Evolution of thermal tolerance and size of the geographic range in closely related species of water beetles

Amparo Hidalgo Galiana

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Evolution of thermal tolerance and size of the geographic range in closely related species of water beetles

Amparo Hidalgo Galiana
PhD THESIS 2014

FACULTAT DE BIOLOGIA
DEPARTAMENT DE GENÈTICA
Programa de Doctorat de Genètica

EVOLUTION OF THERMAL TOLERANCE AND SIZE OF THE GEOGRAPHIC RANGE IN CLOSELY RELATED SPECIES OF WATER BEETLES

EVOLUCIÓN DE LA TOLERANCIA TÉRMICA Y EL TAMAÑO DEL RANGO GEOGRÁFICO EN ESPECIES HERMANAS DE ESCARABAJOS ACUÁTICOS

Memoria presentada por Amparo Hidalgo Galiana para optar al título de Doctor por la Universidad de Barcelona

Trabajo realizado en el Instituto de Biología Evolutiva (CSIC-UPF)

Amparo Hidalgo Galiana
Ciempozuelos, Septiembre de 2014

Director	Directora	Tutor
Dr. Ignacio Ribera Galán	Dra. Alexandra Cieslak	Dr. Julio Rozas Liras

A la memoria de mi madre

A todos a los que quiero,
por su apoyo imprescindible

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Muchas cosas me han gustado de vivir en Barcelona (que maca que ets!), una ciudad a la que siempre me apetecerá volver. Hay una costumbre que me gusta especialmente, la de orientarse al este y el oeste con el mar y la montaña, al norte y al sur con los ríos Besòs y Llobregat. Quedar con alguien, y que el punto de encuentro sea un edificio cualquiera, del lado mar.

Barcelona, 29 de septiembre de 2014

Amparo Hidalgo Galiana

CONTENTS

GENERAL INTRODUCTION

This thesis studies the *Agabus brunneus* complex (Coleoptera: Dytiscidae) (Millán & Ribera, 2001), formed by one insular endemic (*A. rufulus* Fairmaire, 1860) and one widespread (*A. brunneus* (Fabricius, 1798)) and one restricted species (*A. ramblae* Millán & Ribera, 2001). This group was selected because it is a simple system of three species for which we have abundant data and precise ecological and distributional information. Integrant species share part of their distribution ranges, what prevents spurious differences simply by species occurring in different geographic areas. The study of this group allowed us to ask some fundamental questions in evolution and biogeography. The fact that they are closely related species with large differences in distributional ranges, including one insular species, allows studying evolution in a recent speciation processes and the reason why closely related species might show very different distributional ranges. This group of species show different thermal tolerances, which are related to the size of the geographical range (Calosi *et al.* 2008a), allowing the investigation of physiological differences and their role in speciation and the geographical range determination.

Distributional range

One of the most fundamental questions in ecology and evolution is why species have different geographic distributions. The importance of determining what limits geographic ranges, their causes and their consequences has long been recognized and is a key issue in ecology, evolution and physiology (Gaston 2009). While answers have been given at different levels of biological organization (e.g. genetics, physiology, population dynamics), these have tended to take place in relative isolation (Gaston 2003) and for no single species do we yet have a comprehensive understanding (Gaston 2009).

Most species are confined to small areas whilst comparatively few are widespread, and all have limits to their geographic ranges beyond which they are not found (Brown, Steven and Kaufman 1996, Gaston 2009). Numerous factors have been proposed to limit the geographic ranges of species: dispersal strategies, niche breadth, competition for limited resources, role of predation, body size, population abundance, latitude, resource availability, environmental variability, colonization and extinction dynamics and physiological tolerance and capacities (Gaston 2003, Stevens 1989, Gaston 2009, Slatyer *et al.* 2013).

In the case of aquatic beetles, phylogenetic history of the species and geographic location are known to be general factors determining the size of the geographical range (Abellán & Ribera 2011). Habitat persistence is also known to be a factor constraining dispersal ability, and thus the separation between the geologically stable lotic (running) and the more ephemeral lentic (standing) water bodies is also reflected in the size of the geographical range of the species typical of either habitat type (Ribera 2008). Recently, it has been shown that what drives differences in range size between lentic and lotic aquatic beetles is in fact dispersal capability, rather than their ecological tolerances (Arribas *et al.* 2011).

Perhaps the most common explanation of geographic range limits has been that species have limited climatic tolerances, and therefore they cannot persist in areas where environmental demands are beyond their limits. Climate and physiology play determinant roles in limiting geographical distributions of species (Spicer & Gaston 1999) and it is expected that locally abundant and widely distributed species should have broader niches, while species that are locally rare and restricted in distribution should have narrower niches (Brown 1984). Among the hypothesis for the explanation of range patterns across species, Rapoport's Rule (Stevens 1989) hypothesizes that there is an increase in latitudinal range size with latitude in the Northern Hemisphere. This is related to differences in physiological tolerance (Janzen 1967; Stevens 1989; Gaston *et al.* 1998) that might have been selected for as a result of temporal climatic variation at higher latitudes. Widespread taxa will have broader ranges of physiological tolerance and plasticity than their restricted relatives (Calosi *et al.* 2008b, Gaston *et al.* 2009). One of the last hypotheses proposes that species with broader fundamental niches will tend to achieve greater local densities, survive in more places, and so occupy wider geographical areas than narrow-niched relatives (Gaston & Spicer 2001).

Species context

The study of differences on distributional ranges in a group of closely related species that present large differences on this trait and that have diverged recently is very informative. However, in a scenario of early differentiation, the complexity of spatial relationships that occur between diverging populations is not reflected in the traditional separation of speciation processes into allopatric, parapatric or sympatric categories (Butlin *et al.* 2012). The transition from natural populations to species is considered a gradual continuum (Mallet 2008) where populations might be connected by gene flow for some period of time and experience gradually changing environments (Wolf *et al.* 2010).

Any part of a species (individuals and populations) can evolve further without affecting the rest of the lineage. Evolutionary processes might result in descendants without extinction of the parental group, which results automatically in paraphyly of the latter. In these cases a strict application of monophyly as criterion of grouping could artificially split up well-defined older monophyletic groups because of the rise of descendants, or force together groups of descendants with parts of older groups (Hörandl 2006). Recent speciation research is interested in approaches that focus on the causes of initial divergence in populations that are only partly isolated (Via 2009). From such work it has been recognized that barriers to gene flow can evolve as a result of ecologically based divergent or disruptive selection (Wolf *et al.* 2010), and ecological speciation is seen as a prerequisite for the evolution of reproductive isolation (van Doorn 2009, Schluter 2001, Rundle and Nosil 2005).

Ecological factors help to delimit species assuming that each of them is related to its particular niche. Species distributions are constrained by abiotic factors like climate plus biotic interactions, dispersal constraints, anthropogenic effects, stochastic events and other historical factors (Pulliam 2000, Soberón 2007). Nonetheless, abiotic conditions probably play a role in setting at least a part of many species geographic range limits (Gaston 2003).

A combination of ecological, genetic and physiological approaches suggest that speciation in some systems include multiple processes that may be simultaneously necessary for the emergence of discrete clusters (Swanson and Vacquier 2002) and it is desirable to study evolutionary history of a group integrating all those disciplines (Eme *et al.* 2014).

Physiological variation in populations and species

The variability in physiological traits among populations and species physiology is studied through the nature of this variation, the distribution and the pattern of variance (Spicer and Gaston 1999). Physiological variation however has been studied mainly on model organisms, leaving groups, habitats and geographic regions under-represented and there is a need for experimentation on non-model organisms (Klok and Chown 2003).

Temperature is one of the most important abiotic variables that determine the limits of species' distribution. It also influences behaviour, metabolism, growth and reproduction rates (Bale 2002; Angilleta *et al.* 2002), and affects functionality and structural stability of all type of macromolecules including proteins (Barja de Quiroga 1993, Chown and Nicolson 2004).

Recent reviews on the cellular stress response have shown that there is a convergence toward a common set of stress-induced proteins in diverse taxa (Kültz 2005). These include molecular chaperones that stabilize denaturing proteins during cellular stress and might also aid in repair of damage following thermal stress (Krebs and Feder 1997). Proteins that sense and repair DNA and RNA damage and that are involved in fatty acid metabolism are also activated. Together, these proteins indicate that cells sense and respond primarily to macromolecular damage to proteins, DNA, and lipids during acute stress. Proteins involved in energy metabolism are also represented (Kültz 2005). During the cellular homeostasis response, cells adjust in response to the novel environment in order to maintain basic cellular functions over the long term, requiring compensatory responses that are specific to the stress and that limit cellular damage (Wang *et al.* 2009, reviewed in Tomanek 2010).

Nowadays there are different approaches that can be used to explore the physiological variability, its evolution and the underlying mechanisms. Proteomics might help to understand cellular mechanisms at the organism level in response to changes in environment, and the integration with ecological and genetic hierarchies might be very informative. This kind of 'environmental proteomics' has many advantages to assess organism function at the molecular level, relating its results to physiology and ecology.

Proteomic analysis

The protein pattern of a biological sample is much more informative of the physiological state than its genome, so its direct analysis is desirable. A small portion of the genome codifies for proteins and many genes are regulated post-transcriptionally, so RNA levels do not necessarily reflect the abundance or diversity of proteins in the cell (Gygi *et al.* 1999). The mRNA transcripts that codify for proteins might be processed after their synthesis by alternative splicing, and the translated proteins might be modified afterwards by post-translation process. Up to 200 possible modifications have been identified (Dziembowski & Seraphin 2004), many types of them associated with protein function (e.g. phosphorylation).

The proteome is defined as the outfit of proteins that are being expressed in a particular space and moment by a cell or an organism (Pandey and Mann, 2000). Tools for the global study of proteins are available since 1975, when the two-dimensional polyacrylamide gel electrophoresis was developed (Klose 1975). Advances in Proteomics in recent years have been possible due to standardized and more replicable techniques for separation of protein and peptides, to more and more powerful mass spectrometers together with better soft ionization techniques. At the bioinformatics level it has been very important the growing capacity of processing, visualizing, comparing and identifying the big datasets that are produced in proteomic studies (see Tomanek 2010).

The use of population proteomics have been recently underlined as very useful in ecological studies (Biron *et al.* 2006b, Cieslak and Ribera 2009). An advantage of using proteomics for ecophysiological questions is that unexpected responses might be discovered as the research is not directed to a determined set of proteins but to the general expression of the proteome space facilitating the understanding of organismal responses to abiotic stressors.

OBJECTIVES

The main objective of this work is to contribute to the understanding of the factors that limit the geographic range of species. For this purpose I have integrated several approaches, first to unravel the evolutionary and ecological history the group of species selected for the study, and then to analyse the evolution of their response to thermal stress and the underlying mechanisms. This investigation is structured in three chapters, with the following specific goals.

In Chapter 1 we want to understand the role of thermal niche differences in shaping geographical expansion and speciation processes within the *A. brunneus* complex. Specifically, we want to study the speciation process in relation to the evolution of the climatic niche, and the climatic niche divergence among the species of the complex.

In Chapter 2 our main interest is to assess the possibility of comparing the overall protein expression of wild populations subjected to different temperature treatments. We particularly focus on variation observed at the level of technical replicas, biological replicas, temperature treatments and in the response of two populations of the same species.

In Chapter 3 our aim is to trace changes in protein expression through the speciation processes within the complex, and to relate these changes with the evolution of phenotypic traits known to differ between species (morphology, climatic niche, thermal tolerance).

ADVISOR'S REPORT

Advisors report on the publication status of the results and the impact factor of the published papers

Dr Alexandra Cieslak and Dr Ignacio Ribera, co-supervisors of the PhD thesis of Amparo Hidalgo-Galiana, with title "Evolution of thermal tolerance and size of the geographic range in closely related species of water beetles", report that the Thesis is formed by three Chapters consisting in respectively two published papers and a manuscript ready for submission.

Chapter 1

Hidalgo-Galiana, A., Sánchez-Fernández, D., Bilton, D.T., Cieslak, A. & Ribera, I. 2014. Thermal niche evolution and geographic range expansion in a species complex of western Mediterranean diving beetles. BMC Evolutionary Biology, 14: 187.

BMC Evolutionary Biology has in the latest edition of the Journal of Citations Reports (2013) an impact factor of 3.407. This journal is in the second quartile in the categories "Evolutionary Biology" (18th of 46) and "Genetics and Heredity" (59th of 164). BMC Evolutionary Biology is an open access journal that is becoming a reference in systematics and evolutionary biology.

Chapter 2

Hidalgo-Galiana, A., Monge, M., Biron, D.G., Canals, F., Ribera, I. & Cieslak, A. 2014. Reproducibility and consistency of proteomic experiments on natural populations of a non-model aquatic insect species. PLoS ONE, 9(8): e104734

PLoS ONE has in the latest edition of the Journal of Citations Reports (2013) an impact factor of 3.534. This journal is in the first quartile in the category "Multidisciplinary sciences" (8th of 55). PLoS ONE is a multidisciplinary open access journal of increasing importance in all biological disciplines.

Chapter 3

Hidalgo-Galiana, A., Monge, M., Biron, D.G., Canals, F., Ribera, I. & Cieslak, A. Protein expression parallels thermal tolerance and ecologic changes, but not speciation, in the diversification of a diving beetle species complex. To be submitted to Molecular Ecology.

Molecular Ecology has in the latest edition of the Journal of Citations Reports (2013) an impact factor of 5.840. This journal is in the first quartile in the categories “Biochemistry and molecular biology” (40th of 291), “Ecology” (11th of 140) and “Evolutionary biology” (6th of 46). Molecular Ecology is a reference in studies of speciation and phylogeography.

Barcelona, 25th September 2014

Ignacio Ribera Galán Alexandra Cieslak

Advisors report on contribution of the PhD candidate in the papers forming the thesis

Dr Alexandra Cieslak and Dr Ignacio Ribera, co-supervisors of the PhD thesis of Amparo Hidalgo-Galiana, with title “Evolution of thermal tolerance and size of the geographic range in closely related species of water beetles”, report that the contribution of the PhD candidate to the publications forming Chapters 1-3 was as follows:

Chapter 1.

AHG, AC and IR conceived the study. AHG and IR coordinated the sampling. AHG obtained most the sequences and the morphometric and distribution data. AHG and DTB conducted the physiological experiments. AHG, DSF and IR analysed the data. All authors contributed to the writing and improving the manuscript, and approved the final version.

Chapters 2 and 3.

AHG, IR and AC conceived and designed the experiments. AHG, MM, IR and AC performed the experiments. AHG, MM, DGB, FC, IR and AC analysed the data. AHG, IR and AC wrote the paper. All authors contributed to writing and discussion of the results.

We also confirm that none of these publications or the presented data has been used, or will be used, in a different PhD thesis.

Barcelona, 25th September 2014

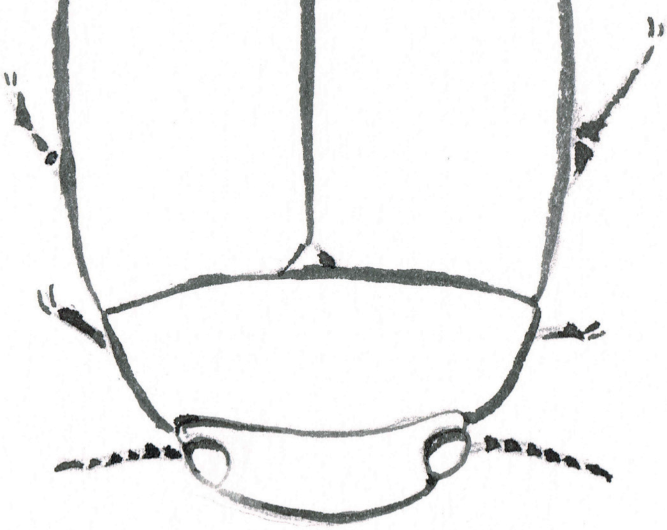
Ignacio Ribera Galán Alexandra Cieslak

ABSTRACT

This thesis studies a group of aquatic beetles (A*gabus brunneus* group) that present important differences in the size of their geographic ranges. This complex is composed by an insular species (*A. rufulus*), a continental species with restricted range (*A. ramblae*) and a widespread continental species (*A. brunneus*), with the aim of study the factors implied in those differences. For this purpose we integrated the phylogeny/ phylogeography of the group and the evolution of the ecological niche together with the study of their morphology and thermal tolerance. This complex of species diversified at the end of the Pleistocene in the Iberian península, probably after the colonization of *A. ramblae* from Morocco. One of the resultant species (*A. brunneus*) at some point of the diversification acquired the ability to resist colder temperatures, and was able to disperse to colder climates. To understand range variability from another perspective we used population proteomics to analyse the response of several populations of *A. ramblae* and *A. bruneus* when subjected to temperatures they might experience in the field. We analysed the variability at several levels in two populations of *A. ramblae*, to test the feasibility of the method when working with natural populations. We obtained consistent and reproducible results, demonstrating that our experimental methodology is appropriate for studying wild populations. When we analysed globally two populations for each species (one from Morocco and one from Iberian peninsula for each) we saw that the diversification observed in the phylogeny was associated with changes in the proteomic response. The more common proteins identified belong to energetic metabolism and stress proteins. The latter were detected to express differentially between the two species studied, showing a different response to thermal stress. This work address the possibility of employing population proteomics in natural populations of non-model species, being able of recovering the stress response facing an environmental factor like temperature. We show as well that differences in range size can be explained by the acquisition of the capacity to face thermal stress.

RESUMEN

Esta tesis parte del estudio de un grupo de especies de escarabajos acuáticos (*Agabus brunneus complex*) que poseen diferencias importantes en el tamaño de sus rangos geográficos, contando con una especie insular (*A. rufulus*), una especie continental de rango restringido (*A. ramblae*) y una tercera especie continental de rango amplio (*A. brunneus*) con el fin de estudiar los factores implicados en esas diferencias. Para ello se integró en un mismo análisis la filogenia/ filogeografía del grupo y la evolución del nicho ecológico junto con el estudio de la morfología y la tolerancia térmica de las especies. Este complejo de especies diversificó a finales del Pleistoceno en la península ibérica, posiblemente tras la colonización de *A. ramblae* desde Marruecos. Una de las especies resultantes, *A. brunneus*, en algún momento de la diversificación desarrolló capacidad de resistencia a bajas temperaturas, lo que le facilitó el poder extender su rango hacia climas más fríos. Para entender este fenómeno desde otra perspectiva se empleó la proteómica de poblaciones para analizar la respuesta de varias poblaciones de *A. brunneus* y *A. ramblae* frente a temperaturas que pueden experimentar en la naturaleza. Para ello, y por utilizar poblaciones naturales, se decidió analizar la variabilidad observada a distintos niveles entre dos poblaciones de una de las especies (*A. ramblae*) obteniendo resultados satisfactorios en cuanto a la reproducibilidad de nuestros experimentos. Al analizar de forma global dos poblaciones para cada especie (una de Marruecos y una de la península ibérica para ambas) descubrimos que la diversificación observada en la filogenia ha ido acompañada de cambios en la respuesta a nivel de expresión proteínica. La mayoría de las proteínas identificadas están relacionadas con el metabolismo energético y con proteínas del estrés, estas últimas detectadas con diferencia de expresión entre las dos especies analizadas, indicando una diferente respuesta al estrés térmico. El presente trabajo abre la posibilidad de realizar este tipo de experimentos empleando poblaciones naturales de especies no modelo y demuestra que la respuesta frente al estrés de un factor ambiental, en este caso la temperatura, puede recuperarse empleando para ello la proteómica. Observamos también que las diferencias en el tamaño del rango pueden ir acompañadas de la adquisición de distinta capacidad de respuesta frente al estrés térmico.



Chapter 1:

Thermal niche evolution and geographical range expansion in a species complex of western Mediterranean diving beetles

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David T Bilton
Alexandra Cieslak
Ignacio Ribera

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RESEARCH ARTICLE **Open Access**

Thermal niche evolution and geographical range expansion in a species complex of western Mediterranean diving beetles

Amparo Hidalgo-Galiana[1*], David Sánchez-Fernández[1], David T Bilton[2], Alexandra Cieslak[1] and Ignacio Ribera[1*]

Abstract

Background: Species thermal requirements are one of the principal determinants of their ecology and biogeography, although our understanding of the interplay between these factors is limited by the paucity of integrative empirical studies. Here we use empirically collected thermal tolerance data in combination with molecular phylogenetics/ phylogeography and ecological niche modelling to study the evolution of a clade of three western Mediterranean diving beetles, the *Agabus brunneus* complex.

Results: The preferred mitochondrial DNA topology recovered *A. ramblae* (North Africa, east Iberia and Balearic islands) as paraphyletic, with *A. brunneus* (widespread in the southwestern Mediterranean) and *A. rufulus* (Corsica and Sardinia) nested within it, with an estimated origin between 0.60-0.25 Ma. All three species were, however, recovered as monophyletic using nuclear DNA markers. A Bayesian skyline plot suggested demographic expansion in the clade at the onset of the last glacial cycle. The species thermal tolerances differ significantly, with *A. brunneus* able to tolerate lower temperatures than the other taxa. The climatic niche of the three species also differs, with *A. ramblae* occupying more arid and seasonal areas, with a higher minimum temperature in the coldest month. The estimated potential distribution for both *A. brunneus* and *A. ramblae* was most restricted in the last interglacial, becoming increasingly wider through the last glacial and the Holocene.

Conclusions: The *A. brunneus* complex diversified in the late Pleistocene, most likely in south Iberia after colonization from Morocco. Insular forms did not differentiate substantially in morphology or ecology, but *A. brunneus* evolved a wider tolerance to cold, which appeared to have facilitated its geographic expansion. Both *A. brunneus* and *A. ramblae* expanded their ranges during the last glacial, although they have not occupied areas beyond their LGM potential distribution except for isolated populations of *A. brunneus* in France and England. On the islands and possibly Tunisia secondary contact between *A. brunneus* and *A. ramblae* or *A. rufulus* has resulted in introgression. Our work highlights the complex dynamics of speciation and range expansions within southern areas during the last glacial cycle, and points to the often neglected role of North Africa as a source of European biodiversity.

Keywords: Thermal niche evolution, Cold tolerance, Demographic expansion, Dytiscidae, Western Mediterranean

Background

Information on the thermal biology of a species is fundamental to understand its ecology, biogeography and evolution, as species are only capable of tolerating a limited range of climatic conditions. Ambient temperature affects all biological processes [1,2], especially in ectotherms [3], and is usually assumed to be one of the main determinants of their spatial distribution [4]. However, in most biogeographical studies the thermal tolerance of species is extrapolated exclusively from their current distributions [5], and even when palaeoclimatic or genetic data are considered (as in e.g. [6-8]), it is rare for these to be combined with experimental data on the actual physiological tolerance of the study organisms [9,10]. Despite this, the need for integrative approaches is increasingly being recognised [11-13], particularly given the limitations of current distributional data for inferring historical or ecological processes [14,15].

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Here we attempt such an integrative approach in a clade of diving beetles that has diversified and expanded its range in the western Mediterranean region during the late Pleistocene, the *Agabus brunneus* complex [16]. Previous work has revealed that thermal tolerance is a good predictor of geographical range extent in these beetles, in which more widespread species have wider thermal windows than their narrow-range relatives [17]. Two species of the complex have partly overlapping distributions in southwest Europe and North Africa, whilst the third is confined to the islands of Corsica and Sardinia [16].

Traditionally, the study of the recent evolutionary history of the European fauna and flora has largely considered the direct effect of the Pleistocene glaciations, particularly the recolonization of previously glaciated areas from unglaciated refugia and the genetic changes resulting from such range movements [18-20]. In most cases, unglaciated areas are simply seen as refugia for northern species, little attention being paid to evolutionary and biogeographical processes in them, other than those which affected these species [21,22]. In contrast to this view, the current diversity of the Mediterranean area is increasingly seen to result from processes which are not directly related to the range movements of northern species during glacial-interglacial cycles [23-26], but our understanding of its origin remains fragmentary, particularly for highly speciose groups such as most insects.

In this study we integrate current and palaeoclimatic information with a molecular phylogeography, morphological analysis and experimentally derived thermal tolerance data to understand the role of thermal niche differences in shaping geographical expansion and speciation processes within the *A. brunneus* complex. Our specific goals are to: 1) test for climatic niche divergence among these species, and associate these differences with their current distribution; 2) test for differences in the estimated ecological niche of each species, and reconstruct the changes in their potential distributions through the last glacial cycle; and 3) evaluate species limits and reconstruct the speciation processes, demographic evolution and range expansion within the *A. brunneus* complex.

We use mitochondrial and nuclear sequence data from populations throughout the extant geographical ranges of all three species of the complex to reconstruct their demographic history and geographic expansion, and explore these within the context of changes in estimated potential distributions through the last glacial cycle. Using the current distribution of the species, we test for differences in climatic niche, and contrast these with experimental data obtained from range edge populations. By integrating these diverse data we are able to reconstruct the evolutionary history of the *A. brunneus* complex in the southwestern Mediterranean region, and illustrate how late Pleistocene climate changes may have shaped its current diversity by promoting ecological differentiation within a southern refuge.

Methods

Taxonomic background on the *Agabus brunneus* complex

The *Agabus brunneus* complex (Coleoptera, Dytiscidae) includes three recognised species of diving beetles with a distribution centred in the western Mediterranean area [16]: *Agabus brunneus* (Fabricius, 1798), *A. rufulus* Fairmaire, 1860 and *A. ramblae* Millán & Ribera [16]. Together with the more distantly related *A. didymus* (Olivier, 1795), which is more widely distributed in the western Palaearctic, they form the *Agabus brunneus* group [27,28]. *Agabus brunneus* has a wide distribution through North Africa and western Europe, including the Iberian and Italian peninsulas, some Mediterranean islands (Elba, Sicily) and France, with isolated populations in southern England ([16]; Figure 1). Some old, isolated records in the eastern Mediterranean (Greece, Syria) with all probability refer to other species (e.g. *A. dilatatus* (Brullé, 1832), unpublished observations). *Agabus ramblae* was recognised based on external morphology and male genitalia as a distinct species previously confounded with *A. brunneus*, and has a disjunct distribution in the South and East of the Iberian Peninsula, the Balearic Islands, Central Morocco and Tunisia [29]. It is usually found in mineralized, temporary running waters [16], and despite its more recent description, exhaustive re-examination of material of *A. brunneus sensu lato* suggests that its apparently restricted range is genuine. The third species, *A. rufulus*, was traditionally considered a colour variety of *A. brunneus* and recorded from various localities in Italy (including the Tyrrhenian islands), Spain and North Africa [30,31]. The revision of Millán & Ribera [16] revealed that it is, instead, a Corsico-Sardinian endemic. *Agabus ramblae* and *A. rufulus* have fully allopatric distributions, but the range of *A. brunneus* completely overlaps with that of *A. ramblae*, and it has also been recorded in Corsica and Sardinia. *Agabus brunneus* and *A. ramblae* are very rarely syntopic, with only 10 reported co-occurrences in the same locality (9 in the province of Albacete and 1 in the nearby area of Jaén, A. Millán, personal communication, 2014). Prior to this study very limited mitochondrial data were available for the three species [32,33], and details of their phylogenetic/phylogeographic relationships, or age of divergence, were lacking.

Morphological identification of species

The main diagnostic difference among species of the *A. brunneus* complex is the shape of the median lobe of the male aedeagus, in particular its relative size, the degree of asymmetry in ventral view and the shape of the apex in lateral view [16], and it is this character suite which was primarily used to assign material to species here.

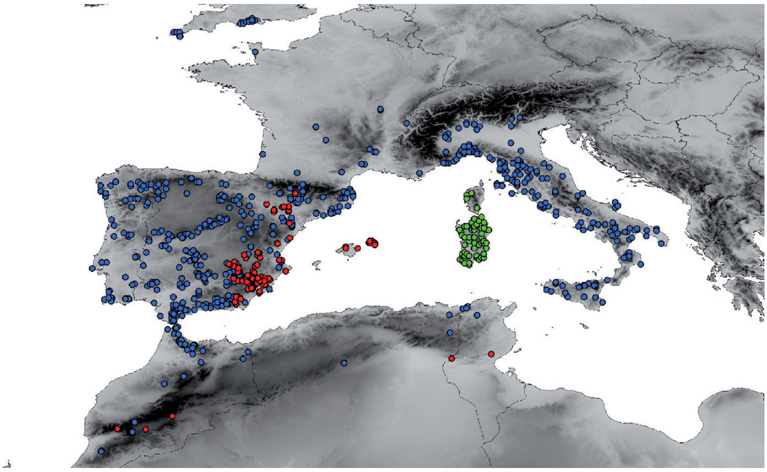

Figure 1 Known distribution of the species of the *Agabus brunneus* complex, including all localities used for DNA sequencing and ecological niche modelling. Blue circles, *A. brunneus*; red circles, *A. ramblae*; green circles *A. rufulus*.

Female specimens were assigned to species by association with males and according to body dimensions and colouration. For 121 males of the three species we measured the maximum length of the median lobe of the aedeagus (AL) and the asymmetry of the aedeagus in ventral view (AD), as these are the main characters separating *A. brunneus* from *A. ramblae*, the two coexisting species [16]. We measured asymmetry (AD) as the difference between the width of the right (RD) and left (LD) sides in the point of its maximum width. We also measured the total body length from the anterior margin of the pronotum to the apex of the elytra (BL), as well as the maximum body width at the widest point (BW) (Additional file 1: Figure S1; Additional file 2: Table S1). Measured males included all specimens used for DNA sequencing (see below) and additional material from a number of sources, including areas for which no fresh material could be obtained (such as Mallorca). Species of the genus *Agabus* have a very uniform shape, and the length of a specimen is a good surrogate of total size [34]. For both *A. brunneus* and *A. ramblae* there is no difference between the body length of males and females [16]. We tested for differences in the length and shape of the aedeagus across the three species using MANOVA, and checked for possible intraspecific geographical variation within *A. ramblae* from Morocco, the Iberian Peninsula and the Balearic Islands, and *A. rufulus* from Corsica and Sardinia. All analyses were performed in IBM SPSS Statistics v. 20 (IBM, Armonk, NY, US).

Thermal tolerance

We determined the thermal tolerance of two populations of different species (Additional file 3: Table S5), one of *A. brunneus* from NE Spain (Girona, Ser river 42° 08'48"N 2°34'48"E) and one *A. ramblae* from central Morocco (Tinghir, Toudgha river 31°33'25"N 5°34'49"W). The experimental procedure followed that used by Calosi et al. [17], from which we extracted data for three additional populations of three different species (*A. brunneus*, *A. ramblae* and *A. rufulus*). Individuals were acclimated for 7 days at 14.5°C in controlled conditions of pH, water composition, light regime and food (red chironomid larvae). Specimens were then separated into sub-groups and thermally ramped ($\pm 1^\circ C\ min^{-1}$) in a computer-controlled water bath (Grant Instruments Ltd, Herts, UK) to obtain measures of their Upper Thermal Limit (UTL) and Lower Thermal Limit (LTL). Temperature was directly measured in one of the wells where individuals were placed for the experiment with a calibrated digital thermometer (Omega HH11; Omega Engineering Inc., Stamford, CT, USA) (see [17] for details). Data were analysed with an ANOVA with species and population as factors and DHS or Tukey post-hoc tests using IBM SPSS Statistics v. 20.

Taxon sampling, DNA extraction and sequencing

Specimens were collected in the field and directly preserved and stored in absolute ethanol. We included molecular data from 68 populations of *A. brunneus*, 22 *A. ramblae* and 6 *A. rufulus*, with up to five individuals per location when available, giving a total of 203 sequenced individuals covering the entire geographical ranges of the three species (Figure 1; Additional file 4: Table S2). As outgroups we used *A. didymus* (the sister of the *A. brunneus* complex [16,27]) together with other published sequences representing a wide range of genera/species of Agabini (Additional file 4: Table S2). Trees were rooted in the genus *Platynectes*, which is within the Agabini but clearly outside the *Agabus* group of genera [32,35,36].

For DNA isolation we employed commercial DNA tissue kits (Additional file 4: Table S2) following the manufacturer instructions. Voucher specimens and DNA aliquots are deposited in the Natural History Museum (NHM, London), Museo Nacional de Ciencias Naturales (MNCN, Madrid) and Institute of Evolutionary Biology (IBE, Barcelona) (Additional file 4: Table S2).

To define the closest outgroups and the general time frame of diversification we used a combination of mitochondrial (a fragment of 827 nucleotides at the 3' end of *cox1*, a continuous fragment between 798–803 (Agabini) or 802 nucleotides (*A. brunneus* complex) including the 3' end of *rrnL* + full *trnL* + 5' end of *nad1*) and two fragments of the nuclear genes *SSU* and *H3* of 608 and 327 nucleotides respectively (see Additional file 5: Table S3 for the primers used and the general PCR conditions). For some specimens in which the *cox1* fragment could not be amplified in a single PCR we used internal primers to obtain two non-overlapping fragments of ca. 400 bp each.

For the detailed phylogeographic and coalescent analyses we sequenced two gene fragments, one mitochondrial (3'-*cox1*) and one nuclear (*H3*). Sequence errors/ambiguities were edited using Geneious Pro 5.3.6 (http://www.geneious.com). New sequences have been deposited in GeneBank with accession numbers LM654767-LM655064 and LM655068-LM655168 (Additional file 4: Table S2).

Phylogenetic analyses

Length-variable sequences were aligned with the on-line version of MAFFT v.7 [37] using the Q-INS-i algorithm, which considers the secondary structure of RNA, and default values for other parameters. The final aligned matrix for the analyses of Agabini was 2579 nucleotide long.

General phylogenetic relationships

To determine the relationships among the main lineages within the *A. brunneus* complex and its phylogenetic relationships within Agabini we used the combined mitochondrial and nuclear sequence from a selection of specimens of the three species. Specimens were selected to cover the geographic range of all species, with a particular focus on potential contact areas (identified through preliminary analyses). Analyses used Bayesian probabilities as implemented in BEAST v1.7 [38] with a partition by genes (except for the *trnL*, pooled with the *rrnL* fragment) and a GTR + I + G evolutionary model for each partition. BEAST was run for 100 million generations, with 10% considered as the burn-in fraction after checking convergence of all parameters with the effective sample size (ESS) as measured in TRACER v1.5 [38].

To establish a temporal framework for the origin and evolution of the *A. brunneus* complex we used the mitochondrial genes only, for which there are recent calibrations for different families of Coleoptera with very homogeneous estimations for the rate of a combination of protein coding and ribosomal mitochondrial regions, calibrated with fossils and different biogeographic events [39-41]. As a prior we used a normal distribution with an average combined rate of 0.01 substitutions/site/million years (MY) and a standard deviation of 0.001, with other settings identical to the above analysis. To ensure that we obtained the same topology as in the analysis employing the full sequence, we constrained the monophyly of ingroup and outgroup, the genera and the *A. brunneus* complex. We used TRACER to calculate the mean and 95% highest posterior density interval for divergence times. We tested different alternative topologies for the relationships amongst species of the *A. brunneus* complex via the use of Bayes factors as estimated with the stepping-stone (SS) and the path-sampling (PS) algorithms in BEAST [42], and with the harmonic mean estimator (HME) in TRACER 1.5 [43] for comparison, in this case requiring an improvement in marginal likelihood of 10 units per additional parameter before accepting a more complex model [44,45]. We tested three different topologies: 1) unconstrained (C0); 2) respective monophyly of *A. brunneus* and *A. ramblae* (C1); and 3) monophyly of *A. rufulus* (C2). We also analysed the *H3* sequences separately in BEAST and RAxML, using a range of outgroups (Additional file 4: Table S2), a single partition with a GRT + G evolutionary model and the previously estimated age of the genus *Agabus* as prior to calibrate the tree in BEAST.

To test for alternative demographic models and to establish haplotype distribution and relationships we used the mitochondrial gene *cox1* of all sequenced specimens of the *A. brunneus* complex, with the same settings as described for the analyses above, but with a mean rate of 0.02 (following [39,40]), with a standard deviation of 0.001. Demographic models were tested first with the haplotypes of the *A. brunneus* complex only (i.e. excluding *A. didymus*), without topological constraints, and then with the haplotypes of *A. brunneus* only (i.e. also excluding *A. ramblae* and A. *rufulus*) (Additional file 4: Table S2).

Four models were tested: 1) constant population size; 2) exponential growth; 3) expansion; and 4) logistic growth. Models were compared through Bayes factors as above, i.e. using the HME, PS and SS. We also constructed a Bayesian skyline plot (BSP, [46]) with the combined results of two independent analyses of the *A. brunneus* complex in TRACER v1.5. The BSP constructs a model of demographic history based on how the number of coalescent events over a given interval differs from that expected under a neutral model for a panmictic population, then summarizes all possible genealogies and provides confidence intervals for all parameters in the model. It estimates changes in effective population size to analyse the population expansion of a species.

Population genetic analysis

We estimated some measures of haplotype diversity and analysed raggedness indices for demographic expansion with Arlequin 3.5 [47] using the *cox1* gene. We tested the validity of the estimated stepwise expansion model using a parametric bootstrap approach.

Ecological niche modelling

We used ecological niche modelling (ENM) based on large-scale climatic data sets and known occurrence points to characterise the environmental niche of all three species, and to test for niche divergence amongst them. Climatic data were obtained at a spatial resolution of approximately 0.08° from WORLDCLIM version 1.3 (19 bioclimatic variables from http://www.worldclim.org; [48]; Additional file 6: Table S4). As records of species occurrences, we employed all known localities for the *A. brunneus* species complex identified according to the morphology of the males (Additional file 7: Table S6) at the same resolution than the bioclimatic variables. Most of the localities used in the ENM were also represented in the molecular analyses.

Bioclimatic values were first subjected to a principal component analysis (PCA) to obtain uncorrelated environmental factors (Varimax rotation). We used the values of the first two PCA factors to represent the climatic space of the whole study area. We plotted the occurrences of the three species in this same space, to visualize the section of the climatic space occupied by each species.

As the results of the thermal tolerance experiments clearly pointed to the importance of lower thermal limits, we used a Kruskal-Wallis ANOVA to compare the minimum temperatures of the coldest month between the localities of the three species. Multiple comparison tests were used to detect significant differences between means. The PCA and the Kruskal-Wallis ANOVA were conducted in Statistica version 8.0 (www.statsoft.com, 2007).

To compare the climatic niche of species we generated ecological niche models using MaxEnt [49], with Schoener's D [50] as a measure of niche similarity between each pair of species as calculated by ENMTOOLS [51]. These values were calculated by comparing the climatic suitability of each grid cell in the study area obtained with MaxEnt. As niche differences may be simply a result of the spatial autocorrelation of the explanatory environmental variables [52], we conducted a background similarity test, also implemented in ENMTOOLS. This test uses randomization to determine whether two species are more or less similar than expected based on the differences in the environmental background in which they occur. A null distribution of 100 niche similarity values was generated by comparing the model suitability values of one species to those generated from random cells drawn from the distribution of the other species. The observed D value of niche similarity between the two species was then compared with the null distribution generated for each of them. The background area of each species should be adjusted to the habitat available, and should be biologically realistic [51]. For the insular *A. rufulus*, the background area was geographically restricted to Corsica and Sardinia, and for *A. ramblae* and *A. brunneus* we defined the background area as the Freshwater Ecoregions in which each species occurs following the classification in Abell et al. [53] (Additional file 8: Figure S2). This method has been used in a number of studies (e.g. [54,55]), including aquatic Coleoptera [56,57].

To provide a climatic context for the interpretation of the demographic models and the changes in distribution of species, we estimated the potential distribution of *A. brunneus* and *A. ramblae* for current climatic conditions, the reconstructed conditions during the last glacial maximum (LGM, 21,000 years before present, YBP) and the last interglacial (LIG, ca. 120,000-140,000 YBP). To estimate potential (not realized) distributions we used a multidimensional-envelope, as it provides a better estimate from observed occurrences (see [58] for details, Additional file 9: Text S1). For both past scenarios we used the same 19 bioclimatic variables at the same resolution as for current climate (see above). For the LGM we used a simulation of the general circulation model (GCM) from the Community Climate System Model (CCSM, http://www.ccsm.ucar.edu/, [59]). The original GCM data were downloaded from the PMIP2 website (http://www.pmip2.cnrs-gif.fr/). For the LIG we used data provided by Otto-Bliesner et al. [60], available at www.worldclim.org.

