Assessing mitochondrial DNA variability in honey bees (Apis mellifera L.) across Europe: inference of introgression and implications for conservation

Sahar Tounakti

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Supervised by

Maria Alice da Silva Pinto

Dora Sofia Martins Henriques

Sana Yakoubi

Bragança

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Abstract

The Western honey bee (*Apis mellifera L.)* diversified into 31 subspecies in its widespread native range in Africa, Europe, and part of Asia. Europe is home to 10 of these subspecies, which are grouped into three mitochondrial lineages; the Western European (M), the Eastern European (C), and the African (A). However, due to the increasing trading of commercial strains, typically of C-lineage ancestry, the genetic integrity of several European subspecies and local populations is threatened. This study assesses the maternal diversity patterns in 225 samples originating from 13 European countries by using the highly polymorphic $tRNA^{leu}$-cox2 intergenic region. Nineteen distinct haplotypes belonging to the M, C, and A lineages were identified, revealing notable regional patterns. In Portugal, A-lineage haplotypes were exclusively identified, suggesting a unique level of conservation within the *A. m. iberiensis* subspecies. Conversely, in countries such as Finland, Estonia, and Sweden, C-lineage haplotypes predominate, indicating a potential replacement of the native M-lineage subspecies, *A. m. mellifera*. This study highlights that Ireland maintains a distinct genetic composition predominantly of *A. m. mellifera*, which together with the Iberian populations, is one of the last M-lineage preserved populations in Europe. These results emphasize the need for nuclear DNA analysis to fully assess genetic introgression and provide a comprehensive baseline for conservation efforts to protect the genetic integrity and diversity of European honey bee populations. Future research should incorporate nuclear DNA markers to complement mtDNA findings, allowing a deeper insight into the degree of introgression and providing a more complete view of the genetic composition of European honey bee populations.

Resumo

A abelha melífera (*Apis mellifera* L.) diversificou em 31 subespécies na sua vasta área de distribuição nativa em África, na Europa e no Asia. A Europa alberga 10 destas subespécies, agrupadas em três linhagens mitocondriais: a europeia ocidental (M), a europeia oriental (C) e a africana (A). No entanto, devido ao crescente comércio de abelhas comerciais, tipicamente de ascendência da linhagem C, a integridade genética de várias subespécies europeias e populações locais está ameaçada. Este estudo avalia os padrões de diversidade materna em 225 amostras provenientes de 13 países europeus, utilizando a região intergénica $tRNA^{leu}$-cox2 altamente polimórfica. Foram identificados 19 haplótipos distintos pertencentes às linhagens M, C e A, revelando padrões regionais notáveis. Em Portugal, foram identificados exclusivamente haplótipos da linhagem A, o que sugere um nível único de conservação dentro da subespécie *A. m. iberiensis*. Inversamente, em países como a Finlândia, a Estónia e a Suécia, predominam os haplótipos da linhagem C, indicando uma potencial substituição da subespécie nativa da linhagem M, *A. m. mellifera*. Este estudo sublinha que a Irlanda mantém uma composição genética distinta, predominantemente de *A. m. mellifera*, que, juntamente com as populações ibéricas, é uma das últimas populações preservadas da linhagem M na Europa. Estes resultados sublinham a necessidade de uma análise do ADN nuclear para avaliar completamente a introgressão genética e fornecer uma base de referência abrangente para os esforços de conservação destinados a proteger a integridade e a diversidade genéticas das populações europeias de abelhas melíferas. A investigação futura deve incorporar marcadores de ADN nuclear para complementar os resultados do mtDNA, permitindo uma visão mais profunda do grau de introgressão e fornecendo uma perspetiva mais completa da composição genética das populações europeias de abelha melífera.

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List of abbreviation

- ATP6: ATP synthase subunit 6
- ATP8: ATP synthase subunit 8
- BLASTn: Basic Local Alignment Search Tool for nucleotides
- COI: Cytochrome oxidase subunit I
- COII: Cytochrome oxidase subunit II
- cyt b: Cytochrome b
- D loop: Displacement loop (control region)
- DNA: Deoxyribonucleic Acid
- EU: European Union
- ESA: European Space Agency
- F_{ST}: Fixation Index; a measure of genetic differentiation among subpopulations relative to the total population
- MAXWELL®: Automated system used for DNA extraction
- mtDNA: Mitochondrial DNA
- Na: Number of Different Alleles
- NADH: Nicotinamide adenine
- ND2: NADH dehydrogenase subunit 2
- Ne: Number of Effective Alleles
- PCR: Polymerase Chain Reaction
- SNP: Single Nucleotide Polymorphism
- tRNA: Transfer RNA
- $tRNA^{leu}$: Transfer RNA specific for leucine
- uHe: Unbiased Expected Heterozygosity
- UV: Ultraviolet
- WGS: wing geometric morphometrics

1. Introduction

The genetic diversity and conservation of native honey bee (*Apis mellifera Linnaeus*, 1758) subspecies are essential for sustaining ecological balance and agricultural productivity across Europe. However, the introduction of commercial honey bee strains has altered the genetic composition shaped by millennia of adaptation to diverse European environments (Jensen et al., 2005; Pinto et al., 2014; Soland-Reckeweg et al., 2009). This genetic change potentially threatens the adaptability of local populations by homogenizing their genetic structures.

This study seeks to assess mitochondrial DNA (mtDNA) variability within European populations and evaluate the prevalence of commercial strains by examining mtDNA variation in the $tRNA^{leu}$-cox2 region, a highly polymorphic region which is valuable for identifying the maternal ancestry of colonies and tracking the impact of the introduction of commercial strains into the native populations in Europe (Garnery et al, 1998; Meixner et al, 2013; Pinto et al, 2014; Pinto et al, 2013). By sequencing this polymorphic intergenic region across a wide array of geographically diverse samples, this study will capture an accurate picture of the extant maternal structure and distribution of commercial strains across Europe.

The methodology employed herein primarily involves sequencing the $tRNA^{leu}$-cox2 mtDNA region, enabling an in-depth analysis of genetic diversity at a continental level. These findings are expected to provide a comprehensive baseline for genetic conservation efforts aimed at preserving the unique subspecific *A. mellifera* diversity in Europe while also offering insights into the potential risks associated with commercial honey bee strains.

1. Literature Review

2.1. *Apis mellifera* diversity in its native range

There are more than 20,000 bee species (Michener, 2000). Bees and the sphecoid wasps belong to the superfamily of *Apoidea*. The western honey bees (*Apis mellifera Linnaeus*, 1758) and 11 other species belong to the *genus Apis.* Among the *Apis* species, only *A. mellifera* now has a worldwide distribution. The remaining *Apis* species are restricted to Southeast Asia (Arias et al, 2005; Raffiudin et al, 2007).

Apis mellifera is native to Europe, Africa, the Middle East, and Western Asia. However, over the last 400 years past, it has been introduced to other parts of the world, including the Americas and Australia, primarily for its superior honey production and pollination capabilities (Figure1), (Kerr et al, 1956; VanEngelsdorp et al, 2010; Ruttner, 1988). *A. mellifera* plays a crucial role in agriculture and ecosystems by providing essential pollination services to various crops and wild plants (Aizen et al, 2009; Hung et al, 2018; Potts et al, 2010). Globally, the value of these pollination services exceeds 153 billion euros annually (Gallai et al, 2009). Additionally, honey bees are managed for hive products such as honey, wax, propolis, royal jelly, and Venom (VanEngelsdorp et al, 2010).

