THE ATLANTIC SIDE OF THE IBERIAN PENINSULA
- A HOT-SPOT OF NOVEL MATERNAL HONEY BEE DIVERSITY -

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INTRODUCTION

➢Mitochondrial DNA has been the marker of choice for assessing Iberian honey bee (*Apis mellifera iberiensis*) variation, particularly the PCR-RFLP of the intergenic tRNA^{leu}-cox2 region, also known as the *Dra*I test (Garnery et al. 1993). Over 2500 colonies (Garnery et al. 1998, Franck et al. 2001, Miguel et al. 2007, Cánovas et al. 2008), mostly sampled in the eastern half of the Iberian Peninsula, have been screened with the *Dra*I test. The data generated by this massive sampling revealed the co-existence of African (A) and western European lineages (M), forming a south-north cline, and unparalleled levels of haplotype diversity and complexity.

➢In comparison with the populations inhabiting the eastern side of the Iberian Peninsula, Atlantic honey bees have been largely undersampled. Yet, the few surveys suggest that Ibero-Atlantic populations harbor a crucial component of the Iberian honey bee diversity. Therefore, a fuller understanding of the Iberian honey bee history has demanded for further surveys of Ibero-Atlantic populations.

➢As part of an ongoing study of the Portuguese honey bee populations, we have detected 16 novel haplotypes with the *Dra*I test. Herein, these haplotypes are fully described by the RFLP approach and by sequence data. Our findings suggest that the Atlantic side of the Iberian Peninsula harbor important genetic resources, especially in face of the escalating threats the honey bee diversity.

METHODS

Samples

The maternal ancestry of over 1000 colonies collected across continental Portugal and the archipelagos of Azores and Madeira (Fig. 1) has been assessed using the *Dra*I test (Garnery et al. 1993). Among the 1000 individuals surveyed, 43 exhibited 16 novel PCR-RFLP patterns.

Sequencing and sequence analysis

➢The tRNAleu-cox2 intergenic region was sequenced and analyzed for a total of 20 individuals carrying the 16 novel haplotypes. This region contains a non-coding sequence which size depends on the forms of the P element and number of repeats of the Q element (Fig. 2). In the African lineage the P element can display two different forms: P0 (sub-lineage A_l and A_{yl}, as defined by Franck et al. 2001) and P1 (sub-lineage A_{ll}, as defined by Franck et al. 2001). The P0 differs from P1 by a 15-bp indel. The Q element can be repeated in tandem one to four times (Fig.2).

➢Following amplification, using the primers E2 and [illegible] and the [illegible] and temperature profile described elsewhere (Garnery et al. 1993), PCR products were purified and sequenced in both directions. Th[illegible]eq for base calling using SeqMan® version 7.0.0.

➢The sequences were aligned using Mega version 5.03 (Tamura et al. 2007). Phylogenetic topology was constructed using the maximum likelihood method implemented in PHYML 3.0 (Guindon et al. 2010), and the nodes were supported by 1000 bootstraps. TN93 + G was used as best-fit model of nucleotide substitution, which was obtained by the program jModeltest 0.1.1 (Posada et al. 2008). The median-joining network algorithm (Bandelt et al. 1999) was implemented in the program Network version 4.6.0.0 (Fluxus Engineering, Clare, UK).

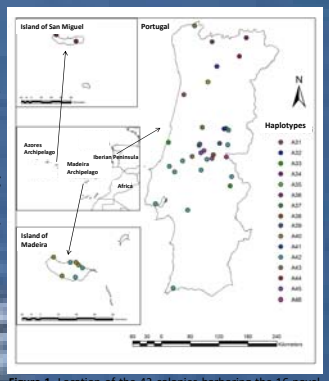

Figure 1. Location of the 43 colonies harboring the 16 novel haplotypes. Most haplotypes were detected in the centre of continental Portugal. A34 and A40 were private to San Miguel and Madeira, respectively. A42 was the only haplotype detected in both mainland and island populations.

