HOLOCENE SEA LEVEL RISE AND RANGE Fragmentation
OF PODARCIS LILFORDI ON MINORCAN ISLETS:
A VICARIANCE SCENARIO REVIEWED THROUGH A mtDNA TREE

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Abstract: A 684 bp fragment from cytchrome-b allowed a coherent arrangement of several Podarcis populations from the Balearic archipelago, in a study focused on the phylogeography of the 15 Minorcan populations of P.lilfordi. A reference site with P.lilfordi from Mallorca clusters with the Minorcan population, although it shares a few variations with P.pityusensis that are not present in the Minorcan clade. Concerning the Minorcan islets, a two steps model is drawn accounting for the observed molecular pattern. An ancient and a derived stock of populations can be discerned, separated by a population event replacing the former populations by the modern ones on the shallowest protoislets, placing it around 7000 to 5000 BP based on bathymetric information and Holocene sea level data. Autapomorphies are present in several sites and could be indicative of separated demes prevailing on peninsular areas of the island, as in Cavalleria, Mola de Fornells, or even on larger regions as in southeast Menorca. Within the framework of a vicariant model, we suggest that islet’s inhabitants have thus retained some of the history of the now extinct mainland population.

Key words: Holocene, fragmented populations, P.lilfordi, Minorcan islets, mtDNA tree.

Resumen: Subida del nivel del mar en el Holoceno y registro de la fragmentación de Podarcis lilfordi en los islotes costeros de Menorca: un escenario vicariante revisado a través del árbol de ADN mitocondrial. La secuenciación de un fragmento de citocromo-b de 684 pares de bases revela un esquema coherente de relaciones entre diversas poblaciones de Podarcis del archipiélago balear, en un estudio dirigido a comprender la filogeografía de las 15 poblaciones de P.lilfordi existentes en Menorca. Una localidad de referencia de P.lilfordi en Mallorca se agrupa con las poblaciones menorquinas, aunque comparte unas pocas variaciones con P.pityusensis, que no están presentes en el grupo de Menorca. En relación a los islotes menorquinas, se propone un modelo de dos etapas para dar cuenta de los resultados moleculares. Se disiente un stock ancestral y otro moderno, separados por un evento poblacional que sustituye las poblaciones antiguas por las modernas en los protoisletes más someros, por lo que ocurriría entre el 7000 y el 5000 BP, atendiendo a la información batimétrica de los islotes y a los datos de nivel del mar en el Holoceno. Las autapomorfías están presentes en diversos islotes y pueden ser indicativas de la existencia de

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poblaciones locales en parte aisladas por efectos península, como en Cap de Cavalleria y Mola de Fornells, o incluso por separaciones que impliquen áreas mayores, como el sureste insular. En el esquema de un modelo vicariante, sugerimos que los habitantes de los islotes han retenido parte de la historia ocurrida en la población principal, ahora extinguida.

Palabras clave: Holocène, fragmentación de poblaciones, *Plioforid*, islotes de Menorca, árbol de ADN mitocondrial.

Resum: Augment del nivell del mar a l’Holòcè i registre de la fragmentació de Podarids *Plioforid* en els illots costaners de Menorca: un escenari vicariant revisat mitjançant l’arbres de mtADN.- La secció- ciació d’un fragment de cirocróm b de 684 parells de bases revela un esquema coherent de relacions entre diverses poblacions de Podaríds de l’Arxipèlag Balear, en un estudi dirigit a comprendre la filogènia de les 15 poblacions de *Plioforid* existents a Menorca. Una localitat de referència de *P. liiforid* a Mallorca s’agrupa amb les poblacions menorquines, encara que comparteix unes poques variacions amb *P. pitiusensis* que no són presents al grup de Menorca. En relació als illots menorquins, es proposa un model de dues etapes per a explicar els resultats moleculars. Es discrimina un stock ancestral i un stock modern, separats per un esdeveniment poblacional que acaba substituint les poblacions antiguas per les modernes als protoillots de menys fondària, cosa que el situaria entre els 7000 i 5000 BP, atenent les b a tèmiques dels illots i les dades de nivell del mar a l’Holòcè. Les autapomorfies detectades a diversos illots podrien ser indicatives de l’existència de poblacions locals en parts aïllades per efectes península, com al Cap de Cavalleria i a la Mola de Fornells, o fins i tot de separacions que impliquessin àrees majors, com el surest insular. Dins l’esquema d’un model vicariant, suggerim que els habitants dels illots han retingut part de la història ocorreguda a la població principal, ara extingida.

