, Italy, Portugal and Belgium), different factors may explain this spreading (Monceau, Bonnard, and Thiéry 2014). One of them is its reproduction system, *V. velutina* is polyandrous (one female mates with several males) and this allowed the first mated queen which appeared in France to produce a generation with enough genetic diversity to stand the different environments in Europe (Arca et al. 2015). In fact, *V. velutina* has immense dispersal and reproductive abilities, it is a predator of many other insects, including honey bees, and adapts rapidly to a new habitat (Moller 1996).

The invasion of *V. velutina* has a huge impact on the loss of honey bees' colonies (*Apis mellifera*). During hunting season which corresponds to the beginning of summer until the end of November, these hornets stand in front of beehives' entrance and catch foraging honey bees when they return to the hive (Monceau, Bonnard, and Thiéry 2014). This hornet can destroy approximately 20% to 30% of a colony (Villemant 2006). *V. velutina* is also harmful to humans, its sting can cause kidney failures or anaphylactic choc due to envenomation (de Haro 2009).

Vespidae contrary to other insects has a low probability of being detected when entering a new territory, methods to lower the population or to contain the species are now crucial but sometimes not efficient or targeting indirectly native endofauna (Kenis et al. 2007; Monceau, Bonnard, and Thiéry 2014). Genetics has been important to study the patterns of colonization of this invasive honey bee predator. When *V. velutina* arrived in Europe it suffered a genetic bottleneck lowering genetic diversity, increasing

in that way the population of sterile diploid males and inversely decreasing the general population (Darrouzet et al. 2015). This diminution is accentuated by natural selection, mutations and genetic drifts (Kyung-Seok and Sappington 2013).

The use of microsatellites is now a popular choice for geneticists and ecologists. Apart from being cost-effective due to minimal equipment requirement, microsatellites are a versatile tool. They are able to provide information about bottlenecks, migration and kinship in the population. Microsatellites are effective markers to study recent migrations (Selkoe and Toonen 2006)**.**

## 2. Objectives

*V. velutina* presents now a major threat to beekeeping in Portugal and in all Europe, this study provides valuable information for the development of strategies to control the population of this alien species and lower its impact on beekeeping. By using microsatellites we are going to evaluate and compare the genetic diversity of *V. velutina* in the district of Bragança and Viana do Castelo.

# II. Literature review

## 1. Characterization of *Vespa velutina*

### a. Entomological characteristics of *Vespa velutina*

*Vespa velutina nigrithorax* belongs to the Hymenoptera order, more specifically the Vespidae family. It is one of the 14 subspecies of *V. velutina* Lepeletier and one of the 22 species of Vespa (Kishi and Goka 2017).

*V. velutina* is predominantly black, with the exception of a wide orange band on the abdomen and a yellow band on the first segment. The front part is yellow-orange and the antennae are black at the ends and brown at the bottom. The ends of the legs are yellow, which explains the common name yellow-legged hornet (Figure 1) (Chauzat and Martin 2009; López, González, and Goldarazena 2011).


**Figure 1**: *Vespa velutina* castes (Monceau, Bonnard, and Thiéry 2014)

The queen's body measures 25 to 30mm, making it one of the smallest *Vespa* species (Kishi and Goka 2017). Size is a component of cast dimorphism with gynes having a well-developed fat body while workers have a less developed one. This difference can be explained by the different feeding behavior of the castes, with the queen receiving higher amounts of food compared to the workers (Rome et al. 2015). Wing shape is also different between castes, allowing gynes to fly longer distances from their nests compared to the workers (Perrard et al. 2012; Cini et al. 2018). The presence of a sting and the length of the antennae also contribute to the dimorphism between

males and females; females have a sting and shorter antennae (Monceau, Bonnard, and Thiéry 2014).

*V. velutina* has a short but efficient life cycle, with a nesting period lasting seven months (Choi, Martin, and Lee 2012; de la Hera, Alonso, and Alonso 2023). From March to June, it is the queen colony phase. During this phase the queen, starts a nest and lays eggs in it. From spring to autumn, the size colony increases, and in autumn it reaches the largest population size both in gynes and males. When the nest becomes too small for the colony, the queen relocates for a second nest, usually in an open area at the top of a tree (Rome et al. 2015). Then, from September to November it is the mating season, where the future queens will be fertilized and hibernate to start a new colony the following season (Monceau, Bonnard, and Thiéry 2014).

*V. velutina's* foraging behaviour is very similar to other Vespidae*,* it gets protein from meat, fish and also honey bees. Pheromones emitted by the honey bees and the hive make it one of the most attractive food (de la Hera, Alonso, and Alonso 2023). *V. velutina* gets carbohydrates from honey, honey bees, sugar candy and grapes that are usually found near human activity (Cini et al. 2018). Moreover, *V velutina* cannot feed directly on caught animals; it needs the larvae from the hive to digest it and produce the carbohydrates and amino-acids required by the hornet (Chauzat and Martin 2009).

When *V. velutina* preys on honey bees, it waits in front of the beehive for workers, it then woops down, catching them (Chauzat and Martin 2009). The bee-carpet and heat-balling are two defense strategies used by honey bees against *V. velutina*. In the bee-carpet honey bees form a large swarm at the hive entrance. In the heat-balling strategy, honey bees form a compact ball around the hornet and by contracting their thoracic muscles, raising the core temperature of the ball to around 45°C, killing the hornet. These strategies are found in *Apis cerana* and *A. mellifera*, but *A. mellifera* is less efficient in protecting itself against the hornet (Chauzat and Martin 2009; Monceau, Bonnard, and Thiéry 2014).

### b. Mating system

In eusocial Hymenopterans monoandry is the most common mating system, but *V. velutina* is polyandrous (Monceau, Bonnard, and Thiéry 2014). *V. velutina* gynes mate with multiple males, this mating system has the advantage of increasing genetic diversity which is beneficial for dispersing into new environments (Keller and Reeve 1994).

Mating usually occurs in autumn, outside the nest or on plants like the ivy (Monceau, Bonnard, and Thiéry 2014). The mating frequency of *V. velutina* is told to be improved by sexual pheromones, produced by the queen's venom, mandibular or sternal glands to attract the male to the chosen place (Monceau, Bonnard, and Thiéry 2014). After mating, *V. velutina* starts its hibernation commonly in woodpiles, shelters and burrows (López, González, and Goldarazena 2011). It has been observed that overwintering could lead to a high mortality rate in queens exceeding 99% (Chauzat and Martin 2009; Kishi and Goka 2017). In spring, after hibernating, *V. velutina* find its embryo nest where it will lay fertilized (diploid:females) and unfertilized (haploid:males) eggs, this will be the annual colony founded by one single mated queen (Rome et al. 2015).

### c. Nesting behavior

*V. velutina* takes into account several factors when selecting a nesting location, including temperature, humidity, light intensity, shelter availability and the presence of a nearby water source, all of which are crucial for the species' survival (Monceau, Bonnard, and Thiéry 2014). The nests presented in Figure 2 are built from vegetal fibers, usually from dead wood mixed with water and saliva (Martin 1995). The colony defend its nest very aggressively being described as a "fearsome" hornet (de la Hera, Alonso, and Alonso 2023).

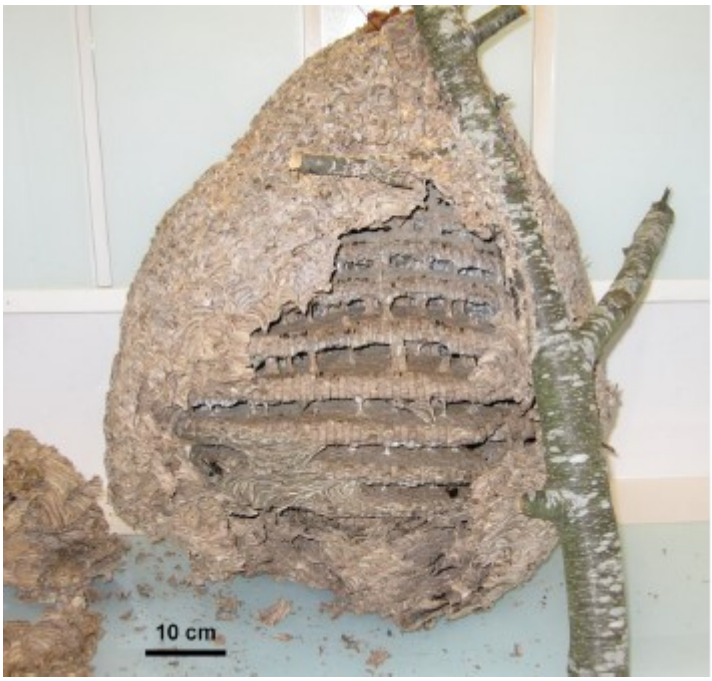


**Figure 2** : *V. velutina*'s nest (Monceau, Bonnard, and Thiéry 2014)

## 2. Course of the invasion

### a. Introduction to Europe

*Vespa* species are found worldwide but most of them are native to Asia. Among the 22 species of *Vespa,* 20 are found in the regions of Asia and Oceania (Arca et al. 2015; Choi, Martin, and Lee 2012). The exceptions are the European species *Vespa crabro* and the sub-Mediterranean species *Vespa orientalis* (Monceau, Bonnard, and Thiéry 2014). Worldwide trade and human mobility were responsible for the introduction of *Vespa* species into new places. This occurred with *V. simillima*, *V. orientalis* and also *V. velutina* (Villemant et al. 2011; Arca et al. 2015).