Results

Morphology

The three species differed in body size, as measured with BL and BW (MANOVA, Roy's greatest root $F = 292.134$, $P < 0.001$), with pairwise differences being significant for all comparisons (Table 1). *Agabus brunneus* was the largest of the three species, and *A. ramblae* the smallest. The measures of size and asymmetry of the aedeagus (AL and AD respectively) were also significantly different (MANOVA, Roy's greatest root $F = 513.120$, $P < 0.001$, Table 1). *Agabus ramblae* and *A. brunneus* were fully separated by AD, with no intermediate specimens, whilst the specimens identified as *A. rufulus* had an intermediate, overlapping shape (Additional file 10: Figure S3). For both

Table 1 Comparison of the measurements of body and male genitalia (aedeagus) among the three species of the *A. brunneus* complex

A) General MANOVA					
Variables	**Effect**	**Value**	***F***	**Sig.**	**Power**
BL, BW (DVs); species (IVs)	Pillai trace	0.85	41.95	<0.001	1
	Wilks' lambda	0.16	84.12	<0.001	1
	Hotelling trace	5.14	143.82	<0.001	1
	Roy's greatest root	5.13	292.13	<0.001	1
AL, AD (DVs); species (IVs)	Pillai trace	0.96	50.62	<0.001	1
	Wilks' lambda	0.09	125.66	<0.001	1
	Hotelling trace	9.48	253.53	<0.001	1
	Roy's greatest root	9.42	513.12	<0.001	1
B) Pairwise *t* comparisons					
t-test comparison	**BL**	**BW**	**AL**	**AD**	
A. brunneus vs *A. ramblae*	<0.001	<0.001	<0.001	<0.001	
A. brunneus vs *A. rufulus*	<0.001	<0.001	<0.001	<0.001	
A. ramblae vs *A. rufulus*	<0.001	<0.001	<0.001	0.1	
C) Mean values					
	BL	BW	AL	AD	
A. brunneus	7.75	4.86	1.44	0.07	
A. ramblae	6.67	4.30	1.15	0.01	
A. rufulus	7.32	4.57	1.33	0.02	

BL, body length; BW, body width; AL, length of the aedeagus; AD, asymmetry of the aedeagus (see Additional file 1: Figure S1). DVs, dependent variables; IVs, independent variables.

A. ramblae (comparing Morocco, Iberian peninsula and the Balearic Islands) and *A. rufulus* (Corsica and Sardinia) there were no significant differences between major geographical areas.

Thermal tolerance

Overall differences in thermal tolerance were highly significant between populations for both LTL (F = 9.87, d.f. = 4, $P < 0.001$) and UTL (F = 27.2, d.f. = 4, $P < 0.001$; Figure 2, Table 2, Additional file 3: Table S5). For UTL the highest differences were between the two populations of *A. brunneus* (north and south Iberian peninsula, post-hoc Tukey $P < 0.001$; higher limit in the southern population), which were also different for LTL (post-hoc Tukey $P < 0.01$; lower limit in the northern population). Differences between the two populations of *A. ramblae* (Morocco and SE Spain) were significant for UTL (post-hoc Tukey $P < 0.05$; higher limit in the northern population) but not for LTL (Figure 2; Table 2).

Between species there were significant differences in LTL ($F = 10.9$, d.f. = 2, $P < 0.001$; Figure 2; Table 2; lower limit in *A. brunneus*, higher in *A. ramblae*), but not UTL. Post-hoc analyses for LTL were highly significant in the case of *A. brunneus* vs. *A. ramblae* ($P < 0.001$) and *A. brunneus* vs. *A. rufulus* ($P < 0.05$), but not for *A. ramblae* vs *A. rufulus* (Table 2).

Phylogeny and phylogeography

Phylogenetic placement of the A. brunneus complex

There were no length differences in the mitochondrial and nuclear protein coding genes (*nad1*, *cox1* and *H3*), and amongst the ribosomal genes length differences were restricted to outgroups, with a maximum difference of 2–5 bp. There were few amino-acid changes in the protein coding genes within the *A. brunneus* complex, and only two in the *H3* fragment: one shared by all *A. rufulus* with the exception of one specimen from Sardinia (AH223), and another shared by all *A rufulus* and all *A. brunneus*.

In the Bayesian analysis of the combined data (mitochondrial plus nuclear) the genus *Agabus* was recovered as monophyletic with strong support (Figure 3), and the *A. brunneus* group also monophyletic but with lower support (posterior probability, pp = 0.61) and with a long stem branch. The monophyly of the *A. brunneus* complex was strongly supported (pp = 1), with *A. ramblae* basal and paraphyletic and a polyphyletic *A. rufulus*: two Corsican specimens were sister to Menorcan *A. ramblae*, while two Sardinian specimens were nested within *A. brunneus.*

Internal phylogeny of the A. brunneus complex, divergence age estimation

The estimation of a time window for the diversification of the *A. brunneus* complex, using the mitochondrial

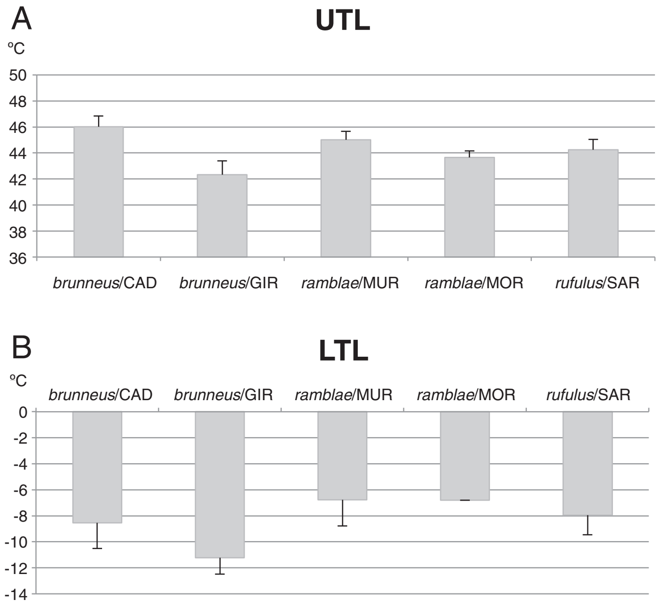

Figure 2 Results of thermal tolerance experiments on the five populations tested. A) upper thermal limit (UTL), **B)** lower thermal limit (LTL). CAD, Cádiz; GIR, Girona; MUR, Murcia; MOR, Morocco; SAR, Sardinia.

Table 2 Differences in thermal tolerance among the species and populations of the *A. brunneus* complex

A) General ANOVA

	Comparison	Sum of squares	d.f.	Quadratic mean	F	Sig.	Power
UTL	species	2.1	2	1.0	0.46	0.6	0.12
	population	72.1	4	18.0	27.20	<0.001	1.00
LTL	species	77.8	2	38.9	10.90	<0.001	0.99
	population	113.8	4	28.4	9.87	<0.001	1.00

B) post-hoc Tukey

							c.i. (95 %)	
	Comparison	sp (I)	sp (J)	Mean difference (I-J)	Std.e.	Sig.	Lower limit	Upper limit
UTL	species	1	2	−0.42	0.50	0.7	−1.63	0.79
			3	0.04	0.57	1.0	−1.33	1.41
		2	3	0.46	0.58	0.7	−0.94	1.86
	population	1	2	3.69	0.37	<0.001	2.62	4.75
		3	4	1.37	0.47	<0.05	0.04	2.69
LTL	species	1	2	−2.96	0.65	< 0.001	−4.53	−1.40
			3	−1.80	0.69	<0.05	−3.47	−0.13
		2	3	1.16	0.73	0.26	−0.61	2.94
	population	1	2	2.70	0.76	<0.01	0.52	4.87
		3	4	0.08	10.96	1.0	−3.11	3.14

In "comparison", "species" refer to the comparison of the three species (pooling the two populations of *A. ramblae* and *A. brunneus* respectively; 1, *A. brunneus*; 2, *A. ramblae*; 3, *A. rufulus*); "population" refers to the comparison of the five populations (1, *A. brunneus* from south Spain, Cádiz; 2, *A. brunneus* from northeast Spain, Girona; 3, *A. ramblae* from southeast Spain, Murcia; 4, *A. ramblae* from central Morocco, Tinghir). UTL, upper thermal limit; LTL, lower thermal limit; d.f., degrees of freedom; Sig., significance; Std.e, standard error; c.i. confidence interval.

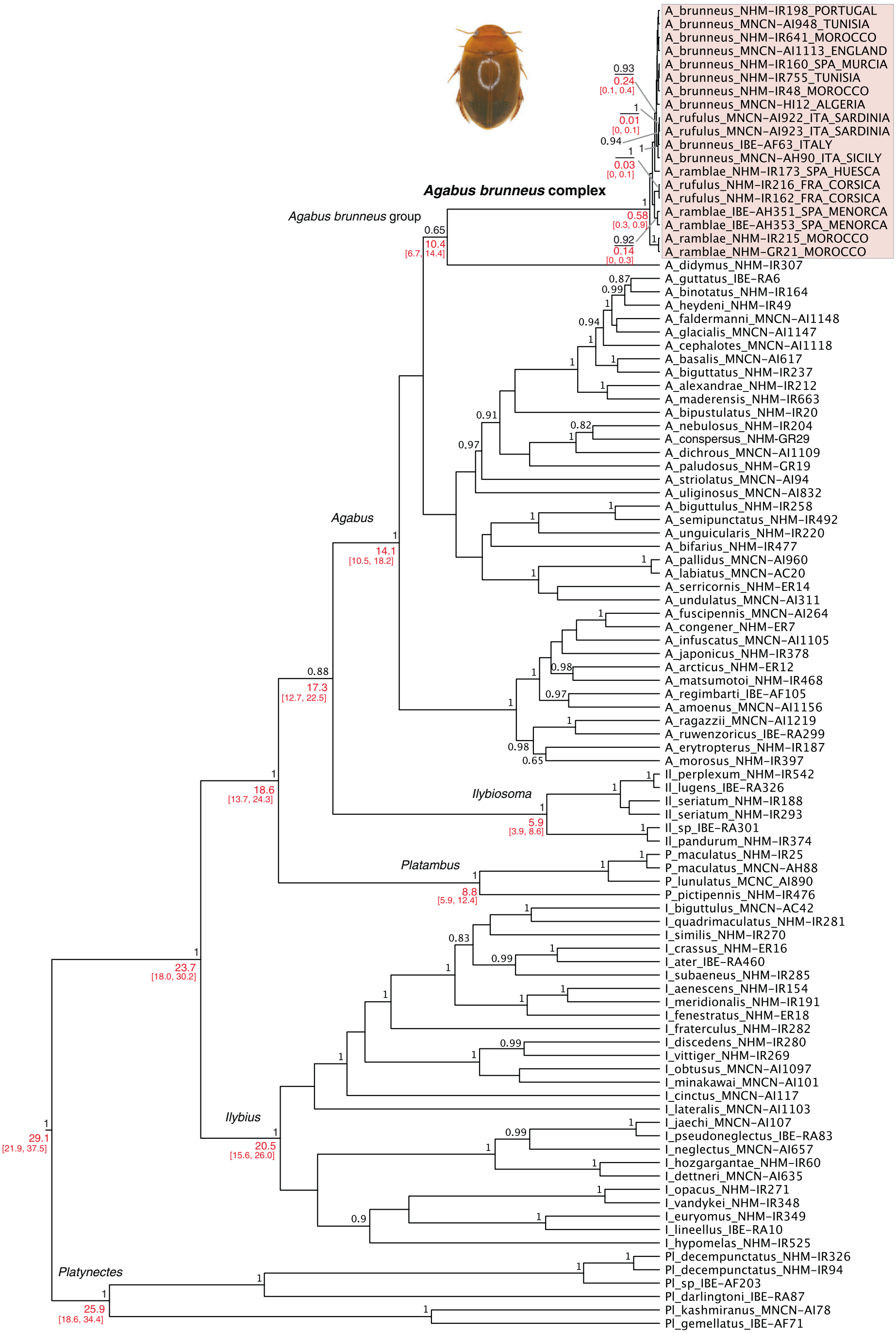

Figure 3 Phylogenetic relationships of the *Agabus brunneus* complex, obtained with BEAST and the combined mitochondrial and nuclear sequence. Numbers in black, node posterior probabilities; numbers in red, estimated age of the nodes (Ma) and 95% confidence intervals, obtained from the analysis of the mitochondrial data only (see Figure 4 and Additional file 11: Figure S4).

sequence from the above specimens and an a priori rate obtained from related groups, gave an age for the stem group of approximately 10.4 Ma (million years ago). The estimated origin of the sampled diversity within the complex was much more recent (0.6 Ma, Figure 3). Models using the two constraints tested (monophyly of *A. ramblae*, C1, and *A. rufulus*, C2) preformed significantly worse than the unconstrained model (C0), with the monophly of A. *rufulus* being the worst of all for all three Bayes factor estimators (HME, PS and SS; with more than 10 units in the difference in –lnLH for the HME, and more than four units in the case of PS and SS, [61], Table 3). The preferred mitochondrial topology had the Moroccan haplotypes of *A. ramblae* sister to the rest of the complex (range of uncorrected mitochondrial p distances, p = 0.004-0.012), Corsican *A. rufulus* sister to Menorcan *A. ramblae* with a divergence of ca. 0.34 Ma (p = 0.008), and Iberian *A. ramblae* sister to *A. brunneus* + Sardinian *A. rufulus*, with a divergence date of ca. 0.35 Ma (p = 0.002; Figure 4, Additional file 11: Figure S4).

We also analysed the *cox1* and *H3* sequences independently. The analysis of the *cox1* gene for all sequenced specimens (203, Additional file 4: Table S2), using *A. didymus* as an outgroup, resulted in a topology very similar to that described above, but with some additional Sardinian *A. rufulus* grouped with Corsican specimens and additional Iberian *A. ramblae* grouped with the Moroccan specimens and nested within *A. brunneus* (Figure 4, Additional file 12: Figure S5), in some cases with identical haplotypes. Two specimens of *A. ramblae* from Menorca (AH348, AH352, the later a male with typical *A. ramblae* aedeagus, Additional file 2: Table S1) and one from Morocco (AH311, female) were also nested within *A. brunneus*.

Table 3 Bayes factors for the topological comparisons

A)

Constraint	**HME**	**PS**	**SS**
C0	−23985	−24419	−24421
C1	−24004	−24424	−24425
C2	−24015	−24443	−24444

B)

Model	**HME**	**PS**	**SS**
C	−1696	−2052	−2053
Ex	−1729	−2041	−2042
Es	−1734	−2043	−2044
L	−1727	−2036	−2038

A) Topological constraints: C0, no constraint; C1, *A. ramblae* and *A. brunneus* respectively monophyletic; C2, *A. rufulus* monophyletic. B) Demographic models of the *A. brunneus* complex: C, constant population size; Ex, exponential growth; Es, expansion; L, logistic growth.
HME, harmonic mean stimator; PS, path sampling; SS, stepping stone (see text for details).

The analysis of the *H3* gene recovered the three species as respectively monophyletic with good support (*A. ramblae* and *A. rufulus* pp = 1, ML bootstrap = 89 and 91 respectively; *A. brunneus* pp = 0.8, MLb = 74; Figure 4, Additional file 13: Figure S6), with only one exception, one female from Albacete identified as *A. ramblae* (AH224) was grouped with *A. brunneus*. Within *A. brunneus* and *A. rufulus* there was some variation (one position), without geographical structure (Figure 4, Additional file 13: Figure S6).

Demographic models of the expansion of the A. brunneus complex

We compared four coalescent demographic models in BEAST using only the *cox1* data of the three species within the *A. brunneus* complex. The best model according to Bayes factors using the PS and SS estimators was logistic growth (with a difference of more than four units of –lnLH), followed by exponential growth (Table 3). On the contrary, marginal likelihood (HME) suggested a constant population size model as optimal (with a difference of more than 20 units of –lnLH), followed by logistic growth. The constant population model performed worst for both PS and SS estimators (Table 3).

The combined two independent runs of the Bayesian skyline plot gave a good convergence when the burn-in fraction was extended to 40 million generations (40%), and suggested exponential population growth at ca. -0.03 Ma, followed by a levelling off at ca. -0.01 Ma with a slight decrease towards the present, i.e. a sigmoidal curve of population growth (Figure 5).

The expansion raggedness indices were lower for populations of *A. brunneus* and *A. rufulus* (from Corsica) than for *A. ramblae*, indicating an expansion in the former two species (Table 4). *Agabus ramblae* also had a higher molecular diversity, as measured with the Theta S and Theta Pi indexes (Table 4).

Ecological niche modelling data

The two first axes of the PCA of climatic variables for all localities of the *A. brunneus* complex jointly accounted for 82.4% of the total variance, and were interpreted as representing ‘aridity’ and ‘seasonality’ gradients respectively. The first axis was positively correlated to maximum temperature of the warmest month, and negatively to precipitation of the driest month, whilst the second axis was negatively correlated with temperature seasonality. The environmental space of *A. brunneus* encompassed almost completely that of the other two species: *Agabus ramblae* occupied the more seasonal and arid extreme of the climatic space of *A. brunneus* complex, and *A. rufulus* was climatically close to *A. ramblae*, although in areas with lower aridity and seasonality (Additional file 14: Figure S7).

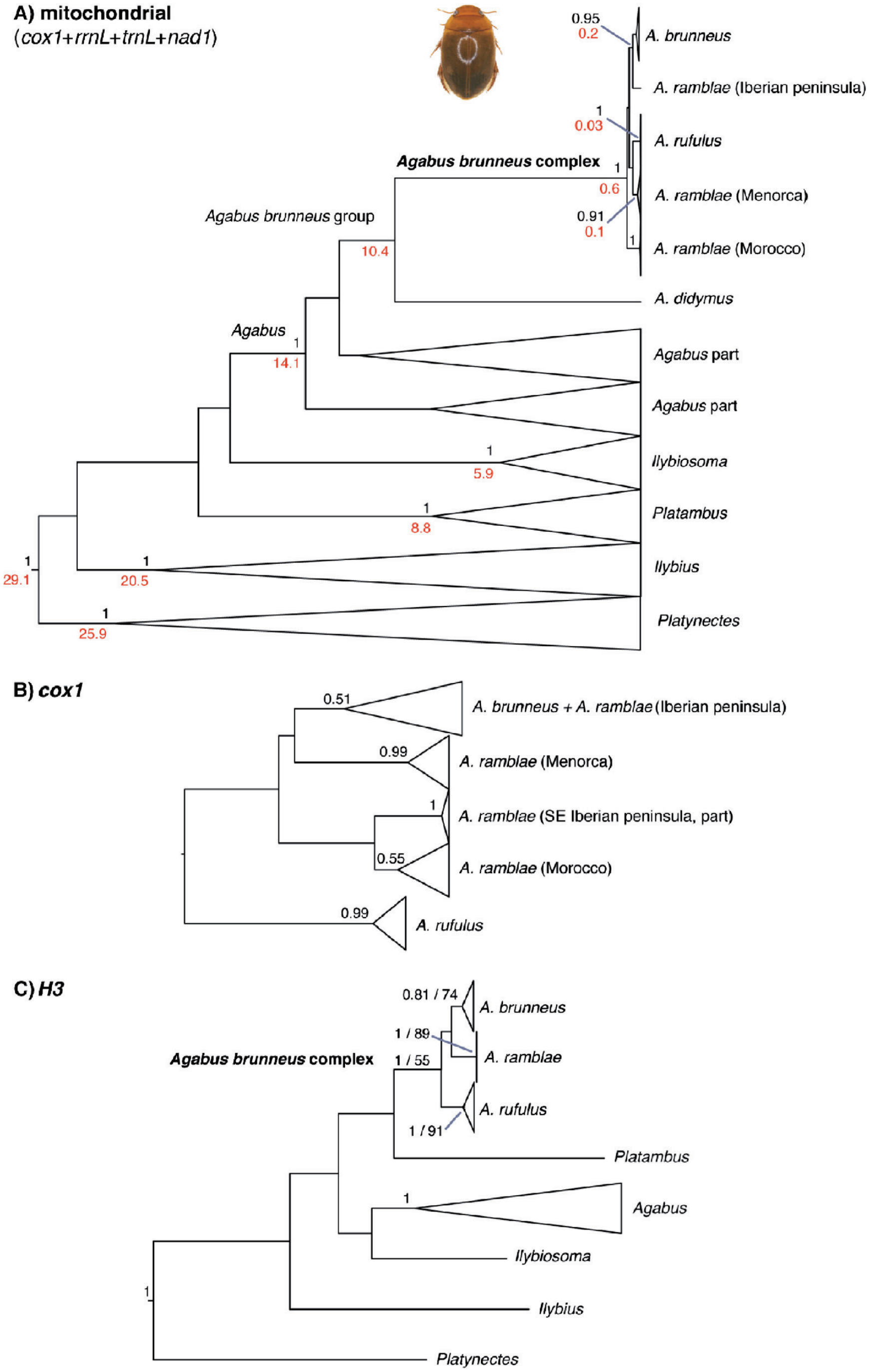

Figure 4 Summary trees obtained from A) combined mitochondrial data (*cox1* + *rrnl* + *trnl* + *nad1*), B) *cox1* and C) *H3*. Node supports in **A)** and **B)**, Bayesian posterior probabilities; **C)** Bayesian pp/ML bootstrap values. In red, estimated age of the nodes (see Additional file 11, Additional file 12 and Additional file 13 for the complete topologies of the trees).

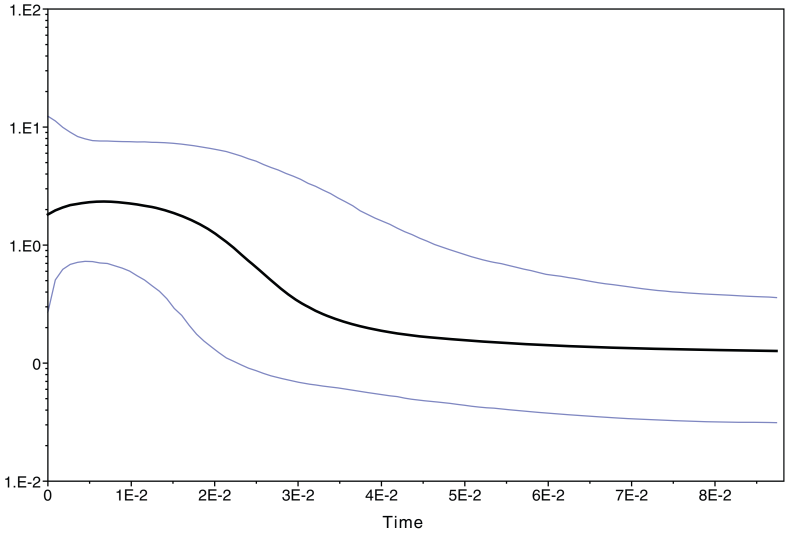

Figure 5 Bayesian skyline plot of the *cox1* sequence data of the 203 studied specimens of the *Agabus brunneus* complex. Blue lines, 95% highest probability density; horizontal axis, time before present (Ma); vertical axis, effective population size (N_eT).

The values of the minimum temperature of the coldest month of the three species (*A. brunneus*: −8.8°C, *A. ramblae*: −4.8°C, *A. rufulus*: −1.9°C) were significantly different, as measured with a Kruskal-Wallis test ($N = 686$; $H = 73.32$, $P < 0.05$). All pairwise comparisons were also significantly different (at $P < 0.05$) except that for *A. brunneus* and *A. ramblae*, which was close to significance ($P = 0.07$).

Results of ENM of each species, estimated with MaxEnt, had low spatial overlap. The degree of niche overlap estimated by Schoener's D statistic was lower than 0.373 for all pairwise comparisons between the three species, suggesting differences in the climatic niche among them ([52]; Figure 6 and Figure 7). The null hypothesis of no differences in ecological niches explained by environmental differences between areas of occupancy alone was accepted (lower p-value = 0.17) for the comparison between *A. ramblae* and *A. rufulus* (despite a low D value, 0.20, this was not significantly more different than expected by chance, Figure 7), and rejected ($p < 0.05$) for comparisons between *A. brunneus* and the other two species.

The potential distributions of *A. brunneus* and *A. ramblae* during the last interglacial (LIG), reconstructed using a climatic envelope, were similar but far more restricted than their current distributions, with most of the Iberian peninsula and North Africa considered unsuitable (Figure 8). This was especially the case for *A. ramblae*, for which, in terms of current range, only the Balearic Islands and some areas in the High Atlas in Morocco appeared appropriate (Figure 8). The largest difference between the LIG reconstruction and the current climate for the studied variables was seasonality, with the areas considered to be unsuitable having values beyond those of their current ranges (Additional file 15: Figure S8).

During the last glacial maximum (LGM) the potential distribution of the two species increased to cover most of their current ranges: for *A. ramblae* only the northernmost

Table 4 Measures of raggedness and molecular diversity of the three species of the *A. brunneus* complex

Species	n. pop.	h	n.pop. >2 ind	n. loci	n.loci <5 % mis.	pol. sites	Ts	Tv	Θ_S	s.d. Θ_S	Θ_π	s.d. Θ_π	RI
A. brunneus	154	28	69	826	717	21	19	2	0.22	0.18	0.22	0.34	0.07
A. ramblae	21	10	6	826	766	12	11	1	1.82	1.19	1.81	1.45	0.31
A. rufulus	9	2	4	826	762	1	1	0	0.37	0.37	0.35	0.48	0.10

n.pop., number of populations; h, number of haplotypes; n.pop. >2 ind, number of populations with more than two individuals; n.loci <5% mis., number of loci with less than 5% of missing data; pol. sites., number of polymorphic sites; Ts, number of transitions; Tv, number of transversions; Θ_S, estimation of the mutation parameter (Theta) from the observed number of segregating sites (S); s.d. standard deviation; Θ_π, estimation of the mutation parameter (Theta) from the mean number of pairwise differences (Pi); RI, average raggedness index.

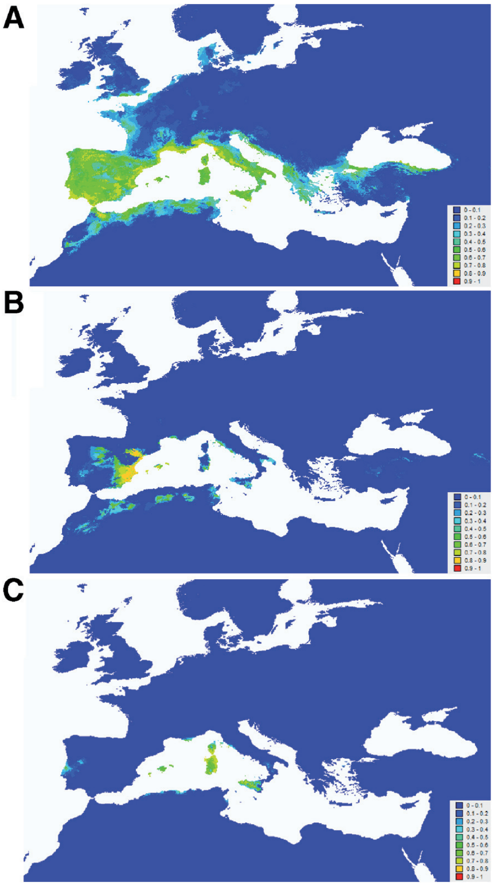

Figure 6 Environmental niche models (ENMs) using MaxEnt for A) *A. brunneus*; B) *A. ramblae*; and C) *A. rufulus*. Higher MaxEnt probabilities (red colours) represent areas more suitable for the species according to the MaxEnt models, lower values (blue) represent less suitable areas. These continuous measures of habitat suitability were used in ENM-based tests of niche similarity.

known locality was outside the potential LGM distribution (Alcampell, in the province of Huesca), which was also an outlier in the representation of the climatic niche of the species (Additional file 14: Figure S7). For *A. brunneus* some Pyrenean localities, those on the north coast of France and the south coast of Britain, north Italy and the French Massif Central were outside the LGM potential distribution (Figure 8), but most of its current distribution corresponded to its potential range in the LGM. On the contrary, for both species the estimated potential present day distribution was much wider than the actual range: for *A. brunneus* it included the whole Mediterranean area and most of Europe, and for *A. ramblae* most of the Mediterranean and central France (Figure 8).

Discussion

Species limits within the *A. brunneus* complex – morphology, molecules and physiology

Our initial criterion for species recognition was the morphology of the aedeagus, in agreement with current taxonomy [16]. The simple measures used were able to unambiguously discriminate between *A. brunneus* and *A. ramblae*, but *A. rufulus*, the insular species, had an intermediate morphology for these characters, although the shape of the aedeagus in lateral view allows the unequivocal identification of this species [16]. The three species were clearly recovered as monophyletic with the nuclear marker (*H3*), with the exception of one female from the southeast of the Iberian Peninsula, characterised as *A. ramblae* but nested with other peninsular *A. brunneus*, which may represent a misclassified individual (in the same area both *A. brunneus* and *A. ramblae* can be found, Additional file 4: Table S2). In contrast to nuclear genes, the mitochondrial markers recovered a paraphyletic *A. ramblae* as ancestral to *A. brunneus* and *A. rufulus.* This paraphyly could be due to incomplete lineage sorting resulting from the recent evolution of the group [62-64], which is estimated to have diverged mostly within the last 0.5 MY –an insufficient time to reach reciprocal monophyly. However, in our uncalibrated tree for Agabini the estimated rate of *cox1* was approximately eight times higher than that of *H3*, which being nuclear, should have a longer coalescent time than mitochondrial genes [65]. A possible explanation could be a complete replacement of the *A. ramblae* mitochondrial genome by that of *A. brunneus* in the Iberian peninsula, due to an early introgression event which did not result in phenotypic change between North African and Iberian populations of *A. ramblae* (either morphological, ecological or in thermal tolerance). The clear morphological separation between the two species argues against continued events of gene flow between them, which if present should have produced a higher frequency of specimens with intermediate morphologies. But given the low variability among the species of the *A. brunneus* complex in the *H3* fragment, this difference in coalescent times could also be simply due to random effects.

There is some additional evidence of a mismatch between morphology and some of the genetic markers used that suggests occasional introgression, but this is only seen in geographically marginal areas: Sardinia (between *A. rufulus* and *A. brunneus*), Menorca (between *A. ramblae* and *A. brunneus*), and possibly Tunisia (also between *A ramblae* and *A. brunneus*). Mitochondrial haplotypes of *A. brunneus* were found in individuals identified by morphology and nDNA as *A. ramblae* in Menorca, and *A. rufulus* in Sardinia. This could be due to a secondary colonisation of the islands by continental *A. brunneus*, similarly to what has been described in a related genus of

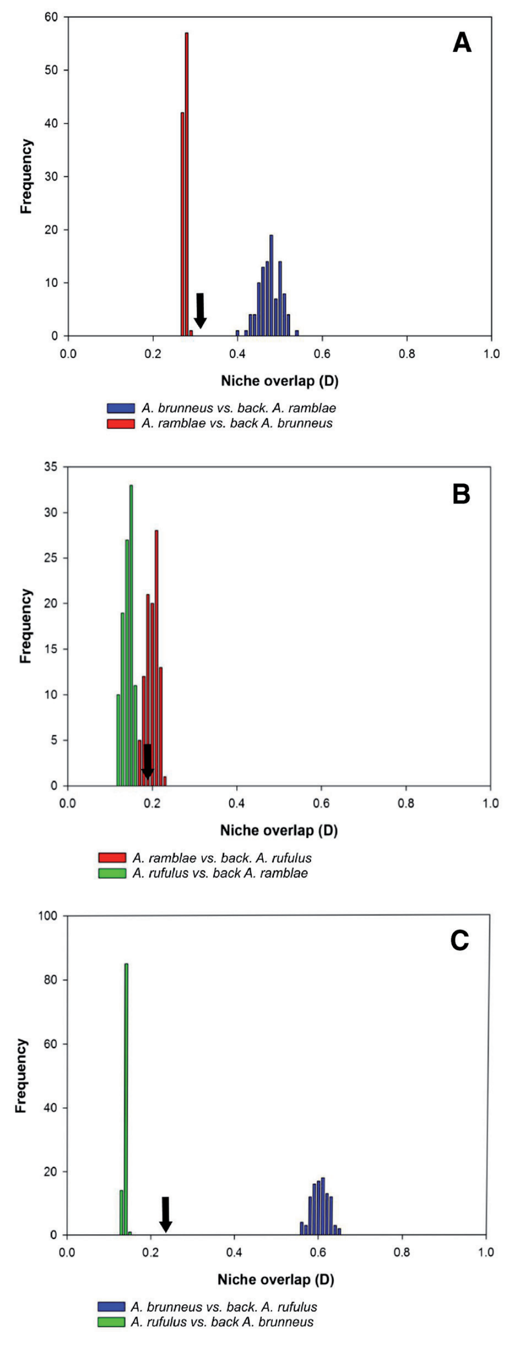

Figure 7 Background similarity test results for species within the *Agabus brunneus* species complex. Observed niche overlap values (arrows) were compared with null distributions (100 replicates) generated by comparing model suitability values of one species to those generated from random cells drawn from the background area of the other species.

diving beetle (*Meladema*) in the Canary Islands [66]. In Tunisia, southern populations were characterized as *A. ramblae* by both morphology and nDNA, but the mtDNA clustered with *A. brunneus* and Iberian haplotypes of *A. ramblae*. Two possible scenarios may account for such results: Tunisian *A. ramblae* could have arrived directly from the Iberian peninsula, which seems unlikely given the geographical distance and the presence of sea barriers; or they could have arrived from elsewhere in North Africa, and then hybridised with northern *A. brunneus* either also from North Africa or from Sicily. A Moroccan origin is supported by the presence of other water beetles typical of arid or saline habitats with a similar distribution through central and south Morocco to south Tunisia, such as *Enochrus risii* Arribas et al. [67] or *Ochthebius salinator* Peyerimhoff [68]. The situation of *A. ramblae* in the Iberian peninsula is more complex and difficult to interpret: some specimens had mitochondrial haplotypes clustering with those of *A. ramblae* from Morocco, suggesting the persistence within Iberia of some of the ancestral Moroccan haplotypes and a derived origin of most of both the Iberian *A. ramblae* and *A. brunneus*. But again, the replacement of the *A. ramblae* mitochondrial genome by that of *A. brunneus* through introgression in secondary contact zones cannot be discarded.

The two species with broadly overlapping ranges, *A. brunneus* and *A. ramblae*, were also ecologically different as measured through ecological niche modelling and the background test. Our experimental results show that the greater resistance of *A. brunneus* to lower temperatures may have been a key feature to allow its range expansion during the LGM (see below). This difference was reflected in the significantly lower minimum temperature of the coldest month of the places in which *A. brunneus* is currently found. This species had also significant differences in thermotolerance between populations, with the northern one (with an average lowest temperature of the coldest month of 2.8 °C) being more resistant to cold than the southern population (average lowest temperature of the coldest month of 6.0 °C). With our data it is not possible to discriminate whether this difference results from local adaptation or phenotypic plasticity, although Calosi et al. [17] found that members of the group did not significantly adjust their LTL after a short period of acclimation in the laboratory. This suggests that *A. brunneus* populations may instead adapt to local temperature conditions,

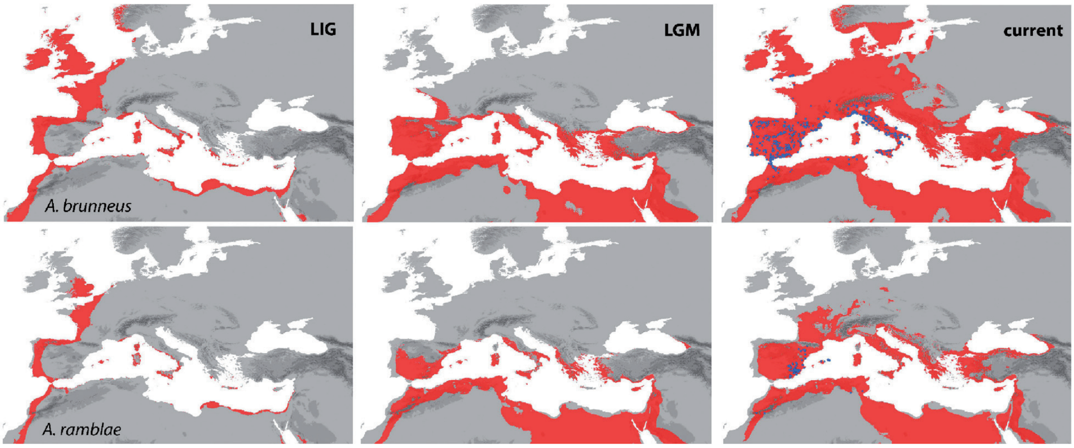

Figure 8 Estimated potential distribution for *A. brunneus* and *A. ramblae* during the last interglacial (LIG), last Glacial Maximum (LGM) and the present. In red, areas considered to be climatically suitable for the species (according to the ecological conditions of the localities in which they are currently found). Blue dots, current localities of the species.

these evolutionary changes possibly facilitating range expansions.

Evolutionary history of the *A. brunneus* complex

We found strong support for the monophyly of the *A. brunneus* complex, and also recovered a monophyletic *A. brunneus* group, albeit with lower support. The long stem branch of the complex is atypical within the rest of Agabini, and as there are no known species worldwide that could be more closely related to it [28], it appears that the *A. brunneus* complex has a very isolated position amongst extant Agabini.

Although the support for the internal relationships of the *A. brunneus* complex was low, the selected mitochondrial topology recovered the Moroccan populations of *A. ramblae* as paraphyletic, suggesting an origin of the complex in western North Africa, with colonization of the Iberian peninsula ca. 0.5 Ma. As seen above, much of the likely recent introgression between the species of the group is restricted to a few populations in secondary contact zones, so we do not expect this to affect the mitochondrial phylogeny. Even if some paraphyly was due to early introgression within the Iberian peninsula this would not affect our biogeographic scenario. The colonization of the Balearic Islands and Corsica and Sardinia happened in a narrow temporal window, possibly from Iberia. Although we cannot discard a direct colonization from North Africa, this seems less plausible due to the longer geographical distances involved. According to our estimation, *A. brunneus* split from SE Iberian *A. ramblae* ca. 0.25 Ma. An alternative scenario is that *A. brunneus* originated in Morocco and colonized the Iberian Peninsula in parallel with *A. ramblae*, but as well as being less parsimonious this hypothesis seems less plausible since some Iberian *A. ramblae* have mitochondrial haplotypes clustering with those from Morocco.

The ecological and physiological differences between *A. brunneus* and *A. ramblae* may have originated during the speciation process or evolved later, with an initial separation only due to isolation. Either way, at some point *A. brunneus* acquired the capacity to resist colder temperatures.

The demographic analyses estimated a population expansion of the complex at the start of the last glaciation 30–40,000 YBP, in agreement with the extension of their potential distributions during the last glacial maximum (LGM, 21,000 YBP). For both widespread species, potential distributions during the LGM covered practically the totality of their current ranges, and were mostly determined by minimum temperatures and climatic seasonality. It is remarkable that only very few current known localities (mostly for *A. brunneus*) are outside the reconstructed potential range of both species during the LGM, despite a large increase in apparently suitable geographical areas both in Europe and north Africa. During the LGM sea levels could have been up to 200 m lower, probably extending the suitable surface and potentially favouring the expansion of the continental species of the group to areas now isolated by sea barriers, such as south Britain.

The current absence of *A. brunneus* from central and northern Europe cannot be attributed to the effect of anthropogenic habitat modification, as there are no historic or Quaternary fossil records of the species outside its current range [24]. The external appearance of species of the *A. brunneus* complex is very characteristic, so that even incomplete remains would be recognizable, and in central and northern Europe the fossil record from the LGM and the Holocene is very complete [24]. Possible explanations for the absence of range expansion in *A. brunneus* and *A. ramblae* after the LGM are the presence of undetected climatic, biotic or ecological limiting factors, or simply a lack of sufficient time for these species to arrive at equilibrium with their potential ranges. All species of the *A. brunneus* complex are exclusively found in running waters, and such lotic taxa have, in general, weaker dispersal abilities than their lentic relatives, leading to a stronger mismatch between their realized and potential distributions in central and north Europe [66,69].

Conclusions

Using a combination of morphology, genetics, ecological niche modelling of current and paleoecological data and physiological experiments we have reconstructed the surprisingly complex evolutionary history of this diving beetle clade in the western Mediterranean. The *A. brunneus* complex diversified ca. 0.6-0.25 Ma, most likely in the south of the Iberian peninsula after the colonization of *A. ramblae* from north Morocco. Whilst insular populations (*A. ramblae* in the Balearic Islands and *A. rufulus* in Corsica and Sardinia) did not apparently differentiate substantially in either morphology or ecology, continental *A. brunneus* evolved the most distinctive morphology within the complex, as well as wider tolerance to cold habitats, something that seems to have facilitated range expansion.

From a reduced potential distribution during the LIG, *A. brunneus* and *A. ramblae* appear to have expanded their ranges during the last glacial (0.03-0.01 Ma) (*A. brunneus* to a much wider area), covering most of their LGM potential rages in the western Mediterranean. This expansion was accompanied by a population expansion, as identified through demographic models. However, despite much wider current potential distributions, both species have not occupied areas beyond their LGM potential distribution except for some isolated populations of *A. brunneus* in France and England. In Sardinia, the Balearic Islands and possibly Tunisia, secondary contact between species of the complex has resulted in introgression, with some specimens showing discordance between mitochondrial haplotypes typical of *A. brunneus* and nuclear sequences and morphology typical of *A. rufulus* or *A. ramblae* respectively.

Our work highlights the complex dynamics of speciation and range expansions within refugia during the last glacial cycle, and the fact that the biota of southern Europe, in addition to being a source of colonisers of formerly glaciated areas in the north, experienced much evolutionary change during this time period. It also highlights the fundamental but often neglected role of North Africa as source of biodiversity in Europe [70-74].

Availability of supporting data

All raw data are included in the Supplementary files with the exception of the sequences, deposited in the EMBL database with accession numbers LM654767-LM655064 and LM655068-LM655168.