Paraules claus: Holocè, fragmentació de poblacions, *Plioforid*, illots de Menorca, arbres mt ADN.

**INTRODUCTION**

Pre human tetrapods of the Balearic Islands are uniquely represented by the endemic midwife toad *Alytes muletensis* and by endemic lizards of the genus *Podarcis* (Squamata, Lacertidae), being the remaining fauna introduced by man (Alcover et al., 1981). Indeed, in the case of *Plioforid* from Mallorca and Menorca, their mainland stock became extinct on historical times, as can be derived from paleontology (Sanders, 1984). Today, only a circle of isolated populations survive on surrounding islets, and at least two of these local settlers became extinct within the XX century: in Rates (Pérez-Mellado & Salvador, 1988) and in Ses Mones, occupied around 1995 by the invader *Psicula* (Pérez-Mellado, unpublished). The status of several individuals in Ses Aligués, previously believed extinct in early eighties, is still unclear (Pérez-Mellado, unpublished). The case study presented here concerns the remaining extant populations around Menorca, consisting of up to 15 islets inhabited by genuine demes. Up to now, 14 of them have been studied and taxonomically named. Only the recently discovered population from Mel islet, within the Albufera des Grau coastal lagoon remains unassigned at a subspecific level.
Minorcan *P. lilfordi* has been dealt with in considerable extent under taxonomical, enzymatic polymorphism, life history, behavioural and ecological studies. Nonetheless, no study has been turned up to date concerning DNA molecular markers, neither screening their genetic polymorphism nor from the phylogeographic point of view. In the present work, a first approach to the characterization of the Menorca clade is done by using a mitochondrial DNA marker. We essay to figure out the scenario and timing of the colonization pattern. In so doing, we find to know the possibilities of inferring some details of the population history in prehuman times.

The period of interest concerns the Holocene, after the breakdown of the Gymnesic or Great Balearic Island in the pleniglacial, into separate Mallorca and Menorca islands. It is generally accepted that postglacial sea level rise should have marked the calendar of isolation events. Supporting that, the clearest morphological convergences and behavioural specializations are found on lizards of islets with the deepest bathymetry, as is the case for the type species *P. lilfordi lilfordi* from Illa del Aire. Nevertheless some other islets separated from the mainland by shallower channels are also inhabited by unique morphologies that have been named too, as in *P.l. porrosicola* from Illa des Porros.

Although there remain substantial uncertainties about the Holocene eustatic curve on the Mediterranean, a relatively fine panorama can be drawn. After a fast rise until middle Holocene, finishing 6000-5000 BP, a slowing rate or even stabilization characterizes the recent millennia. It remains unclear if sea level rose higher than present on mid to late Holocene, as it is shown by erosion terraces and beaches, speleothems (Ginés *et al.*, 2001) and highstand waters inferred from micropaleontology of littoral marshes (Mateu *et al.*, 2001). Based on this geological support, several authors have proposed a timing of islets isolation inspired by the Holocene curves available every time (Pérez-Mellado, 1989), thus generating a thorough, yet untested, colonization hypothesis based on both direct taxonomic and indirect bathymetric evidence.