*Vespa velutina* is naturally found in Southeast Asia, spanning from northern India to the Indochinese peninsula. It is also found in the mountainous regions of Sumatra and Sulawesi, which have a temperate and cold climate, respectively (Vidal 2022; Ibañez-Justicia and Loomans 2011; Choi and Ohseok 2015). The Asian hornet was observed for the first time outside its native range in 2003 in Korea. According to the SRPV (Société Régionale de la Protection des Végétaux), its introduction in Europe through France occurred before 2004 (Villemant et al. 2011; Vidal 2022). However, in

France the first *V. velutina's* nest was discovered in 2005 in the city of Lot-et-Garonne (Villemant 2006). It is suggested that its arrival in Europe has been made by the importation of pottery from China, where the founder queen was fertilized by 3.6 males and was most likely hibernating during the winter (Vidal 2022; Villemant 2006; Arca et al. 2015).

Despite the genetic bottleneck during its introduction to Europe *V. velutina* has exhibited rapid expansion. Since 2004, it has rapidly spread throughout the Southwest of France and by 2009, it has already established itself in 32 French departments (Ibañez-Justicia and Loomans 2011; Villemant 2006; Arca et al. 2015). The invaded European area expanded to 190,000 km² in 2010 and further increased to 360,000 km² by 2012, with a spreading rate of 60 km/year which is much higher than Korea's rate of 10-20 km/ year (Vidal 2022; Arca et al. 2015; Choi, Martin, and Lee 2012). *V. velutina* rapidly extended its range to other countries, including Spain (2010), Portugal (2011), Belgium (2011), Germany (2014), England (2016), Switzerland and Netherlands (2017) (Figure 3). The difference in spreading rates between Korea and Europe can be explained by the absence of competition between *Vespa* species. In Korea *V. velutina* coexists with six other *Vespa* species, whereas in Europe, it competes with only one species (*Vespa crabro*). Furthermore, one of the hornets that exists in Korea is *Vespa mandarinia* which is the biggest predator of wasps and bees, contributing to a balanced ecosystem (Chauzat and Martin 2009; Villemant et al. 2011).

Studies on climate suitability show that all European countries could be colonized with a lower risk of invasion in the driest region, and a higher risk along the Atlantic (Portugal and Spain), Mediterranean coasts and the southern coast of the Black and Caspian Seas (Barbet-Massin et al. 2013).

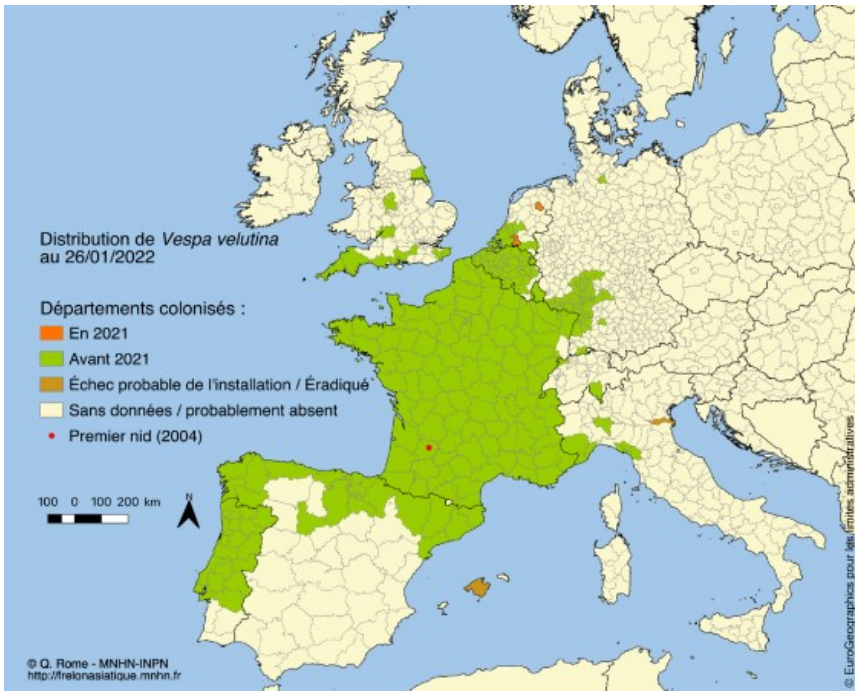


**Figure 3 :** Map showing the distribution of *V.velutina* in Europe in 2022 (INPN, 2022)

Several other factors, including the pre-adaptation to temperate climates (Choi, Martin, and Lee 2012) that exist in different regions of Europe, have been proposed to explain the remarkable capacity of *V.velutina* to spread throughout Europe. Other factors including its eusocial behavior and the polyandrous nature contribute to the diversity and the plasticity of responses, enabling its survival and adaptation in a new environment (Arca et al. 2015; Beggs et al. 2011).

### b. Introduction to the Iberian Peninsula

*V. velutina* was first reported in the Iberian Peninsula in 2010, initially in Navarre (Spain) and then in the Basque Country (Spain), the origin of these populations was France (López, González, and Goldarazena 2011; Castro and Pagola-Carte 2010). Despite the Pyrenees acting as a natural barrier to dispersal from France, the invasion occurred through the northern valleys near the border, which have around 800m of altitude. It was facilitated by a human mediated diffusion, probably through terrestrial transportation of wood (Goldarazena et al. 2015). In the following year, 2012, *V. velutina* was reported for the first time in Galicia (Spain).

Quaresma et al. (2022) investigated the demographic history of *V. velutina* in the Iberian Peninsula using genetic data. They analyzed the mitochondrial DNA and 16

microsatellites markers in 45 individuals from Spain and 190 from Portugal. The findings support the hypothesis that France served as the source population for both Spain and Portugal. This conclusion is based on the presence of a single mitochondrial haplotype, called F, in all individual from these countries. The same haplotype was found in other surveys conducted across Europe (Arca et al. 2015; Budge et al. 2017; Granato et al. 2019; Jones et al. 2020). The nuclear data further support this hypothesis, as the microsatellite alleles identified in the populations of the Iberian Peninsula are a subset of the alleles found in France, which in turn are a subset of the alleles found in Asia (Arca et al. 2015). Although France is the source population for both Portugal and Spain, the colonization patterns differ between the two countries. Spain was colonized predominantly by leading-edge expansion, whereas the Portuguese population originated from a long-distance jump. The Portuguese propagule rapidly established itself southward along the Atlantic coast and northwards into Galiza (Spain). Galiza is a place of secondary contact between the founding population in Portugal and the other is the natural expansion along the Atlantic coast in northern Iberia (Figure 4).

