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INDÍCE

SESSÃO 8 - VÁRIOS EN

EVOLUTIONARY HISTORY OF THE IBERIAN HONEY BEE (*APIS MELLIFERA IBERIENSIS*): A GENOME-WIDE APPROACH

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ABSTRACT
The Iberian Peninsula has been recognized as a hot spot of diversity and endemisms for numerous plant and animal species, and the honeybee is no exception. Honey bees occur naturally in Europe, Africa and the Middle East. In this vast range of habitats, adaptation to the diverse ecological conditions has led to evolution of over 29 subspecies, which have been grouped into five lineages. The Iberian Peninsula harbours two of such lineages (A and M) and the greatest genetic diversity and complexity across Europe. Unraveling the evolutionary forces underlying such complex patterns of diversity has been a major goal of numerous studies and an increasingly important undertaking given the escalating threats to the honey bee populations (e.g. diseases, parasites, pesticides, colony collapse disorder, genetic pollution). Herein we will present an ongoing research project which is using cutting edge molecular and analytical tools to disentangle the evolutionary forces shaping the Iberian honey bee diversity. The genome scan approach that will be used in this study will enable dissection of genome-wide (expansions, contractions, admixture) from genome-specific forces (selection). Furthermore, the honey bee genomic resources will enable exploration of the molecular basis of adaptation. We anticipate that this study will provide unprecedented insights into the history and adaptive divergence of honey bees and the findings can be applied for designing conservation programs to protect locally adapted ecotypes.
Keywords: *Iberian Peninsula; landscape genetics; population genomics; selection; SNPs*

INTRODUCTION
During the Pleistocene ice ages the Iberian Peninsula was one of the most important refugial areas of the European subcontinent. The privileged geographical location and isolation made this southernmost European region a place of speciation and differentiation, which is reflected by the high levels of diversity and endemisms reported for numerous plant and animal species [1, 2]. Additionally, an increasing number of phylogeographic studies of European flora and fauna suggest that the Iberian Peninsula was not only a cradle for genetic differentiation but also a species repository for the northern European latitudes after the retreat of ice sheets [3]. Accordingly, the Iberian Peninsula has been considered one of the most important sites for conservation in Europe.
As reported for many other plant and animal species, the evolutionary history of honey bees (*Apis mellifera*) that currently occupy western and northern Europe is tightly connected to the Iberian Peninsula. Honey bees survived the glacial periods in the Iberian refugium and expanded north following the ice retreat [4]. Currently, honey bees belonging to the western European lineage (lineage M) expand from the Iberian Peninsula to Scandinavia and from the British isles to Poland and Ukraine. At the same time, the proximity of the Iberian Peninsula to the African continent made it a place of contact (hybrid zone) between European and African honey bee lineages [5-7]. In summary, the Iberian Peninsula has served not only as reservoir of honey bees but also as a place of admixture between divergent evolutionary lineages. The high levels of genetic diversity exhibited by the Iberian honey bee (IHB; *A. m. iberiensis*) is a reflection of its complex history. Contemporary human-mediated processes involving movement of colonies, selective breeding, and accidental introductions of parasites, may have further

complicated patterns of variation. While the challenge of deciphering IHBs complexities has prompted numerous studies, its evolutionary history has yet to be fully resolved.

The honey bee genome has recently been sequenced [8] and a high density of single nucleotide polymorphisms (SNPs) [9] is available to conduct genome scans in multiple individuals of multiple populations (population genomics paradigm) [10] in a time and cost effective fashion. These technological advances coupled with increasingly sophisticated analytical tools will enable, for the first time, a fine-scale analysis of patterns and underlying processes (neutral and adaptive) shaping IHB variation. While the population genomics approach will provide more robust inferences on population evolutionary history and phylogeography, the prospects of identifying adaptive variation and exploring its molecular basis is of great significance.

Herein, we will describe a recently started research project, funded by Fundação para a Ciência e Tecnologia (PTDC/BIA-BEC/099640/2008), which uses a genome-wide approach to address the patterns and evolutionary processes of IHBs. To set the stage for the study, we will first provide background information on the honey bee evolutionary history. Then, we will describe the main tasks of the project, including the objectives and expected (and preliminary) results. It is our hope that the cutting edge molecular and analytical tools and approaches that are being used in this project will provide unprecedented insights into history and locally adaptive divergence among IHBs.