Additional files

Additional file 1: Figure S1. Measures used for the identification of the specimens. A) Median lobe of the aedeagus of *A. ramblae* in ventral view, with the measurements used. The global measure of asymmetry used was AD = RD-LD. B) Maximum body length (BL, excluding head) and width (BW).

Additional file 2: Table S1. List of specimens with morphometric measurements. Ref., reference of the specimen: with AH and AI, voucher numbers of extracted specimens (see Additional file 4: Table S2); BM, other material not used for DNA extraction. BL, body length (from anterior side of prototum to apex of elytra); BW, maximum body width; AL, length of the aedeagus; AD, asymmetry of the aedeagus (see Additional file 1: Figure S1).

Additional file 3: Table S5. Thermal tolerance data. Population: SPA-Cádiz: Spain, Cádiz (data from ref.[17]); SPA-Girona: Spain, Girona (42°08'48"N 2°34'48"E); SPA-Murcia: Spain, Murcia (data from ref.[17]); MOR-Tinghir: Morocco, Tinghir (31°33'25"N 5°34'49"W); ITA-Sardinia: Italy, Sardinia (data from ref.[17]).

Additional file 4: Table S2. List of the specimens used for DNA extraction, with accession numbers of the sequences. Extraction method: Charge, Charge Switch gDNA Tissue Kit (Invitrogen, Carlsbad, USA); Invisorb, Invisorb Spin DNA Extraction Kit (Invitek GmbH, Berlin, Germany); Qiagen, Qiagen DNeasy Tissue Kit (Qiagen GmbH, Hilden Germany). In the column 16S, with asterisk: single fragment including the 3′ end of *rrnL*, the full *trnL* and the 5′ end of *nad1*; in the rest either the fragment is divided in two sequences or includes only the *rrnL* fragment.

Additional file 5: Table S3. List of primers used and PCR conditions.

Additional file 6: Table S4. Climatic variables used in the niche modelling.

Additional file 7: Table S6. Localities used for the niche modelling of the three species. BD (BIODIV), unpublished database of the Department of Ecology and Hydrology, University of Murcia (Spain); CKmap, Checklist and distribution of Italian fauna (http://www.faunaitalia.it/ckmap/); ESACIB, database “Escarabajos Acuáticos Ibéricos” (Sánchez-Fernández et al. 2008); GBIF, Global Biodiversity Information Facility (http://www.gbif.org); NBN Gateway, National Biodiversity Network of United Kingdom (http://www.nbn.org.uk).

Additional file 8: Figure S2. Background area used for each species in the background similarity test. A) *Agabus brunneus*; B) *A. ramblae*; C) *A. rufulus*.

Additional file 9 Text S1. Method used to obtain the potential distribution of species.

Additional file 10: Figure S3. Bivariant plot of the measures of the median lobe of the aedeagus. Open circles, *Agabus ramblae*; grey diamonds, *A. brunneus*; black triangles, *A. rufulus*. Open square: male *A. rufulus* from Sardinia with an *A. brunneus* mitochondrial haplotype.

Additional file 11: Figure S4. Ultrametric tree of Agabini obtained in BEAST using only mDNA data, constraining the monophyly of the ingroup and outgroup (genus *Platynectes*), the genera and the *A. brunneus* complex. To calibrate the tree we used an a-priori rate of 0.01 substitutions/site/MY (see text for details). Numbers in nodes in black font, posterior probabilities (above 0.5); in red, estimated age.

Additional file 12: Figure S5. Phylogenetic analyses of the *cox1* data. Calibrated tree obtained in BEAST, using a mean rate of 0.02+/−0.001 substitutions/site/MY. Small numbers in nodes, estimated age (Ma), large numbers in nodes, posterior Bayesian probabilities (pp). Negative branches collapsed in polytomies. In red, specimens likely to have introgressed mitochondrial DNA from *A. brunneus*.

Additional file 13: Figure S6. Phylogram obtained in RAxML with the H3 sequences. Numbers in nodes, posterior probabilities obtained in BEAST (if above 0.5) / bootstrap support (if above 50%). With an asterisk, female from Albacete (SE Spain) of uncertain identity.

Additional file 14: Figure S7. Representation of the scores of the two first axis of the PCA with all climatic variables. Grey surface, climatic space of the western Palaeartic (background). In colours, climatic space occupied by the three species.

Additional file 15: Figure S8. Reconstructed seasonality and minimum temperature of the coldest month during the last glacial interval (upper row) and the last glacial maximum (lower row). Blue circles, current distribution of *A. ramblae*.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

AHG, AC and IR conceived the study. AHG and IR coordinated the sampling. AHG obtained most the sequences and the morphometric and distribution data. AHG and DTB conducted the physiological experiments. AHG, DSF and IR analysed the data. All authors contributed to the writing and improving the manuscript, and approved the final version.

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Supplementary information index:

Additional file 1: Figure S1: http://www.biomedcentral.com/content/supplementary/s12862-014-0187-y-s1.pdf

Additional file 2: Table S1: http://www.biomedcentral.com/content/supplementary/s12862-014-0187-y-s2.xlsx

Additional file 3: Table S5: http://www.biomedcentral.com/content/supplementary/s12862-014-0187-y-s3.xlsx

Additional file 4: Table S2: http://www.biomedcentral.com/content/supplementary/s12862-014-0187-y-s4.xlsx

Additional file 5: Table S3, page 35

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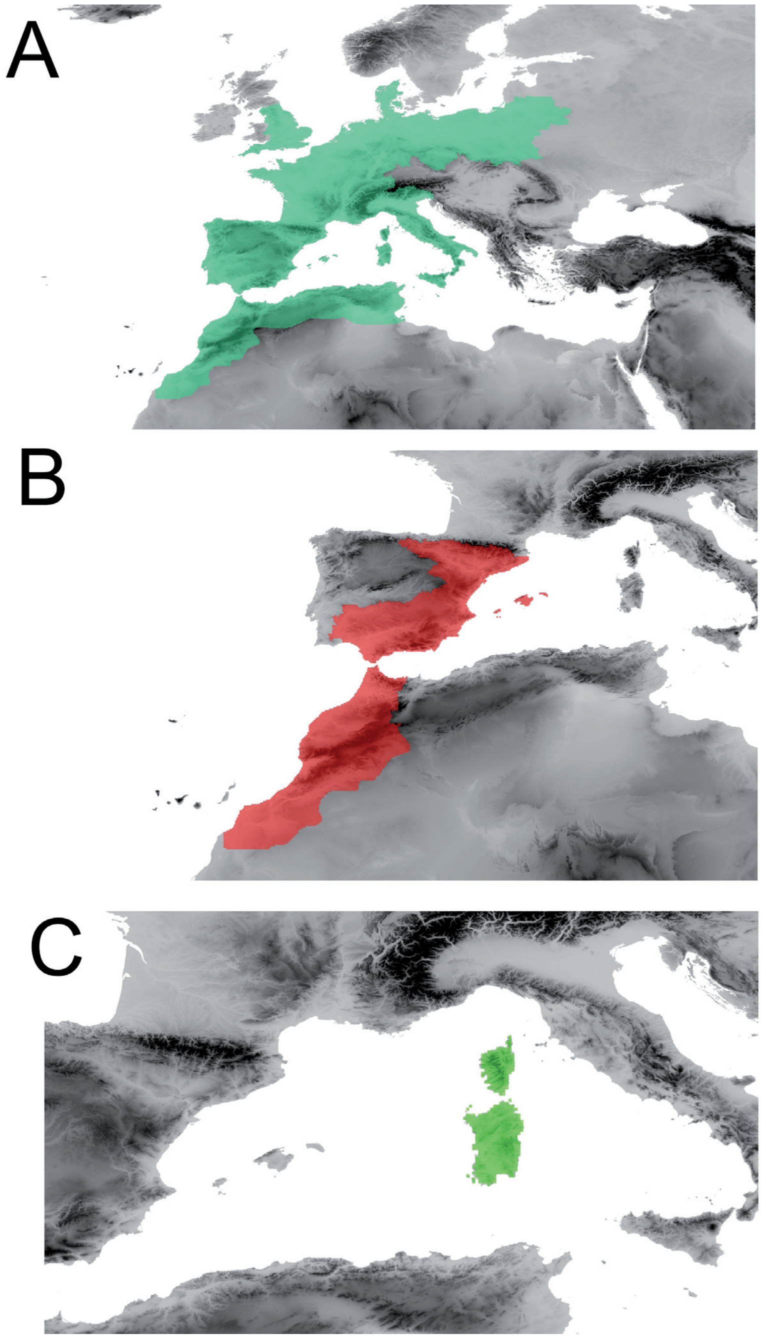
SUPPORTING INFORMATION

Gene	Primer	F/R	Sequence	Reference	PCR conditions
cox1	Jerry (5')	F	5' CAACATTTATTTTGATTTTTTGG	Simon *et al.*, 1994	96º C 3' [94º C 30'' - 47º C 30'' - 72º C 1'] x 40 72º C 10'
	Pat (3')	R	5' TCCAATGCACTAATCTGCCATATTA	Simon *et al.*, 1994	
	Chy1 (5')	F	5' T(A/T)GTAGCCCA(T/C)TTTCATTA(T/C)GT	Ribera et al. 2010	
	Tom1 (3')	R	5' AC(A/G)TAATGAAA(A/G)TGGGCTAC(T/A)A	Ribera et al. 2010	
rrnL-trnL-NAD1	16SaR (=M14)	F	5' CGCCTGTTTAACAAAAACAT	Simon *et al.*, 1994	96º C 3' [94º C 30'' - 48º C 1'' - 72º C 1'] x 35 72º C 10'
	16Sb	R	5' CCGGTCTGAACTCAGATCATGT	Simon *et al.*, 1994	
	ND1A (=M223)	R	5' GGTCCCTTACGAATTTGAATATATCCT	Simon *et al.*, 1994	
H3	H3aF	F	5' ATGGCTCGTACCAAGCAGAC(A/G)CG	Colgan *et al.*, 1998	96º C 3' [94º C 30'' - 50º C 30'' - 72º C 1'] x 40 72º C 10'
	H3aR	R	5' ATATCCTT(A/G)GGCAT(A/G)AT(A/G)GTGAC	Colgan *et al.*, 1998	
SSU	18S 5'	F	5' GACAACCTGGTTGATCCTGCCAGT	Shull *et al.*, 2001	96º C 3' [94º C 30'' - 50º C 30'' - 72º C 1'] x 40 72º C 10'
	18S b5.0	R	5' TAACCGCAACAACTTTAAT	Shull *et al.*, 2001	

Additional file 5: Table S3. List of primers used and PCR conditions.

Code	Variable
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

Additional file 6: Table S4. Climatic variables used in the niche modelling. Source: http://www.worldclim.org (Hijmans et al. 2005)



Additional file 8: Figure S2 Background area used for each species in the background similarity test. A) *Agabus brunneus*; B) *A. ramblae*; C) *A. rufulus*.

Additional file 9: Text S1. Method used to obtain the potential distribution of species.

Assuming that presence localities reflect a subset of the suitable conditions under which a species can maintain viable populations, environmental envelope is an approach directed at maximizing the capacity to represent geographically the potential distribution of species when they are only based on distributional data (see Jiménez-Valverde et al., 2011; Sánchez-Fernández et al., 2013 for an application of this procedure). In this procedure, the maximum and minimum scores (extreme values) for all relevant climatic variables from the entire set of observed presence cells are first calculated for each species. Then, all grid cells with climatic values falling within the mentioned range are designated as suitable, and all cells outside it as unsuitable. In this way, the extreme values are used to derive a binary distributional hypothesis about the areas having climatically suitable conditions (climatic potential distribution), under the assumption that recorded occurrences reflect the spectrum of climatic conditions in which the species can We consider relevant variables as the minimum set of climatic variables needed to explain the occurrence of each species as estimated using an ecological-niche factor analysis (ENFA; Hirzel et al., 2002; Basille et al., 2008). Factors were retained or discarded based on their eigenvalues relative to a broken-stick distribution (Hirzel et al., 2002). Climatic variables selected as relevant were those showing the highest correlation values with the retained ENFA factors. In our case, the same four variables (Temperature Seasonality; Max. Temp of the warmest month; Min. Temp of the coldest month and Thermal Annual Range) were selected for both species.

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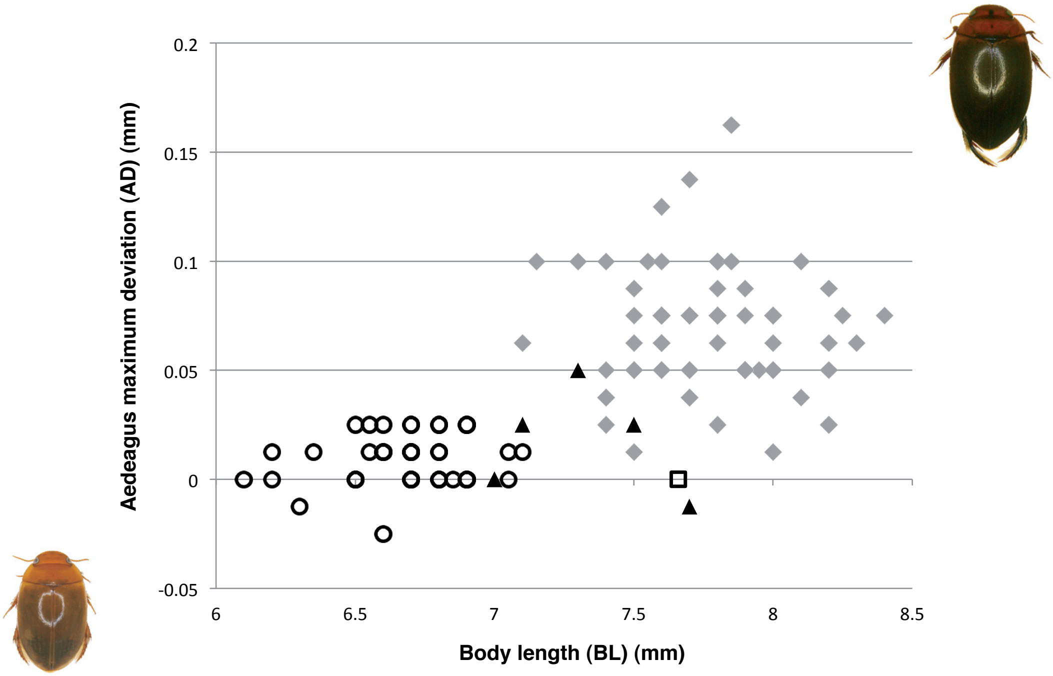
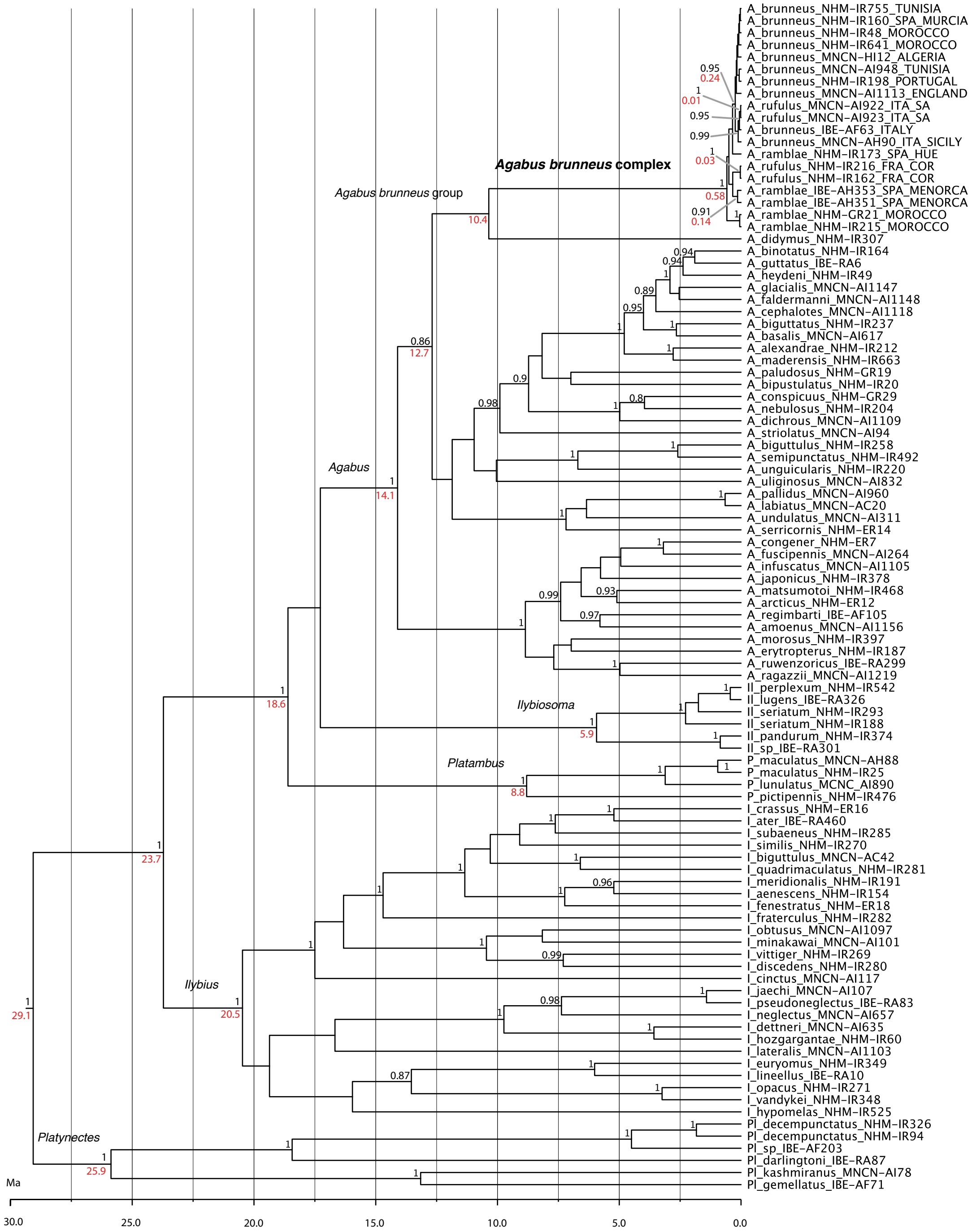
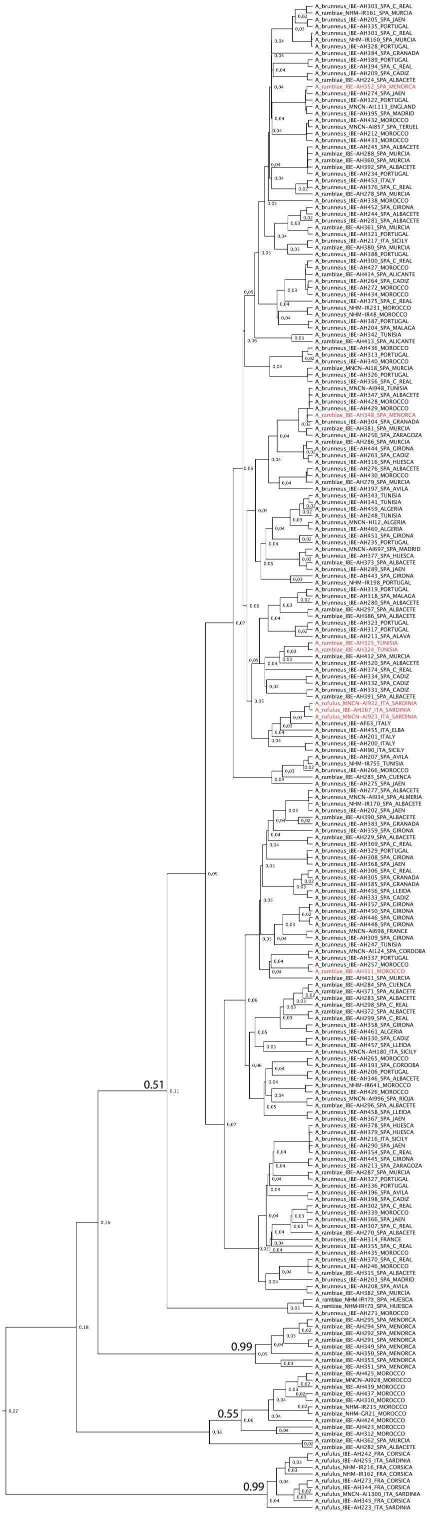
Hirzel, A.H., Hausser, J., Chessel, D. & Perrin, N. (2002) Ecological-niche factor analysis: how to compute habitat suitability maps without absence data? Ecology, 83, 2027–2036.



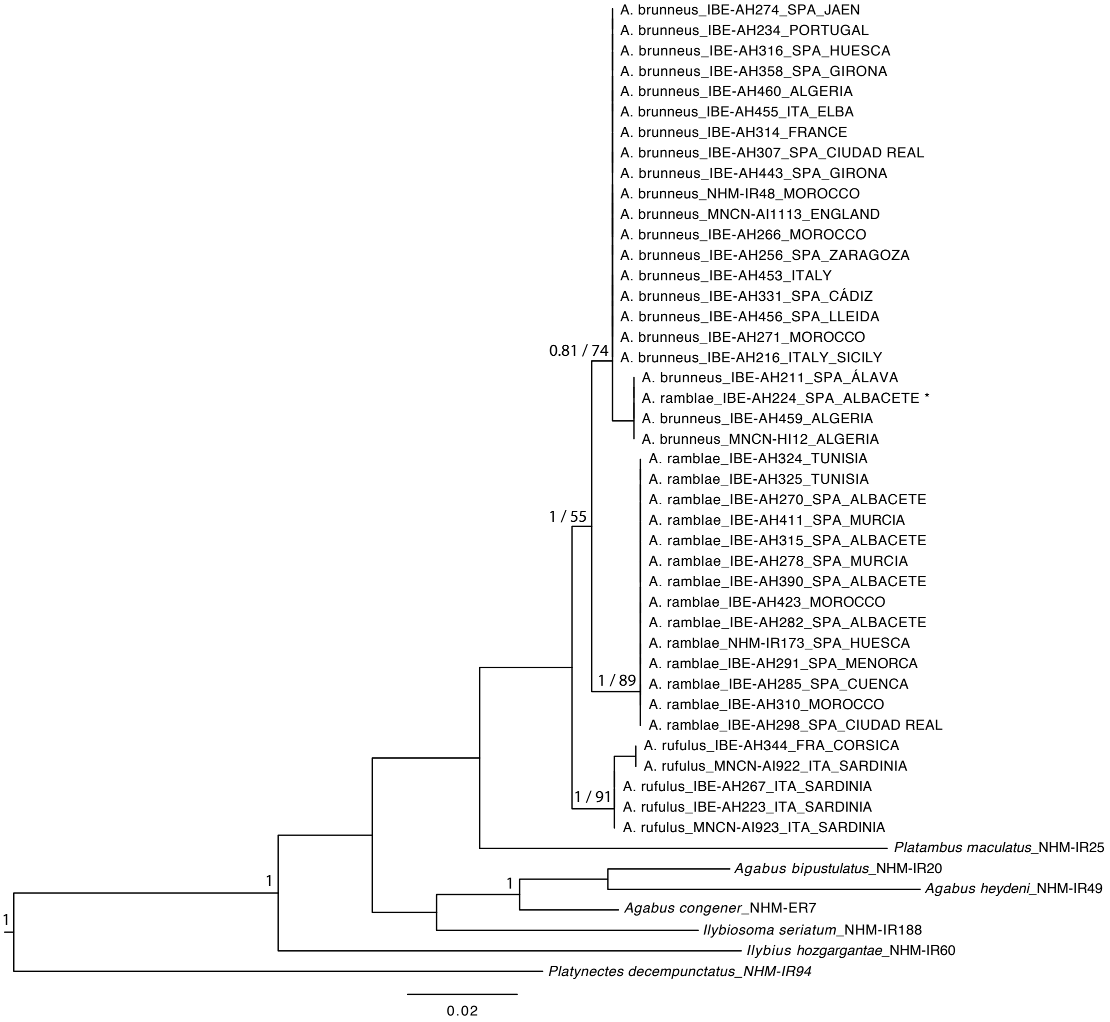
Additional file 10: Figure S3 Bivariant plot of the measures of the median lobe of the aedeagus. Open circles, *Agabus ramblae*; grey diamonds, *A. brunneus*; black triangles, *A. rufulus*. Open square: male *A. rufulus* from Sardinia with an A. brunneus mitochondrial haplotype.



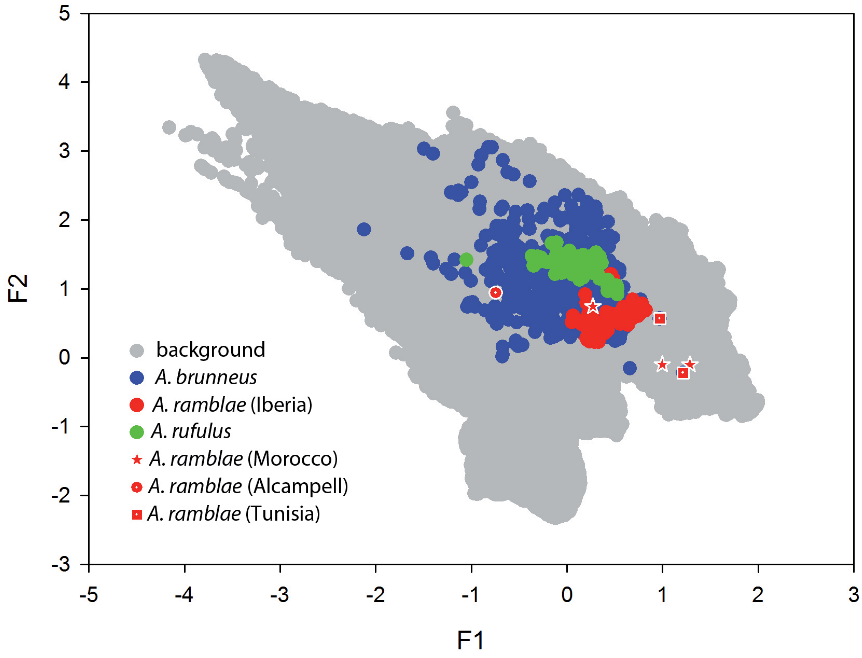
Additional file 11: Figure S4 Ultrametric tree of Agabini obtained in BEAST using only mDNA data, constraining the monophyly of the ingroup and outgroup (genus Platynectes), the genera and the *A. brunneus* complex. To calibrate the tree we used an a-priori rate of 0.01 substitutions/site/MY (see text for details). Numbers in nodes in black font, posterior probabilities (above 0.5); in red, estimated age.



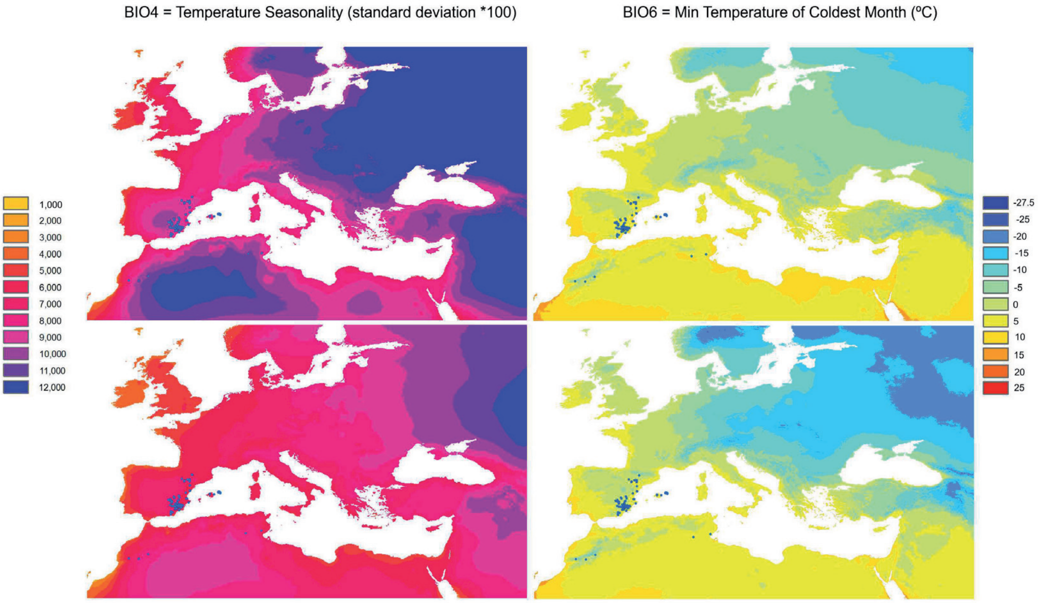
Additional file 12: Figure S5 Phylogenetic analyses of the cox1 data. Calibrated tree obtained in BEAST, using a mean rate of 0.02+/□0.001 substitutions/site/MY. Small numbers in nodes, estimated age (Ma), large numbers in nodes, posterior Bayesian probabilities (pp). Negative branches collapsed in polytomies. In red, specimens likely to have introgressed mitochondrial DNA from *A. brunneus.*



Additional file 13: Figure S6 Phylogram obtained in RAxML with the H3 sequences. Numbers in nodes, posterior probabilities obtained in BEAST (if above 0.5) / bootstrap support (if above 50 %). With an asterisk, female from Albacete (SE Spain) of uncertain identity.



Additional file 14: Figure S7 Representation of the scores of the two first axis of the PCA with all climatic variables. Grey surface, climatic space of the western Palaeartic (background). In colours, climatic space occupied by the three species.



BIO4 = Temperature Seasonality (standard deviation *100)

BIO6 = Min Temperature of Coldest Month (ºC)

Additional file 15: Figure S8 Reconstructed seasonality and minimum temperature of the coldest month during the last glacial interval (upper row) and the last glacial maximum (lower row). Blue circles, current distribution of *A. ramblae.*

Chapter 2:

Reproducibility and consistency of proteomic experiments on natural populations of a non-model aquatic insect species

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Reproducibility and Consistency of Proteomic Experiments on Natural Populations of a Non-Model Aquatic Insect

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Abstract

Population proteomics has a great potential to address evolutionary and ecological questions, but its use in wild populations of non-model organisms is hampered by uncontrolled sources of variation. Here we compare the response to temperature extremes of two geographically distant populations of a diving beetle species (*Agabus ramblae*) using 2-D DIGE. After one week of acclimation in the laboratory under standard conditions, a third of the specimens of each population were placed at either 4 or 27°C for 12 h, with another third left as a control. We then compared the protein expression level of three replicated samples of 2–3 specimens for each treatment. Within each population, variation between replicated samples of the same treatment was always lower than variation between treatments, except for some control samples that retained a wider range of expression levels. The two populations had a similar response, without significant differences in the number of protein spots over- or under-expressed in the pairwise comparisons between treatments. We identified exemplary proteins among those differently expressed between treatments, which proved to be proteins known to be related to thermal response or stress. Overall, our results indicate that specimens collected in the wild are suitable for proteomic analyses, as the additional sources of variation were not enough to mask the consistency and reproducibility of the response to the temperature treatments.

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Introduction

The comparison of natural populations using proteomic methods, which has been termed "population proteomics" [1,2] has a high potential to address fundamental questions in ecology and evolutionary biology, as it allows us to directly link environmental conditions to changes in protein expression [3–6]. Proteomic methods are especially suited to understanding phenotypic changes induced by the environment, since they enable detection of alterations affecting physiologically significant protein expression and modification, rather than changes in mRNA expression levels [7–9]. When applied to different populations exposed to varying environmental conditions, differences in proteome expression are likely to be directly linked to a physiological response [1,10,11].

Although proteomic studies of non-model organisms are increasingly common (see e.g. [8] for review), in most cases specimens are kept in the laboratory under controlled conditions, as the use of specimens directly taken from their natural environment poses an additional challenge [12]. Many unknown factors, such as the genetic background of the individuals, their age, the physiological state, the presence of parasites or other pathogens may introduce variation of unpredictable importance [13–15]. Thus, it seems that previous to any comparative study of wild populations it is necessary to estimate the degree of variability due to unknown or unforeseeable sources of variation, and to assess both the reproducibility and consistency of the protein expression data.

In the study presented here we approach this problem using two natural populations of a diving beetle species (*Agabus ramblae* Millán & Ribera). This species has a disjunct distribution in the South and East of the Iberian Peninsula and Central Morocco, and is usually found in highly mineralized, temporary running waters [16]. It belongs to a complex of three closely related species (the *Agabus brunneus* group [16]) distributed in the western Mediterranean, which most likely diversified during the Pleistocene, within the Iberian peninsula [17]. Variation in protein expression was quantitatively assessed with 2-D Differential Gel Electrophoresis (2D-DIGE). The experimental setup also included investigation of temperature induced protein expression, as temperature is one of the most important abiotic factors known to influence a wide range of physiological reactions [18–20]. In the evolutionary lineage the studied species belongs to, thermal tolerance is known to be related to the size of the geographical range [21]. In the context of a wider study of ecological segregation and speciation in *A. ramblae* and its most closely related species, we studied the response of two geographically separated populations (in Central Morocco and Southern Spain) to two temperatures at the extremes of what they may experience in the field. Our main interest was to assess the possibility of comparing the overall protein expression of wild populations subjected to different temperature treatments, without being overwhelmed by confounding variation. We specifically aimed to determine the variability of 1) technical replicas, by comparing the internal standards of each experiment; 2) biological replicas, by comparing pooled samples of several individuals of the same population exposed to the same treatment (referred to as "replicated samples"); 3) temperature treatment, by comparing the different treatments within the same population; and 4) the response of the specimens of two different populations to the same treatment.

Methodology

Studied populations, acclimation

In order to determine potential differences in response to temperature treatment, two natural populations of the diving beetle *Agabus ramblae* (Coleoptera, Dytiscidae, size range 7–9 mm) were used for the experiments: 1) Spain, Murcia, Corneros stream N37°42′10.7″ W1°55′33.8″ (30 adult specimens); and 2) Morocco, Tinghir, Toudgha river N31°33′25.1″ W5°34′49.5″ (24 adult specimens). *Agabus ramblae* is not included in any national or international list of protected or endangered species, and the two populations were in public land not covered by any special legal protection. No permits or ethical approval were required for the experimental procedures. The two populations were sampled in September 2007 and May 2011 respectively, and all specimens that could be found during a search of 2–3 h in the available microhabitats were collected and transported under similar conditions to the laboratory. Three specimens of the Spanish population were snap frozen in the field in liquid nitrogen, serving as a field control (FC).

Once in the laboratory, individuals were acclimated for one week in aquaria, with mineral water and some vegetation taken from the place of origin. Specimens were kept at room temperature (RT, always below 25°C), which was considered the control for these experiments, and with a natural day/night cycle.

Specimens were fed *ad-libitum* on frozen red Chironomidae larva from commercial sources (sold as fish food). After a week, an equal number of specimens were randomly allocated to each of three treatments for 12 h: 4°C, RT, and 27°C. This is within the range of temperatures the species are likely to experience under natural conditions (WorldClim_2.5 m database). After the treatment, specimens were snap frozen in liquid nitrogen, separated into three samples of 2–3 specimens for each temperature treatment and stored at -80°C. The number of specimens per replicated sample was limited by the total number of specimens available and the need of having at least three replicas per treatment for comparison. By using only 2–3 specimens we increased the potential variability between replicated samples, so it can be expected that by using more specimens this individual variability could be further reduced.

Protein extraction and sample preparation

Proteins of whole specimens were extracted in a solution of 9.5 M urea, 1% Dithiothreitol (DTT), 2% (3-cholamidopropyl)-dimethyl-ammonia (CHAPS) and 2% Pharmalyte$^{\mathrm{TM}}$ (pH gradient 3–11), using a mortar and liquid nitrogen to maintain the low temperature [22]. The samples were sonicated after extraction to break up nucleic acids. After centrifugation at 13,200 rpm for 2 min the supernatant was transferred into a new tube for further processing. Samples were precipitated using 2-D-CleanUp kit (GE/Amersham Biosciences, Freiburg, Germany) to remove interfering contaminants. The total protein was then resuspended in an appropriate volume of DIGE lysis buffer (Tris 30 mM, Urea 7 M, Tiourea 2 M, CHAPS 4%, HCl to reach pH 8.5). Samples were quantified with a Bio-Rad RCDC Protein Assay (Bio-Rad, Hercules, CA, USA).

Experiment design and DIGE analysis

An internal standard for each experiment was generated by pooling equal amounts of protein from each extraction. Five gels per experiment were run with the internal standard and two samples derived from different treatments.

Sample aliquots of 50 µg were labelled with Cy3 and Cy5 NHS ester and the pooled internal standard was labelled with Cy2 (GE Healthcare, Buckinghamshire, UK), according to the Ettan DIGE minimal labelling protocol (GE Healthcare). To avoid any possible bias due to labelling efficiency, the samples of each group were alternately labelled with both Cy3 and Cy5 dyes. The DIGE experiments followed the standard protocol as described in ref. [23]. Gel images were obtained with a Typhoon 9400 scanner (GE Healthcare). Images were scanned at 550/580, 560/620 and 525/555 nm excitation/emission wavelengths for the Cy2, Cy3 and Cy5 dies respectively, at 100 µm resolution. 2D-DIGE image analysis and statistical quantification of relative protein abundance were performed with Progenesis SameSpots v4.0 (Nonlinear Dynamics, Newcastle, UK). This software allows detecting, quantifying and matching of spots between gels after normalization to the internal standard. Statistically significant differences in protein expression between groups (temperature treatments) were tested with one-way ANOVA. To correct for multiple tests we used the false discovery rate correction (FDR) as implemented in SameSpots ($q<0.05$).

Statistical analyses

Gel images were aligned with reference to the internal standard, normalized, and the protein spots verified. For each experiment, pairwise comparisons of the expression level between replicated samples and between treatments were done, selecting those spots which indicated a significant difference according to the ANOVA analysis at $P<0.05$, $P<0.01$ and $P<0.001$ levels. The analyses included: 1) experimental variation due to technical error; 2) variation between replicated samples, in order to detect variability due to individual differences (genetic background, sex, age, physiological state); 3) variation between treatments within the same population; and 4) variation between treatments across populations. To determine the variation due to technical reasons, for each experiment the five images of the internal standard were compared using the 'single stain per gel' option in SameSpots software to detect false positives. We also computed the coefficient of variation (CV) among the spot volumes across the different replicas, averaged for all spots ($\mathrm{CV} = \mathrm{SD}/\mathrm{mean} \times 100$; [24]).

To assess the variability between replicated samples or treatments within the same population we calculated the distribution of the differences in expression of the same protein spot for each comparison, generating a matrix of protein expression data which showed significantly different level of expression at the selected P-level. With these matrixes we did a hierarchical cluster analysis using the Euclidean distance and Ward's amalgamation method (see [1] for comparison). We used the single available field control to have an estimate of the changes

Table 1. Protein yield obtained from each replicated sample.

population	Replicated sample	ind./replica	average prot(µg)/ind
SE Spain (Murcia)	FC	3	498,7
	RT_r1	3	603,6
	RT_r2	3	1343,4
	RT_r3	3	1362,8
	4_r1	3	1113,7
	4_r2	3	1376,7
	4_r3	3	1184,5
	27_r1	3	1130,6
	27_r2	3	1380,7
	27_r3	3	1045,5
C Morocco (Tinghir)	RT_r1	2	3829.3
	RT_r2	2	448.4
	RT_r3	2	1186.1
	4_r1	2	1193.4
	4_r2	2	1400.2
	4_r3	2	1197.9
	27_r1	2	2503.3
	27_r2	2	2969.5
	27_r3	2	1551.7

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introduced by the acclimation process, with a standardised food supply.

For a global comparison of treatments across populations we performed an ANOVA analysis for those protein spots exhibiting significant differences of expression level in the pairwise comparison between treatments within the same population. We considered *P*-level, temperature treatment and population as factors. All statistical analyses were done with Statistica version 7 (http://www.statsoft.com) and JMP v5.1 [25].

Protein selection and identification

Selection of protein spots. We selected and identified protein spots with different levels of expression between experiments as a proof of principle, to test the viability of our approach and methodology for the study of the thermal biology of *Agabus ramblae*. For this purpose, we set up a new analysis in SameSpots v4.0 by directly comparing all images. All proteins spots were first automatically selected and then manually checked for consistency and quality of image, with a final selection of 565 protein spots common to all experiments. We then compared the expression level of the 565 protein spots in the 27°C *vs* 4°C replicas, irrespective of the population of origin, as these were the ones most likely to show strong differences in protein expression. We used the normalized spot volumes to estimate fold changes, and compared the values for each spot using a one-way ANOVA with a cut-off absolute value of$>$1.3-fold ($P<0.05$, with FDR correction). The normalized volume of the protein spots with significant differences in expression was used in a Multiple Discriminant Analysis (MDA) to identify the protein spots that better discriminate between treatments. Finally, the selected protein spots were double-checked again in SameSpots v4.0, where the final selection was made.

Protein identification and Liquid chromatography-Mass spectrometric analysis. A new preparative gel was run to extract and identify target proteins. Three hundred micrograms of a mix of protein extracts from representative samples were Cy labelled and gels were scanned and images analysed as described above. The gel images were matched against the spots referenced in the picking list created after the detection of the significantly up- or down-regulated protein signals in the gels used for the analyses. The selected protein spots were excised from the gel using an automated Spot Picker (GE Healthcare), within-gel digestion with trypsin (Promega, Wisconsin, USA) as described in [26].