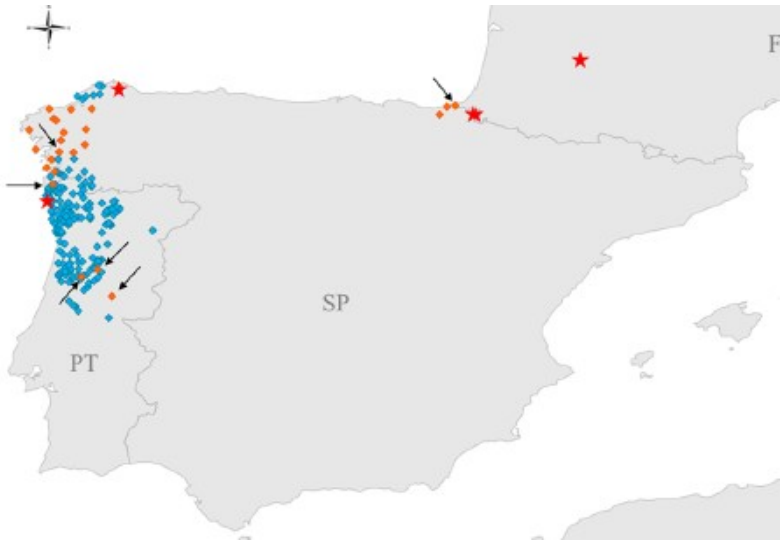


**Figure 4 :** Distribution of *Vespa velutina's* population in the Iberian Peninsula (Quaresma et al. 2022). The red stars indicate the first sightings of *V. velutina*. The arrows point to the first generation migrants. The blue and orange dots are respectively the Portuguese population and the French population

The dispersal rate of *V. velutina* in the Iberian Peninsula is estimated to be 40km/year which is comparatively lower than in other parts of Europe (Carvalho et al. 2020). This slower rate can be attributed to the severe bottleneck that *V. velutina* suffered upon arriving in Portugal. Genetic data indicate a loss of 29.6% in its allelic richness and 53.9% of its heterozygosity (Quaresma et al. 2022; Villemant et al. 2011).

## 3. Impacts and control measures of *Vespa velutina*

*V. velutina* have negative impacts on the environment, as well as on human activities. It represents a serious threat to beekeeping due to its consumption of honey bees, which make up one-third of its diet. In some invaded areas, *V. velutina* feeding behavior has resulted in the loss of between 30% and 80% of honey bee colonies (Monceau and Thiéry 2016; Laurino et al. 2019; Choi and Ohseok 2015). The activity peak of *V. velutina* coincides with the period when honey bees are preparing for hibernation, disrupting the production of overwintering workers. This predation weakens the colonies' ability to survive the winter, which has an effect leading to a decline in honey production and consequently impacting beekeepers' income. According to the European Commission (https://agriculture.ec.europa.eu/) 240 million Euros were allocated to Apiculture programs in Europe. In France alone, beekeepers have lost over 100 million Euros per year (Monceau and Thiéry 2016; Quaresma et al. 2022).

Honey bees play a crucial role as pollinators, contributing to the pollination of 200,000 plant species (Chardonnerau 2014). Therefore, honey bee predation interferes not only with agriculture and apiculture but also to the ecosystem functioning. Additionally *V. velutina* feeds on other insects disrupting the population of several pollinator species (Villemant et al. 2014; Laurino et al. 2019). *V. velutina* competes with the native species *V. crabro* which is already considered an endangered species, as they occupy a similar ecological niche and compete for the same food resources during the same period (Monceau and Thiéry 2016). The absence of predators for *V. velutina* in Europe further increases its competition (Villemant et al. 2011). If the population of the Asian hornet is not controlled, it could replace *V. crabro* leading to a disruption of the whole ecosystem (Cini et al. 2018).

*Vespa velutina* also poses negative health impacts, as its sting can be painful and in some cases, life-threatening, particularly when individuals experience allergic reactions (Villemant et al. 2011; Vidal 2022).

The complete eradication of *V. velutina* in Europe is nearly impossible; therefore methods to control the population are crucial (Chardonnerau 2014). There are numerous approaches, each with their own benefits and drawbacks. One of them is toxic or poisonous bait which combine food and insecticides; typically, they have a slow-acting effect so the hornet may go back to its nest and poison the rest of the colony. However, depending on their constitution it can pose a risk to other non-target species (Monceau and Thiéry 2016). Another approach is sticky traps or baits that can be used to capture the foundress or reproductive males causing a mating disruption (Laurino et al. 2019; Choi and Ohseok 2015), but this method could also affect other species. Besides, the use traps has not made any relevant change in controlling *V. velutina's* population (Monceau, Bonnard, and Thiéry 2014).

Nest destruction is one of the most efficient methods to reduce the population of *V. velutina*, but it is very expensive (Monceau, Bonnard, and Thiéry 2014). This approach involves the application of insecticides inside the hornet's nest, effectively killing the entire colony. However, the insecticide can have adverse effects on biodiversity. Therefore, the destroyed nest must be carefully removed to prevent harm to other species (Chardonnerau 2014; Beggs et al. 2011; Monceau, Bonnard, and Thiéry 2014). The location of the nests is often determined using the triangulation technique, which involves tracking the flight direction of the hornets. Drone-assisted nest tracking, infrared thermal imaging, and radio-telemetry nest tracking methods are still in development (Laurino et al. 2019).

Currently, biological control remains unutilized against this species; however, it holds the potential to be a viable solution. Studies must still be conducted to rate the effectiveness and evaluate the risk on the local endofauna (Monceau, Bonnard, and Thiéry 2014).

## 4. Microsatellite markers

There are several types of markers such as alloenzymes, SNPs (Single Nucleotide Polymorphisms), and microsatellites (Schlötterer 2004). Each marker type

has unique strengths and weaknesses. SNPs, for instance, are highly abundant throughout the genome. However, the information content per SNP is relatively low, limiting their individual utility (Vignal et al. 2002; Schlötterer 2004). In contrast microsatellites exhibit high levels of informativeness due to their large number of alleles and high heterozygosity and they can be easily isolated for analysis. However, microsatellites display complex mutation behavior, and cross-study comparisons require specific preparation steps (Schlötterer 2004).

Microsatellites, also known as SSR (Simple sequences repeats), STR (Short tandem repeats) or SSLP (Simple sequence length polymorphism) (Jacob et al. 1991; Fowler et al. 1988; Rassmann, Schlötterer, and Tautz 1991), were originally developed for human research but they have become a powerful tool for genetic mapping, population genetics and conservation biology (Bruford and Wayne 1993; Oliveira et al. 2006; Ellegren 2004). STRs are ubiquitous and non-randomly spread throughout the genome the sequence goes from one to six pair of bases (Bhargava and Fuentes 2010; Ellegren 2004). They can be classified based on the length of the repeat motif (Di-nucleotide-2 bp motif, Tri-nucleotide-3 bp motif, etc…) but also as perfect when the sequence is not interrupted by any base not belonging to the motif, as imperfect when a pair of bases that does not belong to the motif interrupts it, uninterrupted when a small sequence that does not match the motif is inserted and composite when there are two adjacent distinctive sequence repeats (Table 1) (Oliveira et al. 2006).

**Table 1:** DNA sequences according to the type of microsatellite, the red bases are those which do not belong to the microsatellite motif (Oliveira et al. 2006).

| Types of microsatellites | Sequence |
|---|---|
| Perfect | TATATATATATATATA |
| Imperfect | TATATATACTATATA |
| Uninterrupted | TATATACGTGTATATATATA |
| Composite | TATATATATAGTGTGTGTGT |

Microsatellite loci have been observed to exhibit higher mutation rates compared to other genomic regions. The mutation rate at microsatellite loci can range from $10^{-2}$ to $10^{-6}$ nucleotides per locus per generation (Sia et al. 2000).

These mutations occur as a result of a phenomenon known as strand slippage during DNA replication by DNA polymerase. During replication, there can be dissociation and reassociation of the DNA strands at microsatellite loci, leading to the insertion or deletion of repeats. This process can result in a new DNA strand with a decrease or increase of repeats compared to the parental sequence (Figure 5). The complex nature of microsatellites mutations continues to be an active area of research, and further studies are being conducted to unravel the underlying mechanism involved.

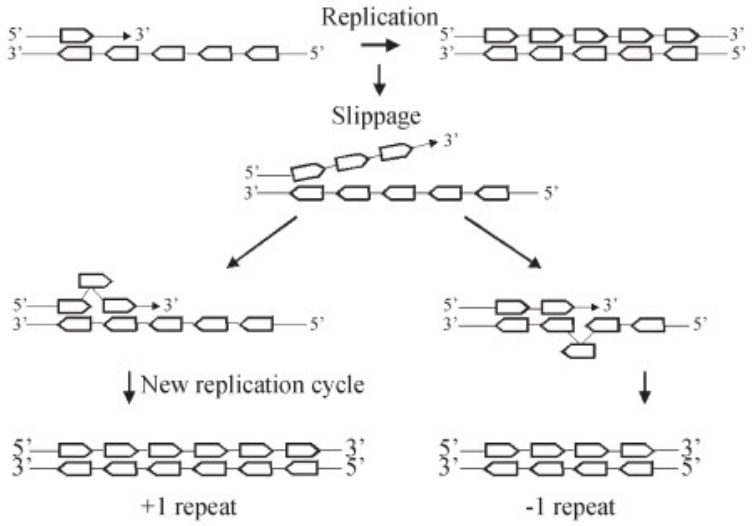


**Figure 5**: Strand slippage during DNA replication leading to a contracted or extended new strand of DNA (Oliveira et al. 2006)

Interpretation of microsatellites analysis was often based on the IAM (Infinite Allele Model) which is more useful in SNP analysis (Putman and Carbone 2014). In IAM each mutation results in a new unique allele and it is not entirely applicable to microsatellites. Instead, SMM (Stepwise Mutation Model) which describes that each mutation results in the gain or loss of a single repeat unit is more suitable to microsatellites (Putman and Carbone 2014). Furthermore, SMM model contrary to the IAM model takes into consideration homoplasy which is often present in microsatellites. Additional models such as GSMM (General Stepwise Mutation Model) or the K-allele models are also used (Kelkar et al. 2011; Di Rienzo et al. 1994).