BACKGROUND

Honey bees occur naturally in Europe, Africa and the Middle East. In this vast range, adaptation to the diverse ecological conditions has led to evolution of over 29 subspecies. Phylogeographical studies using morphology [11], allozymes [12], mitocondrial DNA (mtDNA) [13], microsatellites [14], and more recently SNPs [9] have grouped this wide-ranging diversity into five lineages (A, M, C, O, Y). And yet, while these studies are generally concordant regarding the number and subspecies composition of lineages, the origin of the western European lineage (M), to which the Iberian honey bee belongs, is controversial.

The Iberian Peninsula harbours the greatest honey bee maternal diversity and complexity in Europe [15].The challenge of deciphering the mechanisms underlying such complexity has led to numerous surveys of IHBs using morphology [11], allozymes [16], mtDNA [5-7], and microsatellites [7]. Differential patterns of diversity, revealed by these markers, have led scientists to propose two competing hypotheses for the origin of lineage M. Early phylogeographic studies of morphology [11] and allozymes [16] revealed the existence of a gradient extending from Africa to northern Europe, with IHBs showing intermediate phenotypes. This pattern gave rise to the first hypothesis, an African origin for the honey bee and a mechanism of primary intergradation for lineage M origin. Recently, this hypothesis was resurrected, based on SNPs patterns, although only 11 IHB individuals were examined [9].

Patterns of IHB mtDNA tell a different history. The mtDNA has been the most-used marker in IHB genetic surveys [5-7, 15, 17-21]. These mtDNA studies have shown that the complex pattern of IHB variation is produced by coexisting African and European haplotypes forming a south-north cline. The co-occurrence of highly divergent maternal lineages in Iberia is more compatible with a second hypothesis, secondary intergradation. Adding to, rather than resolving the differences, patterns of microsatellite variation support neither hypothesis. Unlike the other markers, microsatellites showed virtually no differentiation among populations of the two subspecies of the lineage M, and no traces of African genes in IHBs [7].

Historically, the Iberian Peninsula has been described as a major refugium [2] during Pleistocene glaciations. Over the last 3 million years, fluctuations of the climate have promoted recurring phenomena of contraction, fragmentation, expansion, admixture, and local adaptation in many species [2], and IHBs may be no exception. In addition to natural processes, contemporary human-mediated processes involving movement of colonies within (mobile beekeeping) and between lineages (introduction of lineage C queens), selective breeding, along with accidental introductions of exotic pests and diseases, may have further complicated patterns of variation. The conflicting views of the history of the IHB are probably a reflection of such complex interacting natural and artificial processes.

Different genetic markers may capture different parts of an organism's history. While mtDNA can only reveal the maternal component, biparentally inherited markers may capture genome-wide effects (e.g. admixture, expansions, and contractions) or locus-specific effects (e.g. selection). Accordingly, we may expect that the complex demographic and evolutionary patterns of IHBs will be revealed through genome scans which sample genomic regions that are selectively neutral and genomic regions that are under selection. With the publication of the honey bee genome [8] and development of high-density SNPs [9], powerful tools are now available which enable dissection of the relative importance of neutral and adaptive forces underlying spatial structure of IHBs. While early studies did not have the power to unravel the complexities of IHBs, these recent technological advances coupled with increasingly sophisticated analytical tools provide an appropriate framework to more effectively explore complex histories.

PROJECT TASKS: DESCRIPTION, OBJECTIVES, AND RESULTS (PRELIMINARY OR EXPECTED)

Task 1 - Putting together a honey bee tissue collection

Objectives

The objective of this task is to build a spatially referenced large-scale IHB collection of samples taken across latitudinal gradients and a reference collection of other honey bee subspecies.

Methodology and approaches

In order to potentially provide increased power for detecting local signatures of selection and identifying its causes, sampling in the Iberian Peninsula was carried out across a latitudinal gradient. Building upon early studies which showed the existence of mtDNA, morphological, and MDH latitudinal gradients, IHBs were collected across three north-south transects: one extending along the Atlantic Coast, one through the interior, and another along the Mediterranean Coast. Six to eight sampling sites were selected per transect. In each location, 30 colonies, from 10 different apiaries, and approximately 50 haploid males (drones) per colony were collected in absolute ethanol and stored at -20°C. In addition to honey bee collecting, information on the beekeeping activity (e.g. total number of colonies owned by the beekeeper, practice of mobile beekeeping, purchase of queens) and GPS coordinates were taken for each apiary. In addition to the Iberian collection, a reference collection of the African subspecies *A. m. intermissa* (lineage A) from Morocco and Algeria, of the north and western European *A. m. mellifera* (lineage M) from France, Holland, Denmark, Norway, and of the beekeepers favoured *A. m. ligustica* (lineage C) from Italy is being made.