Extracted samples were analysed on a Maxis high resolution Q-TOF spectrometer (Bruker, Bremen), coupled to a nano-HPLC system (Proxeon, Denmark). After evaporation and dissolution in 5% acetonitrile 0.1% formic acid in water, samples were first concentrated on a 100 µm ID, 2 cm Proxeon nanotrapping column and then loaded onto a 75 µm ID, 15 cm Acclaim PepMap nanoseparation column (Dionex). Chromatography was run using a 0.1% formic acid - acetonitrile gradient (5–35% in 10 min; flow rate 300 nL/min). The column was coupled to the mass spectrometer inlet through a Captive Spray (Bruker) ionization source. MS acquisition was set to cycles of MS (1 Hz), followed by MS/MS (0.5–2 Hz) of the 8 most intense precursor ions with an intensity threshold for fragmentation of 5000 counts and using a dynamic exclusion time of 0.5 min. All spectra were acquired on the range 100–2200 Da. LC-MSMS data was analysed using the Data Analysis 4.0 software (Bruker).

Proteins were identified using Mascot (Matrix Science, London UK) by search on the NCBI database limiting the search to Other Metazoa (13,577,271 sequences; 4,662,347,403 residues). MS/MS spectra were searched with a precursor mass tolerance of 10 ppm, fragment tolerance of 0.04 Da, trypsin specificity with a maximum of 2 missed cleavages, cysteine carbamidomethylation set as fixed modification and methionine oxidation as variable modification.

Table 2. Comparison between the five internal standards within each experiment (population) to estimate technical variation.

population	ANOVA	Spots signif. diff. $P<0.05$	max	min	avrg
SE Spain	12*vs*345	97	13.7	1.1	1.9
(Murcia)	14*vs*235	49	3.9	1.1	1.8
	135*vs*24	95	4.0	1.1	1.8
C Morocco	12*vs*345	56	2.6	1.1	1.4
(Tinghir)	14*vs*235	48	2.6	1.1	1.5
	135*vs*24	46	2.1	1.1	1.4

ANOVA, different groupings of the internal standards for the ANOVA test (see main text). Values, fold change.

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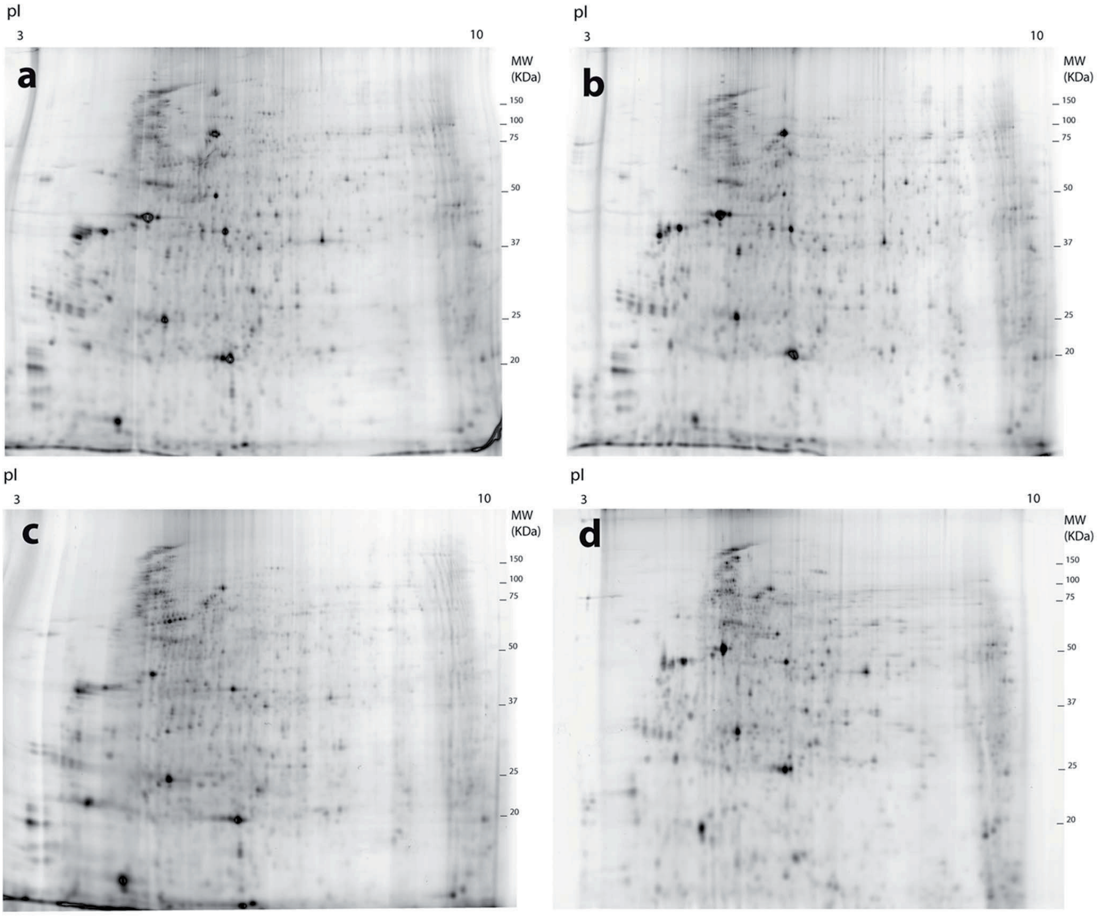

Figure 1. Example images of 2D-DIGE gels representing populations and temperature treatments. a) Spanish population, 4°C treatment, Cy5; b) Spanish population, 4°C treatment, Cy3; c) Spanish population, 27°C treatment, Cy3 and d) Moroccan population, 27°C treatment, Cy3. Differences between a) and b) correspond to variation between replicated samples; a) and c) different treatments within the same population; and c) and d) same treatment between different populations. pI, isoelectric point; MW molecular weight (Kilo Daltons).

doi:10.1371/journal.pone.0104734.g001

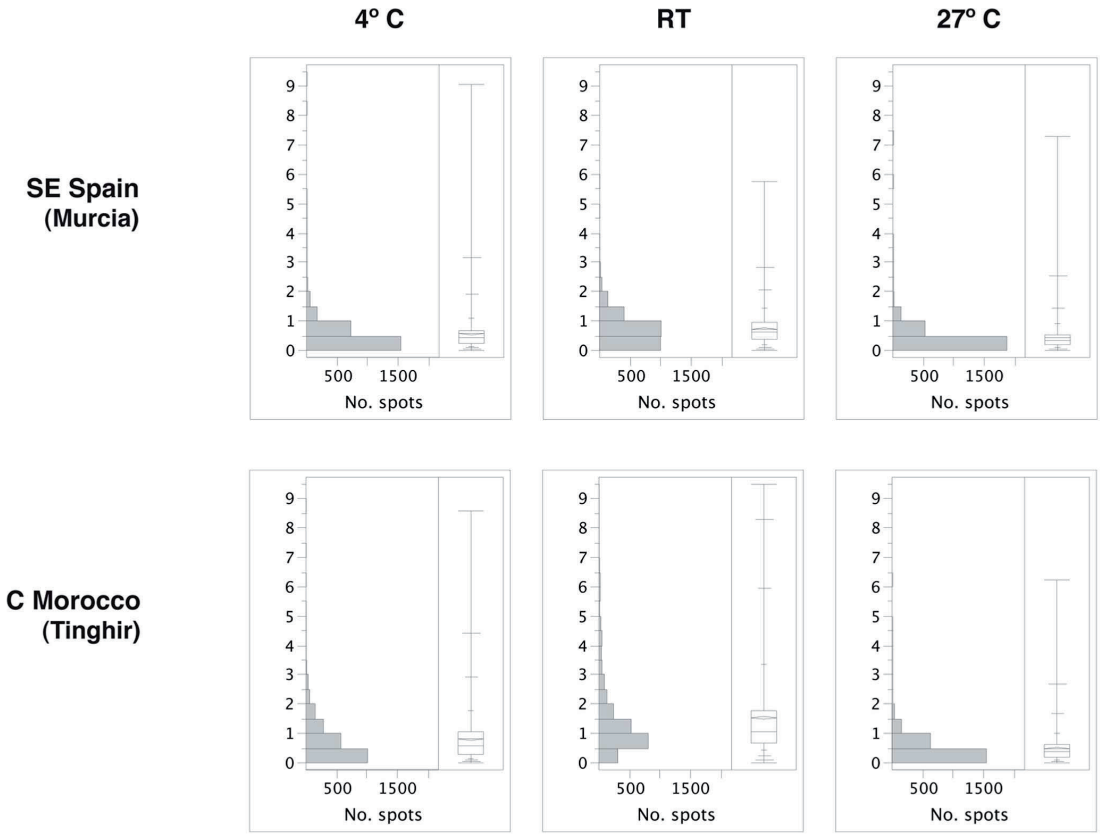

Figure 2. Distribution of differences between the three replicated samples of each treatment. Data reflect normalised protein spot volume. On the right of each graph the quantile box plot reflects the distribution of the variation, with mean (rhomboid) and median. Vertical axis, fold change.

doi:10.1371/journal.pone.0104734.g002

Results and Discussion

Specimens of *A. ramblae* weighed between 35–75 mg. The average amount of total protein per specimen was ca. 1500 µg, with no significant differences between populations (2-tail t-test, $P>0.1$; Table 1).

Technical variation

The five images of the internal standards were compared in both populations to detect the technical variation of the experimental setup using the single stain option. When the images were grouped in different combinations, the number of protein spots with significantly different levels of expression at a statistical threshold of $P<0.05$ ranged between 2–4% (Table 2). This rate of false positives could be considered as technical error in our experimental setup. The technical variation estimated by comparison of the internal standards is in fact an overestimation, as it is not corrected by the normalization. The maximum value of the coefficient of variation (CV) between technical replicas was 35%, within the standard range for 2-DE experiments (20–40%, [27]).

Reverse labelling was used to minimize any possible bias due to preferential labelling with one of the Cy dyes. When images of samples belonging to the same group and labelled alternatively with Cy3 or Cy5 were compared, no significant differences were observed. This behaviour is consistent with previous reports that have shown that labelling is only a very minor source of variability in DIGE experiments [28].

Variation between replicated samples

Differences between individual histories and circumstances are one of the major sources of variation in expressed physiological traits [29]. We tried to minimize this variation by including several specimens per replica [30,31], as we wanted to assess population-level, not individual responses to thermal stress or other environmental factors. As noted in the methods, the final number of specimens per replicated sample was a trade-off between the availability of specimens and the need for replicas in the 2-DE experiments.

The distribution of the differences in protein expression between the replicated samples of the same treatment were very similar in the two populations (Figures 1, 2). Among the samples of the 4°

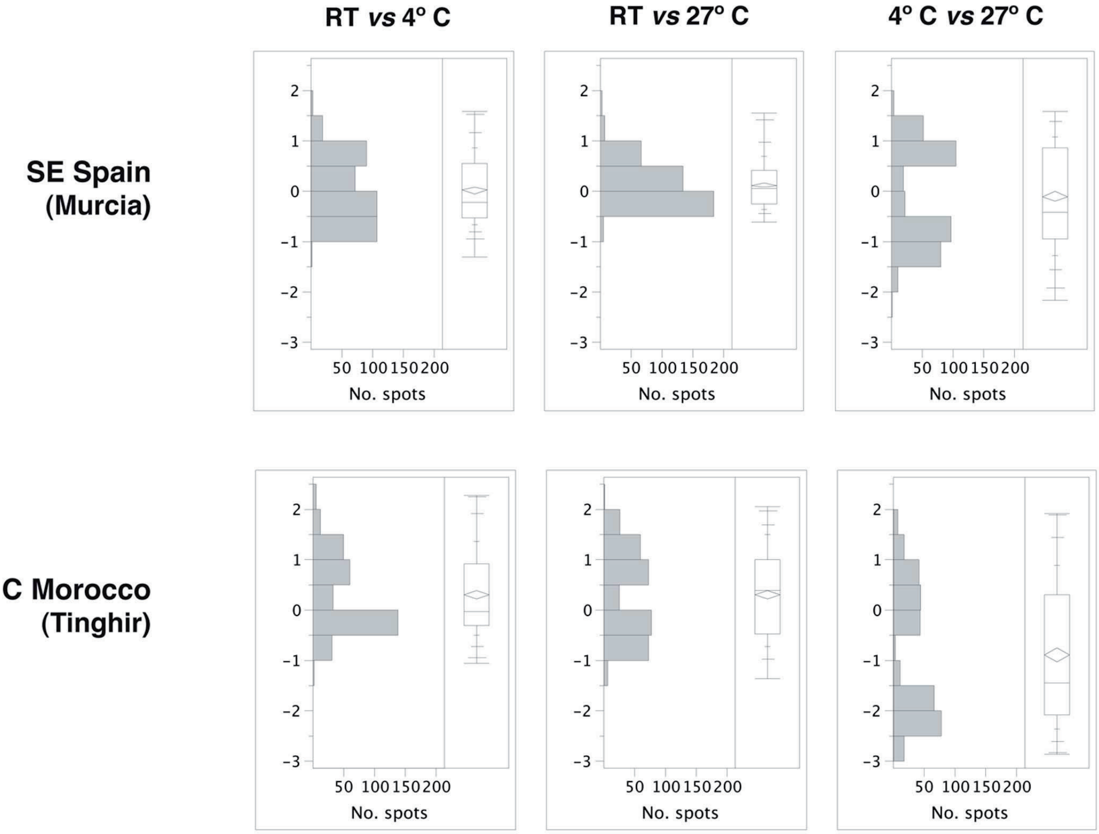

Figure 3. Distribution of pairwise differences in protein spot volume between temperature treatments in each population. On the right, distributions in a quantile box plot, including mean (rhomboid) and median. Vertical axis, fold change.
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and 27°C treatments more than 50% of the protein spots show differences of expression levels of less than 0.5 fold change, although the replicas at RT had a higher overall variation, with the median between 0.5–1 fold for the Moroccan population (Figure 2, Tables S1, S2). The CV among replicated samples ranged between 40–60% for the 4°C and 27°C treatments, and between 75–126% for the replicas at RT, higher than the variation between technical replicas (see above) [32] but similar to other reported measures of biological variation [33]. RT samples were exposed to fluctuating temperatures within a range that can be considered normal for the species, and therefore the spectrum of expressed proteins can be expected to be wider than that of specimens exposed to a constant extreme temperature, with a more selective protein expression.

A potentially important factor may be the existence of cohorts in the studied population, which could reduce the inter-individual variability of specimens collected at the same time in the same area, but may show increased variability throughout the year or between different geographical areas. The life cycle of *Agabus ramblae* is not known in detail [32], and therefore it is not possible to predict the population structure at any given time. Although larvae are more often reported from March to June [34], adults can be found any time of the year, as usual for lowland species in the Southern part of the Iberian Peninsula and Morocco. So it seems likely that a mixture of adults of different origin, age, gender and physiological state were included in the samples. In any case, it has been shown that males and females of the same species had identical values for upper and lower thermal limits [21].

Intra-population analysis on the effect of temperature treatments

Around 40% of all the protein spots had significantly different expression levels between treatments at $P<0.05$ (Table 3). The largest differences were detected between the two extreme temperature treatments (4°C and 27°C). Of all the protein spots with significant differences at $P<0.05$, between 95–99% were different between these two treatments in both populations (Table 3). The false discovery rate correction (FDR) reduced the number of spots with significant differences by 2.6% and 8.1% for the south Spanish and Moroccan populations respectively, but did not change the overall pattern. The distribution of the pairwise differences between treatments was in general bimodal and approximately symmetrical, especially for the comparisons between the 4 and 27°C treatments (Figure 3, Tables S1, S2). Only

Table 3. Number of protein spots with a significantly different level of expression.

Population	*P* level	all PS.	comparison	PS	fold>1.5
SE Spain (Murcia)	<0.05	856	RT *vs* 4°C	81	64
			RT *vs* 27°C	46	35
			4°C *vs* 27°C	811	514
			4°C *vs* RT *vs* 27°C	467	291
	<0.01	402	RT *vs* 4°C	15	11
			RT *vs* 27°C	4	0
			4°C *vs* 27°C	385	267
			4°C *vs* RT *vs* 27°C	84	66
	<0.001	79	RT *vs* 4°C	0	0
			RT *vs* 27°C	0	0
			4°C *vs* 27°C	77	62
			4°C *vs* RT *vs* 27°C	5	5
C Morocco (Tinghir)	<0.05	755	RT *vs* 4°C	63	62
			RT *vs* 27°C	63	61
			4°C *vs* 27°C	716	715
			4°C *vs* RT *vs* 27°C	334	333
	<0.01	451	RT *vs* 4°C	4	4
			RT *vs* 27°C	2	2
			4°C *vs* 27°C	439	439
			4°C *vs* RT *vs* 27°C	96	96
	<0.001	120	RT *vs* 4°C	0	0
			RT *vs* 27°C	0	0
			4°C *vs* 27°C	119	119
			4°C *vs* RT *vs* 27°C	6	6

All PS, overall number of protein spots with significant differences; PS, number of protein spots with significant differences in the pairwise comparison between treatments; fold >1.5, number of protein spots with fold differences above 1.5.

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for the comparison between RT and 27°C of the Spanish population the distribution was unimodal, with modal values between 0 and 0.5 fold change (i.e. most protein spots showed little or no differences) (Figure 3, Tables S1, S2). The range of variation was similar for all comparisons, with most spots between +/−2 fold change, with generally higher values for the comparison between 4 and 27°C.

In the hierarchical cluster analysis of the two populations, the protein spots significantly different in expression at both $P<0.05$ and $P<0.01$ resulted in a clear grouping of the biological replicas of the 4°C and 27°C treatments, with these two treatments showing the split at the deepest level. Differences within treatments were minimal relative to differences between treatments (Figures 1, 3, Figure S1). However, the replicas of RT were inconsistently clustered with the 4°C or the 27°C treatments. At the $P<0.05$ level some RT replicas were nested within the treatments (Figure S1), but at $P<0.01$ level the replicas of the two treatments were clustered together to the exclusion of the RT replicas (Figure 4). At $P<0.001$ the number of protein spots with significant differences was not high enough for a meaningful cluster analyses (Table 3). As already noted, in the RT treatment specimens were not subjected to a particular stress factor after their acclimation period, therefore their protein expression may represent a basic metabolic state with no compensatory reaction, with a wider range of intra-sample variation. In contrast, specimens subjected to extreme temperature treatments (4° and 27°C) reflected the influence of these stressful conditions by a stereotype modification of the protein expression pattern, with ca. 30% of the total number of protein spots significantly varying between these two treatments at $P<0.05$ level (Table 3).

The effect of the transport and acclimation period in the laboratory previous to the experiments and protein extraction could also have resulted in an artificially higher homogenisation of intra-experimental variability. All specimens were kept under the same conditions and fed on a homogeneous diet during one week, potentially reducing variation due to their individual history (starvation, consumption of different preys). This homogeneity in the experimental conditions could have introduced an artefact by modifying the protein expression in a similar way in all specimens. That this was not the case, and that the influence of the transport and acclimation was not reflected in the protein expression pattern, could be shown by the analysis of the field control of the Spanish population. The high overlap in the pairwise comparison of the RT-samples and the field control (Figure S2) indicates that neither the basic metabolism changed significantly nor the uniform food and conditions resulted in a higher homogeneity, although, due to the difficulty in obtaining enough specimens, only one replicated sample of a field control could be studied.

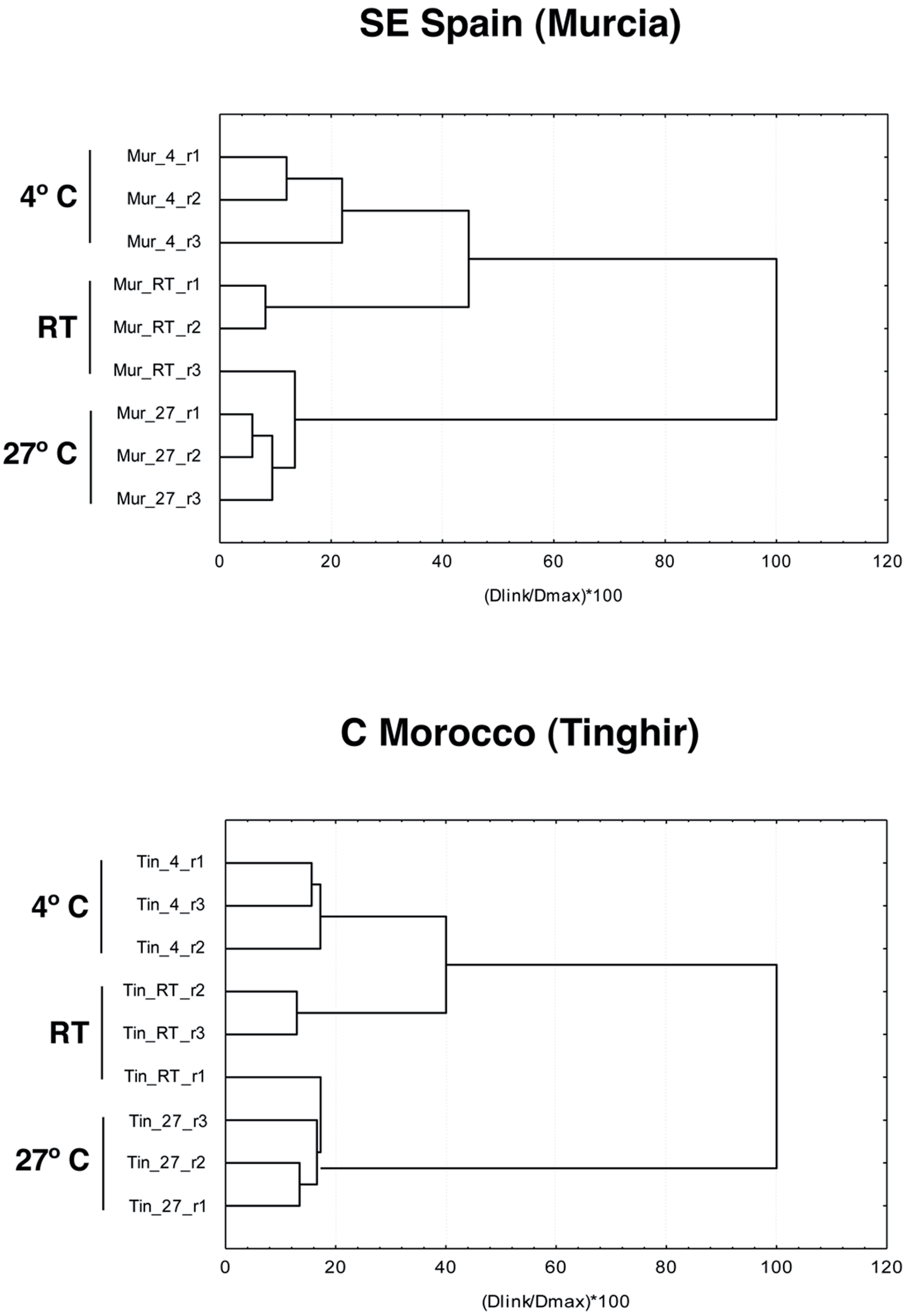
SE Spain (Murcia)
4º C
RT
27º C
Mur_4_r1
Mur_4_r2
Mur_4_r3
Mur_RT_r1
Mur_RT_r2
Mur_RT_r3
Mur_27_r1
Mur_27_r2
Mur_27_r3
0
20
40
60
80
100
120
(Dlink/Dmax)*100
C Morocco (Tinghir)
4º C
RT
27º C
Tin_4_r1
Tin_4_r3
Tin_4_r2
Tin_RT_r2
Tin_RT_r3
Tin_RT_r1
Tin_27_r3
Tin_27_r2
Tin_27_r1
0
20
40
60
80
100
120
(Dlink/Dmax)*100

Figure 4. Cluster analysis of the number of significantly differently expressed protein spots. The analysis includes the proteins with significantly different expression for each replicated sample, as measured with ANOVA at $P<0.01$ (see Tables 3, 4). r1 to r3, replicated samples.
doi:10.1371/journal.pone.0104734.g004

Comparison of the same treatment between populations

The number of protein spots that showed significant differences in the pairwise comparison of treatments was very similar for the two populations at each of the P levels used (Table 3, Figure 1). Differences between populations were not significant (as measured with ANOVA, $P>0.8$, Table 2), while differences for treatments and for the P-levels used for selecting the protein spots included in the comparison were highly significant ($P<0.001$, Table 4).

Despite the geographical distance, the general climatic conditions between Murcia and Tinghir are similar: annual average maximum temperatures are 30.8°C and 37.0°C respectively, and minimum temperatures 2.1°C and 1.2°C (WorldClim 2.5 m database, [35]). Monthly average maximum and minimum temperatures, for the month in which the specimens were collected, are 27.0°C and 14.8°C for Murcia (September) and 30.0°C and 15.2°C for Tinghir (May). General climatic conditions may however not reflect the particular thermal circumstances which the species are exposed to [36], especially for freshwater organisms living submerged [37]. In order to identify potential physiological reaction norms in either of the populations, preliminary data on the thermal tolerance of the Moroccan population were obtained by the same methodology as described in [21]. The average UTL (Upper Thermal Limit) for the Spanish population was 45°C [21], while the Moroccan population only reached 43.6°C. The average LTL (Lower Thermal Limit) of the Moroccan population was −6.8°C, identical to the LTL reported for the Spanish population [21]. There thus seems to be only slight differences between the thermal tolerances of both populations, which were reflected in the similar results of our comparative study of their response to different temperatures.

Protein identification

We selected 10 spots with significantly different expression levels between temperature treatments as measured with ANOVA ($P<0.05$ with FDR correction, cut-off values of >1.3 fold), and with the highest discriminant values in the MDA. Of these, three protein spots were selected for a preliminary analysis. These were identified as a chaperone (heatshock cognate Hsc70), a structural protein (alpha actinin) and a protein involved in the energy metabolism and membrane ion transport (sarco(endo)plasmic reticulum-type calcium ATPase) (Table 5).

Hsc70. This protein was up-regulated at 27°C in both populations. The same effect has been reported for the same or related proteins in several groups of animals (Heteroptera [38], Tunicata [39], leaf beetles [40]), although there are also reports of up-regulation at low temperatures (e.g. [41]). It belongs to Hsp70 family, regulating the ATP-dependent folding of proteins [41]. The expression of proteins of the Hsp70 family might be up-regulated as a response to temperatures routinely experienced in nature, and is related to thermal tolerance. It is considered to be a much more sensitive and ecologically relevant indicator of sub-lethal thermal stress, hence important in establishing the limits of the distribution of species or populations along environmental temperature gradients [40]. Experiments using RNAi to suppress Hsp70 translation prevented completely the recovery from heat shock, and also affected negatively the repair of chilling injury in insects [38].

Alpha actinin. This protein was also up-regulated at 27°C, as reported in other studies (e.g. [39]). The alpha actinins belong to the spectrin gene super-family that represents a diverse group of cytoskeletal proteins. Alpha actinin is an actin-binding protein with multiple roles in different cell types.

Sarco(endo)plasmic reticulum-type calcium ATPase (SERCA). The SERCA protein was found to be down-regulated at 4 °C. It is a protein involved in removing calcium from the cytoplasm to maintain the low concentration necessary for cell signalling, known to be temperature dependent in insects [42–43]. The differences in expression in *Agabus ramblae* may suggest that this species may show cold hardening, something that would require experimental data to confirm.

Concluding remarks

In this work we show that it is possible to conduct proteomic studies on wild populations of non-model organisms to obtain physiologically relevant data with relatively less noise. The reproducibility and uniformity of the results presented here for two distinct populations of a species of water beetle (*Agabus ramblae*) suggest that the experimental setup allowed the detection of a common stress-related response to temperatures at the extremes of the range they experience in their natural environment. We selected and identified some example proteins, and found that, in agreement with previous work [39,41], up-regulated proteins at higher temperatures were involved in structural protection, and down-regulated proteins at low temperatures in the reduction of metabolic activity and energy expenditure. Our work opens the possibility of a wider use of comparative population proteomics in wild populations of non-model organisms, with a vast potential to address a whole range of basic questions in ecology and evolutionary biology. The use of wild populations not only allows the study of species for which common garden experiments are not feasible, but also the study of the interaction with local conditions. If differences in the reaction norm of local populations were due to environmental imprinting, common garden experiments may mask phenotypic variability that could be potentially important to explain evolutionary processes at the edge of the geographical range of species [44–46].

Table 4. Results of the ANOVA comparison between populations.

Factor	DF	Sum of squares	F Ratio	Sig.
population	1	737	0.05	>0.8
P level	2	363344	11.10	<0.0001
treatment	3	644782	13.13	<0.001

See Table 3 for the number of protein spots with significant differences between treatments at each P level.
doi:10.1371/journal.pone.0104734.t004

Table 5. Exemplary identified proteins.

no.spot Protein	2180 Hsc70	1727 alpha actinin	483 sarco(endo)plasmic reticulum-type calcium ATPase
regulation	**up-regulated at 27°C**	**up-regulated at 27°C**	**down-regulated at 4°C**
fold change	3.12	8.5	5.8
sequence	DAGTIAGLNVMR	QTDNSLAGVQK	VGEATETALIVLAEK
score	52	67	85
IP	5.02	5.60	5.32
MW	82	100	64
Acc.No.	AB122065	NP_726784	AF115572
functional category	Chaperone	structural	energy, transport
organism	*Crassostrea gigas*	*Drosophila melanogaster*	*Heliconius virescens*

Included are the amino-acid sequence of the identified fragment with its isoelectric point (IP), molecular weight (MW) and the score of the search in the database, the identification, and the NCBI accession number and organism of the best match.

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Supporting Information

Figure S1 Cluster analysis of the number of significantly differently expressed protein spots ($P<0.05$). The analysis includes the proteins with significantly different expression for each replicated sample, as measured with ANOVA at $P<0.05$ (see Tables 3, 4).
(EPS)

Figure S2 Cluster analysis of the number of significantly differently expressed protein spots including the field control (FC). The analysis includes the proteins with significantly different expression for each replicated sample plus the field control (FC) as measured with ANOVA at $P<0.01$ (see Tables 3, 4) of the Spanish population.
(EPS)

Table S1 Normalised volume of the spots detected in the Spanish population. Included, whether the spot had significant differences for any of the ANOVA comparisons and was included in the subsequent analyses (Yes) or not (No) (all spots were included in the histogram in Fig. 2); SPA (Spain); 4, RT and 27, temperature treatments (4°C, room temperature and 27°C respectively); 1, 2 and 3, biological replicas.
(XLSX)

Table S2 Normalised volume of the spots detected in the Moroccan population. Included, whether the spot had significant differences for any of the ANOVA comparisons and was included in the subsequent analyses (Yes) or not (No) (all spots were included in the histogram in Fig. 2); MOR (Morocco); 4, RT and 27, temperature treatments (4°C, room temperature and 27°C respectively); 1, 2 and 3, biological replicas.
(XLSX)

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Author Contributions

Conceived and designed the experiments: IR AC. Performed the experiments: AHG MM IR AC. Analyzed the data: AHG MM DGB FC IR AC. Wrote the paper: AHG IR AC. Wrote an initial draft of the paper: AHG IR AC. Contributed to writing and discussion of the results: AHG MM DGB FC IR AC.

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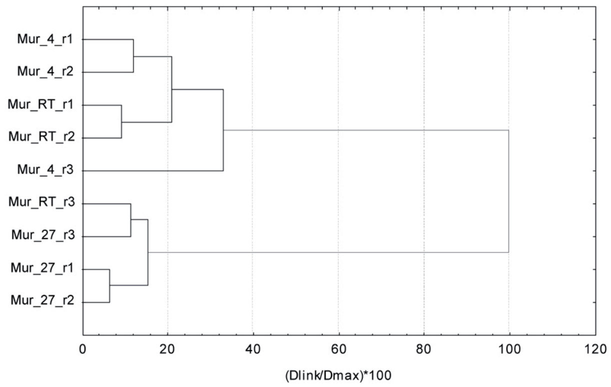
SUPPLEMENTARY INFORMATION

SE Spain (Murcia)



C Morocco (Tinghir)

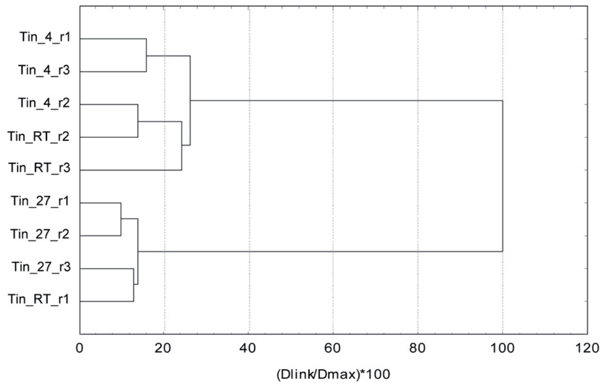


Figure S1 Cluster analysis of the number of significantly differently expressed protein spots (P<0.05). The analysis includes the proteins with significantly different expression for each replicated simple, as measured with ANOVA at P<0.05 (see Tables 3, 4).

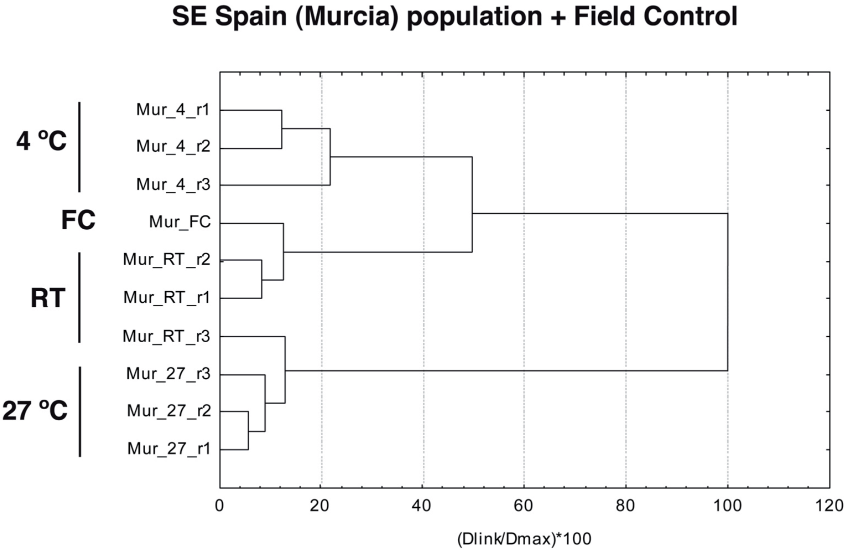


Figure S2 Cluster analysis of the number of significantly differently expressed protein spots including the field control (FC). The analysis includes the proteins with significantly different expression for each replicated sample plus the field control (FC) as measured with ANOVA at P<0.05 (see Tables 3, 4) of the Spanish population.

Table S3. : http://www.plosone.org/article/info:doi/10.1371/journal.pone.0104734#pone-0104734-t003

Table S4 : http://www.plosone.org/article/info:doi/10.1371/journal.pone.0104734#pone-0104734-t004

Chapter 3:

Protein expression parallels thermal tolerance and ecologic changes, but not speciation, in the diversification of a diving beetle species complex

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Manuscript to be submitted

Abstract

Physiological changes associated with evolutionary and ecological processes such as diversification, range expansion or speciation are still incompletely understood, especially for non-model species. Here we study differences in protein expression in response to temperature in a western Mediterranean diving beetle species complex, using 2D-DIGE with one Moroccan and one Iberian population each of *Agabus ramblae* and *A. brunneus*. We selected and identified a number of proteins with significant expression differences after thermal treatments comparing them with a reference EST library build from one of the species of the complex (*A. ramblae*). We found changes in response to 27°C in many proteins related to energy metabolism in association to the colonisation of the Iberian peninsula by North African populations of *A. ramblae* during the Middle Pleistocene, possibly related to a change to lower maximum temperatures and reduced seasonality. The subsequent speciation of *A. brunneus* from within populations of Iberian *A. ramblae* was associated to changes in the protein expression of several stress-related proteins (mostly chaperons) when exposed to 4°C. This is in agreement with the known tolerance to lower temperatures of *A. brunneus*, which occupies a larger geographical area with a wider range of climatic conditions. In both cases protein expression changes paralleled the evolution of the thermal tolerance and distribution of the *A. brunneus* complex, but while in the colonisation of the Iberian peninsula these were not associated to morphological changes, the speciation process of *A. brunneus* resulted in genetic isolation and substantial differences in male genitalia and body size and shape.

Keywords: Agabus brunneus complex, metabolism related proteins, range expansion, stress related proteins, thermal tolerance, western Mediterranean

Introduction

The development of high throughput genomic and proteomic tools has allowed an exponential increase in our knowledge of speciation mechanisms (e.g. Alcaide *et al.* 2014; Soria-Carrasco *et al.* 2014; see van Dijk *et al.* 2014 for an overview), but the relationships between phenotype and the underlying gene- and protein expression changes, and how these changes developed during the speciation processes resulting in the extant taxa, remain poorly understood. Standard phylogenetic methods can trace phenotypic changes associated to speciation events through the evolutionary history of a lineage (e.g. Adams & Collyer 2009; Rabosky *et al.* 2014), but the study of the underlying genetic changes have so far been addressed only in a handful of model systems (e.g. Brawand *et al.* 2014; Carbone *et al.* 2014; Mallarino *et al.* 2012).

Changes in gene expression are known to play a role in speciation, although genetic differences have been rarely identified (Tautz 2000; Wolf *et al.* 2010; Butlin *et al.* 2012). The role of temperature in establishing biogeographical ranges has also been recognized for long, but the physiological and molecular mechanisms responsible for establishing these patterns are not well known (Pörtner 2001) and their study is just emerging (Pörtner & Knust 2007).

The quantification of gene expression is methodologically complex; there are different quantitative methods available to study RNA or the whole proteome, but they may give not consistent results, and some do not measure total accumulated quantity of protein present (Wolf *et al.* 2010). A relatively simple alternative is the direct quantification of the amount of protein with two dimensional differential gel electrophoresis (2D-DIGE, Thiellement *et al.*, 1999; Krogh *et al.* 2007). The study of changes in proteins abundance has become a powerful tool for generating hypotheses on how the environment affects the biology of organisms. Proteomics has the potential to discover unknown cellular effects to environmental

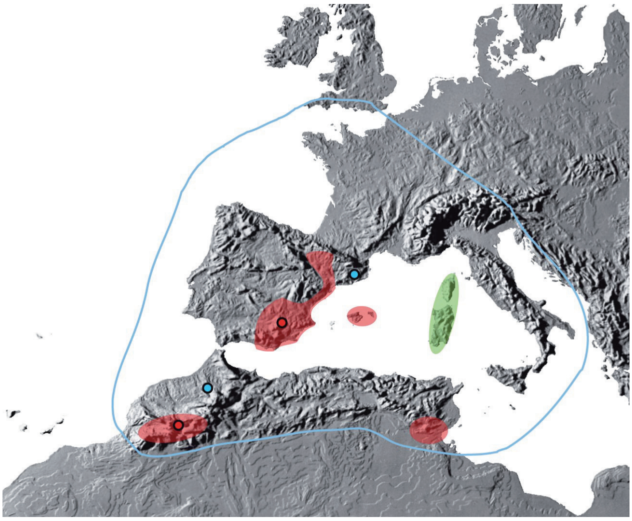

Figure 1. Distribution of the *Agabus brunneus* species complex. Blue: *A. brunneus*; red: *A. ramblae*; green: *A. rufulus*. Spots mark the location of the populations used for proteomic analyses.

stressors, such as changes in temperature (Cravatt *et al.* 2007).

The use of these methods will have to be extended to non-model species if they are to become standard tools in evolutionary biology (Wolf *et al.* 2010; Butlin *et al.* 2012). The use of non-model species suffers from two major drawbacks: first, the lack of genomic data (which is, however, of increasingly less concern, van Dijk *et al.* 2014), and second, and more important, the difficulty of using wild populations. This is necessary when studying species that cannot be bred in the laboratory, but also to address questions that can only be answered looking at the response to natural conditions, or that require comparisons of a wide representation of the gene variation within a species.

The unknown and uncontrolled variation in natural populations is often though to render the study of protein expression unfeasible (Wolf *et al.* 2010). However, we could recently show that in a western Mediterranean diving beetle species variation of protein expression, as determined by 2D-DIGE, was lower between replicated samples of different populations than variation between different temperature treatments (Hidalgo-Galiana *et al.* 2014b). Our results demonstrated that specimens collected in the wild were suitable for proteomic analyses, as the additional sources of variation were far below the expected range and did not mask the consistency and reproducibility of the response to the temperature treatments (Hidalgo-Galiana *et al.* 2014b).