Due to their advantages and high mutation rates, microsatellites are the best marker for analyzing invading population that have undergone a founder effect such as *V. velutina* (Quaresma 2019). Taking the advantage of microsatellite transferability across related taxa, (Bruford and Wayne 1993), seven microsatellites belong to other Vespidae and 15 microsatellites loci previously isolated from *V. velutina* were used by Arca et al. (2015) to analyze the genetic variability and assess the invasion route of the species.

# III. Material and methods

## 1. Sampling and DNA extraction

A total of 120 female *V. velutina* hornets were sampled from four different nests in the following three locations:

- Macedo de Cavalheiros – Bragança (30 samples – 1 nest)
- Mirandela – Bragança (30 samples – 1 nest)
- Vila de Punhe – Viana do Castelo (60 samples – 2 nests)

The samples were kept in ethanol and then stored at -20°C until the molecular analysis.

The DNA extraction was performed from the individuals' thorax using the Macherey-Nagel (Nucleospin®Tissue) extraction kit, following the standard protocol for animal tissue.

After each extraction, a gel electrophoresis was performed using a 1% agarose gel to verify the presence of DNA.

## 2. PCR Amplification and genotyping

The 120 individuals were genotyped using 16 microsatellites loci, which were distributed into three multiplexes with a final volume of 10 µL. We used the primers developed by Hasegawa and Takahashi (2002); Daly et al. (2002); Arca et al. (2012). The forward primers were labeled with 6-FAM, HEX, Atto 550 or Atto 565 fluorescent dyes (Table 2 and 3)

**Table 2:** Composition of the three multiplexes (Quaresma 2019)

| Composition | Quantity in µL |
|---|---|
| DNA | 1 |
| Multiplex PCR kit (Qiagen) | 5 |
| Primer mix (A,B,C) | 1 |
| Water | 3 |
| Total | 10 |

**Table 3:** Multiplex distribution

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Multiplex A | **STR locus** | R1-36 | R1-169 | R4-114 | D3-15 | LIST2015 | LIST2020B | R4-33 | R1-77 |
| | **Fragment size (bp)** | 99-119 | 148-165 | 122-152 | 157-180 | 167-198 | 183-217 | 199-225 | 241-255 |
| | **Dye** | HEX | HEX | FAM | Atto 565 | Atto 565 | FAM | HEX | Atto565 |
| Multiplex B | **STR locus** | LIST2018B | R1-137 | D2-185 | VMA-8 | | | | |
| | **Fragment size (bp)** | 113-161 | 168-202 | 208-228 | 230-271 | | | | |
| | **Dye** | Atto 550 | HEX | FAM | Atto 565 | | | | |
| Multiplex C | **STR locus** | R1-80 | R1-75 | R4-100 | VMA-6 | | | | |
| | **Fragment size (bp)** | 100-174 | 142-154 | 154-194 | 238-242 | | | | |
| | **Dye** | FAM | Atto 565 | Atto 550 | HEX | | | | |

The PCR was carried out for a total of 3 hours in T100 Thermal cycler (BioRad) using the temperature profile described in the (Table 4).

**Table 4:** Description of the PCR steps

| **Step** | **Temperature in °C** | **Time** | **Number of cycles** |
|---|---|---|---|
| **Denaturation step** | 95 | 15 min | 1 |
| **Alignment** | 95 | 30 sec | 40 |
| | 55 | 1 min | |
| | 72 | 1 min | |
| **Extension** | 60 | 60 min | 1 |

The PCR products were sent to STABVIDA Inc. (Portugal) to be genotyped on an ABI 3730xl DNA Analyzer using LIZ500 as the internal size standard.

## 3. STR analysis

Fragments' lengths were determined using GeneMapper 3.7 (Applied Biosystems). The percentage of missing data for each individual and loci was determined through manual calculation. MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) was employed to identify genotyping arising from large allelic dropout, stuttering, and/or the presence of null alleles. Allele frequencies by population and by locus, observed number of alleles (Na), effective number of alleles (Ne), observed and expected heterozygosities (Ho and He) and unbiased heterozygosity (uHe) were calculated first between the four colonies and then between the two population (Bragança and Viana do Castelo) using the Frequency option with the co-dominant data from GENALEX 6.5 (Peakall and Smouse 2012).

Allelic richness was calculated using FSTAT 2.9.4 (Goudet 2001). To infer the population structure, the model-based Bayesian clustering method STRUCTURE 2.3.4 (Pritchard, Stephens, and Donnelly 2000) and the Principal Coordinates Analysis (PCoA) were performed after excluding monomorphic loci. The PCoA was performed in GENALEX. In STRUCTURE, the membership proportion (Q-value) for each ancestral cluster (K) was estimated using the admixture ancestry and correlated allele frequency models, employing the unsupervised option The initial burn-in period comprised 250,000, followed by an additional 750,000 Markov chain Monte Carlo (MCMC) iterations. Each value of K (ranging from 1 to 5) was run 20 times. The results obtained from STRUCTURE were analyzed by STRUCTURE HARVESTER 0.9.94 (Earl and vonHoldt 2012) , this software employs Evanno's method (Evanno, Regnaut, and Goudet 2005) to determine the optimal K. The 20 runs, were aligned with CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) using the Greedy method (M=2). Graphical visualization of the final results was done using DISTRUCT 1.1 (Rosenberg et al. 2002)..

The genetic differentiation between colonies and populations was assessed using ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). The fixation index ($F_{ST}$) was calculated between each population with a significance level of $\alpha$=0.05.

Departure from Hardy-Weinberg equilibrium based on the Fischer's method with a number of independent tests set to k=4 and $\alpha$=0.05, was processed with GENEPOP

4.7 web version (Raymond and Rousset 1995; Rousset 2008) on each locus of the four colonies, using the probability test, the heterozygosity excess test and the heterozygosity deficiency test. The Markov chain parameters were set to default with a dememorization number of 1000, a number of batches of 1000 and a number of iterations per batch of 1000.

# IV. Results

## 1. Quality control

The overall rate of missing data was low. Out of 120 genotyped individuals, only three had missing data (25%). Among the 16 loci, eight had no missing data, four exhibited a 1.7% rate, and the remaining four had a 0.8% rate (Table 5).

MICRO-CHECKER identified the presence of three null-alleles in LIST2015, R1-137 and VMA-6.

**Table 5:** Missing data across loci

| Locus | %MD |
|---|---|
| R1-36 | 0 |
| R1-169 | 0 |
| R4-114 | 0 |
| D3-15 | 0 |
| LIST2015 | 0 |
| LIST2020B | 0 |
| R4-33 | 0 |
| R1-77 | 0 |
| LIST2018B | 1.7 |
| R1-137 | 1.7 |
| D2-185 | 1.7 |
| VMA-8 | 1.7 |
| R1-80 | 0.8 |
| R1-75 | 0.8 |
| R4-100 | 0.8 |
| VMA-6 | 0.8 |

## 2. Genetic analysis

Allele frequencies are presented in Table 6 with loci D3-15, LIST2015, LIST2018B, VMA-8 and R4-100 exhibiting the highest number of alleles, specifically three alleles each. The LIST2020B and R1-77 loci with only one allele were monomorphic for all populations and therefore were removed from the analysis.