Preliminary results

The IHB sampling was carried out between May and July of 2010. We sampled over 660 colonies representing 221 apiaries and 194 beekeepers. The location of each apiary is shown in Figure 1.

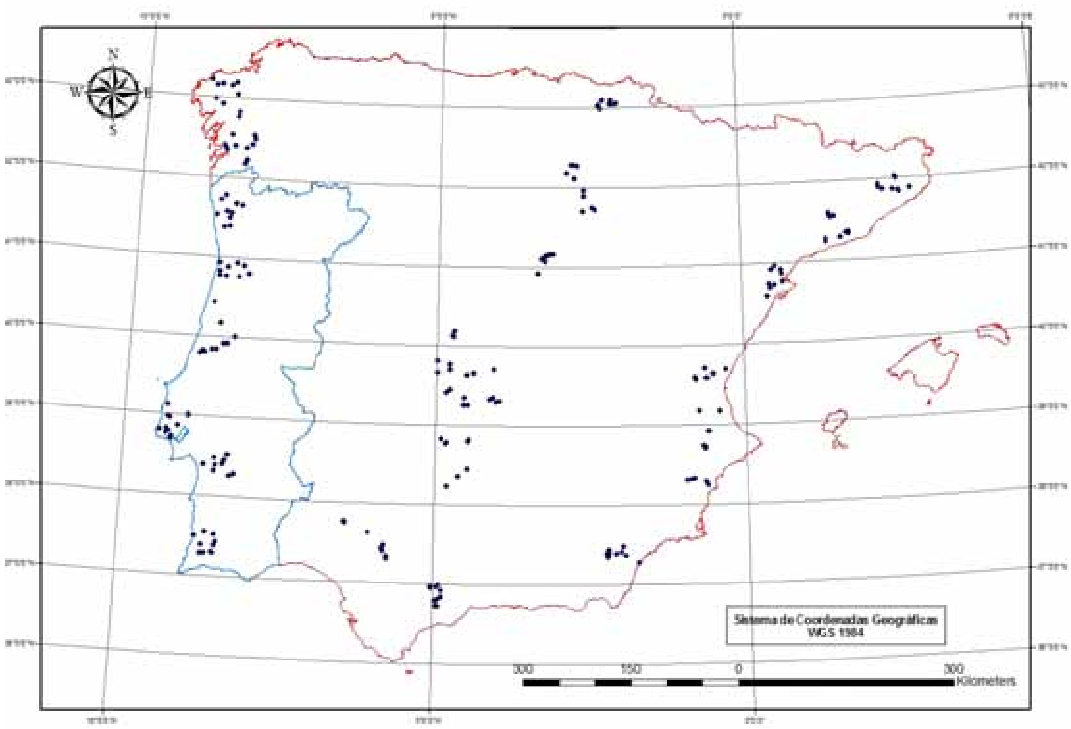

Figure 1: Map showing the locations of the sampled apiaries across the three north-south transects. Each point represents an apiary. In each apiary honey bees were collected from three hives.

Most beekeepers were amateurs (58%). The number of colonies owned by the beekeepers varied between 3 and 2000 with 47,5 % owning less than 100 colonies (Figure 2). Most (60,1%) beekeepers did not practice mobile beekeeping and did not purchase (86,5%) queens (native or exotic, such as Italian).

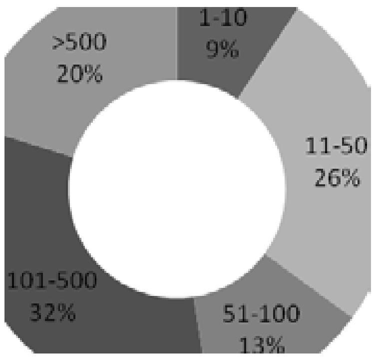

Figure 2: Distribution of beekeepers by classes of total number of owned colonies.

Task 2 - Generating a SNP data set

Objectives

The objective of this task is to generate a database of approximately 760 genome-wide haplotypes comprising 1536 SNP loci.