In this work we use the same diving beetle as in Hidalgo-Galiana *et al.* (2014b) (*Agabus ramblae*), together with its sibling species *A. brunneus*, to study changes in protein expression through the speciation process in relation to other changes in morphology, geographic distribution, ecology and thermal tolerance data. The *Agabus brunneus* complex is a well defined and phylogenetically isolated group of three closely related species of diving beetle with a western Mediterranean distribution (Millán & Ribera 2001). In a recent work the species complex was reconstructed to have first diversified in central Morocco during the Middle Pleistocene, in where the oldest haplotypes of *A. ramblae* were found (Hidalgo-Galiana *et al.* 2014a). Between 0.6-0.25Ma they first colonized the Iberian peninsula and subsequently the Balearic islands, Corsica and Sardinia, in the latter two giving rise to the species *A. rufulus*. The colonisation of Iberia by *A. ramblae* did not result in substantial morphological change, as measured with size of the adults and size and shape of the male genitalia (used to characterise closely related species in the genus Agabus and other diving beetles) (Millán & Ribera 2001; Hidalgo-Galiana *et al.* 2014a). Similarly, no differences were found between their upper and lower experimentally measured thermal tolerances (Hidalgo-Galiana *et al.* 2014a).

The species *A. brunneus* was reconstructed to have originated from Iberian populations of *A. ramblae* ca. 0.35 Ma, and subsequently (mostly during the Last Glacial Maximum, 0.03-0.01 Ma) dispersed to occupy its current range, through North Africa and Western Europe including some Mediterranean islands and the extreme south of Great Britain. The speciation of *A. brunneus* was associated with a significant increase in body size and a change in the shape of the male genitalia, as well as an increase in the cold tolerance. The current climatic niche of *A. brunneus* also differs significantly from that of *A. ramblae*, occupying less arid and seasonal areas, with a lower minimum temperature in the coldest month (Hidalgo-Galiana *et al.* 2014a).

Here we use Moroccan and Iberian populations of each *A. ramblae* and *A. brunneus* to compare their physiological response to extreme temperatures within their natural range, as measured with 2D-DIGE electrophoresis. Our aim was to trace changes in protein expression through the speciation process of the complex, and to relate these changes with the evolution of phenotypic traits known to differ between species (morphology, climatic niche, thermal tolerance) in an integrative approach (Eme *et al.* 2014). Our results will also contribute to the understanding of the origin of differences in size of geographical range between closely related species, and the factors that allow geographic expansion to new areas under different climatic regimes.

Materials and methods

Species and populations

For the experiments we selected two populations of *A. brunneus* and *A. ramblae* from distant regions within their geographic range (Fig. 1). For *A. brunneus* we used one population from the Moroccan Middle

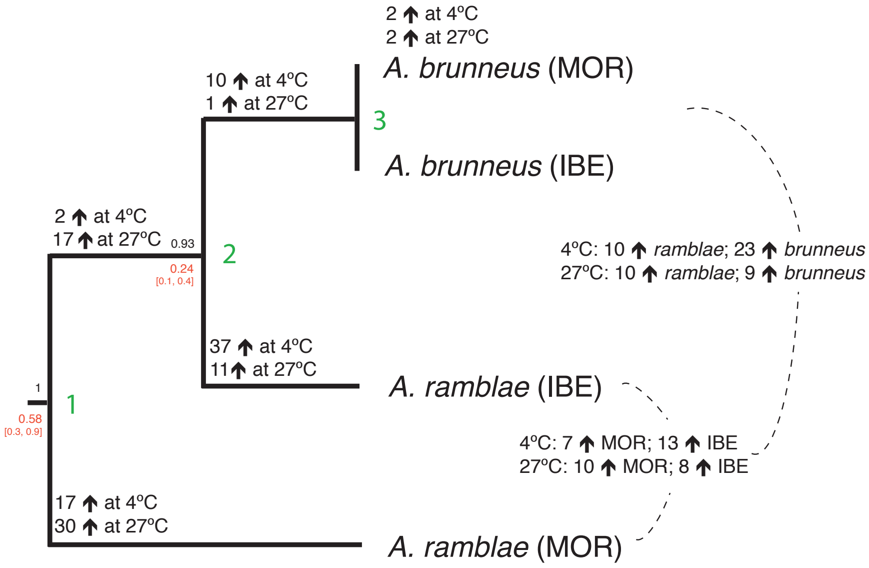

Figure 2. Summary phylogenetic relationships of the *A. brunneus* complex (from Hidalgo-Galiana et al. 2014b). Numbers in nodes: in black, node posterior probabilities; in red, estimated ages of the nodes and 95% confidence intervals (million years). Above branches, number of protein spots with significantly higher expression levels at a P< 0.001 for the comparison of populations at the two branches of the node for each treatment. With dashed lines, comparisons between populations not corresponding to nodes in the phylogeny.

Atlas (Tizi-n'Rechou, Kerrouchèn, 32º47'34.9"N 5º14'33.4"W, 1810m a.s.l., 8.4.2007 P. Aguilera, C. Hernando & I. Ribera leg.); and another from NE Spain (Girona, river Ser ca. Santa Pau, 42º08'48"N 2º34'48"E, 445m a.s.l. 13.10.2010 A. Hidalgo-Galiana, A. Rudoy, R. Alonso & I. Ribera leg.). For *A. ramblae*, we used one population from the south side of the Haut Atlas in Morocco (Tinghir, Toudgha river, 31º33'25.1"N 5º34'49.5"W, 1370m a.s.l., 26.5.2011 A. Hidalgo-Galiana & N. Bennas leg.); and another from SE Spain (Murcia, Lorca, 37º42'10.7"N 1º55'33.8"W, 550m a.s.l., 15.9.2007 P. Abellán, A. Cieslak, A. Millán & I. Ribera leg.). Of each population ca. 30 specimens were collected in the field and transported to the laboratory in small plastic containers with vegetation and some water from the place, in portable cooling boxes.

Thermal treatments

Once in the laboratory, individuals were acclimated for one week in aquaria, with mineral water and some vegetation taken from the place of origin. Specimens were kept at room temperature (RT, always below 25°C) with a natural day/night cycle. These conditions represented the control for the experiments. Specimens were fed ad-libitum on frozen red Chironomidae larva from commercial sources. After a week, an equal number of specimens were randomly allocated to each of three treatments for 12h: 4ºC, RT, and 27ºC. This is within the range of temperatures the species are likely to experience in their natural habitat (Hidalgo-Galiana *et al.* 2014b). After the treatment, specimens were snap frozen in liquid nitrogen, separated into three samples of 2-3 specimens for each temperature treatment and stored at -80ºC. The number of specimens per replica was limited by the total number of specimens available and the need of having at least three replicas per treatment for comparison. The protocol was shown to be appropriate to guarantee reproducibility and consistency of data generation in a preliminary study on *A. ramblae* populations (Hidalgo-Galiana *et al.* 2014a)

2D-DIGE experiments and image analysis

Proteins of whole specimens were extracted with a standard protocol under denaturing conditions as described in Hidalgo-Galiana *et al.* (2014a). Total protein yield of each sample was determined using Bio-Rad RCDC Protein Assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions.

The 2D-DIGE experiments were performed as described in Hidalgo-Galiana et al. (2014a). An internal standard for each experiment was generated by pooling equal amounts of protein from each extraction and labelled with Cy2-dye. Five gels per experiment were run, each loaded with two samples derived from different treatments and with different combinations of the Cy3- and Cy5-dye labelling and the internal standard. 2D-DIGE image analysis and statistical quantification of relative protein abundance were performed with Progenesis SameSpots v4.0 (Nonlinear Dynamics, Newcastle, UK). This software allows detecting, quantifying and matching of spots between gels after normalization to the internal standard.

Analyses of expression patterns

All protein spots identified by SameSpots v4.0 were verified using the scanned gels images. Statistically significant differences in protein expression between groups (temperature treatments) were tested with one-way ANOVA.

We tested for significant differences in protein expression in response to the thermal treatments between the populations grouped by the three key nodes of the reconstructed evolutionary history of the group (Fig. 2; Hidalgo-Galiana et al. 2014b): 1) Moroccan *A. ramblae* vs. the other three populations (*A. ramblae* from the Iberian peninsula and *A. brunneus* from both localities); 2) Iberian *A. ramblae* vs. *A. brunneus*; and 3) Moroccan vs. Iberian *A. brunneus* from NE Spain. We also compared the combined data of populations of *A. ramblae* vs. populations of *A. brunneus.*

For the comparison between populations we first obtained the normalized spot volumes and then standardised them following the SameSpots recommended protocol (Table S1). We analysed the global expression patterns of the spots with significantly different expression levels for the different comparisons with Principal Component Analysis (PCA). We used a significance level of $P < 0.001$, which for the number of points used (less than 600, see below) gave an overall number of expected false positives of ca. 0.6 per comparison. In addition, we used a Hierarchical Cluster Analyses (HCA) to relate the proteins with similar

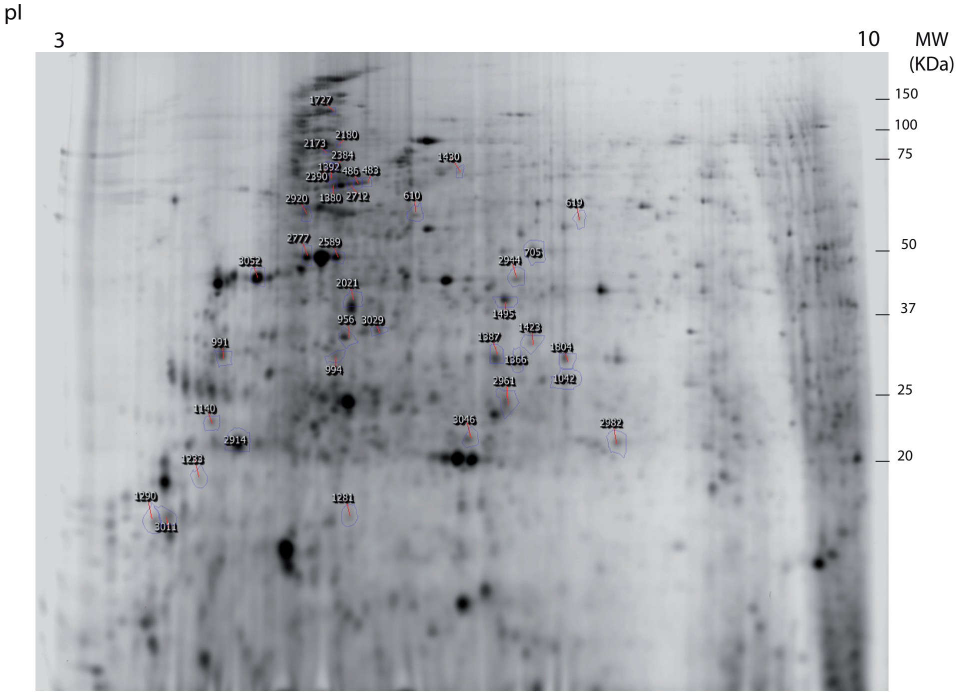

Figure 3. Image of the extraction gel (i.e. with pooled aliquots of all experiments) with the location of the spots selected for identification (see Tables 2,3). Vertical axis, molecular weight; horizontal axis, isoelectric point.

expression patterns across samples in the form of a heat map using PermutMatrix V (Caraux & Pinloche 2005), with Euclidean distances and UPGMA amalgamation method (Biron et al. 2006).

Protein selection and identification

We selected the protein spots to be extracted and identified among the spots with significant differences at a $P < 0.001$ level in any of the comparisons. The selection was based on the reconstructed SameSpots 3-D images of the spots in each gel, to ensure they had highly significant expression differences between treatments and consistent uniform spot formation.

To obtain a sufficient amount of protein for identification of the selected spots a total of three hundred micrograms of a mix of protein extracts from representative samples were labelled, run on a preparative gel and scanned. The gel images were matched against the spots from the list previously generated in SameSpots v4.0 (see above). The selected spots were excised from the gel using an automated Spot Picker (GE Healthcare), within-gel digestion with trypsin (Promega, Wisconsin, USA) as described in Shevchenko *et al.* (1996). Extracted samples were analysed on a Maxis high resolution Q-TOF spectrometer (Bruker, Bremen), coupled to a nano-HPLC system (Proxeon, Denmark). Proteins were identified using Mascot (Matrix Science, London UK) by search on the NCBI database limiting the search to "Other Metazoa" (see Hidalgo-Galiana *et al.* 2014a for details).

EST reference library construction and sequencing

We build a reference EST library using RNA derived from the whole body of adult specimens of *A. ramblae* to identify the cDNAs of the selected candidate proteins. For that we collected specimens from the same Iberian population of *A. ramblae* (Murcia, river Vélez, 18.11.2008 P. Abellán leg.) and repeated the

			p-value	spots no	fold range	% spots fold > 1.3	up-regulated first group	%	up-regulated second group	%
A	*A. ramb.* (MOR) *vs.* rest	**4° C**	p < 0.05	120	1.6-9.8	100	86	71.7	34	28.3
			p < 0.01	54	1.8-9.8	100	43	79.6	11	20.4
			p < 0.001	19	2.3-9.8	100	17	89.5	2	10.5
			p < 10^4	4	2.9-9.8	100	4	100.0	-	
		27° C	p < 0.05	211	1.2-12.3	99.5	102	48.3	109	51.7
			p < 0.01	121	1.2-12.3	99.2	50	41.3	71	58.7
			p < 0.001	47	1.9-12.3	100	17	36.2	30	63.8
			p < 10^4	15	2.6-12.3	100	7	46.7	8	53.3
B	*A. ramb.* (IBE) *vs. A. brunn.*	**4° C**	p < 0.05	285	1.55-23.28	100	164	57.5	121	42.5
			p < 0.01	158	1.63-23.28	100	106	67.1	52	32.9
			p < 0.001	47	2.40-23.28	100	37	78.7	10	21.3
			p < 10^4	14	2.95-23.28	100	13	92.9	1	7.1
			p < 10^5	7	3.14-9.97	100	7	100.0	0	
		27° C	p < 0.05	111	1.38-6.06	100	67	60.4	44	39.6
			p < 0.01	44	1.5-4.24	100	31	70.5	13	29.5
			p < 0.001	12	2.15-4.06	100	11	91.7	1	8.3
			p < 10^4	5	2.35-4.06	100	5	100.0	0	
C	*A. brun.* (MOR) *vs. A. brun.* (IP)	**4° C**	p < 0.05	85	1.4-11.6	100	53	62.4	32	37.6
			p < 0.01	20	1.55-6.23	100	14	70.0	6	30.0
			p < 0.001	2	-		-		-	
		27° C	p < 0.05	81	1.28-8.95	97.5	42	51.9	39	48.1
			p < 0.01	19	1.28-3.16	89.5	8	42.1	11	57.9
			p < 0.001	2	-	-	-		-	
D	pooled *A. ramb. vs.* pooled *A. brun.*	**4° C**	p < 0.05	182	1.45-16.27	100	89	48.9	93	51.1
			p < 0.01	87	1.65-16.27	100	50	57.5	37	42.5
			p < 0.001	33	2.48-16.27	100	23	69.7	10	30.3
			p < 10^4	5	4.46-16.27	100	4	80.0	1	20.0
			p < 10^5	2	-		-		-	
		27° C	p < 0.05	178	1.36-8.51	100	109	61.2	69	38.8
			p < 0.01	83	1.46-7.46	100	56	67.5	27	32.5
			p < 0.001	19	1.56-5.2	100	9	47.4	10	52.6
			p < 10^4	4	2.05-5.2	100	4	100.0	0	
E	*A. ramb.* (MOR) *vs. A. ramb.* (IBE)	**4° C**	p < 0.05	211	1.37-24.6	100	114	54.0	97	46.0
			p < 0.01	103	1.61-24.6	100	55	53.4	48	46.6
			p < 0.001	20	2.05-10.01	100	7	35.0	13	65.0
			p < 10^4	4	2.05-8.2	100	1	25.0	3	75.0
		27° C	p < 0.05	183	1.26-9.31	99.5	92	50.3	91	49.7
			p < 0.01	69	1.44-9.31	100	43	62.3	26	37.7
			p < 0.001	18	1.47-8.08	100	10	55.6	8	44.4

Table 1. Number of spots with significant differences between treatments for each of the comparisons. The P< 0.001 level was used as reference for the results, other levels are given for comparison. See Fig. 2 for the placement of the compared populations in the phylogenetic tree of the *A. brunneus* complex. *A. ramb.* (*A. ramblae*), *A. brun.* (*A. brunneus*), MOR (Morocco), IBE (Iberian Peninsula, spot no. (number of spots)

same experimental procedure as described above. After the temperature treatments specimens were measured and weighted, and their total-RNA was extracted as described by Sambrook *et al.* (1989). The yield of total RNA was measured photometrically and the amount extracted per mg calculated before pooling the RNA with proportionally the same amount for each sample.

An aliquot of 200µg total-RNA was sent to and external service (LGC-genomics, Berlin, Germany) for mRNA-isolation, cDNA-library construction, amplification, normalisation and high-throughput sequencing using the 454 FLX-technology of Roche (Roche/454 life sciences, USA). With the assembled sequence contigs we build a custom database in Geneious v.6 (Drummond *et al.* 2010) and used the amino acid sequence of the identified protein fragments to identify matching transcript-sequences.

Results

The 2D-DIGE map derived from all four experiments corresponding to two populations each of *A. brunneus* and *A. ramblae* contained a total of 2114 protein spots, with molecular masses ranging from 10 to 150 KDa and isoelectric points between 3 and 10 (Fig. 3). Although the protein samples of the Moroccan population of *A. brunneus* contained substantially less spots than their Iberian sister (Table S2), when analysed following the protocol described in Hidalgo-Galiana *et al.* (2014a) the intra- and inter-population variation were similar to those found for *A. ramblae*, and did not mask their response to the different temperature treatments applied (Fig. S1).

For all four populations a common set of 563 protein spots could be identified, which were included in the following analyses. The number of spots up- or down-regulated at 4ºC varied between the 11.3% for the Iberian *A. brunneus* to the 47.9 % for the Iberian *A. ramblae*, while at 27ºC it ranged from 45.7% for Iberian *A. ramblae* to 60.8% for the Iberian *A. brunneus* (Table 1; see Table S3 for the fold change values of all 563 common spots).

Expression changes through the phylogeny of the group

Approximately 35% of the common spots (196 of 563) showed significant differences in at least one of the comparisons with a P< 0.001 (Table S1). In the comparison between the Moroccan *A. ramblae* and the other three populations, corresponding to the first diversification event of the lineage by colonisation of the Iberian peninsula, almost 12% of the 563 spots showed significant differences, most of them in the response to the 27ºC treatment (Fig. 2; Tables 1,S3). In the next node in the phylogeny of the complex, i.e. the comparison between the Iberian *A. ramblae* and the two *A. brunneus* populations (Fig. 2), a similar number of spots showed significant differences (10%), but the main changes were in the response to the 4ºC treatment, with almost four times more varying spots than observed in response to the 27ºC treatment (Fig. 2; Tables 1,S3).

In the intraspecific comparison of the two populations of *A. brunneus* (Iberian and Moroccan) less than 1% of the spots had significantly different expression levels (Fig. 2; Tables 1,S3), with equal number of changes same after 4° or 27ºC treatments. On the contrary, between the two populations of the paraphyletic *A. ramblae* almost 7% of the spots were significantly different, also equally distributed between the 4 and 27ºC treatments. When both populations of each of the two species were combined and compared the number of spots with significant changes (ca. 9%) was twice as high for the 4ºC (6%) than for the 27ºC (3%) treatment.

Global expression patterns

The Principal Component Analysis (PCA) with the fold values of all 563 common spots showed a good discrimination of species in the first axis, and temperature treatment in the second (Fig. 4A; Fig. S2A). The room temperature controls (RT) had always intermediate scores between the 4ºC and 27ºC treatments for each of the populations (Fig. S2B,C), and were not included in some of the following analyses for simplicity. In the first axis (with 76.8% of the

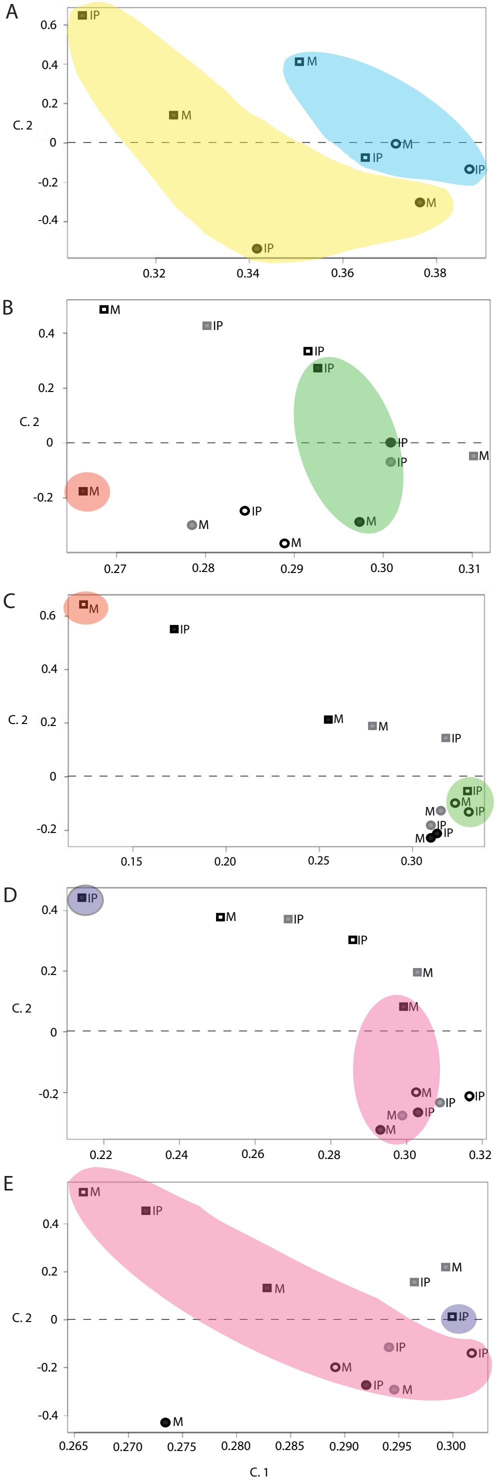

Figure 4. Plots of the two first axes of the PCA analyses of the fold values of the 563 spots common to all experiments. A: all spots, without room temperature (RT) samples; B to E: points with significantly different expression levels (P< 0.001) in the comparison of B: Moroccan *A. ramblae vs.* other populations, 4°C treatment; C: Moroccan *A. ramblae vs.* other populations, 27°C treatment; D: Iberian *A. ramblae vs. A. brunneus*, 4°C treatment; E: Iberian *A. ramblae vs. A. brunneus*, 27°C treatment. Squares: *A. ramblae*; circles: *A. brunneus*; filled symbols: 4°C; empty symbols: 27°C; M: Moroccan; IP: Iberian. Coloured areas group the samples used to define the comparisons. See Table S4 for details on the PCA results.

total variance, Table S4) *A. ramblae* populations had the lowest scores, with the only exception of the 4°C treatment in Iberian *A. ramblae*, which had a higher score than the 27°C Iberian *A. brunneus*. For the second axis (with 10.4% of the total variance, Table S4) the situation was more complex, with the 4°C treatments of *A. ramblae* and *A. brunneus* having the extreme positive and negative values respectively, with intermediate positions occupied by 27°C treatments, again with a single exception (27°C Moroccan *A. ramblae*) (Fig. 4A).

In the PCA of the spots with significant differences in the comparison of Moroccan *A. ramblae* vs. all other populations there was also a good separation between species in the first two axes (Fig. 4B,C), but, as expected, differences were larger between Moroccan and Iberian *A. ramblae* than between Iberian *A. ramblae* and *A. brunneus*. In the comparison between Iberian *A. ramblae* with *A. brunneus* the second axis perfectly separated the two species (with positive values for *A. ramblae* and negative for *A. brunneus* in both cases, Fig. 4D,E). On the contrary, the first axis discriminated Iberian *A. ramblae* only for the 4°C treatment, but not for the 27°C, for which the score was similar but slightly lower than that of Iberian *A. brunneus*, and much larger than that of Moroccan populations of both species (Fig. 4E).

In the PCA of the spots significantly different between the pooled populations of the two species they were perfectly separated by the second axis, but the separation of the different samples along the first axis was less clear (Fig. S2B,C).

The cluster analyses gave similar results to the ordination with PCA, with a clear separation between species and, to a lesser extend, between treatments when the spots with overall significant differences were selected (Fig. S3, differences at a P< 0.001 for *A. ramblae* (MOR) vs. rest; Fig. S4, differences at a P< 0.0001 for *A. ramblae* vs. *A. brunneus*). In the PermutMatrix analysis expression levels of the selected spots could be associated with the different clusters, visualising a primary separation between species and a secondary between temperature treatments, with no clear geographical structure (Figs S3,S4).

Identification of proteins

We sequenced and identified the protein spots best matching the selection criteria, i.e. a significantly different expression between treatments (at a P< 0.001 level), sufficient protein material on the preparative gel and a well-defined spot in the images (Table 2; see Table S5 for the details of the proteins). In some of the spots the targeted proteins could not be identified due to the abundance of highly expressed structural proteins (mostly actines or miosines), and were not further considered (Table S5). Several proteins were also identified in different spots, suggesting

No	Node	spot No.	Protein	Mascot score	type	function	ref
1	1,2	610	Enolase	249	met	glycolysis	13
2	1	705	Acyl-CoA dehydrogenase/oxidase	303	met	ß-oxidation, mitochondria, energy household	17
3	1	956	phosphoglucomutase	233	met	activation of phosphorylated Glucose into active metabolite useful for low energy situations	16
4	1	956	HSP70	44	str	protein folding, general cell function, stress response	8,21
5	2	1042	ATP-binding cassette transporter	41	str	transport function membrane, RNA and DNA repair	18
6	1,2	1140	14-3-3 protein zeta	101	met	Signalling protein binding, functionally diverse group of partner proteins, kinase, phosphatase, transmembrane receptors	19
7	1,2	1140	translationally controlled tumor protein	199	str	protein folding, general cell function, stress response, other, unknown functions?	7,14
8	1,2	1366	Arginine Kinase	40	met	transferase phosphat-residue, ATP-dependent	6
9	2	1387	putative translation elongation factor 2?	110	met	Protein synthesis	
10	2	1392	chaperonine protein HSP60	40	str	Protein folding, general cell function, stress response, other?	3,4,5, 20,21
11	1	1423	Acyl-CoA dehydrogenase/oxidase	111	met	ß-oxidation, mitochondria, energy household	17
12	2	1430	similar to Neural conserved at 73EF CG11661-PF	84	met	oxidation-reduction processes	12
13	2	1495	transferrin	440	met	Transport and Storage protein, binding-protein, Iron-ion-binding	10
14	2	1804	L-lactate dehydrogenase	44	met	L-lactase generation NAD(H) dependent	3,9
15	1	2021	Actinin-type, actin-binding	136	met	Structural protein, cell skeleton, actin-binding	1,2
16	2	2390	HSP 60	141	str	Protein folding, general cell function, stress response	3,4,5, 20,21
17	2	2589	HSP70	56	str	Protein folding, general cell function, stress response	8,21
18	2	2914	glycogen phosphorylase	66	met	glycogenlysis, energy-metabolism	15
19	1	2944	Acyl-CoA dehydrogenase/oxidase	63	met	ß-oxidation, mitochondria, energy household	17
20	1,2	2961	triosephosphate isomerase (tpi gene)	116	met	Metabolism, Glycolysis, energy production	
21	1	3046	ATPase, F1 complex, alpha subunit	264	met	Metabolism, mitochondria, oxidative phosphorylation	11

Table 2. Proteins identified in the selected spots according to the Mascot and EST library identifications (see Table S7 for full details). Nodes (see Fig. 2): 1, Moroccan *A. ramblae vs.* rest of populations; 2, *A. ramblae vs. A. brunneus.* sc: Mascot score. ty. (type): met, metabolic related protein; str, stress related protein. Ref: 1, Carrasco et al. (2011); 2, Carrasco et al. (2012); 3, Chen et al. 2014; 4, Colinet et al. (2010); 5, Cui et al. (2010); 6, Ge et al. (2013); 7, Gnanasekar et al. (2009); 8, Huang et al. (2007); 9, Krebs et al. (2001); 10, Lee et al. (2006); 11, Li et al. (2008); 12, Lim et al. (2011); 13, Liu et al. (2010); 14, Mak et al. (2007); 15, Overgaard et al. (2014); 16, Rank et al. (2007); 17, Reynolds et al. (2012); 18, Sukhai et al. (2000); 19, Tabunoki et al. (2008); 20, Wu et al. (2013); 21, Zhang et al. (1998).

they were residual fragments or contaminations and were discarded from further analyses (Table S5). Two identified proteins best matched a ciliate (which are common ectoparasites of Dytiscidae) and were equally discarded.

Of the final selection of 19 spots with identified proteins 10 were differentially expressed in Moroccan *A. ramblae* with respect to all other populations, two of them with clear signals for two proteins (Tables 2,3). Most of the significant changes (6) were in response to the 27ºC treatment, and most of them were in proteins that could be related to the energy metabolism (Tables 2,3). The only stress-related proteins (mostly chaperons) differently expressed in this node were from two spots that also contained a protein related to energy metabolism, one of them in a spot also differently expressed between *A. ramblae* and *A. brunneus* (i.e. with a significant expression change in node 2 in Fig. 2) (Tables 2,3). On the contrary, of the 9 proteins with expression changes only between *A. ramblae* and *A. brunneus* (node 2 in Fig. 2) 4 were related to stress response and 5 involved in energy metabolism (Table 2). Of these 4 stress-related proteins, one (HSP70, spot No. 2589) had a higher level of expression in *A. ramblae* than *A. brunneus* after the 4ºC treatment, but the other three showed overall different levels of expression between species for both temperature treatments (Table 3).

Between the two populations of *A. brunneus* (node 3 in Fig. 2) the only identified spot (2944) contained a metabolic related protein that was also differently expressed in the comparison of Moroccan *A. ramblae* to the other three populations (Table 3).

Discussion

Protein expression changes associated to the speciation process

The diversification of the *A. brunneus* complex started in the Middle Pleistocene with the colonization of the Iberian peninsula from North Africa by *A. ramblae*, but this range expansion did not result in appreciable changes in morphology (Hidalgo-Galiana *et al.* 2014b). Contrary to this lack of morphological variation, we found a substantial amount of proteins differently expressed between the Moroccan and Iberian populations, mostly in response to the 27ºC treatment and mostly proteins related to energy metabolism. The number of known localities of *A. ramblae* in north Africa (three in Morocco and two in Tunisia) is too low to allow a reliable estimation of their climatic niche, but their conditions may fall outside the main range of the Iberian *A. ramblae*, with more seasonality and highest temperatures in the warmest month (Hidalgo-Galiana *et al.* 2014b). In Hidalgo-Galiana *et al.* (2014b) no significant differences in the thermal limits between the Moroccan and Iberian populations of *A. ramblae* were found, but it must be noted that upper thermal limits were estimated with standard short-term ramping experiments, in which both populations of *A. ramblae* (and also those of *A. brunneus*) tolerated temperatures above 40ºC (Calosi *et al.* 2008; Hidalgo-Galiana *et al.* 2014b), close to the common limit of most eukaryotes and imposed by the denaturation of proteins (Somero 1995). The observed differences in the response to the 27ºC treatment may be related to differences in the long-term capability to sustain temperatures not high enough to induce stress responses, but enough to significantly alter metabolic processes (through, for example, a systemic response to temperature, Hill *et al.* 2008) and affect long term fitness.

The speciation process at the origin of *A. brunneus* was estimated to have taken place within the Iberian peninsula from within populations of *A. ramblae* (Hidalgo-Galiana *et al.* 2014b). This process was associated to significant changes in morphology, both in body size and size and shape of male genitalia, but also to an increased cold tolerance and significant changes in the estimated climatic niche (Calosi *et al.* 2008; Hidalgo-Galiana *et al.* 2014b). These changes allowed the geographical expansion of *A. brunneus* during the Last Glacial Maximum to its current range, completely overlapping that of *A. ramblae* but extending beyond it to occupy much of the western Mediterranean (Fig. 1). The proportion of proteins that showed a significant change in the expression level associated with the origin of *A. brunneus* was similar to the proportion found associated to the colonisation of the Iberian peninsula, but in this case they were mostly proteins with a different response to the 4ºC treatment, and included several proteins known to be associated with thermal stress. The subsequent expansion of *A. brunneus* apparently did not result in further physiological changes, with less than 1% of proteins with significantly

				27ºC				4ºC			
				ramblae		*brunneus*		*ramblae*		*brunneus*	
No	spot	Node	function	Mor	Ibe	Mor	Ibe	Mor	Ibe	Mor	Ibe
1	2021	1	met	5.50	4.91	7.63	11.49	21.71	5.96	7.87	7.47
2	3046	1	met	4.68	2.97	0.67	1.81	14.59	3.00	1.45	1.84
3	956	1	met,str	1.23	6.85	10.87	8.11	4.41	1.21	18.46	15.06
4	705	1	met	8.28	1.74	3.32	2.32	1.96	6.23	2.17	1.23
5	1423	1	met	5.50	1.45	1.38	2.01	5.39	2.12	1.68	0.71
6	2944	1	met	5.61	2.06	1.45	0.96	6.38	2.60	1.56	0.72
7	610	1,2	met	3.94	1.70	1.33	1.05	2.79	3.81	0.93	0.75
8	1140	1,2	met,str	0.76	2.20	3.42	3.40	0.86	1.19	4.44	6.96
9	1366	1,2	met	4.72	2.94	0.72	1.06	4.81	1.93	0.98	0.98
10	2961	1,2	met	9.86	6.70	3.32	5.00	12.59	5.96	4.34	5.63
11	2914	2	met	17.07	46.14	39.46	41.17	19.75	8.52	60.85	67.70
12	2589	2	str	4.93	2.54	12.58	6.86	9.11	11.90	4.55	2.22
13	1042	2	str	7.35	5.28	2.23	3.86	7.20	5.81	3.19	4.29
14	1387	2	met	8.90	3.88	1.12	1.74	6.44	7.81	1.45	1.60
15	1430	2	met	1.18	0.49	0.14	0.34	3.22	1.35	0.21	0.23
16	1495	2	met	9.82	5.38	3.07	4.57	9.74	6.28	3.13	1.49
17	1804	2	met	3.89	2.75	1.47	2.31	2.99	3.03	1.40	1.50
18	1392	2	str	0.49	1.25	2.43	4.30	0.24	0.72	2.14	1.69
19	2390	2	str	0.71	0.78	1.60	1.34	0.14	0.37	0.70	0.58

Table 3. Table 3. Summary of significant changes in expression of the identified protein spots. Values are the average of the three replicas for each population and treatment (RT excluded for clarity, see Table S3 for all values). See Table 2 for the identification of the proteins in each of the spots. Only significant differences (ANOVA, P< 0.001) for the comparison of the populations in each of the nodes of the phylogeny (Fig. 2) are highlighted, in red differences to the 27ºC treatment (paler, lower value; more intense, higher value), in blue differences to be 4ºC treatment, and in grey overall differences irrespective of the temperature treatment. Function: met, metabolic related protein; str, stress related protein (see Table 2 for details on the proteins, in spots 956 and 1140 two proteins were identified). Nodes: 1, Moroccan *A. ramblae vs.* rest of populations;

different expression levels between the two Iberian populations tested. This uniformity is in agreement with the general lack of acclimation in the group and in other Dytiscidae species (Calosi *et al.* 2008, 2010), although there may be interactions with other environmental factors (e.g. salinity, Sánchez-Fernández *et al.* 2010). It may also be simply due to lack of time, given that the geographic and demographic expansion of *A. brunneus* is estimated to have taken place during the Last Glacial Maximum, less than 30,000 years ago (Hidalgo-Galiana *et al.* 2014b).

Differences in the lower thermal limit between *A. ramblae* and *A. brunneus* (including Moroccan and Iberian *A. ramblae*) were also measured with short term ramping experiments, and all were able to sustain temperatures below -4ºC (Calosi *et al.* 2008; Hidalgo-Galiana *et al.* 2014b). However, in this case the temperature used for the treatments (4ºC) is likely to be at the extreme of what the species can tolerate when submerged in water (the physiological experiments were conducted in air, not water), and thus more likely to induce a stress response.

Protein identification

We could identify only a small proportion of all proteins with significantly different expression level, but the selection criteria were fully independent of the comparisons in which the protein spots were differently expressed, and the protein type being selected was unknown. We therefore can assume that any potential bias in our sampling did not affect the proportion of metabolic or stress related proteins in the different comparisons. The agreement of our results with known ecological and physiological differences reinforces our interpretation of the contrasting role that metabolic and stress related proteins have played in the diversification process of the *A. brunneus* complex. However, in must be stressed that 2D-DIGE experiments can only identify targets for further functional studies (Rifai *et al.* 2006; Hamelin *et al.* 2011), and the risk of overinterpreting the data should always be considered (Pavlidis *et al.* 2012; Welch & Jiggins 2014).

The response to cellular stress is known to involve a common set of proteins in diverse taxa (Kültz 2005). These include, among others, molecular chaperons that stabilize denaturing proteins, most notably heat shock proteins (HSP, Krebs & Feder 1997). HSPs are a group of evolutionary highly conserved stress-inducible or constitutive proteins that maintain homeostasis in eukaryotic and prokaryotic cells (Rassow *et al.* 1997). We found four HSPs among the stress-related proteins with significant expression differences, two instances of each HSP70 and HSP60. Although the fingerprint fragments of the two HSP60 were different, both corresponded to the Tribolium HSP60 sequence and were localised very close to each other on the gel (Fig. 3; Table 2), suggesting the presence of some small post-transcriptional modification. HSP60 and similar proteins have been shown to interact with HSP70 in bacteria to modulate the heat shock response (Mogk *et al.* 1997). However, no temperature related function has been found for HSP60 in Drosophila melanogaster (Colinet *et al.* 2010). In our case, the response of the two HSP60 was also the same for both temperature treatments within each species, but the expression level was always higher in *A. brunneus*, suggesting a species-specific response.

In the case of the HSP70 the spots containing the two proteins had a different response: one was less expressed in the Moroccan *A. ramblae* with respect to the other populations in the 27°C treatment (spot 956 in Table 3), and the other was more expressed at 4°C in both populations of *A. ramblae* with respect to the two populations of *A. brunneus* (spot 2589 in Table 3). In other beetle species (Leptinotarsa) it has been shown that in populations exposed to temperatures lower than what they usually experience the HSP70 expression level was higher than in other populations that experience these low temperatures regularly, even when the populations had only recently diverged (Lyytinen *et al.* 2012).

Although the fingerprint fragments of the two identified HSP70s were also different, they matched the same HSP70 reference sequence of Tribolium, but in this case their respective spots had a different molecular weight (32 and 43 KDa respectively). Proteins originated from the same transcript can be modified in the cell compartment and change their chemical and/or physical properties (Schlüter *et al.* 2009), which can be easily identified and characterised using 2D-DIGE (unlike other methods that directly analyse RNA or protein fragments). HSP70 prevents aggregation or premature folding, and it is known to interact with other HSPs. It is the most commonly studied stress protein in cold-related studies (e.g. Sørensen & Loeschcke, 2007; Clark & Worland, 2008). In addition to their general stabilising function, HSPs are also known to play key roles in the origin of phenotypic novelties, suggesting that they may mediate adaptation and speciation in addition to the protective homeostatic effects of the cellular stress response (Williams *et al.* 2009).

Other stress-related proteins were the translationally controlled tumour protein (ICTP) and an ATP binding transporter protein (Sukhai *et al.* 2000) up-regulated in both populations of *A. ramblae* in all treatments. For the former (ICTP) the same spot contained also a protein related to different metabolic functions (14-3-3 protein zeta, Zhang *et al.* 1998). This spot had a significantly different level of expression in two comparisons: Moroccan *A. ramblae* vs. the other populations for the 27°C treatment, and *A. ramblae* vs. *A. brunneus* for both treatments. Without additional data is impossible to know which of the two proteins was responding to which treatment, but it is interesting to note that there is the possibility that the metabolic-related protein was responsible for the differences between the Moroccan *A. ramblae* vs. the rest (node 1 in the tree of Fig. 2), and the stress-related protein for the differences between species (node 2), in agreement with the general pattern. The situation for the only other spot in which two proteins were considered (956, Tables 2,3) was similar, with one stress-related and one metabolic-related protein and with significant response to nodes 1 and 2. This leave open the possibility of a perfect match between differences in metabolic-related proteins in the separation between the Moroccan *A. ramblae* and the rest of populations (node 1), and stress-related proteins between both populations of *A. ramblae* and *A. brunneus* (node 2).

Concluding remarks

We have shown that the protein expression patterns of a complex of closely related species can be associated to parallel changes in their ecology and thermal tolerance, contributing to understand their evolutionary history and their geographical distributions. The colonisation of the Iberian peninsula by North African populations of *Agabus ramblae* during the Middle Pleistocene was accompanied by a change in the response to high temperatures in many proteins related to energy metabolism. The new environments in the Iberian peninsula likely had different climatic conditions, with lower maximum temperatures and seasonality, but if these physiological changes were previous to the colonisation (and likely facilitating it) or posterior (i.e. the result of a local adaptation) is unknown. Once in the Iberian peninsula a speciation process gave rise to *A. brunneus*, with an increased tolerance to cold temperatures that likely allowed its range expansion during the Last Glacial Maximum to a much wider geographical area. These phenotypic changes were paralleled by changes in the protein expression of several stress-related proteins when exposed to low temperatures. Again, whether differences evolved previous or after the geographic expansion is unknown, but the genetic and ecologic uniformity of *A. brunneus* through its known range and its estimated temporal origin (Hidalgo-Galiana *et al.* 2014b) point towards the first possibility.