**Table 6:** Allele frequencies between colonies and populations

| Locus | Allele (bp) | Vila de Punhe 1 | Vila de Punhe 2 | Viana do Castelo | Mirandela | Macedo de Cavaleiros | Bragança |
|---|---|---|---|---|---|---|---|
| **R1-36** | **98** | 0.833 | 1.000 | 0.917 | 0.767 | 0.767 | 0.767 |
| | **108** | 0.167 | 0.000 | 0.083 | 0.233 | 0.233 | 0.233 |
| | **158** | 0.500 | 0.500 | 0.500 | 0.683 | 1.000 | 0.842 |
| **R1-169** | **164** | 0.500 | 0.500 | 0.500 | 0.317 | 0.000 | 0.158 |
| **R4-114** | **129** | 0.483 | 0.000 | 0.242 | 0.500 | 0.233 | 0.367 |
| | **139** | 0.517 | 1.000 | 0.758 | 0.500 | 0.767 | 0.633 |
| **D3-15** | **161** | 0.000 | 0.000 | 0.000 | 0.000 | 0.500 | 0.250 |
| | **167** | 1.000 | 1.000 | 1.000 | 0.733 | 0.250 | 0.492 |
| | **171** | 0.000 | 0.000 | 0.000 | 0.267 | 0.250 | 0.258 |
| **LIST2015** | **179** | 0.000 | 0.000 | 0.000 | 0.250 | 0.000 | 0.125 |
| | **181** | 0.000 | 0.133 | 0.067 | 0.750 | 1.000 | 0.875 |
| | **187** | 1.000 | 0.867 | 0.933 | 0.000 | 0.000 | 0.000 |
| **LIST2020B** | **190** | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| **R4-33** | **205** | 0.683 | 0.500 | 0.592 | 0.717 | 0.717 | 0.717 |
| | **209** | 0.317 | 0.500 | 0.408 | 0.283 | 0.283 | 0.283 |
| **R1-77** | **253** | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| **LIST2018B** | **113** | 0.267 | 0.362 | 0.314 | 0.000 | 0.000 | 0.000 |
| | **121** | 0.000 | 0.345 | 0.169 | 0.759 | 0.500 | 0.627 |
| | **137** | 0.733 | 0.293 | 0.517 | 0.241 | 0.500 | 0.373 |
| **R1-137** | **183** | 0.333 | 0.000 | 0.169 | 0.000 | 0.733 | 0.373 |
| | **187** | 0.667 | 1.000 | 0.831 | 1.000 | 0.267 | 0.627 |
| **D2-185** | **208** | 0.783 | 0.362 | 0.576 | 1.000 | 0.667 | 0.831 |
| | **214** | 0.217 | 0.638 | 0.424 | 0.000 | 0.333 | 0.169 |
| **VMA-8** | **250** | 0.000 | 0.000 | 0.000 | 0.103 | 0.000 | 0.051 |
| | **252** | 0.500 | 0.276 | 0.390 | 0.621 | 0.750 | 0.686 |
| | **267** | 0.500 | 0.724 | 0.610 | 0.276 | 0.250 | 0.263 |
| **R1-80** | **109** | 0.000 | 0.000 | 0.000 | 0.500 | 0.267 | 0.383 |
| | **111** | 1.000 | 1.000 | 1.000 | 0.500 | 0.733 | 0.617 |
| **R1-75** | **153** | 0.217 | 0.500 | 0.356 | 0.000 | 0.283 | 0.142 |
| | **157** | 0.783 | 0.500 | 0.644 | 1.000 | 0.717 | 0.858 |
| **R4-100** | **178** | 0.000 | 0.000 | 0.000 | 0.100 | 0.000 | 0.050 |
| | **180** | 0.783 | 1.000 | 0.890 | 0.900 | 1.000 | 0.950 |
| | **184** | 0.217 | 0.000 | 0.110 | 0.000 | 0.000 | 0.000 |
| **VMA-6** | **243** | 0.000 | 0.000 | 0.000 | 0.800 | 0.500 | 0.650 |
| | **245** | 1.000 | 1.000 | 1.000 | 0.200 | 0.500 | 0.350 |

R1-137, D2-185 and R1-75 were monomorphic for Bragança specifically inside the colony of Mirandela. Similarly, in Macedo de Cavaleiros R1-169, LIST2015 and R4-100 were monomorphic. Additionally, LIST2015 was the sole monomorphic loci identified in Vila de Punhe 1 within the district of Viana do Castelo; also, R1-36, R4-114, R1-137 and R4-100 demonstrated monomorphism in Vila de Punhe 2. Moreover both colonies in Viana do Castelo exhibited monomorphism in the genetic markers D3-15, R1-80 and VMA-6. Number of alleles (Na) varied between 1.000 and 3.000 in the two populations (VMA-8 Mirandela; D3-15 Macedo de Cavaleiros; LIST2018B Vila de Punhe 2), effective number of alleles (Ne) went from 1.000 to 2.696 (D3-15 Bragança) more precisely 2.667 (D3-15 Macedo de Cavaleiros) and it also went from 1.000 to 2.536 (LIST2018B Vila de Punhe 2) specifically 2.977 (Vila de Punhe 2). Ar (Allelic richness) differed between 1.000 and 3.000 in Bragança and Viana do Castelo (VMA-8 Mirandela; D3-15 Macedo de Cavaleiros; LIST2018B Vila de Punhe 2), these values indicate a low genetic diversity in both populations (Table 7 and 8). Observed and Expected heterozygosity (Ho/He) were close among the two populations, with the highest value of 0.427/0.330 in Bragança (0.456/0.297 Macedo de Cavaleiros) and the lowest value being 0.363/0.258 in Viana do Castelo (0.323/0.204 Vila de Punhe 2) (Figure 6). Number of private alleles was higher in Bragança with four private alleles, three were present in Mirandela (LIST2015 allele 179; VMA-8 allele 250; R4-100 allele 178) and one private allele in Macedo de Cavaleiros (D3-15 allele 161) while only one private allele was detected in Viana do Castelo in Vila de Punhe (R4-100 allele 184). Genetic diversity was higher in Bragança where both Mirandela and Macedo de Cavaleiros had 68.75% of the loci polymorphic whereas in Viana do Castelo it was lower with Vila de Punhe (1 and 2) presenting 62.50% and 43.75% of polymorphism respectively.

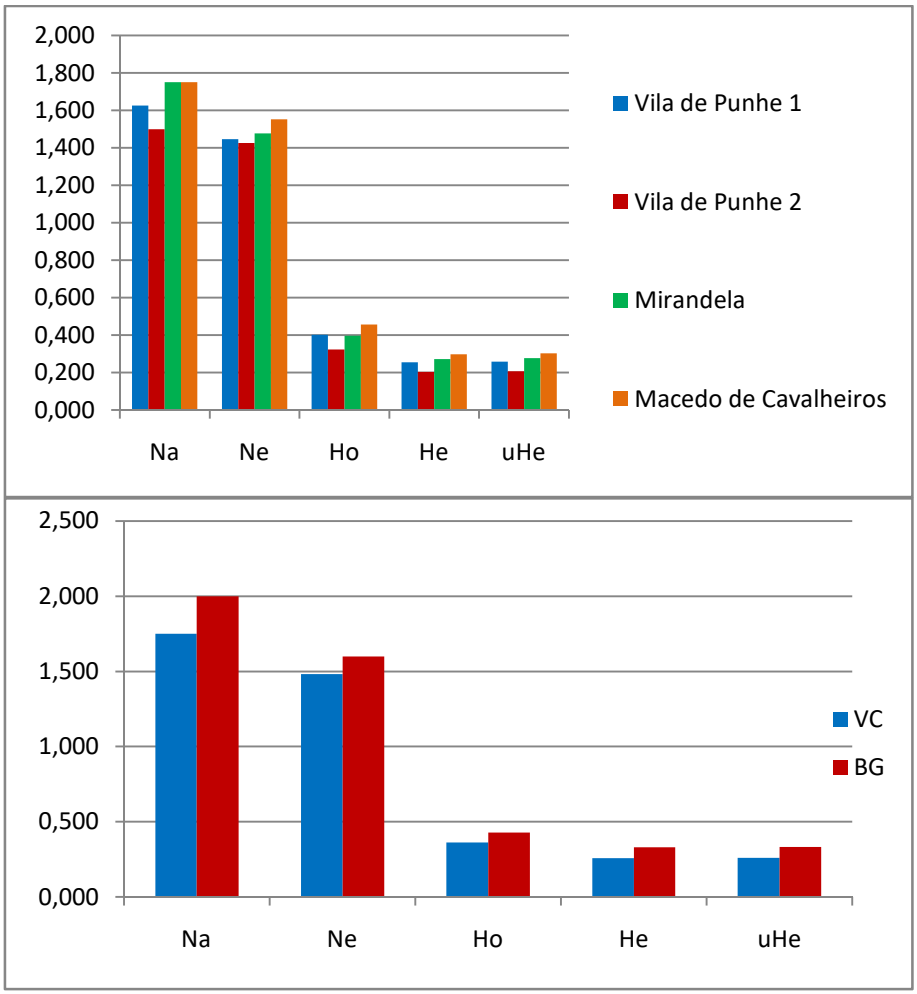


**Figure 6:** Genetic diversity by colony for Vila de Punhe (Red and Blue), Mirandela (Green) and Macedo de Cavaleiros (Orange) and by population for Viana do Castelo (Blue) and Bragança (Red)