Figure 1: Map displaying the geographical distribution of the 31 *A. mellifera* subspecies. The colors represent evolutionary lineages: red for African (A), blue for Western and Northern European (M), orange for Eastern European (C), and green for Middle Eastern and Western

Asian (O). Subspecies *A. sossimai, A. m. taurica*, and *A. m. artemisia* are marked in black due to insufficient information on their evolutionary lineage (Engel, 1999)

In its widespread native range, *A. mellifera* diversified into 31 subspecies, as shown in (Table 1), (Chen et al, 2016; Meixner et al, 2011; Ruttner, 1988; Sheppard et al, 1997). Based on morphological traits, these subspecies have been grouped into four main evolutionary lineages, each native to a distinct region: western and north-eastern Europe and north-western Asia (Lineage M); central and south-eastern Europe (Lineage C); Africa (Lineage A); and the Near East and Central Asia (Lineage O) (Ruttner, 1988).

Table 1: Geographic distribution of honey bee Lineages and subspecies (Ruttner,1988)

Lineage	Region	Subspecies	Subspecies Native Range
M lineage	Western and Northern European	*Apis mellifera mellifera*	Western and northern Europe
		Apis mellifera iberiensis	Iberian Peninsula, Balearic Islands, Macaronesia islands
	China	*Apis mellifera sinisxinyan*	China
A lineage	Africa, Middle East	*Apis mellifera jemenitica*	Chad, Oman, Saudi Arabia, Somalia, Sudan, Yemen
	Africa	*Apis mellifera intermissa*	Northern Africa (Morocco to Tunisia)
		Apis mellifera sahariensis	Southern side of the Atlas range (Morocco to Algeria)
		Apis mellifera adansonii	Western Africa (Niger to Senegal, south to Democratic Republic of Congo)
		Apis mellifera lamarckii	Egyptian Nile Valley

		Apis mellifera scutellata	South Africa to Ethiopia
		Apis mellifera capensis	Cape Region of South Africa
		Apis mellifera monticola	Mountains of Eastern Africa (Kenya and Tanzania)
		Apis mellifera unicolor	Madagascar
		Apis mellifera litorea	Eastern coast of tropical Africa (Southern Kenya to Mozambique)
		Apis mellifera simensis	Mountain systems of Ethiopia
	Europe	*Apis mellifera siciliana*	Island of Sicily in the Mediterranean Sea
		Apis mellifera ruttneri	Island of Malta in the Mediterranean Sea
C lineage	Europe	*Apis mellifera ligustica*	Italian Peninsula
		Apis mellifera carnica	Austria, Slovenia, Croatia, Bosnia-Herzegovina, Albania, Serbia, Hungary, Romania
		Apis mellifera macedonica	Bulgaria, FYROM, Greece, Romania, Ukraine, Turkey
		Apis mellifera cecropia	Southern Greece, including Peloponnese and surrounding Aegean islands

O lineage	Middle East	*Apis mellifera syriaca*	Eastern shores of the Mediterranean Sea (Syria, Lebanon, Jordan, Israel)
	Middle East, Asia	*Apis mellifera meda*	Iran, Iraq, Southeastern Turkey, Northern Syria
	Asia	*Apis mellifera remipes*	Armenia
		Apis mellifera anatoliaca	Turkey
		Apis mellifera caucasia	Caucasus Mountains
		Apis mellifera cypria	Island of Cyprus
		Apis mellifera pomonella	Tien Shan Mountains (Kazakhstan to western China)
	Europe	*Apis mellifera adami*	Island of Crete in the Mediterranean Sea

2.2. *Apis mellifera* diversity in Europe

Europe, with its wide range of environments encompassing Mediterranean areas, as well as colder northern regions, is the cradle of 10 such subspecies (Meixner et al, 2013). These subspecies differ in morphology and behaviour and are adapted to different environmental conditions (Table 2). Moreover, each subspecies is critical in maintaining the ecological balance and supporting agricultural productivity through pollination services (Bretagnolle et al, 2015; Engel et al, 2023).

Among the 10 European subspecies, two belong to lineage M, four to lineage C, two to lineage O, and two to lineage A (Table 2). The native range of the M-lineage subspecies stretches from the Iberian Peninsula to southern Scandinavia and from Great Britain and Ireland to the Ural Mountains (Ruttner, 1988). In contrast, C-lineage subspecies are confined to the Apennine and Balkan peninsulas, bordered to the north by the Alps and Carpathians, and to the south by Sicily and the western Aegean islands (Ruttner, 1988). The remaining four subspecies evolved on

Mediterranean islands, namely: *A. m. adami* on Crete, *A. m. cypria* on Cyprus, *A. m. ruttneri* on Malta, and *A. m. siciliana* on Sicily (Figure 1and Table 2).

Table 2: Characterisation of European *Apis mellifera* subspecies

Subspecies	Lineage	Distribution	Colour	Behaviour	References
Apis mellifera mellifera	M	Northern and Western Europe (UK, Germany, France, Poland, Scandinavia)	Dark brown to black with very little yellow	Hardy, withstands cold climates, moderate to high aggressiveness, strong swarming tendency, good honey producers in cool climates	(Carreck, 2008)
Apis mellifera iberiensis	M	Iberian Peninsula (Spain, Portugal, Balearic Islands)	Variable, often dark with some yellow bands	Adapted to hot, dry climates, moderate to high aggressiveness, high swarming tendency, good honey production, especially from wild plants	(Canovas et al., 2008)
Apis mellifera ligustica	C	Italian Peninsula	Light brown to golden with yellow bands	Gentle, prolific brood production, high honey production, low swarming tendency, good disease resistance	(Franck et al., 2000a)
Apis mellifera carnica	C	Central-Eastern Europe (Austria, Slovenia, Croatia, Bosnia-Herzegovina, Albania, Serbia, Hungary, Romania)	Dark with brown or grey bands	Gentle, excellent overwintering ability, low swarming tendency, efficient foragers, good honey producers, rapid spring build-up	(De La Rúa et al., 2009)
Apis mellifera macedonica	C	Bulgaria, Greece, Romania,	Dark with some yellowish tones	Moderate temperament, good overwintering ability, high	(Bouga et al., 2005)

		Ukraine, Turkey		swarming tendency, efficient foragers, good honey producers	
Apis mellifera cecropia	C	Southern Greece, including the Peloponnese	Dark with some yellow bands	Adapted to Mediterranean climates, moderate aggressiveness, high swarming tendency, good honey producers, especially from early spring blooms	(Bouga et al., 2005)
Apis mellifera cypria	O	Cyprus	Light with yellow bands	Adapted to hot, dry climates, high aggressiveness, high swarming tendency, efficient foragers in arid conditions	(Kandemir et al., 2006; Bouga et al., 2005)
Apis mellifera adami	O	Unknown	Unknown	Unknown	(De La Rúa et al., 2009; Garnery, 1993)
Apis mellifera ruttneri	A	Malta	Dark with yellow bands	Adapted to hot, dry climates, high aggressiveness, high swarming tendency, efficient foragers in arid conditions	(De La Rúa et al., 2009; L. Garnery, 1993)
Apis mellifera siciliana	A	Sicily	Light with yellow bands	Adapted to Mediterranean climates, moderate aggressiveness, high swarming tendency, good honey producers	(Arias and al., 1996 ; Garnery, 1993)

However, in many parts of Europe, this remarkable diversity, which has been shaped by thousands of years of evolution, is endangered by the large-scale introduction of foreign subspecies. This is particularly the case of the C-lineage *A. m. carnica* and *A. m. ligustica*, which are prized by beekeepers due to their gentle behaviour and high productivity (Ruttner, 2013). This beekeeper-mediated gene flow can modify the gene pool of locally adapted

populations through introgressive hybridization(De La Rúa et al., 2009). This process can result in the loss of unique populations, a phenomenon that has been observed in the native range of *A. m. mellifera* (Jensen et al., 2005; Pinto et al., 2014; Soland-Reckeweg et al., 2009), *A. m. siciliana* (Muñoz et al., 2014) and *A. m. cypria* (Papachristoforou et al., 2013; Kandemir et al., 2006). In addition, recent reports suggest that *A. m. adami* is on the brink of extinction (Zampakas et al., 2024).