Whatever the details, the mainstream model of *Podarcis lilfordi* biogeography is a vicariant one, driven by the coastal fragmentation and isolation of lizard populations. Even without excluding secondary dispersal between the closest islets—some of them separated by only a few meters—, the overall pattern predicted would be a cladogenetic process driven by bathymetric resemblance of the channel, more than by geographic proximity. Given the presence of islets on both northern and southern sides of Menorca, the confirmation of this pattern would imply a more or less interconnected mainland source population, furnishing local random samples to the isolating coastline. Figuring out this pattern through some molecular approach would depend strongly on the marker used. Fast changing and highly variable molecular markers would probably mask this pattern and favor detecting independent
local effects—either coming from selection, assortative mating or drift—. On the opposite, slower markers would detect, if any, the main pattern, even at a cost of masking the finest details of drift, founding effects, and the posterior process of local adaptation on every islet. Our approach here is based on the use of an intermediate molecular marker, the family of mitochondrial genes coding for proteins, and specially for cytochrome-b, presumed to faster accumulating silent mutations than other proteinic and ribosomal genes.

MATERIAL AND METHODS

From autumn 1999 to summer 2000, a few specimens of Podarcis spp. were caught for each islet surrounding Menorca, from one reference islet in Mallorca (La Guàrdia), and from the mainland in Eivissa and Formentera. As Podarcis lilfordi and P. pityusensis are firmly believed to group as a homogeneous clade of lacertids (HARRIS & ARNOLD, 1999), this was taken as an advantage to use P. pityusensis as the closest outgroup to root the model, whereas the population of southern Mallorca was left free, pooled within the modeled set, to test the monophyly of the Minorcan demes.

During fieldwork on islets, a piece of the tail was clipped off and animals were released again in the capture place. The material was preserved in 100% ethanol. In the lab a small bit (3-4 mm³) of tail tissue was removed from 3 individuals of each locality. Total genomic DNA was extracted using the extraction kit Qiagen QIAamp. A 684 bp fragment of the mitochondrial cytochrome b gene was then amplified using two underhand regions that are limited by the sites 16321 and 17005 in amphibians. Amplification from genomic DNA was done using different primers (Table 1). The 50-μL PCR mixture contained 2-3 μL of DNA template, 1 μL of each primer, 1 μL of dNTP, 5 μL of Buffer amb 0.5 U of Taq polymerase. After a denaturing step of 4 min at 95°C, samples were processed through 30 cycles at the optimal annealing temperature (51-55°C), and 1

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CB-J-10612</td>
<td>Cyt-b</td>
<td>CCGATCAACCTGCTTCAGCATGATGAAA</td>
<td>(Simon et al., 1994)</td>
</tr>
<tr>
<td>CB-N-10920</td>
<td>Cyt-b</td>
<td>CCCTCAGAATGATTTTCTGCTCA</td>
<td>(Simon et al., 1994)</td>
</tr>
<tr>
<td>B1</td>
<td>Cyt-b</td>
<td>CATCACCACACCTCAGATGATGAAA</td>
<td>(Kocher et al., 1989)</td>
</tr>
<tr>
<td>B4</td>
<td>Cyt-b</td>
<td>TGGGTGGTGGTGAAGTTTCTGGGTCA</td>
<td>(Birt et al., 1992)</td>
</tr>
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Table 1. Primers used in this study
min at 72°C. The last elongation step was lengthened to 6 min. PCR products were cleaned with the cleaner Microcon-PCR kit. DNA sequencing was carried out with the Rhodamine terminator cycle Sequencing kit from Perkin-Elmer. Finally the products of sequencing were cleaned with Sephadex columns.

The sequences obtained were aligned by eye and compared with homologous sequences of lacertids *Podarcis hispanica, Podarcis atrata* (Castilla et al., 1998); *Lacerta media* and *L. caucasica* (Fu et al., 1997). In this first step we used the BioEdit software package, vers.4.7.8. To determine the relationships among taxa, we used maximum parsimony (MP), neighbor-joining (NJ) with LogDet corrected distance, and maximum likelihood (ML), with the GTR model. The maximum parsimony analysis was done with 1000 replicates, and let us arriving to a consensus tree. Analyses were performed with WinClada ver. 1.00.08.