The first of these transitions (Moroccan to Iberian *A. ramblae*) implied the crossing of a geological barrier (the Strait of Gibraltar) and the colonization of a new territory, likely resulting in isolated, allopatric populations, as suggested by molecular data (Hidalgo-Galiana *et al.* 2014b, although the resolution of the markers used in this study did not allow a precise geographical resolution). As seen above, this transition was not accompanied by substantial morphological change or differences in the lower thermal limit. The geographical setting of the origin of *A. brunneus* is less precise, but it involved substantial morphological change in body size and male genitalia as well as genetic isolation (except for some secondary contact zones: Tunisia, some areas in SE Iberia and some Mediterranean islands, Hidalgo-Galiana *et al.* 2014b). It may be hypothesized that the Moroccan and Iberian populations of *A. ramblae* did not evolve morphological differences, despite the physiological and ecological changes between them, because they likely developed in geographic isolation. On the contrary, ecological and physiological changes between *A. brunneus* and Iberian *A. ramblae* may have evolved in close geographical proximity, resulting in the formation of two genetically isolated species through a process of reinforcement (e.g. Pannell 2012; Orsini *et al.* 2013).

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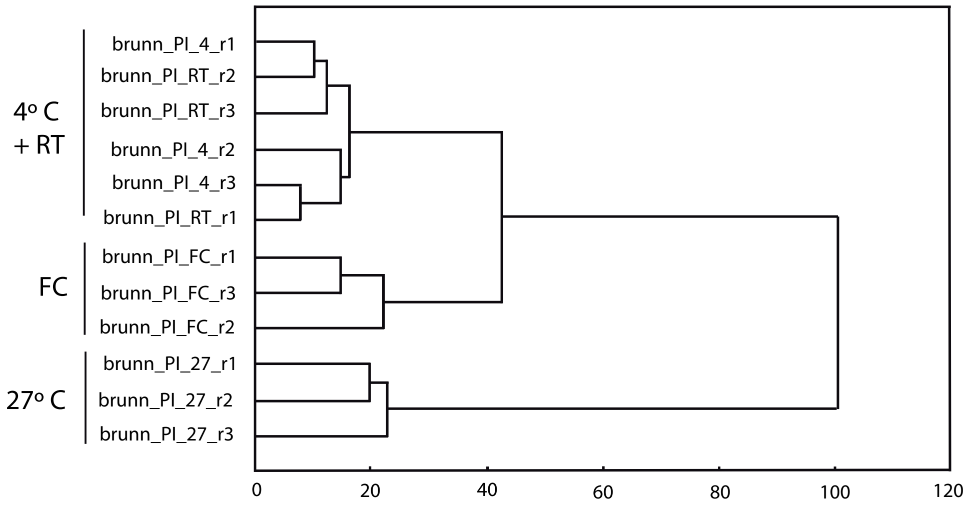
Index for Supporting Information:

SUPPORTING INFORMATION

Iberian Peninsula (NE)



Morocco (C)

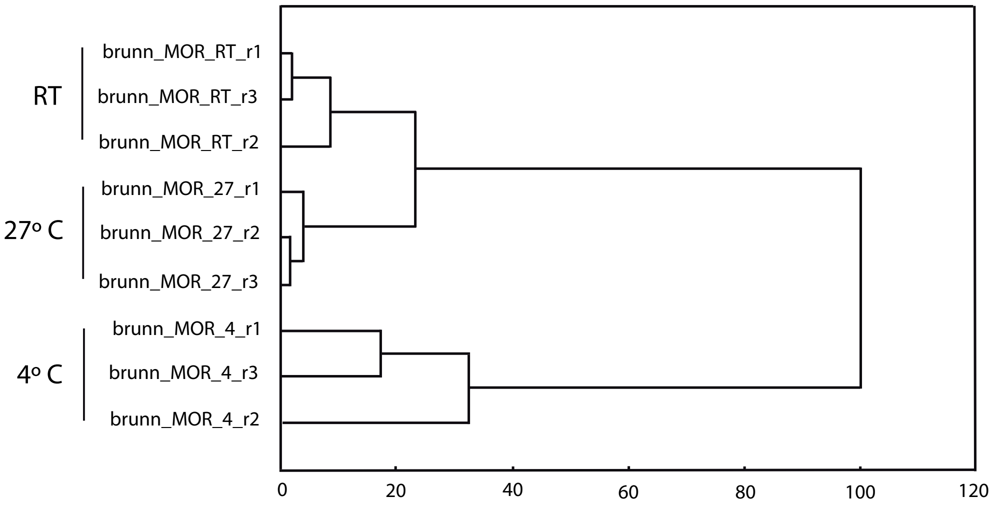


Figure S1. Cluster analysis of the number of significantly differently expressed protein spots for the two *A. brunneus* populations (PI, Iberia; MOR, Morocco). The analysis includes the proteins with significantly different expression for each replicated sample, as measured with ANOVA at $P< 0.01$. RT, room temperature; FC, field control; r1 to r3, replicated samples. See Hidalgo-Galiana *et al.* (2014a) for details of the experimental procedure.

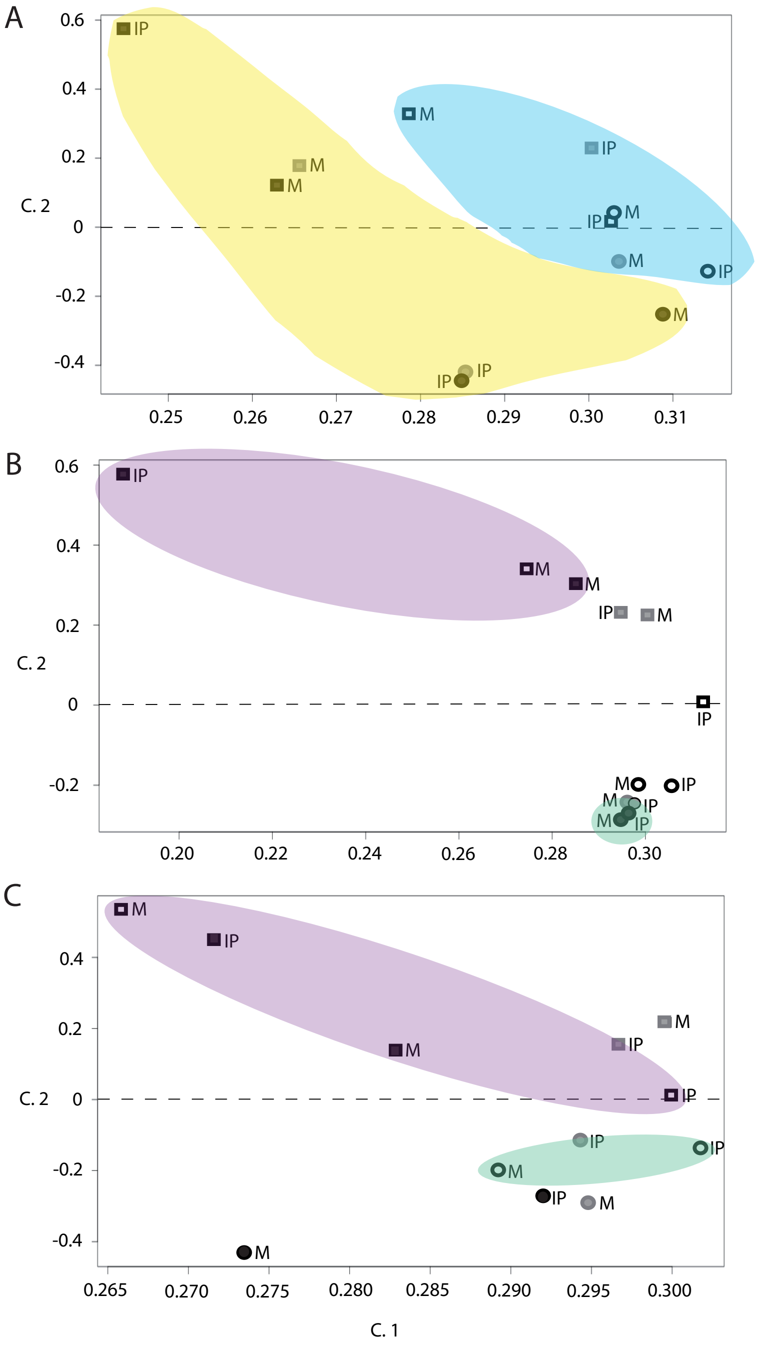


Figure S2. Plots of the PCA analyses of the spots with significantly different expression levels (*P*< 0.001). A: all 563 spots, differences between treatments (including room temperature, RT). B: pooled *A. ramblae* populations *vs.* pooled *A. brunneus* populations, 4°C treatment; C: pooled *A. ramblae* populations *vs.* pooled *A. brunneus* populations, 27°C treatment. Squares: *A. ramblae*; circles: *A. brunneus*; filled symbols: 4°C; empty symbols: 27°C; grey symbols: RT. M: Moroccan; IP: Iberian. See Table S4 for details on the PCA results.

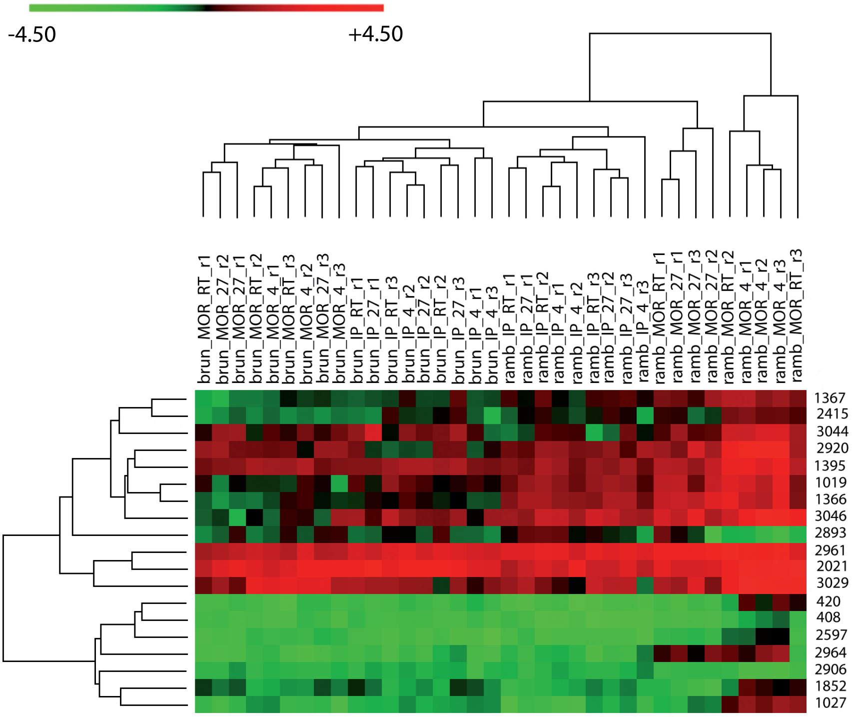


Figure S3. PermutMatrix representation of the cluster of the significantly different expressed spots in the comparison of the Moroccan *A. ramblae vs.* the other populations at a P< 0.001. Colours reflect the level of expression according to the scale.

Figure S4. PermutMatrix representation of the cluster of the significantly different expressed spots in the comparison of the pooled *A. ramblae vs. A. brunneus* at a P< 0.0001. Colours reflect the level of expression according to the scale.

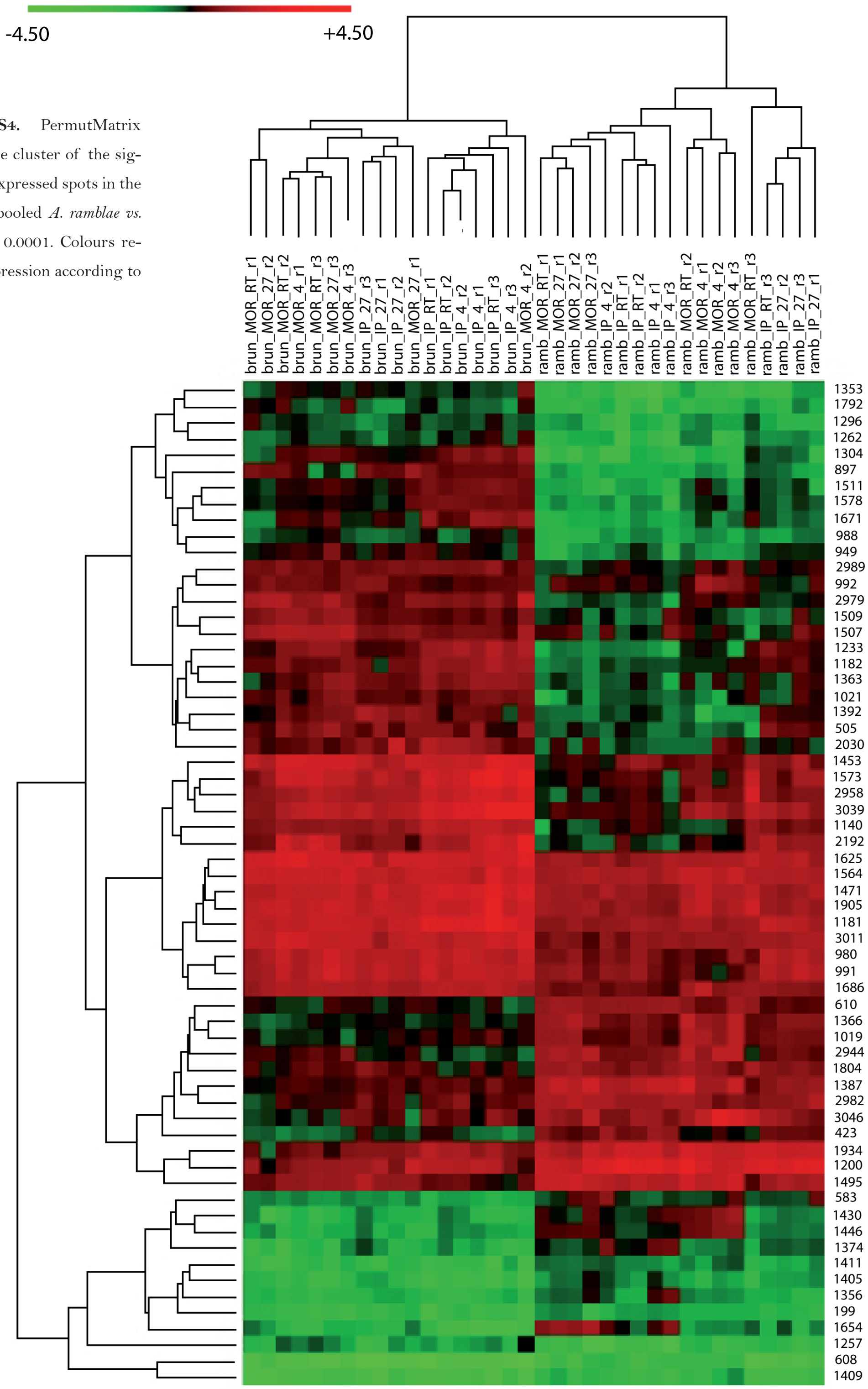


Table S1. Average standardized volumes of the 563 spots common to all experiments for all treatments. Highlighted with a black bar on the side, spots which proteins have been identified (see Tables 2,3). *A. ramb.*: *A. ramblae*, *A. brun.*, *A. brunneus*. M: Morocco; I: Iberia. RT: room temperature treatment (control).

ID	no. spot	*A. ramb.* (M) 4° C	*A. ramb.* (M) RT	*A. ramb.* (M) 27° C	*A. ramb.* (I) 4° C	*A. ramb.* (I) RT	*A. ramb.* (I) 27° C	*A. brun.* (M) 4° C	*A. brun.* (M) RT	*A. brun.* (M) 27° C	*A. brun.* (I) 4° C	*A. brun.* (I) RT	*A. brun.* (I) 27° C
	41	0.024	0.045	0.053	0.146	0.082	0.027	0.025	0.041	0.059	0.051	0.037	0.054
	47	0.016	0.015	0.038	0.032	0.023	0.011	0.005	0.005	0.008	0.022	0.035	0.018
	85	0.092	0.116	0.293	0.117	0.089	0.078	0.095	0.131	0.154	0.291	0.263	0.180
	99	0.163	0.229	0.293	0.231	0.379	0.242	0.270	0.376	0.326	0.421	0.278	0.176
	100	0.040	0.059	0.064	0.198	0.315	0.182	0.147	0.191	0.178	0.214	0.246	0.103
	110	0.019	0.034	0.041	0.168	0.171	0.146	0.071	0.086	0.087	0.040	0.046	0.032
	113	0.007	0.025	0.029	0.103	0.103	0.077	0.026	0.032	0.028	0.070	0.045	0.028
	117	0.002	0.004	0.006	0.015	0.005	0.012	0.003	0.002	0.003	0.006	0.005	0.003
	120	0.008	0.025	0.026	0.123	0.066	0.028	0.014	0.024	0.020	0.037	0.020	0.020
	147	0.115	0.199	0.469	0.575	0.443	0.184	0.111	0.259	0.237	0.178	0.134	0.104
	183	0.235	0.308	1.573	2.229	0.718	0.449	0.211	0.426	0.464	0.269	0.211	0.170
	184	0.089	0.185	0.596	0.180	0.109	0.057	0.072	0.114	0.111	0.078	0.052	0.077
	199	0.228	0.265	0.334	0.290	0.261	0.168	0.080	0.117	0.146	0.113	0.086	0.125
	200	0.179	0.209	0.306	0.165	0.154	0.128	0.060	0.113	0.151	0.115	0.092	0.132
	232	0.007	0.016	0.016	0.012	0.007	0.006	0.005	0.007	0.006	0.005	0.004	0.006
	243	0.089	0.145	0.177	0.865	0.601	0.380	0.307	0.580	0.440	0.165	0.126	0.123
	252	0.056	0.099	0.191	0.158	0.167	0.107	0.056	0.089	0.087	0.056	0.067	0.052
	267	0.617	3.123	3.933	8.459	3.643	1.807	0.428	0.452	0.328	0.299	0.399	0.680
	296	0.065	0.117	0.084	0.571	0.567	0.393	0.445	0.641	0.686	0.148	0.114	0.110
	302	0.140	0.161	0.159	1.172	0.792	0.545	0.602	1.054	1.111	0.161	0.186	0.208
	313	0.216	0.200	0.226	0.440	0.373	0.095	0.226	0.386	0.444	0.080	0.060	0.112
	339	0.093	0.205	0.408	0.344	0.241	0.154	0.169	0.165	0.108	0.082	0.112	0.140
	356	0.249	0.181	0.187	0.766	0.714	0.617	0.400	0.461	0.413	0.209	0.209	0.176
	358	0.080	0.115	0.249	0.155	0.130	0.073	0.095	0.129	0.161	0.066	0.053	0.114

359	0.700	0.526	0.477	0.369	0.523	0.619	0.498	0.495	0.684	0.357	0.567	0.631
367	0.054	0.070	0.071	0.081	0.058	0.043	0.035	0.044	0.052	0.015	0.014	0.045
369	0.425	0.360	0.714	1.112	0.849	0.926	0.693	0.984	0.875	0.298	0.245	0.369
371	0.039	0.059	0.078	0.192	0.132	0.095	0.060	0.070	0.055	0.031	0.021	0.081
375	0.047	0.052	0.058	0.234	0.142	0.089	0.038	0.064	0.063	0.037	0.027	0.053
376	0.118	0.294	0.390	0.473	0.479	0.288	0.210	0.263	0.210	0.076	0.089	0.292
383	0.237	0.418	1.292	1.040	0.605	0.234	0.403	0.482	0.458	0.144	0.118	0.226
389	0.139	0.231	0.269	0.484	0.632	0.327	0.135	0.214	0.217	0.074	0.124	0.133
392	0.120	0.263	0.346	0.374	0.357	0.385	0.196	0.238	0.316	0.089	0.086	0.290
393	0.087	0.208	0.244	0.837	0.193	0.304	0.177	0.188	0.240	0.080	0.064	0.233
408	0.586	0.207	0.137	0.103	0.123	0.111	0.172	0.156	0.191	0.193	0.193	0.173
409	0.022	0.027	0.036	0.033	0.047	0.049	0.014	0.015	0.025	0.021	0.018	0.026
410	0.046	0.061	0.080	0.141	0.177	0.172	0.046	0.074	0.048	0.045	0.032	0.033
415	0.503	0.331	0.381	0.823	0.921	0.585	0.504	0.714	0.647	0.177	0.234	0.282
420	1.247	0.577	0.167	0.125	0.127	0.133	0.208	0.185	0.229	0.305	0.201	0.215
422	0.399	0.439	0.816	1.135	0.997	0.947	0.639	0.749	0.671	0.377	0.358	0.634
423	1.140	1.628	1.758	3.768	2.193	1.897	0.629	0.581	0.626	0.560	0.996	1.099
433	0.106	0.255	0.778	0.495	0.343	0.134	0.155	0.530	0.602	0.158	0.218	0.354
436	0.197	0.163	0.171	0.479	0.641	0.651	0.415	0.521	0.359	0.129	0.147	0.268
437	0.586	0.721	1.081	1.033	1.338	0.636	0.475	0.684	0.780	0.460	0.762	0.617
447	0.073	0.149	0.303	0.198	0.320	0.316	0.196	0.229	0.296	0.051	0.048	0.182
483	0.340	0.744	1.890	1.513	2.664	3.073	3.574	4.102	3.487	0.864	1.381	1.719
486	0.848	1.626	2.465	1.205	3.504	4.490	5.248	6.336	6.777	1.502	1.504	3.555
499	0.133	0.064	0.137	0.464	0.129	0.046	0.080	0.217	0.132	0.096	0.120	0.056
504	0.213	0.122	0.163	0.164	0.191	0.318	0.304	0.356	0.267	0.195	0.205	0.372
505	0.613	0.562	0.807	0.482	1.578	1.791	2.757	2.660	2.896	2.214	2.296	2.309
510	0.292	0.273	0.054	0.078	0.083	0.119	0.172	0.177	0.188	0.326	0.252	0.227
511	0.888	0.653	1.025	0.611	2.429	2.832	4.739	3.703	3.712	2.375	4.380	2.617

	517	0.198	0.226	0.338	0.218	0.283	0.380	0.172	0.174	0.160	0.165	0.212	0.186
	521	0.448	0.258	0.136	0.062	0.110	0.115	0.304	0.290	0.276	0.277	0.262	0.155
	537	4.762	1.637	1.183	0.717	1.285	1.256	2.939	2.016	2.285	1.479	1.669	1.532
	538	0.179	0.118	0.242	0.277	0.276	0.313	0.449	0.362	0.462	0.328	0.348	0.537
	539	0.137	0.238	0.214	0.430	0.288	0.264	0.177	0.234	0.273	0.130	0.079	0.125
	541	0.295	0.368	0.421	1.007	0.624	0.510	0.192	0.383	0.324	0.520	0.402	0.470
	551	0.528	0.432	0.585	0.453	0.394	0.411	0.171	0.336	0.415	0.627	0.751	0.601
	565	0.051	0.040	0.095	0.121	0.100	0.051	0.031	0.032	0.040	0.072	0.036	0.062
	571	0.084	0.204	0.241	0.491	0.200	0.127	0.089	0.142	0.110	0.053	0.048	0.090
	583	1.140	0.672	1.533	1.535	0.773	1.181	0.402	0.407	0.424	0.277	0.218	0.344
	588	0.152	0.282	0.457	0.156	0.325	0.293	0.271	0.403	0.388	0.120	0.120	0.379
	598	1.344	0.973	0.624	0.513	0.525	0.635	0.483	0.484	0.569	0.565	0.538	0.572
	608	0.108	0.053	0.087	0.119	0.057	0.040	0.021	0.026	0.029	0.020	0.018	0.011
■	610	2.794	3.298	3.943	3.812	2.799	1.698	0.933	1.107	1.330	0.750	1.194	1.052
	611	0.241	0.222	0.113	0.185	0.111	0.093	0.044	0.089	0.070	0.061	0.037	0.048
	619	0.773	0.750	1.361	0.660	0.575	0.369	0.302	0.306	0.260	0.334	0.243	0.357
	624	6.678	1.677	0.986	0.736	0.743	1.156	1.768	1.455	1.350	2.226	2.697	2.191
	631	0.748	0.966	0.973	0.662	0.896	0.639	0.856	0.891	1.220	0.687	0.501	1.019
	645	0.450	0.377	0.380	0.304	0.397	0.349	0.369	0.331	0.384	0.348	0.217	0.441
	646	3.864	2.128	3.625	2.516	2.209	2.671	1.856	1.995	2.001	2.793	1.637	2.109
	656	1.104	1.191	1.013	1.359	1.290	1.288	1.269	1.479	1.497	0.676	0.824	1.091
	670	0.376	0.208	0.326	0.329	0.382	0.262	0.204	0.251	0.257	0.473	0.741	0.262
	672	0.040	0.046	0.024	0.042	0.039	0.070	0.159	0.087	0.072	0.366	0.392	0.433
	673	1.507	1.340	1.996	2.552	1.933	0.876	1.244	1.298	1.180	0.327	0.291	1.067
	684	2.019	2.327	2.354	1.442	1.368	1.513	0.897	0.965	1.157	1.754	2.884	1.484
	690	0.388	0.151	0.082	0.176	0.097	0.135	0.062	0.063	0.052	0.074	0.072	0.091
	701	3.531	1.925	1.151	0.755	1.094	1.217	2.634	1.832	2.851	2.556	3.443	2.398
■	705	1.961	3.645	8.284	6.231	3.861	1.741	2.173	3.692	3.319	1.229	1.081	2.324

711	0.024	0.033	0.035	0.053	0.062	0.043	0.033	0.047	0.040	0.032	0.034	0.030
716	0.210	0.290	0.593	0.431	0.317	0.178	0.118	0.118	0.127	0.457	0.180	0.536
725	0.820	0.680	0.909	2.184	1.112	0.702	0.326	0.611	0.584	0.323	0.453	1.076
739	0.949	0.514	0.687	0.526	0.497	0.377	0.603	0.847	0.876	0.392	0.413	0.530
747	1.337	0.850	1.005	1.345	1.119	0.666	1.251	1.870	1.403	0.187	0.240	0.627
755	0.426	0.281	0.221	0.774	0.482	0.422	0.279	0.447	0.440	0.223	0.223	0.419
756	0.012	0.016	0.008	0.010	0.015	0.027	0.134	0.058	0.060	0.060	0.063	0.067
757	0.277	0.606	0.918	0.960	0.518	0.396	0.301	0.392	0.284	0.207	0.239	0.588
763	2.180	1.919	1.049	0.497	0.895	1.152	1.505	1.510	1.517	0.951	0.752	0.929
767	1.273	1.130	0.385	0.251	0.552	0.648	0.757	0.508	0.441	0.551	0.723	0.499
769	1.260	1.123	0.945	0.336	0.648	0.762	1.308	0.791	1.003	0.667	0.659	0.544
777	6.146	3.032	2.119	1.150	1.934	3.012	5.125	3.560	2.888	4.508	4.088	3.417
785	0.800	1.275	1.291	1.553	0.764	0.792	0.491	0.324	0.229	0.504	0.511	0.493
792	0.956	1.934	3.960	3.220	3.107	2.107	1.769	2.933	2.378	0.556	0.465	0.825
797	0.874	0.593	0.643	0.497	0.794	0.778	0.588	0.584	0.400	0.343	0.331	0.523
801	1.405	0.735	0.825	0.882	1.088	0.730	1.364	1.388	1.329	0.967	1.403	1.141
805	0.398	0.324	0.491	0.489	0.487	0.522	0.365	0.356	0.355	0.242	0.224	0.324
808	2.812	2.967	3.617	2.491	2.418	2.183	1.936	2.450	2.242	0.897	1.057	1.271
811	0.277	0.328	0.321	0.350	0.321	0.305	0.244	0.257	0.265	0.131	0.250	0.214
815	2.865	2.856	3.237	2.759	3.230	3.214	1.750	2.121	2.567	1.086	0.831	1.290
818	0.246	0.224	0.223	0.132	0.118	0.128	0.041	0.050	0.090	0.104	0.084	0.113
825	0.503	0.232	0.227	0.104	0.233	0.350	0.891	0.503	0.406	0.735	0.571	0.491
832	0.197	0.232	0.181	0.244	0.338	0.307	0.220	0.207	0.279	1.253	2.084	0.371
834	0.240	0.210	0.276	0.790	0.438	0.177	0.474	0.664	0.646	0.311	0.235	0.123
839	1.107	1.106	0.833	0.431	0.866	1.219	1.814	1.117	1.947	2.630	3.067	1.757
848	1.730	1.181	0.983	0.563	0.925	1.106	1.126	1.063	1.278	2.438	2.093	2.013
850	2.081	1.699	1.271	0.898	0.971	1.208	1.576	1.763	3.287	2.540	3.065	2.952
851	0.107	0.067	0.056	0.082	0.050	0.043	0.032	0.055	0.053	0.039	0.091	0.053

858	0.164	0.149	0.036	0.058	0.145	0.266	0.449	0.307	0.220	0.593	0.717	0.471
860	0.335	0.769	0.936	0.678	0.796	0.646	0.289	0.294	0.412	0.420	0.493	0.368
864	2.777	1.877	1.067	0.645	0.819	1.127	1.657	1.008	1.745	2.969	3.513	3.109
870	1.475	1.504	1.394	1.270	0.908	1.106	1.041	1.133	1.088	1.093	1.239	1.310
871	1.771	0.958	0.981	1.233	1.472	1.289	2.271	2.548	2.609	1.339	1.585	2.250
877	0.181	0.178	0.153	0.314	0.521	0.443	0.359	0.624	0.533	0.348	0.503	0.304
885	0.714	0.492	0.487	0.232	0.310	0.296	0.353	0.196	0.204	0.501	0.681	0.505
886	0.326	0.425	0.520	0.561	0.451	0.292	0.150	0.183	0.228	0.253	0.153	0.160
890	0.657	0.834	0.862	0.277	0.393	0.364	0.603	0.665	0.541	0.176	0.252	0.399
892	0.468	0.197	0.255	0.339	0.345	0.230	0.742	0.875	0.732	0.085	0.100	0.292
893	0.278	0.343	0.263	0.256	0.292	0.361	0.882	0.728	0.703	0.307	0.533	0.465
897	0.306	0.377	0.263	0.255	0.368	0.342	1.373	1.739	2.429	1.918	2.077	2.086
898	0.099	0.061	0.033	0.113	0.224	0.267	0.108	0.114	0.124	0.132	0.198	0.153
904	0.049	0.025	0.027	0.015	0.032	0.050	0.040	0.036	0.049	0.046	0.048	0.058
906	0.368	0.303	0.232	0.260	0.167	0.118	0.144	0.146	0.146	0.136	0.149	0.206
910	0.211	0.331	0.321	0.204	0.169	0.192	0.165	0.131	0.117	0.144	0.153	0.178
917	0.123	0.196	0.166	0.079	0.083	0.057	0.062	0.063	0.078	0.057	0.053	0.082
935	0.231	0.274	0.245	0.275	0.295	0.195	0.170	0.156	0.170	5.018	8.342	7.563
949	0.315	0.438	0.276	0.350	0.797	0.907	1.472	1.120	1.921	1.034	1.232	1.128
951	0.352	0.261	0.316	0.163	0.141	0.213	0.160	0.124	0.131	0.893	1.232	0.564
956	4.412	3.370	1.231	1.214	4.926	6.849	18.456	9.853	10.867	15.057	13.023	8.114
962	0.234	0.273	0.174	0.281	0.542	0.660	0.685	0.527	0.455	1.277	1.389	0.888
980	2.162	2.120	2.595	2.303	4.571	5.932	7.844	7.419	8.033	7.849	8.330	6.682
981	1.637	1.258	0.457	0.627	1.966	1.819	6.639	2.898	2.994	4.108	4.855	1.325
988	0.344	0.309	0.167	0.334	0.338	0.456	1.035	0.684	0.882	0.813	0.903	0.527
991	1.221	2.863	3.308	2.370	4.716	6.710	7.497	7.444	8.130	8.533	8.592	7.254
992	3.786	1.158	1.554	1.190	1.133	1.169	2.351	2.250	2.160	1.682	1.442	2.491
994	4.295	3.324	1.007	1.695	1.636	1.898	2.506	2.416	3.865	2.941	3.198	3.646

1019	5.404	3.860	3.075	1.647	2.130	2.338	0.870	0.832	0.891	1.251	1.517	1.095
1021	0.561	0.801	0.492	0.626	0.750	1.151	2.320	2.440	1.882	2.181	2.235	1.722
1025	0.250	0.192	0.042	0.056	0.064	0.049	0.082	0.076	0.074	0.043	0.058	0.030
1027	2.033	1.238	0.527	0.162	0.268	0.381	0.321	0.305	0.253	0.564	0.402	0.435
1030	0.384	0.307	0.330	0.090	0.268	0.281	0.456	0.259	0.374	0.360	0.498	0.374
1036	5.995	2.783	2.559	1.271	2.036	3.117	2.311	1.605	1.596	2.879	3.675	2.442
1042	7.204	6.474	7.354	5.806	5.422	5.281	3.190	3.123	2.227	4.292	4.072	3.858
1043	0.484	0.578	0.631	1.140	0.951	0.754	1.446	3.208	2.575	1.721	1.140	1.727
1064	1.664	1.894	1.470	2.297	1.695	2.303	0.572	0.535	0.435	2.352	2.406	1.735
1071	0.260	0.408	0.372	0.189	0.349	0.453	0.398	0.271	0.302	0.498	0.545	0.468
1072	1.527	1.474	1.612	0.688	0.787	0.618	1.995	3.299	2.529	1.226	1.337	0.973
1088	0.129	0.212	0.202	0.063	0.136	0.169	0.202	0.108	0.102	0.098	0.146	0.114
1093	10.763	7.488	1.906	2.324	3.514	3.601	6.181	5.269	4.022	4.979	5.348	4.478
1105	7.022	8.504	2.939	2.982	5.469	7.202	10.141	8.883	10.799	13.004	14.071	8.257
1107	2.513	4.262	3.289	1.834	2.396	2.387	5.326	3.804	2.708	3.530	4.288	2.795
1108	0.779	0.867	0.750	0.578	0.592	0.856	0.462	0.362	0.325	1.048	0.674	0.813
1110	4.767	5.582	3.097	1.853	3.118	4.255	4.356	3.817	4.573	3.832	3.491	3.387
1111	2.482	1.863	1.473	0.895	0.965	0.981	1.794	1.309	1.289	1.461	1.559	1.184
1112	8.680	11.799	2.911	1.289	2.337	3.122	6.957	4.738	4.142	6.720	7.705	4.049
1133	0.529	0.462	0.288	0.162	0.142	0.288	0.300	0.181	0.179	0.425	0.427	0.315
1134	0.215	0.142	0.096	0.114	0.179	0.332	0.151	0.089	0.114	0.253	0.266	0.326
1137	0.099	0.087	0.053	0.045	0.058	0.109	0.047	0.028	0.030	0.078	0.056	0.057
1140	0.864	1.627	0.761	1.185	1.860	2.203	4.440	3.228	3.422	6.959	7.908	3.399
1144	0.389	0.301	0.293	0.235	0.399	0.324	0.883	0.764	0.461	4.024	4.805	4.128
1149	0.316	0.364	0.180	1.130	0.975	0.851	0.595	0.763	0.664	0.765	0.573	0.474
1150	4.753	4.444	2.217	1.583	2.447	2.706	5.107	4.553	3.090	5.122	5.400	5.124
1155	1.499	1.767	1.293	0.842	1.050	0.826	1.207	0.764	0.808	2.482	2.161	1.237
1157	2.335	2.506	1.540	1.575	2.820	2.841	1.214	1.229	1.417	2.797	2.625	1.221

1163	2.621	3.013	2.318	2.729	2.329	2.251	2.136	2.252	1.745	3.934	4.492	2.785
1168	0.683	0.895	0.156	0.230	0.701	0.833	1.910	0.995	1.134	3.675	3.689	1.188
1176	9.656	10.720	1.997	1.001	1.914	2.346	4.162	2.379	2.751	8.614	9.532	4.328
1180	2.397	2.355	1.982	2.541	2.652	3.437	3.636	2.667	2.154	2.026	1.917	2.276
1181	4.179	4.228	3.452	3.738	5.953	6.286	10.154	8.758	9.161	15.992	20.227	8.326
1182	1.015	0.837	0.498	0.752	1.069	1.543	2.976	2.473	1.978	3.696	4.379	1.774
1185	0.426	0.324	0.243	0.123	0.294	0.428	0.153	0.131	0.120	0.292	0.329	0.287
1187	3.690	3.165	2.146	1.520	1.598	2.376	0.919	0.594	0.707	3.213	4.677	2.236
1192	1.418	0.984	0.384	0.620	0.718	0.779	1.222	1.548	1.621	0.737	0.730	1.771
1199	1.239	1.013	0.749	0.575	0.680	1.004	0.568	0.409	0.518	0.787	0.722	0.615
1200	10.834	16.576	10.845	18.042	19.677	17.090	3.000	3.617	4.890	5.402	6.926	4.236
1206	2.114	2.451	0.955	0.995	1.092	1.294	2.651	3.015	2.157	3.033	4.641	3.597
1207	47.303	10.153	34.576	6.620	11.333	9.876	53.387	38.336	15.406	43.445	65.400	48.619
1217	1.690	1.108	0.752	0.666	1.014	1.284	1.325	1.063	0.978	1.424	1.154	1.442
1220	1.828	1.284	0.582	0.345	0.596	0.652	1.568	2.776	4.165	5.054	6.523	2.207
1222	3.496	2.930	1.136	0.626	0.788	0.916	2.120	1.316	0.961	3.378	3.236	2.349
1223	1.975	2.199	0.674	0.626	1.019	1.092	2.986	2.309	1.677	2.601	3.720	1.847
1226	0.838	0.279	0.121	0.088	0.148	0.278	0.554	0.266	0.325	0.914	0.615	0.870
1233	0.755	0.888	0.447	0.344	1.223	1.571	3.820	2.301	2.283	4.293	4.945	1.393
1237	2.272	1.748	1.207	1.273	1.412	1.681	2.517	2.052	2.123	2.130	2.418	2.365
1242	8.087	3.000	0.534	0.329	0.432	0.816	1.228	0.544	0.605	3.791	3.865	1.340
1247	1.397	2.357	2.605	2.114	2.875	5.730	1.427	1.298	1.121	3.235	3.977	3.156
1252	0.535	0.724	0.177	0.149	0.220	0.446	0.261	0.152	0.159	1.227	1.176	0.699
1254	1.128	0.986	0.332	0.076	0.158	0.199	0.823	0.512	0.380	1.119	1.174	0.636
1257	0.130	0.132	0.070	0.042	0.119	0.120	0.588	0.390	0.291	0.265	0.329	0.195
1262	0.223	0.400	0.216	0.139	0.193	0.378	1.134	0.685	0.648	0.822	0.973	0.597
1265	0.848	0.985	0.277	0.594	0.786	1.844	0.761	0.499	0.431	2.316	2.968	1.821
1266	0.899	1.207	0.454	0.666	0.858	0.706	1.150	0.981	0.661	0.331	0.272	0.278

1276	7.920	5.822	1.413	0.748	0.841	1.486	2.336	1.130	1.340	4.129	3.888	1.729
1279	0.842	0.987	0.783	0.677	0.883	0.575	0.675	0.618	0.555	1.145	0.793	0.642
1281	11.772	8.085	2.099	0.942	0.988	2.032	3.734	2.223	0.958	1.437	1.358	1.090
1290	1.807	2.156	1.013	2.394	2.759	3.579	8.046	4.609	4.879	7.489	6.883	3.913
1296	0.342	0.468	0.295	0.131	0.195	0.158	0.766	0.998	0.822	0.695	0.822	0.493
1299	2.870	2.443	0.837	0.730	0.593	0.886	1.644	0.920	0.607	3.261	3.365	1.518
1304	0.178	0.362	0.116	0.121	0.317	0.450	2.325	1.242	1.280	2.540	2.162	1.115
1305	0.489	0.644	0.389	0.270	0.556	0.853	0.709	0.413	0.215	0.590	0.646	0.422
1312	12.381	19.643	3.857	5.071	7.482	8.095	14.469	9.232	7.817	9.907	11.232	7.883
1314	0.234	0.214	0.075	0.994	0.589	0.698	0.599	0.527	0.427	0.903	0.806	0.676
1319	2.662	4.407	0.918	0.325	0.510	0.634	0.939	0.636	0.378	1.987	1.730	1.117
1326	3.293	3.306	0.784	1.432	1.633	1.850	3.189	2.078	1.826	4.931	4.978	3.625
1353	0.096	0.179	0.125	0.090	0.063	0.240	1.539	0.874	0.859	0.798	0.811	1.040
1356	0.382	0.402	0.805	1.386	0.235	0.192	0.140	0.157	0.190	0.165	0.144	0.237
1360	3.513	2.005	1.085	0.837	1.629	1.872	4.452	3.870	2.689	2.337	2.237	2.409
1363	0.851	1.088	0.653	0.548	0.859	1.140	2.860	2.086	2.210	3.689	3.357	2.061
1366	4.813	4.636	4.717	1.933	2.167	2.940	0.985	0.763	0.717	0.980	0.960	1.057
1367	2.558	2.241	1.763	0.974	1.323	1.221	0.779	0.613	0.631	0.920	0.812	1.024
1374	0.535	0.519	0.747	2.199	0.865	0.678	0.227	0.183	0.143	0.214	0.310	0.445
1380	1.032	0.756	0.973	1.360	3.326	4.425	6.992	7.045	6.717	4.413	3.625	5.691
1387	6.437	7.218	8.899	7.805	4.993	3.880	1.448	1.247	1.123	1.600	2.052	1.737
1392	0.244	0.540	0.486	0.715	1.128	1.252	2.142	2.068	2.430	1.691	1.940	4.304
1395	7.882	4.882	3.065	1.861	2.400	2.086	2.465	2.078	1.837	2.124	2.282	2.560
1398	1.266	1.980	3.153	2.450	2.236	1.556	2.566	3.234	2.474	0.553	0.394	1.313
1401	0.430	0.299	0.488	0.795	0.904	0.743	0.535	0.849	0.652	0.235	0.206	0.401
1405	0.244	0.218	0.722	0.692	0.336	0.343	0.103	0.135	0.123	0.099	0.072	0.249
1409	0.238	0.097	0.176	0.096	0.081	0.080	0.044	0.042	0.043	0.063	0.032	0.044
1411	0.551	0.408	0.555	0.454	0.360	0.320	0.135	0.130	0.105	0.179	0.084	0.126