**Table 7**: STR analysis overview according to the colonies with the total number of alleles, number of alleles (Na), effective number of alleles (Ne) and allelic richness (Ar)

(MD: Mirandela; MC: Macedo de Cavaleiros; VP1: Vila de Punhe 1; VP2 : Vila de Punhe 2)

| Locus | Na total | Na (MD) | Na (MC) | Na (VP1) | Na (VP2 ) | | Ne (MD) | Ne (MC) | Ne (VP1) | Ne (VP2) | Ar (MD) | Ar (MC) | Ar (VP1 ) | Ar (VP2) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **R1-36** | **7.000** | 2.000 | 2.000 | 2.000 | 1.000 | | 1.557 | 1.557 | 1.385 | 1.000 | 2.000 | 2.000 | 2.000 | 1.000 |
| **R1-169** | **7.000** | 2.000 | 1.000 | 2.000 | 2.000 | | 1.763 | 1.000 | 2.000 | 2.000 | 2.000 | 1.000 | 2.000 | 2.000 |
| **R4-114** | **7.000** | 2.000 | 2.000 | 2.000 | 1.000 | | 2.000 | 1.557 | 1.998 | 1.000 | 2.000 | 2.000 | 2.000 | 1.000 |
| **D3-15** | **7.000** | 2.000 | 3.000 | 1.000 | 1.000 | | 1.642 | 2.667 | 1.000 | 1.000 | 2.000 | 3.000 | 1.000 | 1.000 |
| **LIST2015** | **6.000** | 2.000 | 1.000 | 1.000 | 2.000 | | 1.600 | 1.000 | 1.000 | 1.301 | 2.000 | 1.000 | 1.000 | 2.000 |
| **LIST2020B** | **4.000** | 1.000 | 1.000 | 1.000 | 1.000 | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| **R4-33** | **8.000** | 2.000 | 2.000 | 2.000 | 2.000 | | 1.684 | 1.684 | 1.763 | 2.000 | 2.000 | 2.000 | 2.000 | 2.000 |
| **R1-77** | **4.000** | 1.000 | 1.000 | 1.000 | 1.000 | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| **LIST2018B** | **9.000** | 2.000 | 2.000 | 2.000 | 3.000 | | 1.578 | 2.000 | 1.642 | 2.977 | 2.000 | 2.000 | 2.000 | 3.000 |
| **R1-137** | **6.000** | 1.000 | 2.000 | 2.000 | 1.000 | | 1.000 | 1.642 | 1.800 | 1.000 | 1.000 | 2.000 | 2.000 | 1.000 |
| **D2-185** | **7.000** | 1.000 | 2.000 | 2.000 | 2.000 | | 1.000 | 1.800 | 1.514 | 1.859 | 1.000 | 2.000 | 2.000 | 2.000 |
| **VMA-8** | **9.000** | 3.000 | 2.000 | 2.000 | 2.000 | | 2.118 | 1.600 | 2.000 | 1.665 | 3.000 | 2.000 | 2.000 | 2.000 |
| **R1-80** | **6.000** | 2.000 | 2.000 | 1.000 | 1.000 | | 2.000 | 1.642 | 1.000 | 1.000 | 2.000 | 2.000 | 1.000 | 1.000 |
| **R1-75** | **7.000** | 1.000 | 2.000 | 2.000 | 2.000 | | 1.000 | 1.684 | 1.514 | 2.000 | 1.000 | 2.000 | 2.000 | 2.000 |
| **R4-100** | **6.000** | 2.000 | 1.000 | 2.000 | 1.000 | | 1.220 | 1.000 | 1.514 | 1.000 | 2.000 | 1.000 | 2.000 | 1.000 |
| **VMA-6** | **6.000** | 2.000 | 2.000 | 1.000 | 1.000 | | 1.471 | 2.000 | 1.000 | 1.000 | 2.000 | 2.000 | 1.000 | 1.000 |
| | **Mean Na** | 1.750 | 1.750 | 1.625 | 1.500 | **Mean Ne** | 1.477 | 1.552 | 1.446 | 1.425 | | | | |

**Table 8:** STR analysis overview according to the population with the total number of alleles, number of alleles (Na), effective number of alleles (Ne) and allelic richness (Ar)

| Locus | Na total | Na (Bragança) | Na (Viana do Castelo) | | Ne (Bragança) | Ne (Viana do Castelo) | Ar (Bragança) | Ar (Viana do Castelo) |
|---|---|---|---|---|---|---|---|---|
| **R1-36** | **4,000** | 2.000 | 2.000 | | 1.557 | 1.180 | 2.000 | 2.000 |
| **R1-169** | **4.000** | 2.000 | 2.000 | | 1.363 | 2.000 | 2.000 | 2.000 |
| **R4-114** | **4.000** | 2.000 | 2.000 | | 1.867 | 1.579 | 2.000 | 2.000 |
| **D3-15** | **4.000** | 3.000 | 1.000 | | 2.696 | 1.000 | 3.000 | 1.000 |
| **LIST2015** | **4.000** | 2.000 | 2.000 | | 1.280 | 1.142 | 2.000 | 2.000 |
| **LIST2020B** | **2.000** | 1.000 | 1.000 | | 1.000 | 1.000 | 1.000 | 1.000 |
| **R4-33** | **4.000** | 2.000 | 2.000 | | 1.684 | 1.935 | 2.000 | 2.000 |
| **R1-77** | **2.000** | 1.000 | 1.000 | | 1.000 | 1.000 | 1.000 | 1.000 |
| **LIST2018B** | **5.000** | 2.000 | 3.000 | | 1.879 | 2.536 | 2.000 | 3.000 |
| **R1-137** | **4.000** | 2.000 | 2.000 | | 1.879 | 1.392 | 2.000 | 2.000 |
| **D2-185** | **4.000** | 2.000 | 2.000 | | 1.392 | 1.955 | 2.000 | 2.000 |
| **VMA-8** | **5.000** | 3.000 | 2.000 | | 1.842 | 1.907 | 3.000 | 2.000 |
| **R1-80** | **3.000** | 2.000 | 1.000 | | 1.897 | 1.000 | 2.000 | 1.000 |
| **R1-75** | **4.000** | 2.000 | 2.000 | | 1.321 | 1.847 | 2.000 | 2.000 |
| **R4-100** | **4.000** | 2.000 | 2.000 | | 1.105 | 1.244 | 2.000 | 2.000 |
| **VMA-6** | **3.000** | 2.000 | 1.000 | | 1.835 | 1.000 | 2.000 | 1.000 |
| | **Mean Na** | 2.000 | 1.750 | **Mean Ne** | 1.600 | 1.482 | | |

## 3. Genetic structure

According to Evanno's method, the optimal K is estimated to be 2. In our analysis, colonies from Viano do Castelo comprise one distinct population (green cluster), while colonies from Bragança district form another population (pink cluster; see Figure 7-A). A similar separation into two main groups is evident in the Principal Coordinate Analysis (PCoA) plot (see Figure 7-B). Notably, colonies from Bragança exhibit some separation, whereas those from Viana do Castelo form a cohesive and tight cluster. The PCoA analysis indicates that 40.57% and 14.50% of the observed variation can be attributed to the first and second components (Axis 1 and Axis 2, respectively).