**RESULTS**

A total of 684 base pair sequences were resolved and aligned for all 18 localities corresponding to all islets around Menorca, one from Mallorca, and two from mainland Eivissa and Formentera. No gaps, insertions or deletions were found. The largest pairwise difference was found between *P. lilfordi* and *P. pityusensis*, and the smallest was obtained comparing populations around Menorca. There are 2 synapomorphic and 4 autapomorphic mutations, defining 10 haplotypes, 7 of them characteristic of Minorcan populations. The resulting MP, NJ and ML trees show a strong coherence. A final single most parsimonious tree is shown (Figure 1).

**DISCUSSION**

The resulting trees are supported on the basis of a very scarce number of mutations, so additional data are clearly needed to firmly establish any definite conclusion. However, haplotype arrangements appear highly ordered and fit pretty well with the vicariant scenario (Figure 2). With both the classical vicariance model and the new molecular data at hand, one is able to better detect their mutual inconsistencies, yet exploring more deeply the potential significance of the results. In so doing, we explicitly separate direct empirical inferences from further common sense interpretations. We present the latter as new working hypothesis, seeking the most likely facts by which the observed pattern could be produced.

As all tree types have the same topology, they support the monophyly among the Gymnesic (Mallorca and Menorca) *P. lilfordi*. Further, the reference population of *P. lilfordi* from La Guardia (Mallorca) shows a degree of divergence in relation to *P. pityusensis* lower than the populations from Menorca, indicating a closer affinity between this Majorcan site and the Pityusic clade. Our results roughly suggest a clinal differentiation pattern.
Figure 1. Single most parsimonious tree resulting from an heuristic search with 1000 iterations of random addition of taxa (holding 10 trees per iteration) of the data matrix (50 parsimony informative characters). Tree length 62 steps (52 excluding uninformative characters), consistency index 0.96 and retention index 0.98 (both with uninformative excluded). Zero-length branches collapsed. Unambiguous changes mapped on tree (assuming hard polytomies), full circles indicate single instances of change and open circles denote homoplastic changes.
within *Podarcis lilfordi*, with ancestral Majorcan clades closer to *Podarcis pityusensis* than Minorcan clades (see Terrassa et al., this volume, as a complementary approach).

**Interpretation**

We place the starting scenario in a time point when Menorca and its protoislands became isolated from Mallorca, since no Minorcan islet channel is deepest than that between Mallorca and Menorca. Several aspects of the molecular tree are to be taken apart. Specially remarkable is the fact that a great majority of islets are inhabited by lizards that are replicates of identical haplotypes. Exceptions come only from Colom, Codrell Petit and Carbó, occupied by two haplotypes. But in these cases, and for all 3 pairs of haplotypes, one is taken as the most recent and geographically closest ancestor of the other, given the resolution of the tree. Further on, exception made of a possible introgression in Colom island,
haplotype history and islet development are here synonymized, since we conclude each deme is monotypic in essence.

Autapomorphies are present in 4 independent cases. They didn’t necessarily should have appeared after islets isolation. Some of them could reflect reduced exchange on mainland due to the existence of a peninsular effect, as could be in Tosqueta islet, placed in the outer border of Mola de Fornells, and Sanitja, placed in the northern extreme of Cap de Cavalleria. The possibility of regional differentiation within the mainland context wins in strength when interpreting the synapomorphy that combines Aire and Rei sites. These islets are placed at the east and southeast side of Menorca, and would have originated from some locally differentiated mainland’s common ancestor. The range of this local deme should not be necessarily restricted to the southeastern of the island. But most probably was not living in the north and northeast, since the oldest demes there, covering a wide coastline range (Colom, Grau, Addaia Gran and Sanitja), does not share the synapomorphic character, thus indicating different land ancestors. The hypothetic north-northeast and southeast mainland areas would then be dwelled by populations with different genetic dominances in the early times prior to the formation of the oldest islets.