1415	2.806	2.714	1.885	3.230	2.798	1.717	1.721	1.997	2.175	0.623	0.729	1.261
1416	2.920	2.843	2.084	2.846	2.371	1.695	1.856	2.322	2.271	0.808	0.626	1.209
1421	1.978	0.746	0.799	1.684	1.233	0.534	1.955	3.568	3.543	0.487	0.588	0.721
1423	5.390	4.800	5.504	2.121	2.445	1.453	1.681	1.500	1.384	0.706	0.877	2.010
1426	0.157	0.113	0.140	1.960	0.892	0.486	0.470	1.135	0.873	0.119	0.138	0.135
1430	3.223	1.166	1.184	1.351	0.659	0.492	0.205	0.256	0.140	0.234	0.282	0.342
1436	0.910	0.711	0.706	1.594	1.067	0.554	1.279	1.814	1.521	0.374	0.337	0.476
1437	0.313	0.171	0.191	0.149	0.168	0.154	0.144	0.230	0.281	0.081	0.069	0.195
1441	0.480	0.612	0.908	0.580	0.521	0.420	0.477	0.705	0.597	0.156	0.130	0.396
1446	2.799	1.248	1.593	1.013	0.651	0.511	0.225	0.223	0.277	0.357	0.313	0.348
1452	2.533	1.446	1.370	0.297	0.363	0.843	1.038	0.715	0.799	1.053	1.189	0.984
1453	2.054	2.527	1.381	1.923	3.343	3.451	13.697	11.825	9.381	11.718	11.878	6.280
1456	0.292	0.142	0.065	0.148	0.146	0.248	0.418	0.439	0.276	0.076	0.107	0.096
1462	0.632	0.495	0.550	0.694	1.115	1.129	1.780	2.158	2.220	1.054	0.999	1.791
1465	0.846	1.534	1.942	3.647	2.174	0.751	1.004	2.003	1.722	0.160	0.220	0.450
1471	6.834	6.290	4.077	4.015	4.052	4.599	11.534	8.445	7.346	12.277	15.537	8.413
1472	1.318	1.121	1.096	1.040	1.087	1.071	1.893	1.602	1.401	2.088	2.720	1.544
1473	0.242	0.264	0.330	0.958	0.681	0.775	0.131	0.209	0.523	0.815	1.121	1.343
1480	0.061	0.041	0.079	0.405	0.328	0.155	0.125	0.207	0.224	0.030	0.027	0.030
1483	0.239	0.289	0.307	0.236	0.248	0.215	0.546	0.529	0.526	0.281	0.295	0.404
1491	1.591	1.546	1.929	1.164	1.416	1.314	0.954	1.050	0.939	0.642	0.713	0.918
1495	9.742	8.003	9.823	6.283	5.644	5.383	3.127	3.216	3.067	1.487	1.689	4.569
1498	1.051	0.615	0.346	0.421	0.392	0.735	0.321	0.210	0.171	0.640	0.571	0.461
1499	4.543	2.522	2.834	2.358	2.242	1.436	2.131	3.419	3.855	0.653	0.690	1.257
1507	1.049	1.128	1.264	1.835	0.861	1.698	6.035	5.883	4.617	2.152	2.234	2.542
1509	0.798	0.798	0.858	1.194	0.563	1.085	3.653	3.521	2.963	1.604	1.795	1.839
1511	0.738	0.636	0.237	0.156	0.444	0.536	1.494	1.148	1.354	2.390	2.398	1.039
1523	0.600	0.513	0.523	0.262	0.500	0.553	0.837	0.672	0.548	1.090	0.988	1.047

1527	0.373	0.452	0.440	0.909	0.507	0.298	0.757	1.214	1.157	0.215	0.216	0.282
1533	0.078	0.110	0.484	0.783	0.317	0.141	0.148	0.272	0.205	0.100	0.076	0.085
1535	2.498	2.520	1.563	1.993	1.864	1.923	2.622	2.125	2.051	4.557	4.769	2.905
1546	4.449	3.877	2.835	1.830	2.174	2.468	4.194	2.940	3.775	6.741	9.112	6.101
1555	1.484	3.582	7.306	10.979	8.591	6.399	7.961	14.617	12.676	0.848	1.465	5.032
1557	1.319	2.634	5.407	5.818	4.675	3.264	4.582	8.813	6.967	0.376	0.723	2.877
1564	8.530	6.430	5.372	5.160	5.520	5.553	11.831	10.845	9.595	8.043	12.010	7.479
1566	2.188	2.936	0.805	0.801	1.216	1.505	4.856	1.901	2.340	9.484	10.393	3.072
1568	0.956	1.018	1.010	0.627	0.662	0.861	0.316	0.292	0.276	0.700	0.609	0.783
1572	0.117	0.161	0.197	0.369	0.317	0.267	0.148	0.193	0.227	0.069	0.068	0.155
1573	1.856	4.436	1.220	1.115	2.099	3.788	15.114	7.303	5.554	15.984	17.572	6.244
1578	0.794	0.589	0.333	0.304	0.462	0.671	1.398	1.061	1.332	2.331	2.360	0.949
1583	0.401	1.501	2.004	6.046	2.200	1.224	0.295	0.283	0.252	0.263	0.262	0.546
1586	6.700	5.356	2.232	1.633	2.077	2.239	5.711	4.938	4.026	4.758	5.186	4.851
1606	0.327	0.313	0.237	0.092	0.287	0.404	0.503	0.291	0.573	0.749	0.721	0.469
1625	5.478	5.715	4.374	4.875	4.158	5.672	15.108	12.411	12.277	10.601	11.810	9.885
1630	0.619	0.456	0.681	1.189	0.790	0.371	0.929	1.159	1.164	0.188	0.135	0.227
1643	0.434	1.108	2.253	2.781	1.815	0.557	1.162	2.353	1.971	0.393	0.291	0.534
1654	0.404	1.207	4.096	1.923	0.571	0.467	0.136	0.313	0.359	0.045	0.076	0.177
1660	0.078	0.360	0.956	0.445	0.214	0.189	0.128	0.186	0.254	0.037	0.055	0.107
1671	0.606	0.681	0.213	0.258	0.455	0.561	2.234	0.912	1.037	2.991	3.059	1.170
1677	1.103	0.969	0.708	0.527	0.792	0.697	1.305	1.211	1.189	0.813	0.966	0.840
1679	2.033	1.714	1.043	0.874	0.797	1.075	2.390	1.738	1.818	2.662	3.087	1.854
1683	15.500	17.819	2.740	1.773	2.440	3.712	8.042	6.296	4.915	13.785	14.806	9.811
1684	4.856	6.166	0.668	0.657	0.855	1.348	2.684	2.149	1.958	3.354	4.226	2.718
1685	5.840	6.758	3.728	2.412	5.896	6.108	11.265	11.527	9.235	8.708	8.918	9.723
1686	4.912	3.850	2.209	1.628	2.741	2.860	5.444	5.233	5.120	6.345	7.260	5.261
1688	0.604	0.339	0.453	0.416	0.404	0.358	0.272	0.256	0.289	0.224	0.166	0.342

1693	1.394	0.890	1.103	0.795	0.694	0.558	0.692	0.860	0.858	0.395	0.625	0.495
1707	1.397	1.889	0.495	0.686	0.957	1.669	1.881	1.239	1.266	2.480	2.269	2.020
1713	5.959	3.979	0.853	0.354	0.862	1.009	2.524	1.201	1.726	3.996	3.646	1.639
1719	2.794	1.350	1.568	0.783	2.055	2.737	3.742	3.425	3.316	1.233	1.305	1.701
1727	0.049	0.270	0.669	0.698	0.754	0.457	0.498	1.312	1.436	0.056	0.083	0.331
1730	1.261	1.439	0.928	0.828	0.745	1.189	0.942	0.698	0.654	2.418	2.556	1.338
1736	0.357	0.795	0.748	0.773	1.415	1.266	2.106	2.320	1.786	0.182	0.321	1.230
1743	0.427	0.450	0.709	0.382	0.745	0.810	1.102	1.074	1.288	1.058	0.545	0.971
1771	0.050	0.070	0.235	0.101	0.151	0.120	0.151	0.193	0.228	0.032	0.024	0.165
1774	1.002	0.865	1.428	1.188	1.065	0.631	1.424	2.535	1.973	0.624	0.566	0.570
1776	0.334	0.300	0.578	0.541	0.400	0.246	0.543	0.878	0.674	0.157	0.146	0.138
1788	0.873	1.618	0.796	0.911	0.389	0.184	0.155	0.268	0.278	0.148	0.212	0.262
1790	0.416	0.588	0.728	1.175	0.732	1.488	4.152	6.716	4.894	1.214	1.633	1.107
1792	0.146	0.197	0.130	0.161	0.123	0.279	1.284	1.581	0.816	0.554	0.709	0.383
1804	2.985	4.620	3.893	3.030	2.717	2.753	1.398	1.447	1.470	1.498	1.049	2.310
1806	0.593	0.887	0.696	0.995	0.898	0.838	0.509	0.598	0.475	0.475	0.395	0.602
1812	0.158	0.175	0.400	0.254	0.133	0.077	0.058	0.078	0.194	0.021	0.017	0.019
1815	1.067	1.930	3.554	4.927	4.295	3.422	2.100	3.510	3.594	1.230	2.163	2.500
1829	0.621	0.460	0.531	0.717	0.648	0.466	0.703	1.068	1.004	0.404	0.385	0.362
1834	1.212	1.218	1.309	1.311	1.852	3.141	1.128	0.761	0.534	2.108	2.570	1.672
1835	0.357	0.311	0.199	1.143	2.141	1.732	0.398	0.547	0.438	0.575	0.474	0.465
1842	0.221	0.513	0.716	0.861	0.551	0.215	0.201	0.240	0.171	0.099	0.134	0.204
1845	0.138	0.916	2.331	1.440	0.708	0.430	0.529	0.990	1.160	0.143	0.193	0.394
1847	0.199	0.348	1.304	0.935	0.330	0.249	0.158	0.396	0.486	0.097	0.090	0.154
1849	0.238	0.194	0.298	0.467	0.204	0.061	0.074	0.205	0.221	0.053	0.106	0.069
1852	1.196	0.686	0.190	0.417	0.385	0.458	0.524	0.631	0.767	0.609	0.549	0.714
1854	3.918	2.090	0.701	1.433	1.560	1.803	3.441	1.609	1.999	6.987	7.452	3.249
1855	0.198	0.366	0.675	0.768	0.589	0.318	0.271	0.866	0.919	0.046	0.064	0.190

1858	0.045	0.024	0.071	0.141	0.202	0.210	0.138	0.125	0.122	0.038	0.055	0.090
1862	5.064	2.150	0.569	0.336	0.683	0.838	1.698	1.074	1.029	1.792	2.210	1.337
1869	4.265	3.302	1.776	0.743	2.075	2.724	3.099	1.747	1.820	4.963	4.708	3.725
1872	2.004	1.271	0.901	0.956	1.629	1.480	2.196	2.200	1.725	2.546	2.128	1.944
1881	0.579	0.424	0.442	0.509	0.370	0.286	0.389	0.432	0.659	0.250	0.191	0.327
1883	2.225	1.483	0.973	0.392	0.970	1.149	3.369	1.486	1.484	4.835	4.216	3.323
1889	0.288	0.128	0.216	0.197	0.246	0.191	0.273	0.290	0.243	0.070	0.106	0.130
1893	0.637	0.525	0.478	0.543	0.779	0.591	0.935	0.965	0.914	0.378	0.251	0.439
1895	1.174	1.332	4.053	5.440	2.230	0.770	1.306	3.617	2.356	0.493	0.434	0.481
1898	2.667	2.127	1.068	0.753	1.187	1.323	2.207	1.714	1.453	1.173	0.933	1.345
1899	1.295	1.752	1.231	0.882	0.805	0.650	0.951	0.587	0.769	0.609	0.642	0.742
1905	6.603	7.689	4.328	2.803	3.937	4.064	10.403	9.310	9.688	10.308	15.440	8.362
1917	0.713	0.600	0.732	0.691	0.675	0.557	0.831	0.957	0.774	0.315	0.338	0.359
1925	3.019	2.945	1.595	0.542	0.767	0.680	2.195	1.301	1.144	1.805	2.068	1.380
1926	7.271	3.700	1.823	1.209	1.142	1.187	3.751	2.454	3.586	5.024	6.676	3.879
1929	5.967	4.918	3.015	0.614	1.170	2.015	2.122	1.056	1.049	3.335	5.159	3.134
1932	10.142	10.865	5.580	1.433	2.337	4.300	2.701	1.970	1.969	6.708	7.705	6.985
1934	9.492	11.363	6.683	8.337	11.885	9.614	2.393	2.031	1.785	3.778	5.885	2.943
1941	3.969	2.259	2.003	1.200	2.012	2.202	3.501	3.549	3.734	3.282	4.372	4.009
1946	1.766	1.536	1.128	1.311	1.616	1.732	3.075	2.455	2.530	3.339	3.372	3.228
1950	0.310	0.480	0.607	0.599	0.474	0.462	0.295	0.361	0.348	0.234	0.223	0.276
1952	0.051	0.049	0.072	0.069	0.043	0.047	0.040	0.037	0.032	0.049	0.035	0.031
1955	1.536	0.859	0.361	0.443	0.270	0.306	0.475	0.408	0.736	0.645	0.857	0.717
1964	0.021	0.021	0.034	0.038	0.033	0.035	0.010	0.013	0.011	0.017	0.011	0.017
1966	0.271	0.055	0.125	0.375	0.164	0.136	0.043	0.086	0.073	0.058	0.035	0.078
1970	0.688	0.691	0.747	0.488	0.139	0.217	0.104	0.187	0.332	0.032	0.047	0.085
1982	0.917	1.364	1.887	2.568	1.265	0.800	1.302	1.694	1.282	0.314	0.355	0.717
2001	0.178	0.464	1.356	1.411	0.995	0.417	0.201	0.555	0.514	0.179	0.154	0.165

2008	13.996	12.229	8.290	4.071	7.258	8.907	7.895	5.442	5.831	7.022	5.973	8.085
2018	0.767	0.970	0.667	0.344	0.523	0.610	0.495	0.503	0.504	0.281	0.265	0.433
2019	3.172	4.829	1.252	0.687	0.959	2.733	4.425	3.252	2.371	10.871	10.133	3.836
2020	2.069	3.138	0.676	0.581	0.466	1.258	4.069	1.949	1.455	6.886	5.828	3.039
2021	21.708	15.768	5.503	5.956	4.308	4.907	7.868	6.258	7.634	7.470	9.584	11.490
2023	18.612	13.020	4.278	5.451	3.756	4.071	5.754	4.419	5.886	8.211	8.858	9.443
2024	6.696	3.476	1.740	0.995	1.712	2.251	5.756	3.738	4.046	5.935	5.728	3.705
2027	0.354	0.387	0.441	0.937	0.564	0.469	0.242	0.355	0.285	0.200	0.168	0.558
2030	1.453	0.565	1.206	0.510	0.915	0.946	1.910	2.064	1.686	3.382	4.846	5.006
2031	3.157	2.160	1.283	1.063	1.697	1.955	2.739	2.361	2.036	1.193	1.236	2.762
2033	0.092	0.140	0.413	0.311	0.402	0.286	0.230	0.247	0.273	0.108	0.084	0.272
2035	0.115	0.210	0.234	0.456	0.417	0.455	0.120	0.100	0.082	0.207	0.070	0.155
2040	1.102	1.708	1.750	0.875	1.088	0.713	0.503	0.471	0.627	0.570	0.948	0.709
2044	0.287	0.200	0.259	0.779	0.427	0.192	0.177	0.436	0.287	0.090	0.052	0.117
2046	4.405	1.717	0.850	1.452	1.959	3.015	4.862	3.056	2.827	5.156	3.920	4.693
2047	10.013	9.502	16.455	26.447	23.505	23.455	26.079	35.280	32.181	14.116	13.886	23.256
2055	0.119	0.110	0.149	0.560	0.345	0.162	0.146	0.201	0.193	0.064	0.044	0.139
2058	0.169	0.212	0.098	0.166	0.323	0.599	0.291	0.138	0.174	0.446	0.899	0.604
2061	0.129	0.227	0.304	0.544	0.357	0.216	0.158	0.240	0.205	0.073	0.053	0.139
2068	0.127	1.312	2.533	1.032	1.661	1.180	0.257	0.348	0.341	0.152	0.159	0.291
2078	0.939	0.927	1.130	0.761	0.468	0.696	0.479	0.255	0.203	0.871	0.952	0.558
2080	0.866	0.417	0.141	0.226	0.375	0.452	0.884	0.687	0.578	0.600	0.712	0.909
2088	1.682	1.088	1.633	1.278	1.501	2.012	2.482	2.223	1.715	2.427	3.641	2.375
2092	28.334	37.769	3.696	1.463	2.523	4.580	5.725	4.384	3.341	7.037	5.386	6.138
2098	4.578	2.819	1.116	1.332	1.541	2.569	4.202	2.619	2.104	4.658	5.572	3.188
2107	0.250	0.192	0.190	0.197	0.357	0.430	0.664	0.614	0.385	0.260	0.358	0.562
2110	1.043	0.493	0.564	1.002	0.978	0.933	1.610	1.950	1.309	0.539	0.663	1.084
2122	2.257	3.567	0.446	0.600	1.605	2.424	5.273	3.107	2.122	5.052	5.848	3.101

2127	0.037	0.051	0.116	0.087	0.116	0.084	0.106	0.165	0.145	0.080	0.056	0.071
2143	0.054	0.049	0.298	0.504	0.201	0.089	0.112	0.163	0.255	0.088	0.088	0.079
2153	0.126	0.205	0.454	0.431	0.293	0.200	0.175	0.227	0.239	0.118	0.183	0.425
2159	0.312	0.333	0.577	0.697	0.752	0.455	0.525	0.726	0.623	0.206	0.200	0.455
2166	4.060	5.733	4.063	5.398	6.796	7.188	6.620	5.817	5.433	7.677	10.488	7.754
2169	6.701	2.246	1.693	1.641	0.837	1.225	3.588	3.119	2.267	1.221	1.390	2.056
2173	0.256	0.359	0.907	0.506	0.507	0.429	0.578	1.073	1.263	0.189	0.348	1.638
2180	0.121	0.314	0.630	0.453	0.554	0.586	0.518	0.969	1.206	0.188	0.171	0.756
2192	1.253	1.905	0.830	0.582	2.962	3.488	12.104	6.211	4.350	8.211	7.782	2.716
2195	0.612	0.546	0.238	0.125	0.219	0.359	0.261	0.209	0.230	0.640	0.626	0.480
2200	0.152	0.233	0.391	0.362	0.349	0.229	0.090	0.102	0.111	0.071	0.073	0.147
2210	0.814	1.452	1.413	1.251	0.478	0.560	0.329	0.340	0.307	0.423	0.575	0.288
2211	0.441	0.899	0.843	0.632	0.316	0.241	0.172	0.153	0.187	1.208	0.924	0.361
2214	1.967	2.680	3.834	4.892	2.530	0.890	0.744	1.310	1.011	0.620	0.839	1.083
2220	0.418	0.410	0.202	0.453	0.463	0.470	0.212	0.274	0.358	0.139	0.113	0.162
2247	0.044	0.662	2.589	0.946	0.918	0.566	0.471	1.342	2.143	0.049	0.113	0.656
2249	0.074	0.766	2.967	0.943	0.975	0.598	0.512	1.141	1.900	0.076	0.147	0.901
2278	0.268	0.154	0.205	0.182	0.165	0.144	0.173	0.332	0.324	0.149	0.131	0.158
2287	16.625	19.318	18.209	16.307	8.326	12.629	40.060	38.752	35.234	12.749	13.016	18.012
2290	1.929	1.710	2.337	3.794	3.405	2.529	3.853	5.683	6.254	2.648	2.421	2.687
2293	2.419	2.963	3.626	3.630	4.848	4.238	4.923	6.419	7.371	5.038	4.603	4.230
2300	0.244	0.257	0.383	0.653	0.477	0.419	0.315	0.370	0.291	0.134	0.148	0.203
2304	0.601	1.011	1.408	1.615	0.942	0.542	0.749	1.114	0.935	0.175	0.174	0.532
2306	0.430	0.531	0.617	1.401	0.746	0.399	0.697	0.823	0.773	0.144	0.193	0.356
2316	0.900	0.850	2.030	1.668	1.015	0.577	1.520	2.216	1.857	0.499	0.512	0.401
2318	1.471	1.842	1.705	0.980	1.575	1.406	1.061	1.226	1.385	0.779	0.780	0.614
2326	0.695	0.916	0.299	0.396	0.388	0.453	1.312	0.948	0.804	1.245	1.312	0.679
2328	0.067	0.306	0.386	0.396	0.236	0.158	0.120	0.148	0.167	0.108	0.095	0.132

2329	0.120	0.374	0.515	0.606	0.380	0.283	0.155	0.189	0.284	0.175	0.109	0.187	F
2333	0.341	0.256	0.511	0.597	0.373	0.241	0.343	0.516	0.402	0.178	0.139	0.140	
2336	2.252	2.894	2.489	0.733	1.107	1.417	1.095	1.032	0.757	1.140	0.982	1.342	
2352	0.046	0.082	0.087	0.313	0.253	0.106	0.092	0.126	0.113	0.025	0.019	0.073	
2360	0.307	0.179	0.223	0.347	0.372	0.310	0.259	0.317	0.223	0.237	0.190	0.161	
2367	0.682	0.950	1.048	0.511	0.912	0.890	1.070	1.089	0.950	0.245	0.301	0.522	
2377	0.022	0.016	0.018	0.049	0.052	0.023	0.039	0.063	0.042	0.019	0.012	0.016	
2380	2.450	0.835	0.841	0.631	0.652	0.946	2.169	1.457	1.384	0.923	1.107	1.198	
2384	0.088	0.332	0.621	0.574	0.917	1.062	0.846	1.143	1.650	0.464	0.648	0.868	
2390	0.140	0.375	0.708	0.371	0.721	0.776	0.701	0.986	1.604	0.580	1.342	1.335	
2392	0.027	0.035	0.065	0.100	0.067	0.061	0.048	0.059	0.064	0.035	0.022	0.057	
2396	3.822	3.414	4.962	4.958	3.618	3.670	8.760	9.156	10.846	4.189	2.625	5.419	
2402	0.632	0.543	1.497	1.531	0.752	0.358	0.405	1.337	1.006	0.356	0.292	0.303	
2404	0.310	0.206	0.492	0.659	0.392	0.184	0.587	1.500	1.267	0.162	0.209	0.206	
2406	0.548	0.656	1.191	0.824	0.355	0.193	0.191	0.261	0.332	0.175	0.171	0.265	
2413	0.695	1.826	0.372	0.491	0.507	0.810	0.491	0.420	0.571	1.283	1.564	0.494	
2415	1.663	1.388	0.900	0.733	0.947	1.104	0.674	0.564	0.613	0.671	0.951	0.900	
2422	2.482	2.175	1.779	0.689	1.188	1.734	1.015	0.759	0.828	1.041	1.300	0.920	
2427	0.180	0.811	1.072	1.766	1.285	0.428	0.084	0.128	0.166	0.073	0.119	0.105	
2439	0.134	0.190	0.528	0.340	0.288	0.328	0.174	0.254	0.210	0.125	0.094	0.118	
2440	0.335	0.534	0.414	0.526	0.497	0.486	0.848	0.818	0.783	0.410	0.626	0.858	
2445	0.216	0.477	0.369	0.462	0.195	0.299	0.511	0.499	0.446	0.132	0.238	0.271	
2446	0.277	0.231	0.237	0.384	0.310	0.244	0.410	0.723	0.651	0.133	0.115	0.159	
2449	2.746	2.793	1.140	1.142	1.905	2.595	4.584	2.526	2.013	3.770	3.587	2.288	
2453	0.426	0.407	0.308	0.250	0.297	0.406	0.226	0.218	0.149	0.157	0.126	0.252	
2465	1.962	2.107	0.868	0.842	1.236	1.470	1.999	1.111	0.982	4.127	4.211	2.493	
2473	0.565	0.509	0.339	0.420	0.344	0.312	0.098	0.098	0.090	0.179	0.126	0.206	
2478	0.015	0.014	0.029	0.085	0.034	0.027	0.011	0.014	0.011	0.024	0.032	0.023	

2488	0.257	0.290	0.100	0.158	0.289	0.302	0.704	0.301	0.226	0.238	0.234	0.169
2521	0.575	0.289	0.322	0.388	0.439	0.394	1.055	0.822	0.681	0.276	0.293	0.433
2524	2.221	1.993	1.713	0.774	1.139	1.379	2.013	1.465	1.585	1.630	1.660	1.326
2536	0.765	0.793	0.893	0.508	0.477	0.275	0.272	0.324	0.345	0.131	0.158	0.449
2538	0.514	0.606	0.778	0.570	0.419	0.283	0.265	0.255	0.328	0.120	0.115	0.243
2539	0.351	0.406	0.563	0.308	0.424	0.333	0.341	0.475	0.387	0.149	0.131	0.213
2546	0.042	0.043	0.088	0.072	0.054	0.064	0.020	0.022	0.027	0.057	0.043	0.073
2547	0.034	0.041	0.066	0.151	0.089	0.073	0.016	0.041	0.037	0.059	0.036	0.111
2549	0.264	0.268	0.230	0.336	0.378	0.439	0.601	0.613	0.522	0.244	0.240	0.253
2555	0.042	0.052	0.052	0.151	0.106	0.059	0.026	0.035	0.035	0.036	0.019	0.067
2572	0.163	1.029	1.071	1.303	1.552	1.014	0.288	0.509	0.525	0.180	0.156	0.409
2576	0.320	0.182	0.186	0.347	0.246	0.101	0.319	0.577	0.499	0.127	0.129	0.379
2578	1.866	3.816	3.757	2.145	2.366	2.619	3.577	3.231	3.637	3.088	4.420	3.450
2580	0.449	0.254	0.248	0.396	0.500	0.452	0.739	0.548	0.908	0.787	0.698	0.875
2589	9.111	4.953	4.934	11.900	4.140	2.544	4.549	10.395	12.577	2.215	3.857	6.858
2597	0.919	0.385	0.300	0.126	0.140	0.184	0.246	0.243	0.225	0.121	0.157	0.130
2613	0.067	0.272	0.657	0.269	0.355	0.356	0.203	0.306	0.564	0.044	0.081	0.567
2623	0.634	0.825	0.752	0.658	0.618	0.528	0.390	0.482	0.469	0.174	0.118	0.302
2625	2.809	2.642	1.016	1.425	1.615	3.712	1.958	1.497	1.335	1.601	1.019	1.633
2629	5.806	4.339	4.800	2.262	2.859	3.250	2.826	2.841	3.133	2.003	2.748	4.020
2637	4.664	4.098	2.896	1.647	1.474	1.594	2.409	2.733	2.995	1.500	2.009	2.754
2642	0.555	0.392	0.633	1.141	0.894	0.541	0.317	0.677	0.864	0.410	0.332	0.388
2647	1.133	0.996	0.482	0.573	0.604	0.650	0.769	0.585	0.752	0.883	1.302	0.947
2652	4.249	4.287	3.543	4.901	3.675	1.940	2.534	3.314	3.379	2.429	1.569	2.076
2661	0.506	0.601	1.081	0.888	0.410	0.394	0.237	0.296	0.297	0.193	0.435	0.465
2671	0.506	0.880	1.406	1.658	1.558	1.261	0.526	0.803	0.857	0.260	0.379	0.314
2679	0.047	0.108	0.118	0.088	0.141	0.165	0.143	0.199	0.366	0.036	0.070	0.171
2690	0.412	0.845	1.018	1.240	1.711	1.499	1.009	1.151	1.192	0.608	0.581	1.142

2694	0.160	0.223	0.271	0.235	0.124	0.071	0.083	0.105	0.112	0.120	0.068	0.112
2696	1.274	1.378	0.920	0.585	1.252	1.452	1.750	1.572	2.111	0.903	1.490	1.526
2710	0.177	0.187	0.143	0.130	0.178	0.233	0.131	0.091	0.082	0.138	0.125	0.105
2711	0.282	0.255	0.230	0.183	0.183	0.247	0.210	0.139	0.144	0.123	0.106	0.121
2712	0.670	1.531	2.266	1.554	2.814	3.245	3.401	3.848	4.292	1.456	2.656	2.914
2716	0.498	0.321	0.479	0.331	0.404	0.495	0.948	1.116	1.012	0.317	0.270	0.720
2717	0.315	0.383	0.591	0.311	0.445	0.571	0.492	0.614	0.770	0.230	0.293	1.013
2723	1.751	1.706	0.364	1.025	1.126	0.977	0.922	0.547	0.534	0.984	1.140	0.661
2730	7.474	6.577	7.489	5.649	4.044	2.874	5.040	6.521	7.802	3.718	4.599	9.709
2742	0.240	0.415	0.395	0.468	0.485	1.094	0.309	0.306	0.279	0.518	0.849	0.859
2747	1.424	0.811	0.922	0.516	0.805	0.772	1.834	1.498	1.200	1.721	1.535	0.809
2753	2.703	2.389	1.100	1.488	1.132	1.994	1.389	0.867	0.457	1.557	2.385	2.512
2757	0.026	0.041	0.044	0.177	0.214	0.173	0.060	0.075	0.080	0.148	0.205	0.068
2758	13.386	14.105	9.071	7.215	8.807	7.677	15.434	13.343	11.782	9.662	10.870	15.061
2763	0.346	0.373	0.844	1.507	0.513	0.197	0.480	0.926	1.038	0.952	0.341	0.304
2773	1.847	1.690	1.943	4.512	2.666	2.384	2.640	2.019	1.886	2.338	2.502	2.379
2777	17.469	10.761	9.149	13.951	6.845	3.776	6.079	9.816	10.984	5.118	6.265	9.360
2779	2.257	1.838	2.666	2.633	2.526	2.951	3.095	3.693	3.930	3.306	3.187	3.265
2781	0.213	0.206	0.198	0.225	0.210	0.205	0.112	0.117	0.084	0.155	0.169	0.291
2788	1.664	0.846	1.178	1.010	0.827	0.473	0.592	1.100	1.019	0.283	0.375	1.110
2795	0.735	1.093	0.649	2.799	2.116	2.292	1.955	1.426	1.660	4.033	3.875	1.653
2798	0.167	0.231	0.324	0.809	0.769	0.493	0.179	0.319	0.429	0.142	0.132	0.146
2809	0.039	0.045	0.073	0.065	0.108	0.135	0.152	0.227	0.257	0.117	0.186	0.144
2813	2.487	2.312	1.871	3.252	2.418	2.394	4.348	3.894	4.230	2.398	2.603	4.813
2824	0.373	0.731	1.785	1.592	0.984	0.642	0.640	1.589	1.664	0.307	0.477	1.905
2825	0.107	0.079	0.089	0.137	0.181	0.185	0.138	0.124	0.116	0.105	0.094	0.118
2829	0.831	1.347	2.062	1.836	1.582	1.665	0.730	0.790	0.911	1.296	1.700	1.187
2840	0.273	0.379	0.609	0.351	0.575	0.798	0.546	0.626	1.238	0.317	0.565	0.883

2842	0.302	0.494	0.460	0.518	0.631	0.744	0.556	0.738	1.150	0.310	0.610	0.700
2843	0.490	0.771	0.895	0.589	1.196	1.135	1.608	2.154	2.474	0.582	1.244	1.582
2847	0.642	0.987	1.542	0.535	0.803	1.046	0.965	0.789	0.990	0.735	0.692	1.344
2852	0.480	0.681	0.963	0.745	0.804	1.136	0.921	1.086	1.163	0.233	0.315	0.593
2853	2.532	3.930	4.186	1.955	2.580	2.176	2.289	2.309	5.022	1.796	2.272	1.736
2872	1.165	0.607	1.029	0.712	0.709	0.711	1.193	1.171	1.180	1.006	1.157	0.824
2875	0.040	0.066	0.054	0.122	0.043	0.035	0.033	0.045	0.057	0.030	0.024	0.046
2887	0.777	1.017	1.065	2.146	1.519	1.281	1.208	1.448	1.341	0.998	1.334	1.094
2890	1.744	1.355	1.282	1.012	1.056	0.979	1.722	1.740	1.378	1.173	1.361	1.015
2893	0.272	0.802	0.686	0.977	1.236	1.104	0.913	0.789	1.022	0.759	1.005	0.744
2901	2.505	1.300	1.720	0.763	0.840	1.152	0.946	0.850	0.932	1.532	1.453	0.984
2906	0.118	0.238	0.287	0.473	0.347	0.320	0.348	0.318	0.369	0.337	0.358	0.416
2914	19.754	28.802	17.070	8.518	27.909	46.137	60.847	50.643	39.464	67.701	66.195	41.170
2918	2.330	1.308	2.429	2.795	2.190	2.117	2.476	2.711	2.315	1.427	1.382	2.471
2919	0.773	0.769	1.490	2.267	0.842	0.486	0.424	0.544	0.451	0.518	0.408	0.754
2920	15.472	4.724	3.276	2.237	1.925	1.639	1.616	2.068	2.172	0.903	1.372	1.119
2921	0.175	0.862	1.006	1.137	1.714	1.015	0.298	0.464	0.400	0.185	0.186	0.266
2922	0.068	0.063	0.243	0.204	0.093	0.055	0.051	0.091	0.073	0.050	0.030	0.084
2930	6.442	5.291	3.738	3.895	2.855	2.810	10.260	10.080	10.898	4.548	4.021	5.192
2932	0.204	0.562	1.866	1.305	0.821	0.333	0.290	0.892	1.029	0.102	0.077	0.138
2933	0.414	0.377	0.562	0.804	0.351	0.218	0.134	0.146	0.114	0.208	0.156	0.354
2934	2.602	2.173	1.942	1.885	2.026	2.125	1.766	1.859	1.272	4.161	4.175	2.357
2936	0.472	0.493	0.360	0.453	0.381	0.473	0.259	0.243	0.197	0.256	0.678	0.219
2937	0.217	0.195	0.283	0.357	0.231	0.247	0.164	0.183	0.209	0.102	0.126	0.176
2938	0.416	0.537	0.607	0.304	0.581	0.604	0.351	0.317	0.229	0.881	0.817	0.402
2940	4.287	2.309	1.622	0.694	0.898	1.032	1.882	1.690	1.522	0.430	0.294	1.566
2944	6.384	4.172	5.608	2.601	2.067	2.056	1.563	1.737	1.455	0.723	0.809	0.956
2945	0.510	0.717	0.363	0.163	0.219	0.202	0.195	0.134	0.323	0.235	0.338	0.180

2946	1.423	0.818	0.961	0.943	1.103	0.912	0.883	1.052	0.988	0.186	0.238	0.555
2950	1.376	1.184	1.606	0.702	0.900	0.891	0.792	0.933	0.928	0.540	0.656	0.710
2952	0.075	0.099	0.137	0.209	0.145	0.183	0.046	0.084	0.101	0.086	0.070	0.054
2957	1.918	1.142	0.743	2.064	1.292	1.068	0.980	1.354	1.337	0.432	0.404	0.972
2958	2.516	5.688	0.783	0.961	1.881	2.464	9.072	4.032	4.717	11.360	11.195	4.651
2959	0.341	0.278	0.266	0.299	0.643	0.612	0.651	0.773	0.534	0.340	0.369	0.871
2961	12.592	10.755	9.856	5.963	6.956	6.704	4.341	3.442	3.317	5.632	5.953	5.001
2964	1.814	0.948	1.474	0.444	0.270	0.204	0.205	0.200	0.146	0.182	0.237	0.314
2965	0.134	0.176	0.580	0.491	0.301	0.165	0.750	0.903	0.748	0.159	0.160	0.197
2967	0.063	0.044	0.079	0.631	0.374	0.146	0.103	0.187	0.199	0.035	0.031	0.096
2971	2.939	4.418	3.130	2.090	2.815	2.852	2.202	1.880	1.517	1.264	1.216	1.532
2972	0.940	1.103	1.243	1.744	1.356	1.261	1.040	0.912	0.891	0.473	0.465	0.879
2977	0.996	0.949	0.357	0.272	0.506	0.748	0.769	0.483	0.396	0.697	1.306	1.240
2978	0.220	0.271	0.835	1.006	0.478	0.286	0.213	0.347	0.293	0.139	0.122	0.223
2979	1.414	1.025	0.708	0.554	0.724	0.955	5.043	4.748	4.876	2.435	3.074	1.680
2980	6.186	9.440	3.389	2.559	8.916	10.192	12.136	8.222	7.978	21.175	25.371	8.455
2981	0.627	0.385	0.419	1.468	0.509	0.226	0.162	0.392	0.381	0.193	0.152	0.336
2982	2.910	4.160	5.954	4.763	3.620	4.254	1.558	1.235	0.959	1.948	1.947	2.045
2984	2.029	0.956	0.780	0.724	0.599	0.792	1.687	1.482	1.702	2.348	2.695	2.370
2985	1.404	0.826	0.257	0.361	0.580	0.676	1.201	0.913	0.840	1.173	1.425	1.096
2986	0.108	0.073	0.058	0.294	0.170	0.067	0.145	0.265	0.212	0.048	0.035	0.042
2987	0.219	0.155	0.234	0.866	0.712	0.295	0.668	1.387	0.943	0.139	0.107	0.149
2988	1.501	0.692	0.526	0.614	0.666	0.777	0.849	0.952	1.058	0.644	0.767	0.727
2989	1.897	0.828	0.724	0.956	1.318	1.209	2.350	2.696	2.125	2.173	2.955	2.345
2992	0.710	0.602	0.306	0.177	0.226	0.288	0.648	0.455	0.692	0.373	0.314	1.011
2993	1.415	1.156	0.532	0.244	0.182	0.267	0.555	0.504	0.782	0.548	0.260	1.962
2994	6.239	4.545	2.604	0.760	1.018	1.236	1.388	0.939	0.862	2.478	3.037	1.282
2995	3.307	2.716	1.582	0.565	0.868	0.969	0.619	0.429	0.416	1.855	1.741	0.674

2997	1.197	1.024	1.271	0.914	1.153	1.122	1.067	1.097	1.493	0.663	0.820	1.218
2998	1.931	1.588	1.490	1.606	1.685	1.610	1.209	1.165	1.090	0.550	0.629	1.621
2999	7.803	7.392	4.580	2.901	4.156	3.592	8.409	6.827	5.796	5.551	6.747	5.146
3001	0.154	0.242	0.348	0.417	0.163	0.185	0.195	0.140	0.204	0.139	0.192	0.356
3002	0.318	0.576	0.717	0.480	0.371	0.434	0.426	0.305	0.506	0.387	0.494	0.658
3005	0.871	0.935	0.580	0.339	0.497	0.541	1.660	1.034	0.958	1.509	1.398	0.853
3006	0.948	1.072	0.665	0.195	0.722	0.819	3.235	1.242	1.445	2.805	3.010	1.183
3008	2.247	1.719	0.702	0.597	0.980	1.226	1.982	1.509	0.780	1.773	1.724	1.543
3009	1.686	1.132	1.496	0.471	0.622	0.855	0.620	0.888	0.840	0.372	0.635	1.008
3010	1.360	0.671	0.731	0.475	0.613	0.570	0.495	0.708	0.806	0.343	0.528	0.781
3011	3.657	3.212	2.331	3.532	3.473	5.111	12.140	10.504	8.579	8.644	8.536	6.602
3014	0.362	0.757	1.240	1.139	1.210	1.221	1.027	1.744	1.790	0.359	0.689	1.129
3015	3.920	4.094	7.907	8.473	4.173	1.573	1.860	3.044	2.372	0.165	0.251	1.395
3016	1.486	1.576	3.414	3.673	2.110	0.627	0.770	1.255	0.728	0.135	0.149	0.688
3017	0.830	0.485	0.302	0.432	0.599	0.778	1.908	1.524	1.145	0.995	1.456	1.149
3019	0.824	0.739	0.627	0.512	0.712	0.762	1.333	0.981	0.895	1.129	1.068	0.939
3020	0.741	0.543	0.384	0.206	0.367	0.353	0.883	0.655	0.467	0.824	0.947	0.671
3021	0.110	0.216	0.371	0.157	0.107	0.068	0.189	0.302	0.219	0.062	0.072	0.135
3022	0.150	0.275	0.834	0.534	0.317	0.211	0.271	0.493	0.368	0.056	0.062	0.188
3024	5.031	6.518	2.423	1.070	3.935	4.994	5.981	3.926	3.501	11.833	15.935	5.920
3025	8.654	8.605	3.103	2.273	4.784	6.417	17.548	10.381	8.662	6.167	7.122	6.161
3026	1.138	1.072	0.539	0.430	0.562	0.775	1.443	1.135	1.156	2.039	1.983	1.610
3027	1.650	1.817	1.775	0.782	0.882	1.226	0.794	0.652	0.647	0.922	0.705	1.122
3029	23.237	9.055	3.451	0.945	2.716	3.235	5.315	4.101	3.593	1.724	2.129	2.269
3031	0.126	0.191	0.385	0.414	0.214	0.135	0.217	0.409	0.319	0.163	0.094	0.146
3032	0.160	0.196	0.242	0.164	0.077	0.082	0.118	0.232	0.197	0.149	0.128	0.122
3033	0.749	0.887	0.444	0.720	0.935	1.981	0.482	0.353	0.329	0.787	1.115	1.305
3034	0.447	0.482	0.150	0.235	0.280	0.646	0.381	0.224	0.200	0.499	0.776	0.597

3036	0.567	0.715	0.612	0.806	0.881	0.767	1.076	1.105	0.947	0.485	0.662	0.433
3037	4.283	6.309	5.224	3.986	3.323	4.395	3.109	2.947	3.146	3.328	3.107	3.867
3039	4.452	4.088	1.534	1.266	3.007	4.100	12.888	4.979	5.945	18.478	17.866	6.602
3044	5.207	2.815	1.718	1.145	0.800	1.064	1.558	1.219	1.944	1.304	1.638	3.238
3046	14.586	6.315	4.684	2.995	3.913	2.966	1.454	1.427	0.669	1.836	2.642	1.808
3049	0.168	0.608	0.803	0.362	0.596	0.564	0.552	0.695	0.533	0.124	0.157	0.201
3052	31.422	24.461	40.957	23.536	19.455	19.346	22.246	25.501	27.327	8.271	14.110	30.538

Population	*P* level	all PS	comparison	PS	%	fold>1.5
A. brun. (MOR)	$P < 0.05$	75	RT *vs.* 4°C	25	33,33	41
			RT *vs.* 27°C	34	45.33	
			4°C *vs.* 27°C	29	38.66	
			total	27	36	
	$P < 0.01$	4	RT *vs.* 4°C	3	75	2
			RT *vs.* 27°C	2	50	
			4°C *vs.* 27°C	3	75	
			total	4	100	
	$P <0.001$	/	RT *vs.* 4°C	/		/
			RT *vs.* 27°C	/		
			4°C *vs.* 27°C	/		
			total	/		
A. brun. (IBE)	$P < 0.05$	629	RT *vs.* 4°C	12	2	620
			RT *vs.* 27°C	372	59.14	
			4°C *vs.* 27°C	435	69.15	
			total	292	46,42	
	$P < 0.01$	99	RT *vs.* 4°C	4	4.04	99
			RT *vs.* 27°C	79	79.79	
			4°C *vs.* 27°C	72	72.72	
			total	99	100	
	$P <0.001$	8	RT *vs.* 4°C	0		8
			RT *vs.* 27°C	7	87.5	
			4°C *vs.* 27°C	6	75	
			total	7	100	

Table S2. Results of the comparison between treatments for each of the two populations of *Agabus brunneus*. MOR: Morocco; IBE: Iberia. allPS: total number of spots with significant differences. PS: spots with significant differences at each P level. fold<1.5, number of spots with a fold change larger than 1.5.

