

## XXI EuroFoodChem Conference

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# DNA-based methods as a powerful tool for the entomological authentication of honey

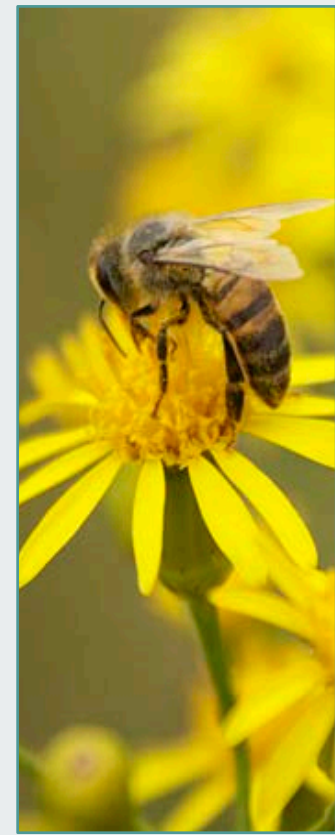
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# Honey authenticity



Natural distribution of *A. mellifera* subspecies, and corresponding evolutionary lineages, in Europe based on mtDNA.

- Honey is a food widely consumed and much appreciated worldwide
- However, it is also considered one of the foods **most prone to be adulterated** in the EU.
- Increased attention has recently been paid to honey entomological origin as it also relates to its geographical origin since honeybees carrying mitochondrial DNA (mtDNA) of distinct ancestries can be found across Europe.

# Natural distribution of *A. mellifera* subspecies



**MIEL DE CORSE - MELE DI CORSICA**

**Méthode d'obtention :**

Le miel bénéficiant de l'appellation d'origine contrôlée « Miel de Corse - Mele di Corsica » doit être exclusivement récolté et décaité en Corse.

Les miels doivent provenir de nectars et/ou miellats butinés par les abeilles d'écotype corse *Apis Mellifera Mellifera* (L.) sur les associations végétales spontanées et naturelles de la Corse. A l'exception des plantations d'agrumes, sont exclus les miels issus d'espèces cultivées.

2.9.2017		Official Journal of the European Union		C 292/7	
1. Title	"SLAVONSKI MED"				
"Slavonski med"	EU No: PDO-HR-02187 - 26.9.2016				
2. Member State or Third Country	PDO (X) PGI ( )				
	Croatia				
3. Description of the agricultural product or foodstuff					
3.1. Product type	Class 1.4. Other products of animal origin (eggs, honey, various dairy products except butter, etc.)				
3.2. Description of product to which the name in (1) applies	"Slavonski med" is honey produced by <b>native grey honeybees (<i>Apis mellifera carnica</i>, Pannonian subtype)</b> from the nectar of melliferous plants or the secretions of living parts of plants or excretions of plant-sucking insects on the				

- In the EU some honeys with protected designation of origin (PDO) specify the honeybee subspecies that should be used in their production
- In the Italian region of Emilia-Bologna is forbidden by law to rear colonies other than *A. m. ligustica* (lineage C)

**LEGGE REGIONALE 04 marzo 2019, n. 2**

**NORME PER LO SVILUPPO, L'ESERCIZIO E LA TUTELA DELL'APICOLTURA IN EMILIA-ROMAGNA. ABROGAZIONE DELLA LEGGE REGIONALE 25 AGOSTO 1988, N. 35 E DEI REGOLAMENTI REGIONALI 15 NOVEMBRE 1991, N. 29 E 5 APRILE 1995, N. 18**

**Art. 7**  
**Tutela dell'Apis mellifera sottospecie ligustica**


1. La Regione Emilia-Romagna tutela l'Apis mellifera, sottospecie ligustica, diffusa nel territorio regionale con le disposizioni di cui ai commi 2, 3 e 4, volte ad assicurare la conservazione di questa sottospecie autoctona e finalizzate al miglioramento genetico, alla successiva diffusione del materiale selezionato e a ridurre i fenomeni di erosione genetica derivanti dall'ibridazione.
2. Nel territorio della Regione Emilia-Romagna gli apicoltori non possono svolgere attività di selezione e moltiplicazione di api regine e di materiale apistico vivo di sottospecie diverse da *Apis mellifera ligustica*. Non è comunque consentito introdurre api appartenenti a sottospecie diverse da *Apis mellifera ligustica*.
3. Gli allevatori che producono e commercializzano materiale apistico vivo della sottospecie *Apis mellifera ligustica*, iscritti all'Albo nazionale degli allevatori di api italiane o ad altra Associazione di allevatori di api regine, possono richiedere l'istituzione di zone di conservazione dell'ampiezza massima di 10 km di raggio attorno ai propri apiari destinati all'allevamento, riproduzione e fecondazione del materiale selezionato. In tali zone **non è consentito allevare api diverse dalla sottospecie ligustica.**

# Main goal

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Main goal  
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 - Develop new tools to discriminate the honey produced by the native A-lineage *A. m. iberiensis* from others of different lineages

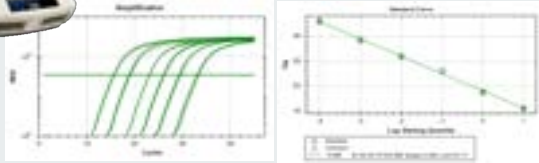


# Molecular analysis

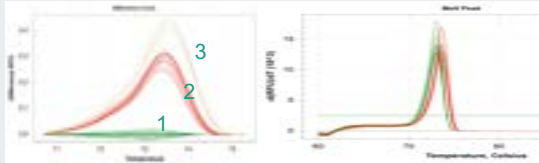
3



RT-PCR coupled with HRM analysis



Optimized conditions with an absolute limit of detection (LOD) of 0.1 pg of honeybee DNA, a reaction efficiency of 93.4% and a  $R^2$  of 0.998.