Methodology and approaches

Over 1536 SNPs are available for use in high-throughput genotyping making it possible to conduct a genome-wide sampling of the IHB populations in a time and cost effective fashion. The sheer number of available loci will allow for the first time robust inferences on the IHB's historical demography. Two different approaches were implemented to obtain the SNPs [9]. One part was derived by comparing the published genome sequence of the honey bee, which was generated from European-derived honey bees, with shotgun genome traces from Africanized honey bees. The other part was obtained by comparative alignments of expressed sequence tag (EST) sequences. Therefore, the SNP panel covers coding and non-coding regions. The mean spacing of the 1536 SNPs is 61.7 kb, which is approximately 1cM/per site, giving reasonably high resolution genomic coverage. The 1536 SNP loci (the maximum allowed in one Illumina Golden-Gate analysis pool) are being genotyped for 760 honey bee samples with the Illumina Bead Station 500G using a custom Oligo Pool Assay (OPA).

Preliminary results

Over 384 IHB drone samples were genotyped until now. These are preliminary data which need to be further optimized. While the level of polymorphism for the 384 samples is lower than expected, it will likely increase with the inclusion of the reference collection (honey bees of other subspecies). About 1/3 of the loci are highly polymorphic, and 1/3 are monomorphic. Of the 1536 scored SNPs, 97 were unscored in 40 of the 384 drones. These 97 SNPs will be re-examined later. 376 SNPs were scored for every drone with another 344 scored for all but one drone. For the remaining 1439 SNPs the minimum allele frequencies (MAF) were as follows: 541 are monomorphic; 307 are polymorphic MAF > 0,1; 364 are polymorphic MAF > 0,05; 487 are polymorphic MAF > 0,01; 864 are polymorphic MAF > 0,002; 899 are polymorphic MAF > 0,001.

Task 3 - Unraveling patterns of adaptive variation

Objectives

The ultimate objective of this task is to identify and interpret the spatial structure generated by loci under selection and potentially reveal the molecular basis of the observed adaptive differentiation in IHBs. In accomplishing this objective we will address several questions:

1) How comparable are the available methods for detecting selection? Do they find the same genomic regions? Do the haplotype-based methods perform better than the more popular F_{ST}-based methods?

2) Do we have increased power in detecting selection and do we gain further insights into the basis of adaptive divergence by using a landscape genetics approach?

3) How concordant are the patterns of adaptive and neutral variation?

Methodology and approaches

Detection of signatures of selection across genomes and identification of their role in adaptive population divergence is a central issue in evolutionary biology. While analytical approaches for detecting selection are becoming increasingly powerful, they face the problem of excluding positives and identifying false positives, owing to violations of assumptions and oversimplified models. To alleviate this problem, Luikart [10] and Storz [22] recommend employing different approaches when searching for selection. In this task we will use F_{ST}-based approaches, haplotype-based approaches, and a landscape genetics approach.

The multiple-population F_{ST}-based approaches identify loci under selection as outliers in the extreme tails of theoretical or empirical null distributions of F_{ST}. In this study, we will employ both theoretical and empirical approaches, although the latter promises to be more powerful as it controls for demographic effects. Several software packages (e.g. DetSel, Fdist, BayesScan) are available to perform the F_{ST}-based theoretical test.

Haplotype-based approaches test for haplotype homozygosity, which is based on the probability that any two haplotypes that are chosen from a common population will be identical. Such methods exploit the relationship between the extensive linkage disequilibrium that surrounds a new mutation and the time it takes for recombination to degrade it, allowing detection of recent selective events. These approaches have proved to be powerful in detecting recent selective

sweeps on humans (23, 24, 25), and promise to be even more powerful in our study. Unlike in human studies, by analysing the haploid males, the haplotypes will be determined from phased data for the whole genome, which provides an increased power for detecting selection.
Using the landscape genetic tools (e.g. software SAM) [26], we will further validate candidate loci for selection. Geographic Information System (GIS) will be used to superimpose landscape and environmental variables onto the genotype data and to test for correlations. This approach will allow hypotheses testing about the cause of spatial patterns. Signatures of selection will be detected by comparing parallel patterns of locus specific variation among replicated populations and by finding non-random associations between locus specific variation and landscape and environmental variables [26-27]. Given that the IHB sample was collected along clines, this approach promises to provide insightful information for detecting adaptive variation and identifying potential causes.
The honey bee genomic resources will be crucial in this task. First, they will allow further confirmation that outliers represent signatures of selection by considering their genome position and gene function. Second, they will help in deciphering the molecular basis of the polymorphism and the potential basis of its adaptive divergence.
In this task, the samples showing evidence of lineage C introgression (e.g. Italian queens) will be removed as they will confound the signal when testing for selection. These outlier samples will be identified using principal component analysis and a model-based clustering algorithm implemented in the software Structure [28].