RESULTS

➢The RFLP patterns (Fig. 2) and sequence data (Fig. 3) revealed the novelty of the 16 haplotypes.

➢The distribution of the 16 haplotypes, which were named sequentially from A31 to A46 following the standard nomenclature, is shown in Fig.1 . Haplotype A42 was the most common (16 colonies) whereas haplotypes A31, A32, A35, A37, A38, A41, A44, and A46 were singletons.

➢The 16 haplotypes are all of African maternal ancestry (Figs. 2, 3, 4). Fifteen haplotypes contained the P1 element whereas only one (A46) exhibited the P0 element (Figs. 2 and 3). Accordingly, 15 haplotypes fit the African sub-lineage A_{lll} (as defined by Franck et al. 2001) whereas only one belongs to the most common sub-lineage A_l. Fifteen haplotypes contained either two (A31, A32, A33, A34, A35, A36, A37) or three Q elements (A38, A39, A40, A41, A42, A43, A44, A46). Only haplotype A45 displayed a sequence with four Q elements (Fig. 2, 3).

➢The median-joining network (Fig. 5), based on 36 variable sites (18 indels and 18 substitutions), illustrates the relationships among the novel and previously described haplotypes of sub-lineages A_l (A3) and A_{lll} (A14, A16, A29, A30). Two distinct clusters, separated by the number of Q elements, are represented in the network.

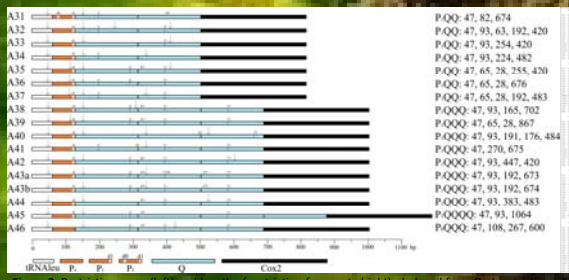

Figure 2. Restriction maps (left) and length of restriction fragments (right), deduced from *Dra*I restriction patterns and sequences of the tRNA^{leu}-cox2 intergenic region, of the 16 novel haplotypes. The *Dra*I site is in bold. *Dra*I site is marked in bold. Indels are denoted by the letters *a-n* while substitution sites by numbers 1-16. denoted with an arrow while deletions and insertions by the letters *d* and *i*, respectively. Position of the specific of the P1 element which defines sub-lineage A_{lll}, as in Franck et al. (2001).

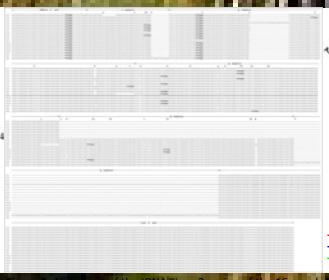

Figure 3. Alignment of the tRNA^{leu}-cox2 region of the 16 novel haplotypes. *Dra*I site is marked in bold. Indels are denoted by the letters *a-n* while substitution sites by numbers 1-16.

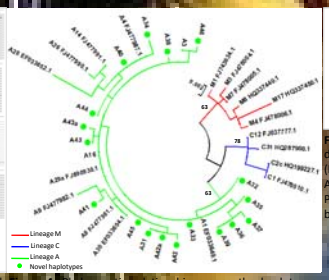

Figure 4. Phylogenetic relationship among the haplotypes representing the three lineages that have been reported for the Iberian Peninsula: A, M, and C (recent human-mediated introductions).

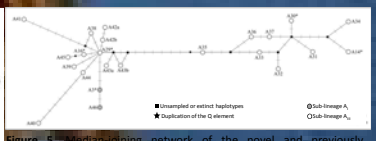

Figure 5. Median-joining network of the novel and previously described haplotypes. Sequences of A14 (FJ477991.1), A29a (FJ890930.1), and A30 (EF033654.1) were downloaded from GenBank. A3 and A16 sequences were obtained from individuals collected in Portugal. The cross lines along branches represent mutational steps between nodes.