Difference curves (A) and melt peak (B) of honeybees and honey samples.

Lineage of <i>Apis mellifera</i>	Cluster	Confidence level (%)	Mealt peak °C
A	1	99.6 ± 0.3	73.00
C	2	98.7 ± 0.9	73.60
M	3	96.9 ± 1.9	73.80

2 DNA extraction

Pretreatment + Nucleospin Plant kit



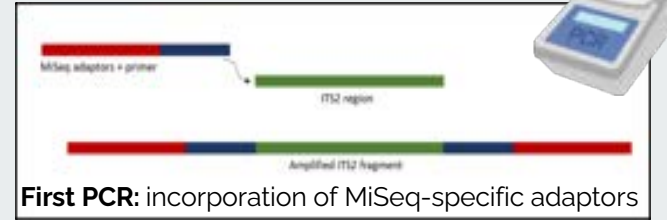
1 Primer design



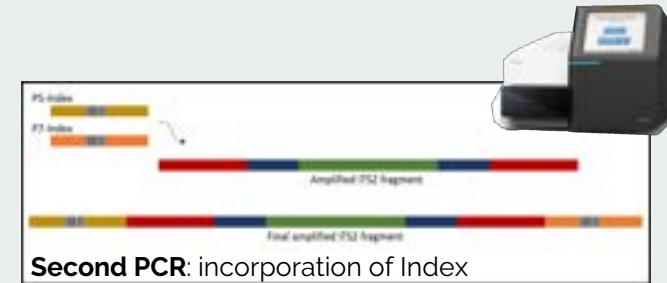
3



Next Generation Sequencing (NGS)



First PCR: incorporation of MiSeq-specific adaptors



Second PCR: incorporation of Index

Bioinformatic analysis



Honey	Next generation sequencing (%)				COI HRM
	Lineage A	Lineage M	Lineage C	Lineage C	
	<i>A. m. iberiensis</i>	<i>A. m. mellifera</i> , <i>A.m. iberiensis</i>	<i>A. m. ligustica</i>	<i>A. m. carnica</i>	
<b>Authentic samples from beekeepers</b>					
Alentejo 1, Multifloral	100	0	0	0	A
Alentejo 2, Multifloral	100	0	0	0	A
Alentejo 3, Multifloral	100	0	0	0	A
Alentejo 4, Multifloral	100	0	0	0	A
Alentejo 5, Multifloral	100	0	0	0	A
Alentejo 6, Multifloral	100	0	0	0	A
Terceira, Multifloral	100	0	0	0	A
Faial, Multifloral	32.4	0	67.6	0	n.c
Trás-os-Montes, Multifloral	100	0	0	0	A
<b>Commercial samples</b>					
Trás-os-Montes, Multifloral	21.6	78.4	0	0	n.c
Trás-os-Montes, Rosmary	47.3	52.7	0	0	n.c
Trás-os-Montes, Rosmary	46.2	53.8	0	0	n.c
Lousã, Laranjeira	100	0	0	0	A
Lousã, urze	100	0	0	0	A
Lousã, eucalipto	100	0	0	0	A
Lousã, urze DOP	100	0	0	0	A

- ✓ In HRM, honeys containing mixtures of different ancestries do not group with reference clusters (A, M or C)
- ✓ Contrary to RT-PCR/HRM analysis, NGS allowed identifying the honeybee lineages in samples containing mixtures of honeys.
- ✓ Most of the honeys from Portugal were clustered with *A. m. iberiensis* (lineage A), consistent with their geographical origin.
- ✓ Honeys from Trás-os-Montes produced near the border with Spain, presented a mixture of A and M lineages

Honey	Next generation sequencing				COI HRM
	Lineage A	Lineage M	Lineage C	Lineage C	
	<i>A. m. iberiensis</i>	<i>A. m. mellifera</i> , <i>A.m. iberiensis</i>	<i>A. m. ligustica</i>	<i>A. m. carnica</i>	
<b>Samples from beekeepers:</b>					
Bologna, Acacia	0	0	1	0	C
Bologna, Lime	0	0	1	0	C
Bologna, Multifloral	0	0	1	0	C
Bologna, Multifloral	0	0	1	0	C
<b>Commercial samples:</b>					
Italy, Multifloral	0	0	0.29	0.71	n.c
Italy, Multifloral	0	0.07	0.93	0	n.c

- ✓ The honeys provided by beekeepers from Italy were clustered with *A. m. ligustica* (lineage C), consistent with their geographical origin.

# France



Honey	Next generation sequencing				COI HRM
	Lineage A	Lineage M	Lineage C	Lineage C	
	A. m. iberiensis	A. m. mellifera, A.m. iberiensis	A. m. ligustica	A. m. carnica	
<b>Commercial samples:</b>					
France, corse	0	1	0	0	M
France, hera-maquis	0	1	0	0	M
France, maquis spring	0	1	0	0	M
France, maquis autumn	0	1	0	0	M

- ✓ The commercial samples from France were clustered with *A. m. mellifera* (lineage M), consistent with their geographical origin and PDO label.



## Final remarks:

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- ✓ Overall, RT-PCR followed by HRM analysis proved to be a simple, fast and cost-effective approach, although it does not allow for the identification of honeybee lineages in case of honey mixtures.
- ✓ In contrast, this can be achieved by NGS that also allows for high-throughput analysis despite being a more laborious approach, requiring the availability of expensive equipment.
- ✓ The evaluated PDO honeys were in good agreement with the honeybee subspecies specifications
- ✓ All other honeys were also consistent with their production origin

# Thank you for your attention

Questions?

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