The most relevant mutation, a cornerstone supporting the vicariance model, is a synapomorphy shared by 10 islets. It excludes the remaning 5 unshared islets in such a way that two kinds of sites appear. Shared islets are separated from mainland by shallow channels, not deeper than 5m at its maximum depth. Unshared islets have both deep and shallow channels, ranging from 0.8m (Mel) to 22m (Sanitja) at maximum depth, so they cover all the bathymetric range. As the haplotype found in Plisfori from Mallorca and in Epityusensis is the common line spread within these last class of islets, this demic group is defined as ancestral. Indeed, it could have reached the overall Minorcan range, that is, all 15 future islets, since these were still proto-islets attached to the mainland. Chronologically, this situation roots in deep time on early Holocene, when sea level was decens of meters below the current one.

All other sites have synapomorphic populations. The ancestral haplotype is completely substituted by a mutant type. This genetic novelty did not reach the older islets, most probably because they were already unavailable when the mutation spread. Sea level rose until most of the ancestral sites became isolated. That point is easily fitted by Sanitja (22m), Aire (12m), Rei (9m) and also probably by Mel, the single occupied island placed outside the open sea, within a coastal lagoon. Radiocarbon dating of its muddy sediments show that they accreted faster than rose sea level in last millennia (PRETUS, unpublished), and the current channel depth is much shallower than was in the older times focussed here. Only Addaia Gran (4m) has a shallower channel but still is settled by ancient haplotypes.

Derived demes sharing the synapomorphy are geographically
widespread. It’s not the case of a local mutation. Their most recent common ancestor had a range as wide as the former type it substituted. It spread abroad, covering the southern and northern coastlines, to reach the shallow-water protoislands, in a time period prior to its physical isolation. Why we now find only derived haplotypes on the modern islets, instead of both ancient and derived types? The only exception is in Colom islet, the largest (40 ha), highest shallow-water site of the Minorcan islands set. To the remaining cases, one is forced to wonder if the remaining ancestral populations were brought to extinction. Although sampling biases have to be considered, since only 3 individuals are kept on each island, all 25 individuals studied did retained the derived character, thus 100% of the material randomly sampled.

**A two-steps vicariance model**

In order to complete a mechanistic model supporting the molecular pattern found, and under the vicariance scenario, a working hypothesis is herewith exposed. It focusses in two facts. First, the molecular findings. These are of two types, those characterising the evolution of the currently extinct mainland population, that are retained in the genetic background, and those that happened on each separated islet as posterior divergence of populations by drift or selection. We are concerned in this paper with the first type, associated to the main synapomorphy. Second, the bathymetric characteristics of each island is an approximate marker of the time of isolation, thus a putative chronology for the mechanisms needed to have some reference picture to further discussion.

As an ongoing working hypothesis we propose that the pattern of isolation that finally brought to the current distribution did not proceed gradually, following the rising sea level, but it followed a two-steps model, with two distinct waves of isolation from the coast of Menorca, separated by some kind of population drawback (Figure 3). Several so-called here ancestral populations became isolated in the first step, they are those from Aire, Rei, Mel, Sanitja and Addia Gran. A second group becomes independent more recently. The islets that carry on an ancestral population are isolated from Menorca by a channel deepest than the others. Gathering available data of eustatic sea level during the Holocene, and making an age translation, we point out the transition period being neither older than 7 ky nor younger than 5 ky. Addia Gran and Adda Petita certainly disagree with the scheme, since both islets are shallow, but harbour an ancient and a modern type, respectively, and require a specific history. But the big picture suggests the ancient islets being separated from the mainland, retaining the genetic ancestral variant, prior to the event replacing the mainland stock.