The growing recognition of the importance of using native subspecies as a source of genetic material for sustainable beekeeping has led to the establishment of several protected areas in northern and central Europe as well as in Sicily aimed at conserving the genetic integrity of *A. m. mellifera* and *A.m. siciliana* (Muñoz et al., 2014). These conservation efforts require tools to identify pure-bred colonies before they are moved to protected areas.

2.3. Tools to study genetic diversity

Genetic diversity is crucial for honey bees to adapt and thrive in diverse environments. Studies have demonstrated that high intra-colony diversity enhances disease resistance, productivity, survivorship, thermoregulation, and homeostasis (Jones et al., 2004; Oldroyd et al., 2007; Oldroyd et al., 2003; Tarpy, 2003). This is further supported by reciprocal translocation experiments, where local honey bees with higher genetic diversity showed longer survivorship and lower pathogen loads compared to introduced subspecies (Büchler et al., 2014; Francis et al., 2014).

In a global world, protecting local subspecies has become an increasing challenge due to the gene flow between local and imported lineages driven by the movement and breeding of commercial bees. To recover and protect native honey bees, several conservation programs have been established across multiple European countries (De la Rúa et al., 2009). Various tools have been employed to monitor honey bee populations, including wing morphometry, microsatellites, single nucleotide polymorphisms (SNPs), and the intergenic $tRNA^{leu}$-cox2 mtDNA region (reviewed by Meixner et al., 2013).

2.3.1. Wing morphometry

Ruttner (1988) applied traditional morphometrics to 42 characters to identify and classify honey bee diversity, successfully distinguishing 24 *Apis mellifera* subspecies. However, traditional morphometrics is very time-consuming, and only a subset of wing characters has proven to be informative for subspecies discrimination (Meixner et al., 2013).

One robust and reliable method for assessing wing shape variation is wing geometric morphometrics (WGM). Widely used in honey bee subspecies identification for various purposes, including conservation (Aglagane et al., 2022; Barour et al., 2016; Henriques et al., 2020; Oleksa et al., 2015; Tofilski et al., 2021; Tofilski, 2008). WGM utilizes coordinates defined by 19 landmarks located at vein junctions to capture variation in wing shape (Bookstein, 1992). The most recent advancement in WGM employs deep learning to automatically extract these 19 landmarks from the right forewing of honey bee workers. This approach is implemented by the software DeepWings©, which allows fully automated identification of honey bees (Rodrigues et al., 2022). WGM is cost-effective and easy to use, as it does not require specific instruments or specialized personnel.

On the other hand, molecular markers provide higher taxonomic resolution (Rodrigues et al., 2022), a deeper understanding of evolutionary processes, and are stable and detectable independently of growth, differentiation, and development.

2.3.2. Microsatellites

Microsatellites are short sequences of DNA, ranging from 1 to 6 bases, that are repeated between 4 to probably 100 or more times (Tautz, 1993). They are among the most popular nuclear markers used in the study of genetic variation in honey bees (Coroian et al., 2014; Cánovas et al., 2011; Franck et al., 1998; Miguel et al., 2007; Pentek-Zakar et al., 2015). Additionally, microsatellites have been the marker of choice in conservation centers to identify C-lineage ancestry in *A. m. mellifera* populations (Jensen et al., 2005; Soland-Reckeweg et al., 2009; Strange et al., 2008).

The popularity of microsatellites is due to several factors: they are biparentally inherited, co-dominant (allowing heterozygotes to be distinguished from homozygotes), and abundant throughout the genome. Furthermore, they can be easily amplified by PCR. However, the most significant drawback is the need for cross-calibration between laboratories due to inconsistencies in allele size calling, which arise from differences in gel migration (Schlötterer, 2004).

2.3.3. Single nucleotide polymorphisms

Single-nucleotide polymorphisms (SNPs) are single nucleotide variations in a DNA sequence that manifest with a minimum frequency of 1%. They are typically bi-allelic and exhibit a lower mutation rate (10-8 to 10-9) relative to microsatellites (10-4), rendering them stable markers for genetic research. Due to advancements in SNP discovery and genotyping technologies,

SNPs now provide high-resolution data and are increasingly utilized in honey bee genetic studies (Vignal et al., 2002). Advances in next-generation sequencing (NGS) platforms now enable the identification of thousands to millions of SNPs directly from sequencing data in both model and non-model organisms (Baird et al., 2008).

Like microsatellites, SNPs are biparentally inherited. However, SNPs are more abundant and widespread in the genome than microsatellites (Weinstock et al., 2006). They offer advantages such as lower genotyping errors, higher data quality, and suitability for automated analysis. Standardization allows for easy replication of experiments across different laboratories (Vignal et al., 2002).

SNPs have been extensively used to study population evolutionary events (Whitfield et al., 2006; Zayed & Whitfield, 2008; Chávez-Galarza et al., 2013; Hen et al., 2016; Cridland et al., 2017; Fuller et al., 2015; Harpur et al., 2014; Wallberg et al., 2018; Nelson et al., 2017; M. Parejo et al., 2017; Parejo et al., 2020; Wallberg et al., 2014). Additionally, they serve as valuable tools for estimating introgression levels and monitoring populations (Henriques, Browne, et al., 2018; Muñoz et al., 2017).

2.3.4. Mitochondrial DNA

Mitochondrial DNA has been a valuable marker for studying the genetic diversity and evolutionary history of honey bee populations. MtDNA is maternally inherited and provides insights into the maternal lineage of populations. It has been used to identify different subspecies and to understand the patterns of diversity in honey bee populations (Galtier et al., 2009; Eimanifar et al., 2016; Mikheyev et al., 2015; Wragg et al., 2017).

The mitochondrial genome of *A. mellifera* (Figure 2) is a circular DNA molecule comprising approximately 16,343 bp and includes 37 genes: 13 protein-coding, 22 transfer RNA (tRNA), and two ribosomal RNA (rRNA). This genome is crucial for energy production and metabolism, containing genes associated with oxidative phosphorylation such as cytochrome oxidases (cox1, cox2, cox3), cytochrome b (cytb), ATPases, and NADH dehydrogenases (Crozier, 1993; Henriques et al., 2019).