Expected results

By using a powerful combination of analytical approaches for detecting selection and the honey bee genomic resources, we expect to provide a robust representation of the spatial patterns of adaptive IHB variation and help gain insight into the molecular basis of the observed divergences. This novel approach will provide a sensitive measure of the extent and distribution of co-adapted complexes in the IHB.

Task 4 - Unraveling patterns and processes of neutral variation

Objectives

The ultimate objective in this task is to reconstruct the IHB human-mediated contemporary and evolutionary history and demography using neutral loci. In accomplishing this objective we will address several questions:
1) What is the impact of contemporary human-mediated forces in shaping IHB structure? Do they obscure historical patterns?
2) Do we find environmental or landscape variables (e.g. barriers to gene flow) that correlate with spatial neutral genetic structure?
3) Can patterns of neutral variation be distinguished from patterns of adaptive variation?
4) How concordant are the patterns of neutral and mtDNA variation?

Methodology and approaches

Robust inferences on history and demography of populations require identification and exclusion of loci that show evidence of selection (outlier loci) as they obscure patterns and processes shaping genome-wide variation [10]. Therefore, to obtain more accurate IHB population parameters we will constrain our data set to the neutral loci identified in the previous task.
We will reconstruct the IHB history and demography using a combination of population genetics methods, phylogenetic methods, and spatial statistical tools. We will use several methods [27] to identify spatial genetic patterns, including correlograms for testing isolation by distance, interpolation of the major principal components of principal component analysis for generating synthesis maps, among others. Once a spatial genetic pattern is identified, we will use Mantel's test, partial Mantel tests, canonical correspondence analysis, among others, to test for correlations with environmental and landscape variables. A GIS will be used to superimpose genetic data with landscape and environmental data. This approach will enable visualization of spatial genetic patterns at a fine scale and at the same time will provide a powerful framework for hypothesis testing of potential causes of the observed neutral diversity.

Importation of Italian honey bees by beekeepers is becoming increasingly common (especially in Spain). The reconstruction of this human-mediated contemporary history requires inclusion in the "neutral data set" of previously identified outlier samples. The software Structure [28] will be used to estimate introgression of lineage C into IHB populations and identify Iberian regions that are impacted by human-mediated gene flow.

Expected results

Interacting historical processes such as population contractions and expansions, admixture, differential rates of sex-related dispersal, and local adaptation have likely shaped IHB diversity. Contemporary human-mediated processes have undoubtedly added to naturally shaped complexity. By dissecting neutral from adaptive variation and from recent human-introduced variation, we expect to produce robust inferences on the history and demography of the IHB. In addition, we expect to provide a more complete picture of the present-day IHB diversity by adding the contemporary human-mediated component.

SUMMARY OBSERVATIONS

Perceiving patterns and processes of genetic variation is an important step towards understanding the basis of biological diversity and provides a stronger scientific foundation for conservation decisions. In identifying spatial patterns of neutral diversity and the genome components that are potentially adaptively important, we expect our study to have major repercussions by providing baseline data for the long term conservation of native populations. IHBs harbour the highest genetic diversity among the European lineages, yet this diversity has been increasingly threatened by introduced pests and diseases, genetic introgression from imported queens, among others (see for example the recent disaster caused by the "colony collapse disorder"). While reductions of effective population sizes (through severe mortality caused by the introduction of *Varroa destructor* in Europe, for example), may lead to a loss of genetic diversity, gene flow (through introductions of Italian queens, for example) may increase it. Nevertheless, both evolutionary forces may compromise honey bee survival and local adaptation; the former by reducing the raw matter on which selection operates and the latter by disrupting co-evolved gene complexes.
Conservation of genetic diversity is important because variation is a pre-requisite for long term adaptive change and necessary to avoid fitness decline through inbreeding depression. On the other hand, preventing incremental changes of genetic diversity by human-mediated gene flow (queen importations) is important because introgressive hybridization may cause outbreeding depression through the loss of important local adaptations. To counteract the detrimental effects of population bottlenecks and human-mediated gene flow, several European countries (Denmark, Switzerland, Norway, France, Austria) have implemented conservation measures to protect locally-adapted *A. m. mellifera* populations [29]. As mentioned before, the Iberian Peninsula has served as a honey bee reservoir for the northern European latitudes during glacial periods. Therefore, given its history and in a rapidly changing and increasingly demanding world, the IHB is certainly a subspecies deserving special attention regarding protection.

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