DISCUSSION

➢These results highlight the Atlantic side of the Iberian Peninsula as an important repository of Iberian honey bee maternal diversity. The 16 novel haplotypes join the 27 previously described African haplotypes of which 17 have been found in the Iberian Peninsula (Cánovas et al. 2008), representing an increase of 59% and 94%, respectively. The 15 haplotypes of sub-lineage A_{lll} ancestry were added to the 8 previously reported (Garnery et al. 1998, Franck et al. 2001, Collet et al. 2006), representing an increase of 188% for sub-lineage A_{lll} variation. The Iberian honey bee has been by far the most intensively surveyed honey bee subspecies with the *Dra*I test (Franck et al. 1998, Miguel et al. 2007, Cánovas et al. 2008). Therefore, detection of such a remarkable number of novel haplotypes was unexpected and suggests that prior studies missed an important component of Iberian honey bee diversity.

➢This study further expands on the complexity of *A. m. iberiensis* patterns and reinforces the importance of this southernmost European territory as a reservoir of *A. mellifera* genetic diversity. There is a growing alert for protecting honey bee genetic resources across its natural range and an increasing number of conservation programs, specially across Europe, to protect *A. m. mellifera* (reviewed by De la Rúa et al. 2009). Preservation of honey bee genetic variation is a pre-requisite for long term adaptive change and avoidance of fitness decline, through inbreeding depression, and thereby a guarantee of a sustainable apiculture. The Iberian Peninsula has been a stage for evolutionary events that have shaped the evolutionary history of western European honey bee lineage. Therefore, this territory certainly deserves special attention in a large scale conservation program.

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REFERENCES CITED

Bandelt, H. J., P. Forster and A. Rohl, 1999 Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16: 37-48.
Cánovas, F., P. De La Rúa, J. Serrano and J. Galián, 2008 Geographical patterns of mitochondrial DNA variation in Apis mellifera Iberiensis (Hymenoptera : Apidae). Journal of Zoological Systematics and Evolutionary Research 46: 24-30.
Collet, T., K. M. Ferreira, M. C. Arias, A. E. E. Soares and M. A. Del Lama, 2006 Genetic structure of Africanized honeybee populations (Apis mellifera L.) from Brazil and Uruguay viewed through mitochondrial DNA COI-COII patterns. Heredity 97: 329-335.
De La Rua, P., R. Jaffe, R. Dall'Olio, I. Munoz and J. Serrano, 2009 Biodiversity, conservation and current threats to European honeybees. Apidologie 40: 263-284.
Franck, P., L. Garnery, A. Loiseau, B. P. Oldroyd, H. R. Hepburn *et al.*, 2001 Genetic diversity of the honey bee in Africa: microsatellite and mitochondrial data. Heredity 86: 420-430.
Garnery, L., P. Franck, E. Baudry, D. Vautrin, J. M. Cornuet *et al.*, 1998 Genetic diversity of the west European honey bee (Apis mellifera mellifera and A. m. Iberica) I. Mitochondrial DNA. Genetics Selection Evolution 30: 531-547.
Garnery, L., M. Solignac, G. Celebrano and J. M. Cornuet, 1993 A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. Experientia 49: 1016-1021.
Guindon, S., J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk *et al.*, 2010 New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biology 59: 307-321.
Miguel, I., M. Iriondo, L. Garnery, W. S. Sheppard and A. Estonba, 2007 Gene flow within the M evolutionary lineage of Apis mellifera: role of the Pyrenees, isolation by distance and post-glacial re-colonization routes in the western Europe. Apidologie 38: 141-155.
Posada, D., 2008 jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25: 1253-1256.
Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei *et al.*, 2011 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution doi: 10.1093/molbev/msr121.