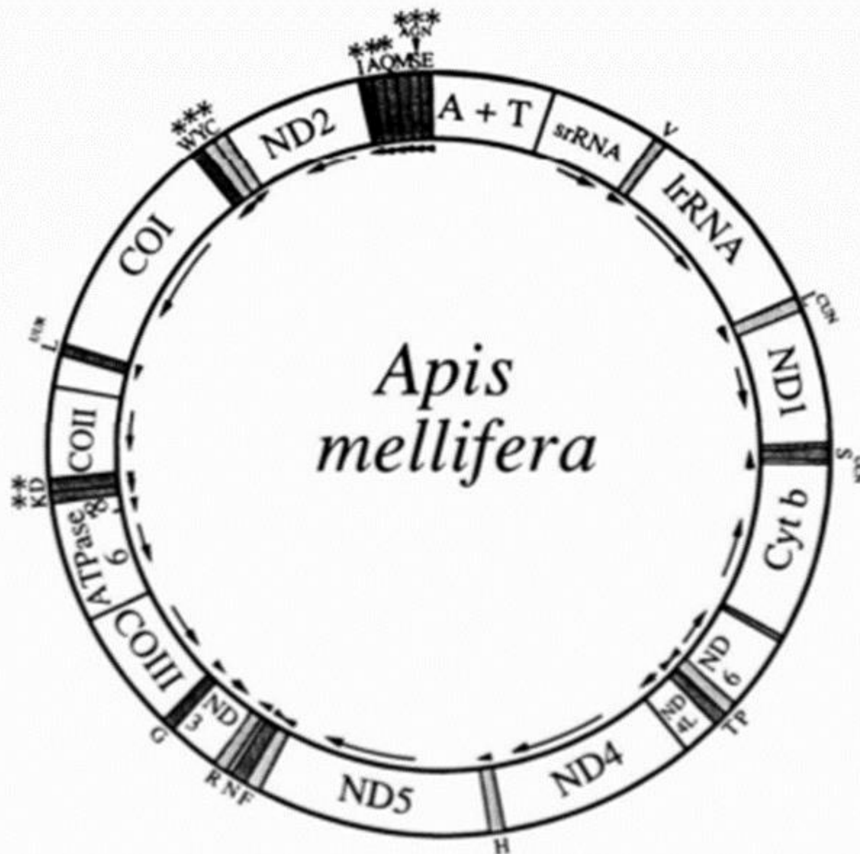

Figure 2: Map of the circular mitochondrial genome of the honey bee *A. mellifera* (Crozier, 1993)

The mtDNA has been widely used to study genetic diversity in honey bees (Evans et al., 2013; Galtier et al., 2009; Meixner et al., 2013). There are several practical reasons for its popularity. Firstly, mtDNA has a maternal inheritance, meaning that a single worker or drone from a colony can reveal the entire colony's mtDNA type (mitotype or haplotype) (Evans et al., 2013). Secondly, mtDNA does not recombine, meaning that it passes intact from the mother to its progeny. Thirdly, mtDNA has a faster mutation rate and a four times smaller effective population size than nuclear DNA (Galtier et al., 2009; Meixner et al., 2013). These features contribute to a more rapid accumulation of genetic variation in mtDNA, making it a well-suited marker for detecting recent changes in populations.

Among various regions for analysing the mtDNA in *A. mellifera*, the most popular is the tRNA^{leu}-cox2 intergenic region. This region has been used to study maternal variation of honey bees across the native and introduced ranges (Garnery et al., 1998; Meixner et al., 2013; Pinto et al., 2014; Pinto et al., 2013). Many published studies assessed variation in the tRNA^{leu}-cox2 intergenic region using the popular DraI test (Garnery et al., 1993). This is a PCR-RFLP assay

consisting of PCR amplification of the tRNA^{leu} and cox2 intergenic region, followed by digestion with the restriction enzyme DraI to cut the DNA at the specific motif TTT/AAA.

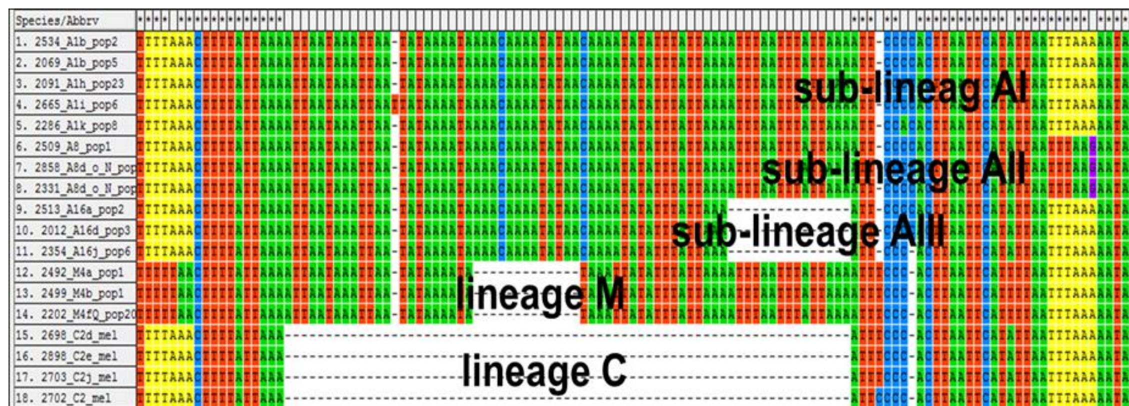

Figure 3: MtDNA partial alignment of honey bee sequences highlighting the TTT/AAA DraI motif and the different indels and point mutations characteristic of the different lineages and sub-lineages present in Europe

The length variation of this mtDNA intergenic region is related to its architecture. This region encompasses the 3' end of the tRNA^{leu} gene, the 5' end of the cox2 gene, and two intergenic elements called P and Q. The P element exhibits size variation, ranging from approximately 53 to 68 bp, and comes in three forms: P0, P, and P1. The Q element is 194-196 bp long and can be repeated in tandem one to five times. This combination of P element variation and the number of Q element repeats creates distinct length polymorphisms in the mtDNA. For instance, honey bees from the C lineage have the shortest intergenic region due to the absence of the P element and the presence of a single copy of the Q element (Garnery et al., 1993). Conversely, honey bees from lineages M and A have longer intergenic regions due to the presence of a variable P element (Figure 3) and one to five copies of the Q element (Rortais et al., 2011; Chávez-Galarza et al., 2017).

While most studies in the past used the PCR-RFLP DraI test to identifiy the maternal ancestry of the colonies, with the cost of sequencing being increasingly affordable, it is now more common to sequence the intergenic region, which allows a greater resolution of sequence variation (Collet et al., 2006; Franck et al., 2001; Pinto et al., 2014; Pinto et al., 2012; Techer et al., 2015). Herein, the honey bee DNA samples collected across Europe were identified by sequencing the intergenic region.

2. Materials and Methods

3.1. Sampling

Between 2023 and 2024, 225 honey bee colonies were sampled from 13 European countries as part of the BETTER-B project (Figure 4). The distribution of the sample size per country was as follows: 14 for Belgium, 8 for Bulgaria, 10 for Cyprus, 8 for Estonia, 10 for Finland, 21 for Greece, 38 for Hungary, 17 for Ireland, 21 for Italy, 17 for Lithuania, 21 for Poland, 24 for Portugal, and 16 for Sweden. From each colony, over five worker bees were sampled by collaborators and preserved in absolute ethanol. The samples were later shipped to the Mountain Research Centre (CIMO) at the Polytechnic Institute of Bragança, where they were stored at -20ºC in absolute ethanol until molecular analysis could be conducted.