Modern islets with shallower channels should be certainly occupied by ancestral populations at the time when deep-channel islets separated from the mainland. When the mutation appeared, the still protoislets should have yet been colonized by the
newcomers. So, we would expect a mixture of them. But it is not at all the general case. The data found suggest a severe population depletion on mainland, or at least on the littoral area, posterior to the isolation of the shallowest of the deep-channel group of islets, and a recolonization of the entire island, comprising its northern and southern littoral margins, prior to the isolation of the shallower islets, and, more exactly, prior to the isolation of the deepest of those. Of course it should happen, also, prior to the extinction of the mainland stock some 2000 years ago. The deepest islets of the secondary group have channels of about 5 meters depth. This places the upper time limit no younger than 5 ky, when sea level starts stabilizing with much lower changes (< 5 m).

We suggest that, within a period no longer than some 2000 years, and probably much shorter, a severe demographic depletion of the mainland population happened (Figure 3), followed by a recolonization with a mutational novelty distributed abroad the mainland range. This bottleneck-like process did not concerned the islets already separated, because no deep-channel islet in Menorca is empty of ancestral forms. The process, similar in essence to the factual evidence of the definite historical extinction of the mainland stock, could be associated to a biological or land-associated factor, driven but not caused proximally by a climatic shift, that would have involved islets and mainland. It is opportune to note that a combination of competition or predation intensification specific over
the mainland, driven by a rapid and stressing climatic change, would mark the difference explaining how populations living on islets were more prone to survive than those on mainland, a situation that proved definitive in their current distribution.

Taxonomy

Finally, it seems opportune remarking how the new molecular data fits the taxonomy currently accepted at the subspecific level. Each one of the 7 subspecies described in Menorca matches some of the following three categories: (1) identifies demes of the ancestral clade, as *P. l. lilfordi* and *P. l. fenni*; (2) identifies demes of the derived clade, as *P. l. sargentanae*, *P. l. porrosicola*, and *P. l. codrellensis*; and (3) identifies mixed demes, with *P. l. balearica* and *P. l. addaiae*. Given the low resolution of the molecular approach, one should be absolutely cautious when interpreting the few inconsistencies that can be drawn, and even remark the broad agreement fitting the expectation of a much clear differentiation of the older isolates (50% of the independent demes named) than modern ones (33% named). Difficulties would be centered in such apparently paraphyletic taxa as *P. l. balearica* (Rei and Colom) and *P. l. addaiae* (Addaia Gran, Addaia Petita and Carbó), since they include ancestral and derived populations as defined here. In the first case, the solution would be to resurrect the subspecies *Podarcis lilfordi brauni* for Colom population, leaving *P. l. balearica* for lizards inhabiting Rei islet. In Carbó islet, the recent study of museum material and specimens from the populations confirms the validity of *Podarcis lilfordi carbonerae* as a different subspecies for this locality, in spite of its proximity to Addaia islands (Pérez-Mellado et al., 2002).

*P. l. balearica* shows more clearly the apparent inconsistency with the molecular tree. The type population of Rei islet clusters together with *P. l. lilfordi* by a synapomorphy. The mutation suggests a former geographic segregation of the southeastern populations of mainland Menorca, when the islets were still attached to the coastline. Once isolated, Rei and Aire demes derived independently and became distinctive at subspecific level. It is difficult to combine the population of Colom islet within this model. Early taxonomists distinguished separately the Colom lizards under the name *P. l. brauni* (Müller, 1927). But detailed reanalysis let modern authors to synonymize it with *P. l. balearica* (Salvador, 1986; Pérez-Mellado & Salvador, 1988; Pérez-Mellado, 1998). Keeping the necessary caution, we conclude that if something could be added to the natural history of Minorcan *P. lilfordi*, would be probably the need to reconsider the status of *P. l. brauni* for the populations of Ila den Colom. This would agree much better with the picture emerging from the molecular approach.
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REFERENCES