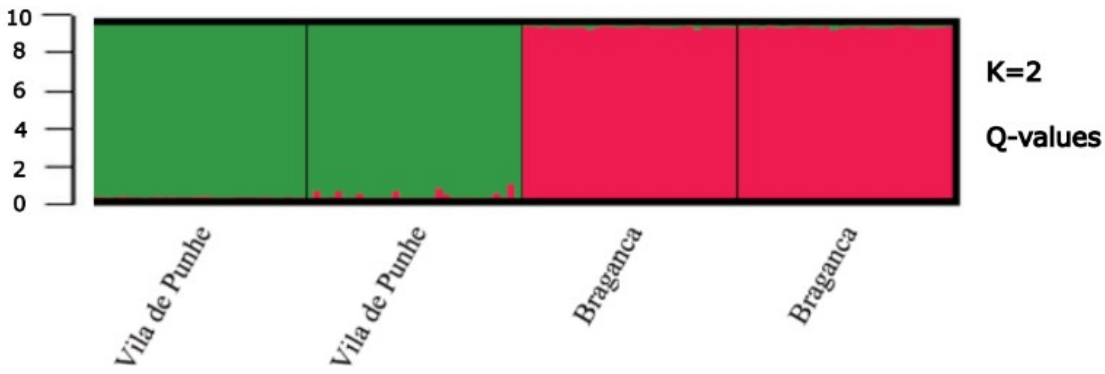


**Figure 7-A:** Graphical output from STRUCTURE ,showing the membership partionning into two clusters. Each vertical line represents an individual and vertical black lines separate individuals from populations, and the color displays the probability of belonging to a cluster

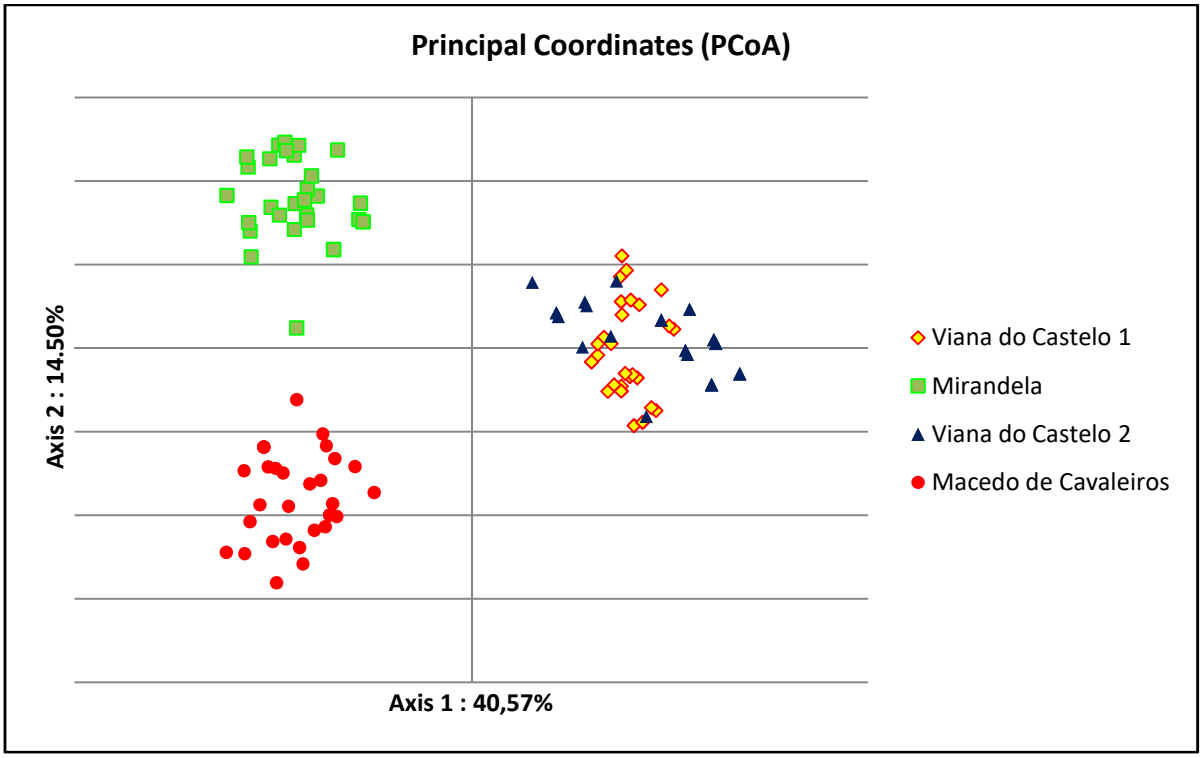


**Figure 7-B:** Principal coordinates analysis (PCoA) of *Vespa velutina*

## 4. Population differentiation

The Pairwise $F_{ST}$ method was used to compare the differentiation between each colony (Table 9). The $F_{ST}$ was lower between the populations of the same district with $F_{ST}$ values of 0.18892 and 0.22354 between the colonies of Vila de Punhe and Bragança respectively (Table 9). On the other hand, the highest level of differentiation was observed between the colony of Vila de Punhe 2 and Mirandela ($F_{ST}$ =0.42333).

**Table 9:** Pairwise Fst (Fst values below diagonal; P-values above diagonal) between the four colonies of *Vespa velutina*

| | **Vila de Punhe 1** | **Vila de Punhe 2** | **Mirandela** | **Macedo de Cavaleiros** |
|---|---|---|---|---|
| **Vila de Punhe 1** | | 0.00000 | 0.00000 | 0.00000 |
| **Vila de Punhe 2** | 0.18892 | | 0.00000 | 0.00000 |
| **Mirandela** | 0.36364 | 0.42333 | | 0.00000 |
| **Macedo de Cavaleiros** | 0.35586 | 0.41041 | 0.22354 | |

## 5. Hardy-Weinberg equilibrium

The HWE tests with heterozygosity excess and heterozygosity deficiency along with the standard errors for each test are presented in (Table 11). Following Bonferroni correction, it is observed that over the 14 loci across four populations, 27 P-values were consistent with Hardy-Weinberg equilibrium (7 in Vila de Punhe; 4 in Vila de Punhe 2; 8 in Mirandela; 8 in Macedo de Cavaleiros). All populations had loci that were out of HWE. Loci R1-36, LIST2015, R1-137, D2-185 and R4-100 exhibit no departure from HWE in all populations while; VMA-8, R4-114 and R1-169 exhibited the greatest P-values out of HWE. There were values out of HWE present in heterozygosity excess; on the contrary all loci were significant with HWE in the heterozygosity deficiency test.

**Table 8:** HWE probability test, heterozygosity excess (Ht exc) and heterozygosity deficiency (Ht def) test with Standard errors (S.E.)

| Locus | | Vila de Punhe | Vila de Punhe 2 | Mirandela | Macedo de Cavaleiros |
|---|---|---|---|---|---|
| R1-36 | HWE | 0.5629 | - | 0.2966 | 0.2941 |
| | S.E. | 0.0004 | - | 0.0007 | 0.0007 |
| | Ht exc | 0.4081 | - | 0.1380 | 0.1366 |
| | S.E | 0.0007 | - | 0.0006 | 0.0006 |
| | Ht def | 1.0000 | - | 1.0000 | 1.0000 |
| | S.E | 0.0000 | - | 0.0000 | 0.0000 |
| R1-169 | HWE | **0.0000** | **0.0000** | 0.0276 | - |
| | S.E. | 0.0000 | 0.0000 | 0.0003 | - |
| | Ht exc | **0.0000** | **0.0000** | 0.0137 | - |
| | S.E | 0.0000 | 0.0000 | 0.0002 | - |
| | Ht def | 1.0000 | 1.0000 | 1.0000 | - |
| | S.E | 0.0000 | 0.0000 | 0.0000 | - |
| R4-114 | HWE | **0.0000** | - | **0.0000** | 0.2965 |
| | S.E. | 0.0000 | - | 0.0000 | 0.0007 |
| | Ht exc | **0.0000** | - | **0.0000** | 0.1378 |
| | S.E | 0.0000 | - | 0.0000 | 0.0006 |
| | Ht def | 1.0000 | - | 1.0000 | 1.0000 |
| | S.E | 0.0000 | - | 0.0000 | 0.0000 |
| D3-15 | HWE | - | - | 0.0756 | **0.0000** |
| | S.E. | - | - | 0.0005 | 0.0000 |
| | Ht exc | - | - | 0.0639 | **0.0000** |
| | S.E | - | - | 0.0005 | 0.0000 |
| | Ht def | - | - | 1.0000 | 1.0000 |
| | S.E | - | - | 0.0000 | 0.0000 |