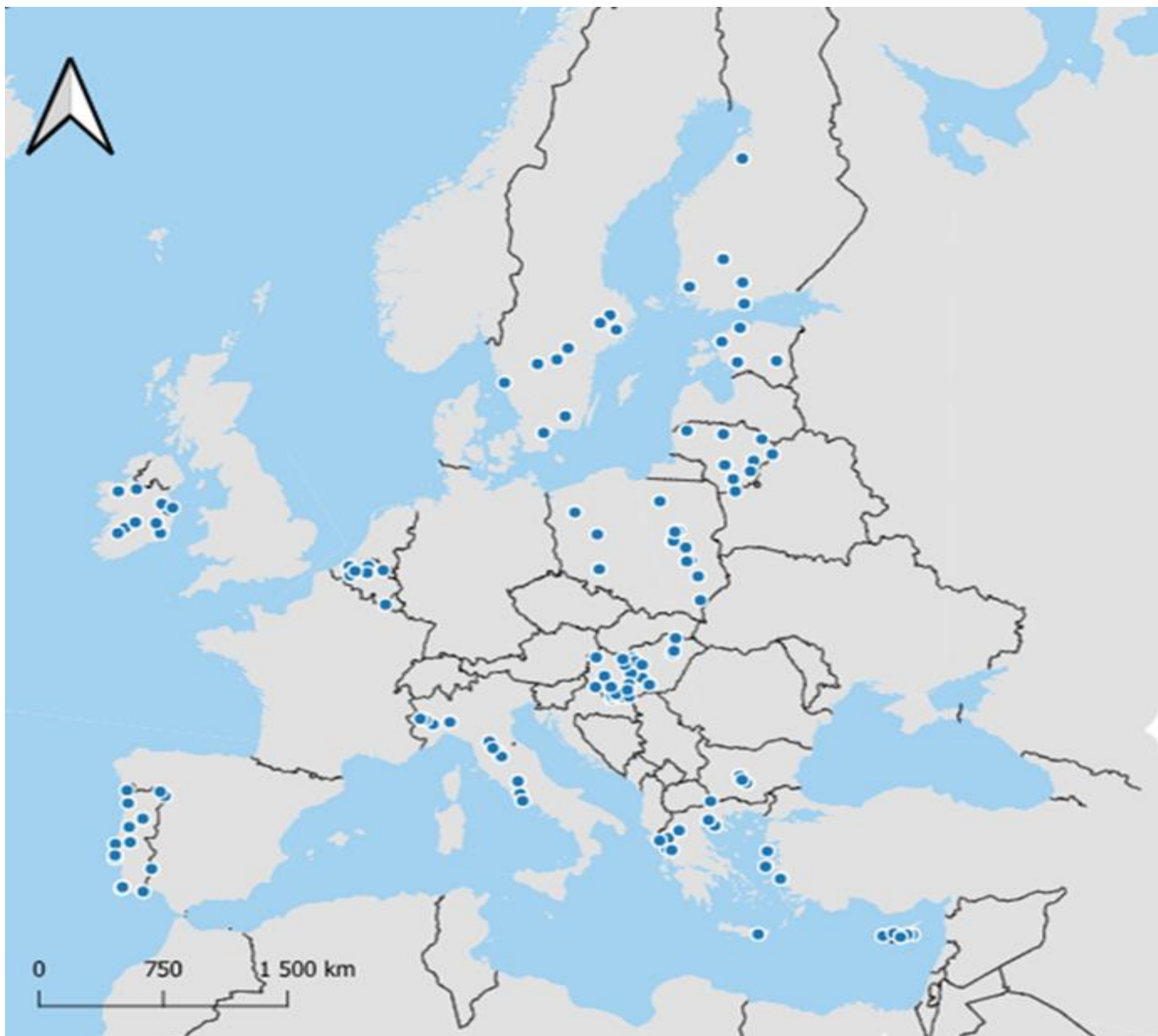

Figure 4: Geographic distribution of the 225-honey bee (*A. mellifera*) samples collected in 13 European countries

3.2. Molecular analysis

DNA extraction was performed on a single worker bee per colony. The honey bee thorax was dissected and placed in a 2.0-mL screw-cap tube containing two 3-mm zirconia beads. Tissue homogenization was achieved using a Precellys 24 tissue homogenizer (Bertin Instruments) with three cycles at 6200 rpm for 5 seconds each. Genomic DNA was extracted using the Maxwell® RSC Instrument (Promega) and the Maxwell® RSC PureFood GMO and Authentication Kit. To confirm the success of the DNA extraction, 1% agarose gel electrophoresis was conducted using a 1 kb DNA marker (New England BioLabs) (Figure 5). Gel images were captured with the Molecular Imager® Gel Doc™ XR System (Bio-Rad) and analyzed with Image Lab™ 2.0 software.

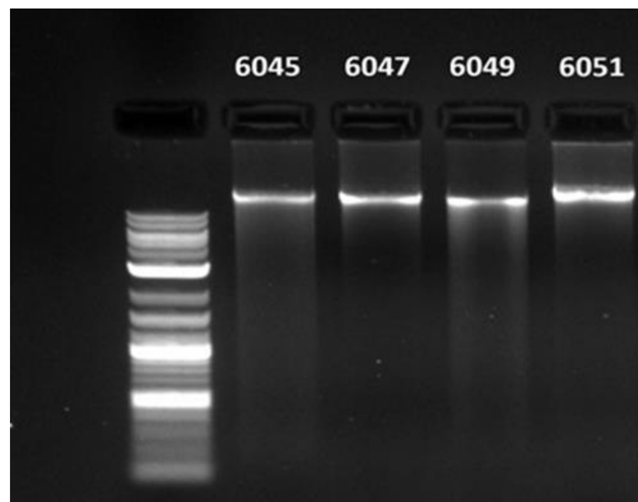

Figure 5: Agarose gel (1%) loaded with 2 µL of DNA extracts obtained for four samples from Belgium. The first lane is loaded with the 1 kb DNA marker. The gel image was captured with the Molecular Imager® Gel Doc™ XR System (Bio-Rad)

The intergenic $tRNA^{leu}$-cox2 region was then PCR-amplified using the primer pair E2 5'-GGCAGAATAAGTGCATTG-3' and H2 5'-CAATATCATTGATGACC-3' and the PCR conditions published by (Garnery et al., 1993), with minor modifications. The PCR reaction was performed in a total volume of 10 µL using SuperTaq DNA Polymerase (Bioron). The reaction mixture contained 1 µL of Buffer Complete (with $MgCl_2$), 1 µL of each primer (0.2 µM), 1 µL of dNTPs (0.2 mM), 0.06 µL of Taq DNA Polymerase, and 6 µL of nuclease-free water. The PCR program included an initial denaturation step at 94 ºC for 5 minutes, followed by 35 cycles of 45 seconds at 92 ºC, 45 seconds at 48 ºC, 2 minutes at 62 ºC, and a final extension at 65 ºC for 20 minutes. The reaction was performed using a T100™ Thermal Cycler (Bio-Rad). The sizes of the PCR products were resolved by 1% agarose gel electrophoresis using a 1 kb DNA ladder (New England BioLabs). Gel images were captured with the

Molecular Imager® Gel Doc™ XR System (Bio-Rad) and analyzed with Image Lab™ 2.0 software. The resulting PCR amplicons were sent for Sanger sequencing to Macrogen, a commercial sequencing service based in Spain.

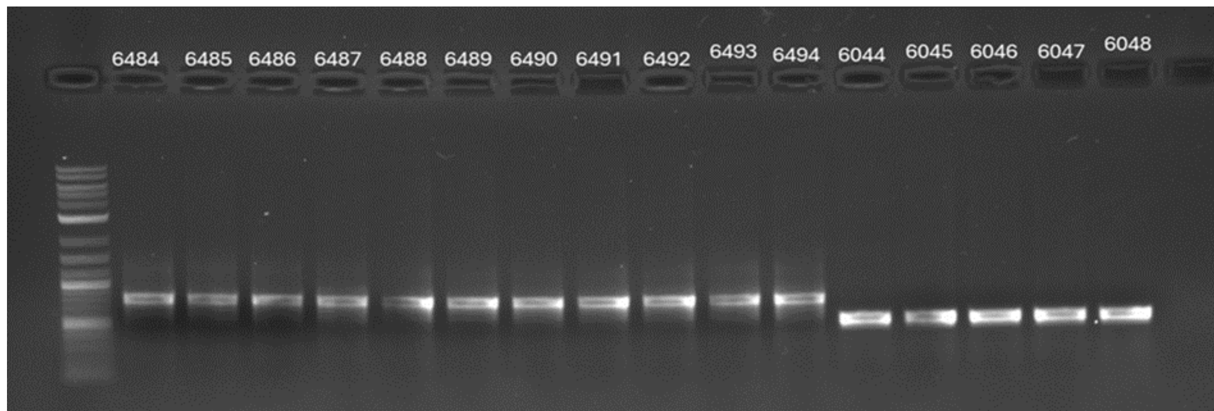

Figure 6: Agarose gel (1%) loaded with 2 µL of PCR product obtained for samples from Belgium. The size polymorphism is evident in the gel. The first lane is loaded with the 1 kb DNA marker. The gel image was captured with the Molecular Imager® Gel Doc™ XR System (Bio-Rad)

3.3. Data analysis

The chromatograms received from Macrogen were analyzed and edited using the BioEdit software (version 7.2.5; Hall et al, 1999). After verifying and editing the sequences, they were subjected to a BLASTn (Basic Local Alignment Search Tool for nucleotides) search in the National Center for Biotechnology Information (NCBI) database to identify the most likely haplotype. The sequences and their corresponding probable haplotypes were then aligned using MEGA11 (version 11.0.13; Tamura et al., 2021) to confirm the precise haplotype and evolutionary lineage.

GenalEx 6.5 (Peakall et al., 2012) was used to calculate the number of different alleles (Na), the number of effective alleles (Ne), unbiased expected heterozygosity (uHe), the number of private alleles (Pa), and the pairwise F_{ST}. A neighbour-joining (Saitou and Nei, 1987) tree was constructed with the F_{ST} values using Python's SciPy library.

3. Results and Discussion

The analysis of the $tRNA^{leu}$-cox2 mtDNA region from 13 countries (Figure 7) identified a total of 20 haplotypes (Table 3), including four from the C-lineage, six from the M-lineage, and nine from the A-lineage.

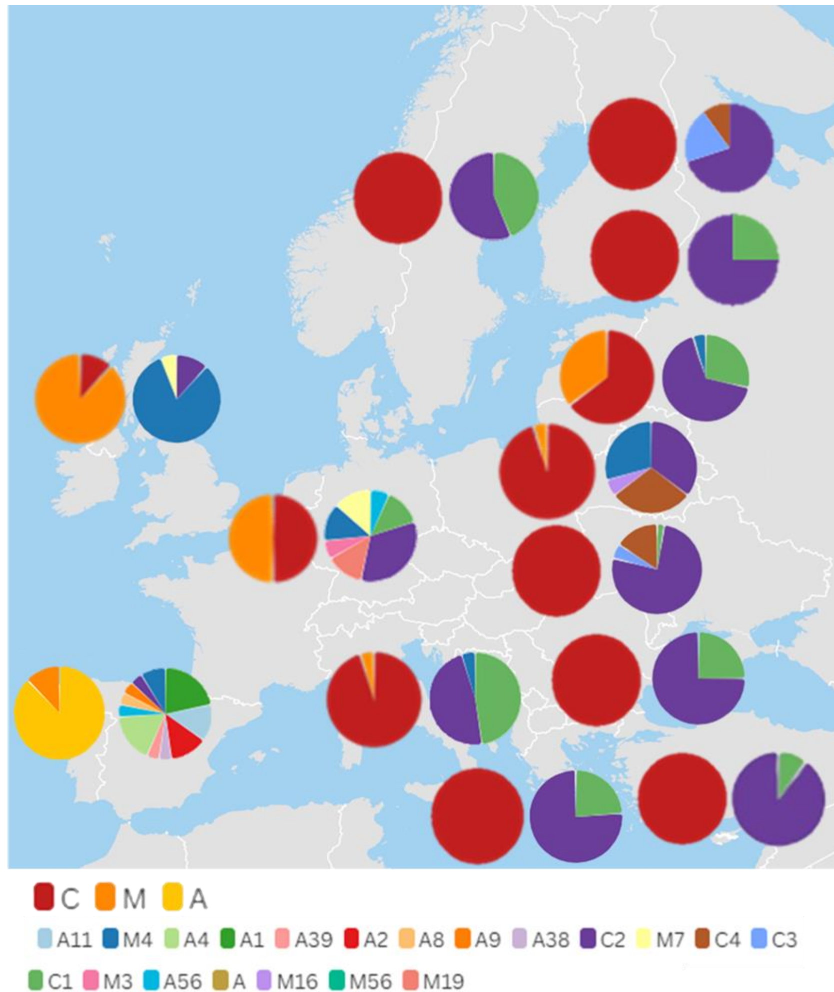

Figure 7: Map showing the pie charts with the distribution of lineages (left pie-chart) and haplotypes (right pie-chart) detected in the samples collected across 13 European countries

C-lineage haplotypes were detected in all 13 countries (Figure 7, Table 3), likely due to the global importation of honey bees from the subspecies *A. m. ligustica* and *A. m. carnica*, both of which belong to the C lineage, which native distribution is in eastern Europe (Ruttner, 1988), as shown in (Figure 1). These subspecies are popular among beekeepers worldwide because of their docile nature and supposed high productivity (Ruttner, 1988). Our results indicate that countries where *A. m. mellifera* is native (Western and North Europe; Figure 1) exhibit varying proportions of C-lineage haplotypes, with the lowest values found in Ireland (12%) and

Belgium (50%). In contrast, and contrary to expectations, all individuals sampled from Finland, Estonia, and Sweden belong to the C-lineage (Figure 7, Table 3).

These findings suggest that *A. m. mellifera*, which spans a wide range from France in the south to Scandinavia in the north, and from the British Isles in the west to the Ural Mountains in the east (Ruttner, 1988), may be the most threatened European subspecies. In Finland, Estonia, and Sweden *A. m. mellifera* appears to have been replaced (Jensen et al, 2005; Soland-Reckeweg et al, 2009), although this finding should be cautiously interpreted due to the small sample size per country. Moreover, it is crucial to study these populations using nuclear markers, as mtDNA is maternally inherited and thus only reflects the history of the queen (De La Rúa et al. 2009).