**Table 8:** Continued

| | | | | | |
|---|---|---|---|---|---|
| LIST2015 | HWE | - | 1.0000 | 0.1438 | - |
| | S.E. | - | 0.0000 | 0.0006 | - |
| | Ht exc | - | 0.5843 | 0.0950 | - |
| | S.E | - | 0.0007 | 0.0005 | - |
| | Ht def | - | 1.0000 | 1.0000 | - |
| | S.E | - | 0.0000 | 0.0000 | - |
| R4-33 | HWE | 0.0277 | **0.0000** | 0.0652 | 0.0653 |
| | S.E. | 0.0003 | 0.0000 | 0.0005 | 0.0005 |
| | Ht exc | 0.0137 | **0.0000** | 0.0400 | 0.0402 |
| | S.E | 0.0002 | 0.0000 | 0.0004 | 0.0004 |
| | Ht def | 1.0000 | 1.0000 | 1.0000 | 1.0000 |
| | S.E | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| LIST2018B | HWE | 0.0755 | 0.0040 | 0.1547 | **0.0000** |
| | S.E. | 0.0005 | 0.0002 | 0.0006 | 0.0000 |
| | Ht exc | 0.0640 | **0.0034** | 0.1258 | **0.0000** |
| | S.E | 0.0004 | 0.0001 | 0.0006 | 0.0000 |
| | Ht def | 1.0000 | 0.9967 | 1.0000 | 1.0000 |
| | S.E | 0.0000 | 0.0001 | 0.0000 | 0.0000 |
| R1-137 | HWE | 0.0109 | - | - | 0.0759 |
| | S.E. | 0.0002 | - | - | 0.0005 |
| | Ht exc | 0.0076 | - | - | 0.0638 |
| | S.E | 0.0002 | - | - | 0.0004 |
| | Ht def | 1.0000 | - | - | 1.0000 |
| | S.E | 0.0000 | - | - | 0.0000 |

**Table 8:** Continued

| | | | | | |
|---|---|---|---|---|---|
| D2-185 | HWE | 0.2910 | 1.0000 | - | 0.0110 |
| | S.E. | 0.0007 | 0.0000 | - | 0.0002 |
| | Ht exc | 0.1900 | 0.7480 | - | 0.0076 |
| | S.E | 0.0007 | 0.0010 | - | 0.0002 |
| | Ht def | 1.0000 | 0.5543 | - | 1.000 |
| | S.E | 0.0000 | 0.0011 | - | 0.000 |
| VMA-8 | HWE | **0.0000** | 0.0695 | **0.0000** | 0.1431 |
| | S.E. | 0.0000 | 0.0004 | 0.0000 | 0.0006 |
| | Ht exc | **0.0000** | 0.0558 | 0.9839 | 0.0949 |
| | S.E | 0.0000 | 0.0004 | 0.0004 | 0.0005 |
| | Ht def | 1.0000 | 1.0000 | 0.0256 | 1.0000 |
| | S.E | 0.0000 | 0.0000 | 0.0005 | 0.0000 |
| R1-80 | HWE | - | - | **0.0000** | 0.0752 |
| | S.E. | - | - | 0.0000 | 0.0004 |
| | Ht exc | - | - | **0.0000** | 0.0639 |
| | S.E | - | - | 0.0000 | 0.0004 |
| | Ht def | - | - | 1.0000 | 1.0000 |
| | S.E | - | - | 0.0000 | 0.0000 |
| R1-75 | HWE | 0.2911 | **0.0000** | - | 0.0656 |
| | S.E. | 0.0006 | 0.0000 | - | 0.0005 |
| | Ht exc | 0.1898 | **0.0000** | - | 0.0403 |
| | S.E | 0.0007 | 0.0000 | - | 0.0004 |
| | Ht def | 1.0000 | 1.0000 | - | 1.0000 |
| | S.E | 0.0000 | 0.0000 | - | 0.0000 |

**Table 8:** Continued

| | | | | | |
|---|---|---|---|---|---|
| R4-100 | HWE | 0.2919 | - | 1.0000 | - |
| | S.E. | 0.0007 | - | 0.0000 | - |
| | Ht exc | 0.1914 | - | 0.7605 | - |
| | S.E | 0.0007 | - | 0.0006 | - |
| | Ht def | 1.0000 | - | 1.0000 | - |
| | S.E | 0.0000 | - | 0.0000 | - |
| VMA-6 | HWE | - | - | 0.5600 | **0.0000** |
| | S.E. | - | - | 0.0006 | 0.0000 |
| | Ht exc | - | - | 0.2544 | **0.0000** |
| | S.E | - | - | 0.0006 | 0.0000 |
| | Ht def | - | - | 1.0000 | 1.0000 |
| | S.E | - | - | 0.0000 | 0.0000 |

# V. Discussion

The high mutation rate and polymorphism of microsatellites makes them excellent markers for studying recent migrations including an ongoing biological invasion (Schlötterer 2004). In the context of *V. velutina nigrithorax's* invasion, microsatellites have been successfully used (Quaresma et al. 2022; Herrera et al. 2023; Dillane et al. 2022). The arrival of this alien-species in Europe has caused great economic and ecological damages especially in the beekeeping area (Villemant et al. 2011). That is why, understanding population's migration routes and dynamics could help us in the development of strategies to prevent future invasions as well as evaluate and manage the current one (Rollins et al. 2009).

The low missing data detected here, shows a high genotyping rate success, however technical issues cannot be set aside as MICRO-CHECKER identified null alleles in three loci (LIST2015; R1-137; VMA-6). The null alleles can be generated by a mutation on the primer binding sites that could have influenced the PCR process (Arca et al. 2015).

## 1. Genetic diversity

*Vespa velutina* arrived in Europe in 2004, initially in France, and has since established a widespread and continuous presence in western countries, from Portugal to the Netherlands, including Great Britain. Its range is expanding eastwards into Italy (Bertolino et al. 2016). This expansion underscores that the efforts to curb the establishment and spread of *V.velutina* in France were insufficient and inefficient (Turchi and Derijard 2018).

In 2011, *V.v.nigrithorax* was initially reported in Portugal; specifically, in the region of Viana do Castelo. Subsequently, its presence has extended across the country, reaching as far as Bragança, marking the northeastern edge of its distributional range (Quaresma et al. 2022). Founder or range expansion events often coincide with losses of genetic diversity, and the extent of these losses can vary depending on factors such as propagule size (Nei, Maruyama, and Chakraborty 1975) or the migration of individuals from the core to edge populations (Swaegers et al. 2013). Therefore, it was expected that the colonies from Bagança would have lower genetic diversity than the colonies from Viana do Castelo. The diversity results show that both nests from both origins have low genetic diversity, as expected because they derive

from the French population (Quaresma et al. 2022). However, Bragança shows higher genetic diversity (Na=2; Ne=1.600; Ho=0.427; He=0.330; uHe=0.333) than Viana do Castelo (Na=1.750; Ne=1.482; Ho=0.363; He=0.258; uHe=0.260). The results suggest that the yellow-legged hornet population in the Bragança region may represent a result of expansion from multiples sources, introducing new alleles to the Bragança population. Additionally, the population structure analysis revealed greater differentiation between the two colonies from Bragança compared to those from Viana do Castelo, supporting this inference. A similar pattern has been described in Galiza by Quaresma et al. (2022), indicating that Galiza serves as a secondary contact between the front derived from the primary propagule introduced in France and the front derived from the secondary propagule introduced in Portugal.

## 2. Inbreeding and Hardy-Weinberg equilibrium

Hardy-Weinberg test is a crucial analysis in our study as it provides insights about the inbreeding proportion into our populations. Yet, it is essential to acknowledge that the HWE test is still a theoretical model with assumptions. HWE assumes a large population size, absence of mutations, genetic drifts or natural selection which is not possible in reality. Also, we should take into account the possible occurrence of genotyping errors (Chen, Cole, and Grond-Ginsbach 2017). While HWE is a valuable tool, it should not be viewed as an absolute measure, but be used in parallel with other structure and dynamics tools.

Both excessive and deficient heterozygosity tests were conducted, revealing that P-values in populations with excessive heterozygosity were deviated from HWE, while significant P-values in deficient heterozygosity populations were observed to be in HWE. This assumes that the population of *Vespa velutina* has a high proportion of homozygotes. This could be attributed to the small population size present in the Iberian Peninsula, potentially leading to elevated levels of inbreeding within the colonies.

# VI. Conclusion

In summary, this study contributes valuable insights into the invasion genetics of *V. velutina* in Portugal. It is crucial to acknowledge the limitation of analyzing only two colonies from each district. To gain a more comprehensive understanding of the genetic dynamics, future studies should involve a larger number of colonies. Notably, the observed low genetic diversity in the colonies from Viana do Castelo and the higher genetic diversity in those from Bragança suggest that the yellow-legged hornet population in the Bragança region may have expanded from multiple sources, introducing new alleles to the local population.

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