The most widespread C-lineage haplotype was C2 (53,33%; Table 3), typically found in *A. m. carnica* (Utzeri et al., 2022; Ušnik et al., 2004; Muñoz et al., 2009; Nedić et al., 2009). In Italy, where C1 haplotypes are usually predominant (Garnery et al., 2001), about half of the colonies carried C2 haplotypes. Moreover, one colony exhibited an M-lineage haplotype, the M4, which was not expected as the most common haplotype in Italy was the M7 (Franck et al., 2000).

Similar to the findings of (Browne et al., 2021) and (Hassett et al, 2018), most of the colonies from Ireland carried M-lineage haplotypes (88,23%), with M4 being the most widespread (82,36%). While this result suggests that Ireland's honey bee population probably comprises mostly *A. m. mellifera*; further analysis using nuclear markers (e.g., microsatellites or SNPs) is warranted for further confirmation at the nuclear level of the observed low maternal introgression. If confirmed, Ireland probably bears one of the best-preserved populations of *A. m. mellifera* in Europe.

Despite the observed high proportion of C-lineage haplotypes in Belgium (50%), this country exhibits the greatest number of M-lineage haplotypes among the studied populations in Western Europe, totalling five, three of which are private (Table 3). Historically dominated by *A. m. mellifera*, Belgium's populations have undergone considerable changes due to the introduction of *A. m. carnica* and *A. m. ligustica* for commercial purposes (Miguel et al., 2007; Pinto et al., 2019). While *A. m. mellifera* once thrived in Belgium, due to its cold-weather adaptations, hybridization with non-native C-derived subspecies has significantly compromised its genetic purity (Gregori et al., 2020). Conservation programs are now in place to protect *A. m. mellifera* populations in several countries (De la Rúa et al., 2009).

A-lineage haplotypes were exclusively found in Portugal, where only negligible levels of C-lineage (one colony with the C2 haplotype, 4,1%) and M-lineage (two colonies with M4

haplotypes, 8,33%) proportions were detected (Table 3). The native subspecies in Portugal is *A. m. iberiensis* (Ruttner, 1988). This subspecies exhibits a complex pattern of genetic diversity, characterized by a cline formed by M-lineage haplotypes predominating in the north-eastern half of Iberia and A-lineage haplotypes predominating in the southwestern half (Chávez-Galarza et al., 2015; Chávez-Galarza et al., 2017; Henriques et al., 2018; Pinto et al., 2012; Pinto et al., 2013). As predicted from previous surveys and consistent with the Iberian cline, A-lineage haplotypes were the most common in Portugal (87,5%), and all haplotypes identified in this study were also detected by others (Pinto et al., 2012; Pinto et al., 2013 Chávez-Galarza et al., 2017). The most common haplotype, A1, accounted for 21% of the samples and was also the most prevalent in other studies (Pinto et al., 2013; Pinto et al., 2012; Chávez-Galarza et al., 2017). These results suggest that the genetic integrity of the Iberian honey bee remains well preserved. Furthermore, Portugal exhibits the highest genetic diversity and the greatest number of private alleles (Table 3, Figure 7), supporting the assertion that Portugal is a hotspot of genetic diversity, typical of glacial refugia populations (Pinto et al., 2013).

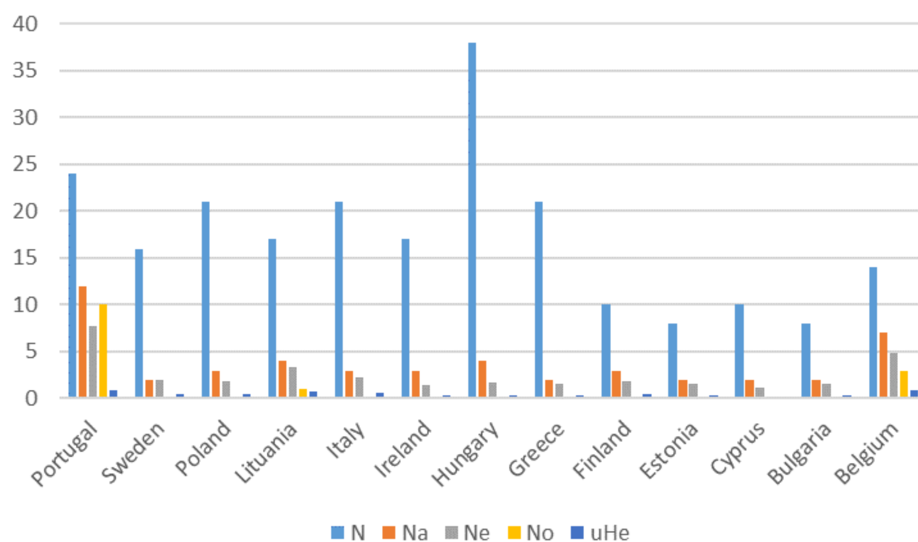

Figure 8: Comparison of diversity measures across honey bee populations. N - sample size; Na - number of different alleles; Ne - number of effective alleles; Np - number of private alleles; uHe - unbiased expected heterozygosity

In Poland, *A. m. mellifera* is native to most of the country, while *A. m. carnica* was natively found only in the southern region. Our results indicate that C-lineage haplotypes have nearly replaced M-lineage haplotypes throughout the country, with only one M-lineage haplotype identified. Highly hybridized populations have also been reported by (Oleksa et al., 2011).

Greece is notable for its rich subspecific diversity, hosting several subspecies that are adapted to local environments, including *A. m. macedonica*, *A. m. cecropia*, and *A. m. adami* (Bouga et al, 2005; Ruttner, 1988), all of which belong to the C lineage. In this country, C1 haplotypes accounted for 24% of the haplotypic diversity, while C2 haplotypes made up 76% (Table 3), indicating that this population has one of the lowest genetic diversities. This reduced diversity may be attributed to the C-lineage haplotypes having shorter intergenic sequences as they lack the P element and carry only a single copy of the Q element (Meixner et al., 2013). Bulgaria and Hungary are also home to C-lineage subspecies, *A. m. macedonica* and *A. m. carnica*, respectively (Ruttner, 1988), although C2 is the most common haplotype in both countries. On the other hand, Lithuania, a country where *A. m. mellifera* is the native subspecies, shows a balanced distribution of C2 and C4 (Figure 7, Table 3).

Table 3: Haplotype counts (left), percentage (right), and genetic diversity measures (Na, Ne, Np, uHe) for 13 European honey bee populations. N - sample size; Na - number of different alleles; Ne - number of effective alleles; Np - number of private alleles; uHe - unbiased expected heterozygosity

		Portugal	Sweden	Poland	Lituania	Italy	Ireland	Hungary	Greece	Finland	Estonia	Cyprus	Bulgaria	Belgium	Total
	C1		7/43,75%	6/28,57%		10/47,62%		1/2,63%	5/23,8%		2/25%	1/10%	2/25%	2/14,28%	36/16%
	C2	1/4,17%	9/56,25%	14/66,66%	6/35,3%	10/47,62%	2/11,76%	29/76,31%	16/76,19%	7/70%	6/75%	9/90%	6/75%	5/35,71%	120/53,33%
	C3							2/5,27%		2/20%					4/1,77%
	C4				5/29,41%			6/15,79%		1/10%					12/5,33%
C	TOTAL	1 /4,17%	16/100%	20/95,23%	11/77,71%	20/95,23%	2/11,76%	38/100%	21/100%	10/100%	8/100%	10/100%	8/100%	7/50%	172/76,44%
	M3													1/7,15%	1/0,44%
	M4	2/8,33%		1/4,77%	5/29,41%	1/4,76%	14/82,36%							2/14,28%	25/11,11%
	M7						1/5,88%							1/7,15%	2/0,88%
	M16				1/5,88%										1/0,44%
	M19													2/14,28%	2/0,88%
	M56													1/7,15%	1/0,44%
M	TOTAL	2/8,33%	0	1/4,77%	6/35,29%	1/4,76%	15/88,23%	0	0	0	0	0	0	7/50%	32/14,22%
	A	1/4,17%													1/0,44%
	A1	5/20,82%													5/2,22%
	A2	3/12,5%													3/1 ,33%
	A4	4/19,04%													4/1,77%
	A8	1/4,17%													1/0,44%
	A9	1/4,17%													1/0,44%
	A11	3/12,5%													3/1 ,33%
	A38	1/4,17%													1/0,44%
	A39	1/4,17%													1/0,44%
	A56	1/4,17%													1/0,44%
A	TOTAL	21/87,5%	0	0	0	0	0	0	0	0	0	0	0	0	21/9,33%
	N	24	16	21	17	21	17	38	21	10	8	10	8	14	225
	Na	12	2	3	4	3	3,	4	2	3	2	2	2	7	
	Ne	7,78	1,97	1,89	3,32	2,19	1,44	1,64	1,57	1,85	1,60	1,22	1,60	4,90	
	No	10	0	0	1	0	0	0	0	0	0	0	0	3	
	uHe	0,89	0,51	0,48	0,72	0,56	0,31	0,39	0,37	0,48	0,40	0,19	0,40	0,83	

Table 4 shows the pairwise F_{ST} values between all studied populations. The most differentiated population is Ireland ($F_{ST} \geq 0.4$), which is the country with the lowest proportion of C-lineage haplotype (after Portugal) and the highest proportion of M-lineage haplotypes. The genetically closest populations are those of Bulgaria and Greece (F_{ST} = 0,000) and Greece and Estonia (F_{ST} = 0,000).

Table 4 : Pairwise F_{ST} values indicating genetic differentiation between honey bee populations from 13 European countries.

	Portugal	Sweden	Poland	Lituania	Italy	Ireland	Hungary	Greece	Finland	Estonia	Cyprus	Bulgaria	Belgium
Portugal	0,000												
Sweden	0,178	0,000											
Poland	0,181	0,018	0,000										
Lituania	0,101	0,147	0,124	0,000									
Italy	0,159	0,005	0,034	0,136	0,000								
Ireland	0,223	0,402	0,389	0,176	0,361	0,000							
Hungary	0,211	0,119	0,058	0,114	0,144	0,448	0,000						
Greece	0,221	0,044	0,008	0,159	0,072	0,464	0,046	0,000					
Finland	0,186	0,120	0,068	0,111	0,141	0,412	0,017	0,063	0,000				
Estonia	0,217	0,039	0,006	0,156	0,065	0,457	0,049	0,000	0,064	0,000			
Cyprus	0,293	0,145	0,065	0,216	0,183	0,574	0,044	0,034	0,072	0,039	0,000		
Bulgaria	0,217	0,039	0,006	0,156	0,065	0,457	0,049	0,000	0,064	0,000	0,039	0,000	
Belgium	0,077	0,067	0,060	0,053	0,060	0,206	0,100	0,090	0,089	0,087	0,153	0,087	0,000

To facilitate the visualization of the differentiation patterns, a Neighbour-Joining tree was constructed using the F_{ST} values for the 13 European Countries. With Ireland being the most basal country, two large clusters are evident: one groups Portugal, Belgium and Lithuania, and the other groups the remaining countries, which are dominated by C-lineage haplotypes. This clustering pattern suggests that *A. m. ligustica* is the likely source of honey bee importations into Sweden whereas *A. m. carnica* is the likely source of honey bee importations into Finland. Greece and Bulgaria are closely related, which is expected given that these countries are home of *A. m. macedonica*. However, the genetic proximity between these two countries with that of Estonia was unexpected given that *A. m. macedonica* is not a commonly traded subspecies.

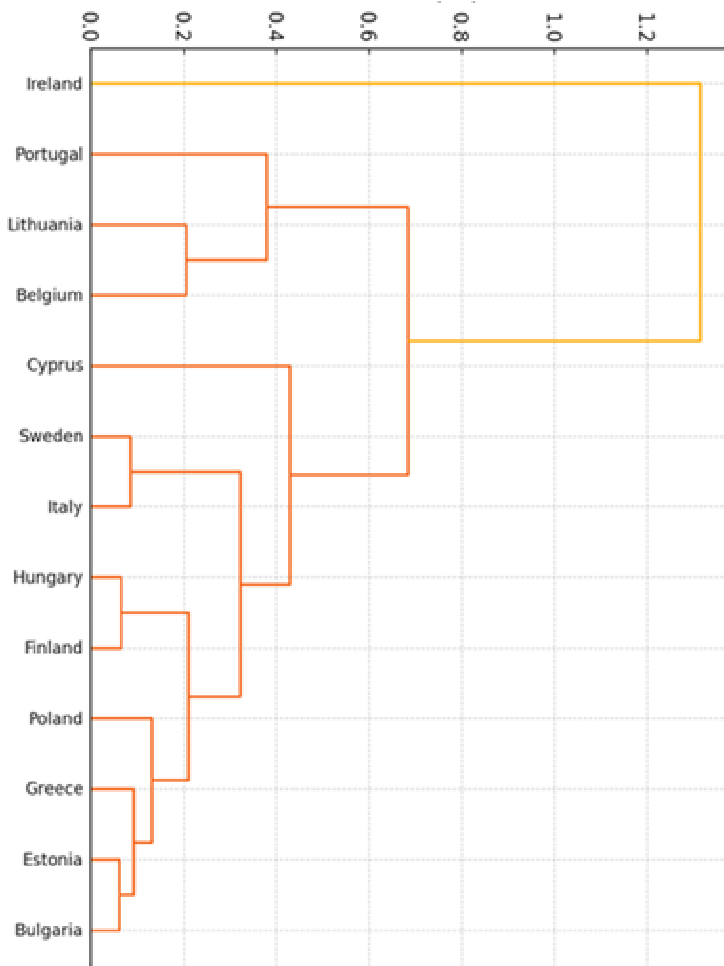

Figure 9: Neighbour-joining tree constructed using the F_{ST} values for 13 European Countries

4. Conclusions

This study provides a snapshot of mtDNA variability in Europe, revealing significant introgression from commercial strains across European honey bee populations. The persistence of unique A-lineage haplotypes in Portugal and the conservation of *A. m. mellifera* in Ireland highlight unique areas of genetic diversity in Europe. These findings emphasize the pressing need to safeguard the local population from genetic homogenization, which could compromise resilience to environmental pressures.

In alignment with the delineated objectives, this study confirms the widespread presence of commercial strains within native populations in Europe.

The inability of the $tRNA^{leu}$-cox2 region to distinguish among C-lineage populations due to their shorter intergenic sequences suggests that structural limitations within this region may influence lineage discrimination. Future research should incorporate nuclear DNA markers to complement mtDNA findings, allowing a deeper insight into the degree of introgression and providing a more complete view of the genetic composition of European honey bee populations. Conservation programs should prioritize populations with higher genetic integrity, as those in Ireland and Portugal, while policy frameworks should focus on limiting movements of commercial to protect native genetic pools

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