Table S3. Significance values of the comparisons of the expression level of the 563 common spots between treatments and populations. Column headings specify the populations compared in each case. *A. ramb.*: *A. ramblae*; *A. brun.*, *A. brunneus*. M: Morocco; I: Iberia. RT: room temperature treatment (control). n.s.: not significant; *, P< 0.05; **, P< 0.01; ***, P< 0.001. Identified spots are highlighted with black squares.

			A. ramb. (M) - rest 4° C	*A. ramb.* (M)- rest 27° C	*A. ramb.* (I) - *A. brun.* 4° C	*A. ramb.* (I) - *A. brun.* 27° C	*A. brunn.* (M) - *A. brun.* (I) 4° C	*A. brunn. (M)* - *A. brun. (I)* 27° C	A. ramb. - *A. brun. 4° C*	*A. ramb.* - *A. brun. 27° C*	*A. ramb.* (M) - *A. ramb.* (I) 4° C	*A. ramb. (M)* - *A. ramb. (I)* 27° C
ID	no. spot	Absolut fold change	sign.	sign.	sign.	sign.	sign.	sign.	sign.	sign.	sign.	sign.
	41	3.030	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	**	**
	47	1.253	n.s.	**	*	n.s.	**	n.s.	n.s.	n.s.	n.s.	**
	85	1.822	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	99	1.888	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	100	4.688	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	110	4.813	*	n.s	**	*	n.s.	*	n.s.	n.s.	**	n.s.
	113	9.326	**	n.s	n.s.	**	n.s.	n.s.	n.s.	n.s.	**	n.s.
	117	3.901	*	n.s	*	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
	120	7.428	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	147	2.508	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
	183	3.851	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	184	1.232	n.s.	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	199	1.419	n.s.	n.s	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
	200	1.585	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
	232	1.141	n.s.	**	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
	243	5.028	*	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
	252	1.615	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
	267	4.962	n.s.	n.s	***	***	n.s.	*	*	*	**	n.s.
	296	5.958	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	***
	302	4.623	*	*	**	n.s.	*	*	n.s.	n.s.	***	*
	313	1.156	n.s.	n.s	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
	339	2.124	n.s.	***	**	n.s.	n.s.	n.s.	n.s.	*	**	*
	356	1.841	n.s.	n.s	*	*	n.s.	n.s.	n.s.	n.s.	*	**
	358	1.320	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
	359	1.716	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

367	1.243	n.s.	n.s	**	n.s.	*	n.s.	**	n.s.	n.s.	*
369	1.649	n.s.	n.s	**	n.s.	*	n.s.	n.s.	n.s.	**	n.s.
371	2.422	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.
375	2.181	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
376	2.142	n.s.	n.s	***	n.s.	*	n.s.	n.s.	n.s.	**	n.s.
383	2.229	n.s.	***	**	n.s.	**	*	n.s.	n.s.	*	*
389	1.668	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
392	1.836	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
393	4.199	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
408	3.751	***	n.s	n.s.	*	n.s.	n.s.	n.s.	**	**	n.s.
409	1.027	n.s.	n.s	*	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
410	1.689	n.s.	n.s	*	***	n.s.	n.s.	n.s.	*	n.s.	*
415	1.002	n.s.	n.s	*	n.s.	n.s.	n.s.	*	n.s.	*	n.s.
420	5.867	***	n.s	n.s.	*	n.s.	n.s.	n.s.	*	**	n.s.
422	1.799	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
423	1.449	n.s.	n.s	***	**	n.s.	n.s.	*	***	**	n.s.
433	2.539	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
436	1.729	n.s.	n.s	n.s.	*	*	n.s.	n.s.	n.s.	*	**
437	1.120	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
447	2.029	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
483	5.842	**	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	**	n.s.
486	3.127	n.s.	*	n.s.	n.s.	*	n.s.	*	n.s.	n.s.	*
499	1.608	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
504	1.036	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
505	2.967	n.s.	**	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	*
510	1.522	n.s.	**	*	n.s.	*	n.s.	n.s.	**	***	*
511	2.900	n.s.	**	n.s.	n.s.	n.s.	n.s.	*	n.s.	*	**
517	1.072	n.s.	n.s	n.s.	**	n.s.	n.s.	n.s.	***	n.s.	n.s.
521	2.089	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

537	2.782	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
538	1.967	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
539	1.790	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
541	1.942	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
551	1.266	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
565	1.455	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
571	2.519	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	*	n.s.	*
583	1.545	n.s.	*	n.s.	*	n.s.	n.s.	*	**	n.s.	n.s.
588	1.202	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
598	2.583	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
608	2.022	n.s.	*	***	n.s.	n.s.	n.s.	***	*	n.s.	n.s.
610	1.525	n.s.	***	***	*	n.s.	n.s.	***	*	n.s.	**
611	2.481	n.s.	n.s	***	n.s.	n.s.	n.s.	*	*	n.s.	n.s.
619	1.787	n.s.	***	*	n.s.	n.s.	n.s.	**	*	n.s.	**
624	4.235	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
631	1.018	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
645	1.322	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
646	1.618	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
656	1.003	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
670	1.121	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
672	4.782	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
673	1.096	n.s.	*	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
684	1.480	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
690	3.729	*	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
701	1.782	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	**	*	n.s.
705	1.637	n.s.	***	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
711	1.643	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
716	1.596	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	*
725	1.151	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.

739	1.871	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*
747	1.441	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
755	1.001	n.s.	**	***	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
756	5.542	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
757	1.764	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	*
763	2.214	*	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
767	2.449	*	n.s	*	*	n.s.	n.s.	n.s.	n.s.	**	*
769	1.635	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.
777	1.710	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
785	1.062	n.s.	*	**	*	n.s.	*	*	**	n.s.	n.s.
792	1.933	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	*
797	1.836	*	n.s	n.s.	**	n.s.	n.s.	n.s.	**	n.s.	n.s.
801	1.312	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
805	1.090	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
808	1.584	n.s.	**	*	n.s.	n.s.	n.s.	**	*	n.s.	**
811	1.147	n.s.	n.s	*	n.s.	*	n.s.	*	n.s.	n.s.	n.s.
815	1.536	n.s.	n.s	*	*	n.s.	*	**	**	n.s.	n.s.
818	2.672	*	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
825	1.146	n.s.	*	*	n.s.	n.s.	n.s.	*	n.s.	*	n.s.
832	2.906	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
834	2.187	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	*
839	1.468	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
848	1.257	n.s.	n.s	*	n.s.	*	n.s.	n.s.	n.s.	***	n.s.
850	1.245	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
851	2.099	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
858	2.242	n.s.	***	**	n.s.	n.s.	n.s.	***	n.s.	*	*
860	1.379	n.s.	**	*	**	n.s.	n.s.	n.s.	**	*	n.s.
864	1.580	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	*	**	n.s.
870	1.300	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*

871	1.097	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.
877	1.881	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
885	1.974	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
886	1.016	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
890	1.868	n.s.	**	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	**
892	1.206	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
893	1.734	n.s.	n.s	n.s.	n.s.	**	n.s.	*	*	n.s.	n.s.
897	3.865	n.s.	*	*	*	n.s.	n.s.	***	**	n.s.	n.s.
898	1.198	n.s.	**	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	***
904	1.444	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
906	2.049	*	n.s	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
910	1.236	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
917	1.856	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
935	7.871	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
949	3.021	n.s.	***	**	n.s.	n.s.	n.s.	***	*	n.s.	**
951	1.151	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
956	2.624	n.s.	***	*	n.s.	n.s.	n.s.	**	n.s.	*	***
962	3.195	n.s.	*	*	n.s.	*	n.s.	**	n.s.	n.s.	n.s.
980	2.775	n.s.	***	**	n.s.	n.s.	n.s.	***	*	n.s.	*
981	2.317	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***
988	2.111	n.s.	***	*	n.s.	n.s.	n.s.	**	*	n.s.	*
991	4.293	n.s.	**	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
992	5.023	**	**	*	*	*	n.s.	n.s.	*	*	n.s.
994	2.174	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	**
1019	4.303	***	*	n.s.	***	n.s.	n.s.	*	**	*	n.s.
1021	3.049	*	**	*	n.s.	n.s.	n.s.	***	*	n.s.	n.s.
1025	4.143	**	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.
1027	5.828	***	n.s	*	n.s.	n.s.	*	n.s.	n.s.	**	n.s.
1030	1.269	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.

1036	2.784	*	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
1042	1.627	*	*	*	*	n.s.	n.s.	**	**	n.s.	n.s.
1043	2.966	**	n.s	n.s.	*	n.s.	n.s.	n.s.	**	n.s.	n.s.
1064	1.046	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
1071	1.392	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
1072	1.172	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	**
1088	1.067	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1093	2.395	**	**	***	n.s.	n.s.	n.s.	n.s.	*	**	n.s.
1105	1.240	n.s.	***	**	n.s.	n.s.	n.s.	**	*	*	**
1107	1.418	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
1108	1.119	n.s.	n.s	n.s.	n.s.	**	**	n.s.	n.s.	n.s.	n.s.
1110	1.424	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
1111	1.794	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
1112	1.740	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
1133	1.789	n.s.	n.s	*	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.
1134	1.245	n.s.	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	*
1137	1.754	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1140	4.856	*	***	**	*	n.s.	n.s.	***	**	n.s.	**
1144	4.403	n.s.	n.s	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	n.s.
1149	2.624	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
1150	1.207	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
1155	1.008	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1157	1.254	n.s.	n.s	n.s.	***	*	n.s.	n.s.	*	n.s.	*
1163	1.119	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1168	2.838	n.s.	***	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	***
1176	2.103	n.s.	n.s	*	n.s.	*	n.s.	n.s.	n.s.	**	n.s.
1180	1.141	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	*
1181	2.384	n.s.	***	**	n.s.	*	n.s.	***	**	n.s.	*
1182	2.438	n.s.	***	**	n.s.	n.s.	n.s.	***	*	n.s.	*

1185	2.250	n.s.	n.s	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.
1187	1.958	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
1192	1.649	n.s.	**	n.s.	**	n.s.	n.s.	n.s.	***	n.s.	n.s.
1199	1.926	*	n.s	n.s.	**	n.s.	n.s.	n.s.	*	n.s.	n.s.
1200	1.229	n.s.	n.s	***	***	n.s.	n.s.	***	**	*	n.s.
1206	1.053	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.
1207	1.372	n.s.	n.s	*	n.s.	n.s.	**	n.s.	n.s.	***	n.s.
1217	1.485	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	*
1220	1.270	n.s.	n.s	n.s.	n.s.	*	n.s.	*	*	*	n.s.
1222	1.712	n.s.	n.s	**	n.s.	n.s.	**	n.s.	n.s.	*	n.s.
1223	1.048	n.s.	**	**	n.s.	n.s.	n.s.	*	**	n.s.	*
1226	1.614	n.s.	*	*	n.s.	n.s.	*	n.s.	*	**	n.s.
1233	3.735	n.s.	***	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	**
1237	1.152	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
1242	4.536	*	n.s	n.s.	n.s.	*	*	n.s.	n.s.	**	n.s.
1247	1.617	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1252	1.021	n.s.	n.s	n.s.	n.s.	**	*	n.s.	n.s.	n.s.	*
1254	1.677	n.s.	n.s	**	*	n.s.	n.s.	n.s.	*	*	n.s.
1257	2.299	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
1262	3.134	n.s.	**	**	n.s.	n.s.	n.s.	***	**	n.s.	n.s.
1265	1.442	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1266	1.256	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1276	3.294	*	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
1279	1.012	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1281	5.777	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	**	n.s.
1290	3.306	n.s.	***	*	n.s.	n.s.	n.s.	***	*	n.s.	**
1296	1.550	n.s.	n.s	***	*	n.s.	n.s.	***	**	*	n.s.
1299	1.528	n.s.	n.s	*	n.s.	n.s.	**	n.s.	n.s.	**	n.s.
1304	9.318	n.s.	**	**	*	n.s.	n.s.	***	**	n.s.	n.s.

	1305	1.069	n.s.	n.s	n.s.	**	n.s.	*	n.s.	n.s.	n.s.	*
	1312	1.261	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
	1314	3.562	**	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	**
	1319	2.457	*	n.s	*	n.s.	*	**	n.s.	n.s.	***	n.s.
	1326	1.034	n.s.	**	*	n.s.	n.s.	**	n.s.	*	n.s.	*
	1353	8.411	n.s.	*	*	***	n.s.	n.s.	**	***	n.s.	n.s.
	1356	1.476	n.s.	***	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
	1360	1.382	n.s.	***	*	n.s.	n.s.	n.s.	n.s.	**	**	*
	1363	2.780	n.s.	*	**	n.s.	n.s.	n.s.	***	**	n.s.	n.s.
■	1366	3.705	***	*	**	***	n.s.	n.s.	*	***	n.s.	n.s.
	1367	2.870	***	*	n.s.	n.s.	n.s.	n.s.	*	*	*	n.s.
	1374	1.644	n.s.	n.s	***	n.s.	n.s.	n.s.	*	**	***	n.s.
	1380	4.124	*	***	**	n.s.	n.s.	n.s.	***	*	n.s.	***
■	1387	1.780	n.s.	**	***	***	n.s.	*	***	**	n.s.	*
■	1392	6.202	**	**	*	n.s.	n.s.	n.s.	***	**	*	***
	1395	3.666	***	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
	1398	1.467	n.s.	*	n.s.	n.s.	*	*	n.s.	n.s.	*	**
	1401	1.213	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	*
	1405	1.220	n.s.	**	**	*	n.s.	n.s.	*	*	n.s.	n.s.
	1409	3.530	**	**	*	*	n.s.	n.s.	*	*	n.s.	n.s.
	1411	2.154	n.s.	**	*	**	n.s.	n.s.	**	***	n.s.	*
	1415	1.510	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
	1416	1.590	n.s.	n.s	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
	1421	1.439	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
■	1423	3.587	**	***	n.s.	n.s.	n.s.	n.s.	*	n.s.	**	*
	1426	5.401	n.s.	n.s	**	n.s.	*	*	n.s.	n.s.	*	*
■	1430	5.405	**	*	***	*	n.s.	n.s.	**	*	n.s.	n.s.
	1436	1.190	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
	1437	2.509	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

1441	1.187	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
1446	5.263	***	***	***	n.s.	*	n.s.	**	*	**	***
1452	3.182	*	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
1453	4.436	n.s.	**	***	n.s.	n.s.	n.s.	***	**	n.s.	n.s.
1456	1.362	n.s.	n.s	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	*
1462	1.860	n.s.	**	n.s.	n.s.	n.s.	n.s.	*	**	n.s.	*
1465	1.895	n.s.	n.s	***	n.s.	*	n.s.	*	n.s.	**	*
1471	1.357	n.s.	n.s	**	n.s.	n.s.	n.s.	**	**	n.s.	n.s.
1472	1.270	n.s.	n.s	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
1473	2.618	n.s.	*	n.s.	n.s.	**	*	n.s.	n.s.	***	n.s.
1480	3.038	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
1483	1.482	n.s.	n.s	n.s.	**	n.s.	*	n.s.	*	n.s.	n.s.
1491	1.729	*	**	n.s.	*	n.s.	n.s.	**	**	n.s.	***
1495	2.682	*	**	**	n.s.	n.s.	n.s.	***	*	n.s.	*
1498	2.282	*	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1499	2.650	*	n.s	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
1507	3.184	*	n.s	n.s.	n.s.	**	*	*	**	n.s.	n.s.
1509	2.694	n.s.	n.s	n.s.	n.s.	*	n.s.	*	**	n.s.	n.s.
1511	1.824	n.s.	*	**	*	*	n.s.	***	**	n.s.	n.s.
1523	1.217	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	**	n.s.
1527	1.682	n.s.	n.s	n.s.	n.s.	*	*	n.s.	n.s.	n.s.	n.s.
1533	4.403	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1535	1.224	n.s.	n.s	n.s.	n.s.	*	n.s.	*	n.s.	n.s.	n.s.
1546	1.046	n.s.	n.s	*	*	n.s.	*	n.s.	**	n.s.	n.s.
1555	4.444	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	**	n.s.
1557	2.723	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	**	n.s.
1564	1.022	n.s.	n.s	*	**	*	**	*	***	*	n.s.
1566	2.307	n.s.	**	*	*	n.s.	n.s.	**	**	n.s.	*
1568	1.746	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.

1572	1.662	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
1573	5.787	n.s.	***	**	n.s.	n.s.	n.s.	***	*	n.s.	n.s.
1578	1.694	n.s.	**	**	n.s.	*	n.s.	**	**	n.s.	*
1583	5.496	n.s.	n.s	***	*	n.s.	n.s.	n.s.	*	*	n.s.
1586	1.661	n.s.	n.s	***	*	n.s.	n.s.	n.s.	**	*	n.s.
1606	1.367	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	*	**
1625	1.861	n.s.	*	**	*	n.s.	n.s.	***	**	n.s.	n.s.
1630	1.241	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
1643	3.332	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	**
1654	1.736	n.s.	***	***	n.s.	n.s.	n.s.	*	*	*	**
1660	2.613	n.s.	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	**
1671	3.016	n.s.	**	*	n.s.	n.s.	n.s.	**	*	n.s.	*
1677	1.251	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
1679	1.029	n.s.	n.s	**	*	n.s.	n.s.	*	**	n.s.	n.s.
1683	1.970	n.s.	n.s	**	n.s.	*	*	n.s.	*	n.s.	n.s.
1684	2.176	n.s.	***	***	*	n.s.	n.s.	n.s.	**	*	*
1685	1.278	n.s.	**	***	n.s.	n.s.	n.s.	**	*	n.s.	*
1686	1.098	n.s.	**	***	**	n.s.	n.s.	*	***	n.s.	n.s.
1688	1.989	*	n.s	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
1693	2.222	*	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	*	**
1707	1.205	n.s.	***	**	n.s.	n.s.	n.s.	**	n.s.	*	*
1713	2.601	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
1719	1.456	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	*
1727	8.452	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
1730	1.107	n.s.	n.s	n.s.	n.s.	**	*	n.s.	n.s.	n.s.	n.s.
1736	2.854	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	*	n.s.
1743	1.982	n.s.	n.s	*	n.s.	n.s.	n.s.	**	*	n.s.	n.s.
1771	1.882	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1774	1.076	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*

1776	1.240	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	*
1788	2.157	n.s.	**	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	*
1790	5.244	*	n.s	n.s.	n.s.	*	n.s.	*	n.s.	n.s.	n.s.
1792	4.553	n.s.	*	n.s.	n.s.	n.s.	*	**	*	n.s.	n.s.
1804	1.511	n.s.	*	**	n.s.	n.s.	n.s.	**	*	n.s.	n.s.
1806	1.112	n.s.	n.s	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1812	1.425	n.s.	*	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	**
1815	2.580	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.
1829	1.022	n.s.	n.s	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.
1834	1.250	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	*
1835	1.973	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
1842	1.756	n.s.	***	***	n.s.	*	n.s.	*	n.s.	*	*
1845	5.082	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	*
1847	1.997	n.s.	**	***	n.s.	n.s.	*	*	n.s.	*	**
1849	1.204	n.s.	*	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	*
1852	2.315	***	***	n.s.	*	n.s.	n.s.	n.s.	**	**	n.s.
1854	1.009	n.s.	***	*	n.s.	n.s.	*	n.s.	*	n.s.	*
1855	1.828	n.s.	n.s	**	n.s.	n.s.	*	n.s.	n.s.	*	*
1858	2.338	n.s.	n.s	n.s.	*	*	n.s.	n.s.	n.s.	**	n.s.
1862	3.972	*	*	**	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
1869	1.453	n.s.	n.s	**	n.s.	n.s.	*	n.s.	n.s.	***	n.s.
1872	1.055	n.s.	***	**	n.s.	n.s.	n.s.	n.s.	*	n.s.	*
1881	1.513	n.s.	n.s	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
1883	1.288	n.s.	n.s	*	n.s.	n.s.	n.s.	*	*	n.s.	n.s.
1889	1.598	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
1893	1.029	n.s.	n.s	n.s.	n.s.	*	*	n.s.	n.s.	n.s.	n.s.
1895	2.055	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
1898	1.935	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
1899	1.591	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

1905	1.187	n.s.	n.s	***	**	n.s.	n.s.	**	***	*	n.s.
1917	1.164	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	*
1925	1.994	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*
1926	2.185	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	*	**	n.s.
1929	2.949	*	n.s	*	n.s.	n.s.	*	n.s.	n.s.	**	n.s.
1932	2.806	*	n.s	n.s.	n.s.	*	*	n.s.	n.s.	**	n.s.
1934	1.963	*	n.s	**	***	n.s.	n.s.	***	***	n.s.	n.s.
1941	1.492	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
1946	1.459	n.s.	**	n.s.	*	n.s.	n.s.	*	**	n.s.	*
1950	1.212	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
1952	1.037	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
1955	2.947	**	n.s	n.s.	**	n.s.	n.s.	n.s.	***	**	n.s.
1964	1.041	n.s.	n.s	**	***	n.s.	n.s.	*	***	n.s.	n.s.
1966	1.705	n.s.	n.s	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
1970	3.308	n.s.	**	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	**
1982	1.521	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	***
2001	3.343	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	***
2008	2.211	*	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	**	n.s.
2018	2.053	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2019	1.680	n.s.	n.s	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2020	1.858	n.s.	n.s	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
2021	3.058	***	n.s	n.s.	*	n.s.	*	n.s.	**	**	n.s.
2023	2.876	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	**	*	n.s.
2024	1.583	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2027	1.297	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2030	1.331	n.s.	n.s	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2031	1.896	*	*	n.s.	n.s.	**	n.s.	n.s.	n.s.	*	*
2033	2.349	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2035	2.279	n.s.	n.s	**	***	n.s.	n.s.	n.s.	**	*	*

2040	1.697	*	***	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	*
2044	1.213	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2046	1.152	n.s.	***	*	n.s.	n.s.	n.s.	n.s.	*	*	**
2047	2.219	*	*	n.s.	n.s.	n.s.	*	n.s.	*	**	n.s.
2055	2.153	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2058	1.781	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
2061	2.007	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2068	3.781	n.s.	*	***	**	n.s.	n.s.	n.s.	*	**	n.s.
2078	1.333	n.s.	*	n.s.	n.s.	n.s.	**	n.s.	**	n.s.	n.s.
2080	1.520	n.s.	***	*	n.s.	n.s.	n.s.	n.s.	**	n.s.	*
2088	1.226	n.s.	n.s	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2092	5.975	**	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2098	1.347	n.s.	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2107	1.497	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
2110	1.007	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
2122	1.614	n.s.	***	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	**
2127	2.474	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2143	4.380	*	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2153	1.920	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	*
2159	1.527	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2166	1.617	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
2169	3.117	**	n.s	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
2173	1.655	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	*
2180	3.196	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	**	n.s.
2192	5.558	n.s.	**	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	***
2195	1.790	n.s.	n.s	n.s.	n.s.	*	*	n.s.	n.s.	n.s.	n.s.
2200	1.147	n.s.	**	***	*	n.s.	n.s.	*	*	*	n.s.
2210	1.219	n.s.	**	*	n.s.	n.s.	n.s.	*	*	n.s.	*
2211	1.522	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*

2214	1.060	n.s.	***	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	***
2220	1.560	n.s.	n.s	**	*	n.s.	*	**	n.s.	n.s.	*
2247	10.978	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	**
2249	6.917	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	**
2278	1.596	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2287	1.386	n.s.	n.s	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
2290	1.779	*	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	**	n.s.
2293	1.872	**	n.s	n.s.	n.s.	n.s.	*	**	n.s.	n.s.	n.s.
2300	1.504	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2304	1.408	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	*
2306	1.738	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
2316	1.366	n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	***
2318	1.565	n.s.	n.s	n.s.	n.s.	n.s.	***	n.s.	*	n.s.	n.s.
2326	1.417	n.s.	**	**	*	n.s.	n.s.	**	**	n.s.	n.s.
2328	3.117	n.s.	**	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.
2329	2.604	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.
2333	1.093	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	*
2336	2.276	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2352	3.145	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.
2360	1.093	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2367	1.120	n.s.	n.s	n.s.	n.s.	*	*	n.s.	n.s.	n.s.	n.s.
2377	1.591	n.s.	n.s	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.
2380	1.974	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2384	7.170	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	**
2390	3.929	**	n.s	n.s.	n.s.	n.s.	n.s.	*	**	n.s.	n.s.
2392	2.299	n.s.	n.s	**	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2396	1.562	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	*	n.s.	n.s.
2402	1.209	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
2404	1.516	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*

2406	1.381	n.s.	***	**	n.s.	n.s.	n.s.	**	n.s.	n.s.	**
2413	1.087	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
2415	2.400	***	n.s	n.s.	n.s.	n.s.	n.s.	*	n.s.	**	n.s.
2422	2.712	*	n.s	n.s.	**	n.s.	n.s.	n.s.	*	n.s.	n.s.
2427	3.563	n.s.	*	**	**	n.s.	n.s.	n.s.	*	*	n.s.
2439	1.594	n.s.	**	*	**	n.s.	**	n.s.	***	n.s.	**
2440	1.774	*	*	n.s.	*	*	n.s.	n.s.	*	n.s.	n.s.
2445	1.705	n.s.	n.s	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.
2446	1.116	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
2449	1.153	n.s.	**	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
2453	2.018	*	n.s	n.s.	**	n.s.	n.s.	*	*	n.s.	n.s.
2465	1.184	n.s.	n.s	*	n.s.	*	*	*	n.s.	n.s.	n.s.
2473	2.435	n.s.	n.s	*	*	n.s.	n.s.	n.s.	**	n.s.	n.s.
2478	2.675	n.s.	n.s	*	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.
2488	1.429	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2521	1.003	n.s.	n.s	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
2524	1.509	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2536	2.518	*	*	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	**
2538	1.614	n.s.	*	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	*
2539	1.319	n.s.	*	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	*
2546	1.186	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2547	2.220	n.s.	n.s	**	n.s.	*	n.s.	n.s.	n.s.	*	n.s.
2549	1.494	n.s.	*	n.s.	n.s.	**	*	n.s.	n.s.	n.s.	*
2555	1.668	n.s.	n.s	***	n.s.	n.s.	n.s.	*	n.s.	*	n.s.
2572	3.620	n.s.	n.s	***	**	n.s.	n.s.	n.s.	*	*	n.s.
2576	1.208	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
2578	1.574	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2580	1.428	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	**	n.s.	*
2589	1.464	n.s.	n.s	***	n.s.	n.s.	n.s.	***	*	n.s.	*

2597	5.592	***	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2613	2.549	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2623	1.557	n.s.	n.s	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2625	1.691	n.s.	n.s	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	**
2629	2.456	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2637	2.519	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	**
2642	1.121	n.s.	n.s	***	n.s.	n.s.	n.s.	**	n.s.	**	n.s.
2647	1.527	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
2652	1.292	n.s.	n.s	*	n.s.	n.s.	*	**	n.s.	n.s.	n.s.
2661	1.153	n.s.	**	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
2671	1.611	n.s.	n.s	**	n.s.	n.s.	**	n.s.	*	n.s.	n.s.
2679	1.900	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2690	2.310	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2694	1.093	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
2696	1.181	n.s.	**	n.s.	n.s.	**	n.s.	n.s.	*	n.s.	*
2710	1.332	n.s.	n.s	n.s.	**	n.s.	n.s.	n.s.	*	n.s.	n.s.
2711	1.640	*	n.s	n.s.	**	n.s.	n.s.	n.s.	***	n.s.	n.s.
2712	3.189	*	n.s	n.s.	n.s.	*	n.s.	*	n.s.	**	n.s.
2716	1.068	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
2717	1.093	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2723	1.792	n.s.	**	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	*
2730	1.556	n.s.	n.s	n.s.	**	n.s.	n.s.	n.s.	*	n.s.	***
2742	1.797	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2747	1.049	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2753	1.829	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2757	4.920	*	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	n.s.
2758	1.243	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
2763	2.833	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2773	1.713	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

2777	2.084	*	n.s	**	*	n.s.	n.s.	**	n.s.	n.s.	**
2779	1.334	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2781	1.299	n.s.	n.s	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	n.s.
2788	2.649	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2795	3.985	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2798	2.258	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2809	2.872	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2813	1.340	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	**	n.s.	n.s.
2824	2.270	n.s.	n.s	***	*	n.s.	n.s.	n.s.	n.s.	**	**
2825	1.186	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2829	1.548	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
2840	1.483	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2842	1.526	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2843	1.890	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.
2847	1.160	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2852	1.319	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2853	1.258	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2872	1.200	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2875	1.533	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2887	1.868	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2890	1.339	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2893	3.246	***	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2901	2.319	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2906	3.261	***	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2914	2.313	n.s.	**	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	*
2918	1.044	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2919	1.384	n.s.	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2920	9.762	***	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	***	n.s.
2921	3.087	n.s.	n.s	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	n.s.

2922	1.487	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2930	1.033	n.s.	n.s	n.s.	*	*	**	n.s.	**	n.s.	n.s.
2932	2.770	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	***
2933	1.084	n.s.	*	**	n.s.	**	n.s.	*	n.s.	n.s.	n.s.
2934	1.001	n.s.	n.s	n.s.	n.s.	*	**	n.s.	n.s.	*	n.s.
2936	1.464	n.s.	n.s	*	*	n.s.	n.s.	*	*	n.s.	n.s.
2937	1.045	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
2938	1.230	n.s.	n.s	n.s.	*	*	n.s.	n.s.	**	n.s.	n.s.
2940	4.279	**	n.s	n.s.	**	*	n.s.	n.s.	n.s.	*	*
2944	3.919	**	***	*	**	n.s.	***	*	*	n.s.	**
2945	2.580	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2946	2.122	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2950	2.030	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2952	1.523	n.s.	n.s	***	**	n.s.	*	*	**	**	n.s.
2957	1.655	n.s.	n.s	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	**
2958	2.835	n.s.	***	*	**	n.s.	n.s.	**	***	*	**
2959	1.262	n.s.	**	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	*
2961	2.371	***	**	n.s.	**	n.s.	*	*	***	**	*
2964	6.545	***	***	*	n.s.	n.s.	n.s.	*	n.s.	*	**
2965	3.491	*	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
2967	4.072	n.s.	n.s	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	*
2971	1.587	n.s.	n.s	n.s.	**	n.s.	n.s.	n.s.	*	*	n.s.
2972	1.155	n.s.	n.s	**	n.s.	*	n.s.	*	*	*	n.s.
2977	1.719	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2978	2.054	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2979	1.894	n.s.	*	n.s.	n.s.	n.s.	*	*	*	*	n.s.
2980	1.933	n.s.	***	*	n.s.	n.s.	n.s.	**	n.s.	n.s.	*
2981	1.032	n.s.	n.s	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
2982	1.056	n.s.	*	**	**	n.s.	n.s.	**	***	n.s.	n.s.

2984	1.279	n.s.	n.s	*	**	n.s.	n.s.	n.s.	***	**	n.s.
2985	1.541	n.s.	***	*	*	n.s.	n.s.	n.s.	**	**	**
2986	1.502	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2987	2.546	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
2988	2.137	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
2989	1.039	n.s.	**	**	**	n.s.	n.s.	*	***	n.s.	n.s.
2992	1.778	n.s.	n.s	*	*	n.s.	n.s.	n.s.	**	*	n.s.
2993	3.150	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	*	**	**
2994	4.045	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2995	3.263	*	*	n.s.	*	*	n.s.	n.s.	**	n.s.	n.s.
2997	1.359	n.s.	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
2998	1.721	n.s.	n.s	n.s.	n.s.	**	n.s.	*	n.s.	n.s.	n.s.
2999	1.388	n.s.	n.s	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3001	1.626	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3002	1.356	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
3005	1.343	n.s.	n.s	*	n.s.	n.s.	n.s.	*	*	n.s.	n.s.
3006	2.193	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	*	n.s.	*	n.s.
3008	1.549	n.s.	n.s	*	n.s.	n.s.	*	n.s.	n.s.	n.s.	*
3009	3.458	**	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
3010	3.106	*	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3011	2.216	n.s.	***	**	n.s.	n.s.	n.s.	***	**	n.s.	*
3014	2.326	n.s.	n.s	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.
3015	1.120	n.s.	**	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
3016	1.027	n.s.	**	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
3017	1.339	n.s.	***	*	n.s.	*	n.s.	*	**	n.s.	*
3019	1.204	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
3020	1.162	n.s.	n.s	*	n.s.	n.s.	n.s.	n.s.	*	**	n.s.
3021	1.241	n.s.	*	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	***
3022	1.911	n.s.	***	*	n.s.	n.s.	*	n.s.	n.s.	*	***

3024	1.251	n.s.	n.s	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	*
3025	1.001	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
3026	1.146	n.s.	*	*	*	n.s.	n.s.	n.s.	**	n.s.	n.s.
3027	1.982	**	*	n.s.	n.s.	n.s.	*	n.s.	*	*	n.s.
3029	8.731	***	n.s	n.s.	n.s.	*	n.s.	n.s.	n.s.	**	n.s.
3031	2.103	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
3032	1.118	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3033	1.130	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
3034	1.202	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
3036	1.391	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3037	1.233	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
3039	2.443	n.s.	***	*	n.s.	n.s.	n.s.	**	**	*	*
3044	3.899	***	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.
3046	6.962	***	*	n.s.	*	n.s.	*	*	*	*	n.s.
3049	2.064	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3052	1.744	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*

Table S4. Principal Component Analyses (PCA) of the spot volumes with significant differences (P< 0.001) in the tested comparisons.. a) Eigenvalues and percentage of variation; b) Correlations between variables and first 3 factors; c) Factor loadings (i.e. standard coordinates); d) Eigenvectors.

a)

	All spots with no RT		*A. ramb.* (MOR) *vs.* rest 4º C P<0. 001		*A. ramb.* (MOR) *vs.* rest 27º C P<0. 001		*A. ramb.* (IP) *vs. A. brun.* 4º C P<0. 001	
	Eigenvalue	% var	Eigenvalue	% var	Eigenvalue	% var	Eigenvalue	% var
component 1	6.145	76.817	10.165	84.712	8.584	71.533	8.400	69.999
component 2	0.832	10.404	0.998	8.316	1.911	15.929	2.408	20.070
component 3	0.533	6.657	0.617	5.139	0.678	5.653	0.471	3.929
component 4	0.190	2.380	0.126	1.048	0.320	2.667	0.354	2.951
component 5	0.148	1.853	0.041	0.342	0.199	1.659	0.171	1.422
component 6	0.081	1.010	0.028	0.230	0.118	0.982	0.081	0.676
component 7	0.045	0.560	0.012	0.102	0.109	0.912	0.047	0.395
component 8	0.025	0.319	0.008	0.066	0.039	0.325	0.031	0.256
component 9			0.003	0.024	0.018	0.152	0.029	0.245
component 10			0.001	0.012	0.011	0.092	0.004	0.032
component 11			0.001	0.006	0.010	0.080	0.002	0.016
component 12			0.000	0.003	0.002	0.016	0.001	0.010

	A. ramb. (IP) *vs. A. brun.* 27º C P<0. 001		All spots		pooled *A. ramb. vs.* pooled *A. brun.* 4º C P<0. 001		pooled *A. ramb. vs.* pooled *A. brun.* 27º C P<0. 001	
	Eigenvalue	% var	Eigenvalue	% var	Eigenvalue	% var	Eigenvalue	% var
component 1	10.728	89.397	9.189	76.576	9.810	81.747	10.728	89.397
component 2	0.634	5.286	1.103	9.188	1.726	14.386	0.634	5.286
component 3	0.296	2.464	0.730	6.086	0.225	1.876	0.296	2.464
component 4	0.191	1.590	0.398	3.321	0.121	1.010	0.191	1.590
component 5	0.079	0.660	0.294	2.449	0.052	0.431	0.079	0.660
component 6	0.054	0.451	0.101	0.842	0.030	0.249	0.054	0.451
component 7	0.010	0.083	0.073	0.607	0.020	0.170	0.010	0.083
component 8	0.008	0.064	0.050	0.416	0.009	0.072	0.008	0.064
component 9	0.000	0.002	0.028	0.232	0.004	0.030	0.000	0.002
component 10	0.000	0.001	0.018	0.154	0.002	0.018	0.000	0.001
component 11	0.000	0.000	0.010	0.080	0.001	0.007	0.000	0.000
component 12			0.006	0.050	0.000	0.003		

b)

	All spots with no RT			*A. ramb.* (MOR) *vs.* rest 4° C P<0. 001			*A. ramb.* (MOR) *vs.* rest 27° C P<0. 001			*A. ramb.* (IP) *vs.* *A. brun.* 4° C P<0. 001		
	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3
4° C *A. ramb.* (M)	0.802	0.123	0.537	0.849	-0.174	0.442	0.747	0.297	-0.495	0.868	0.125	-0.248
RT *A. ramb.* (M)				0.989	-0.046	0.078	0.817	0.263	-0.437	0.878	0.301	-0.328
27° C *A. ramb.* (M)	0.870	0.371	0.170	0.857	0.486	0.111	0.362	0.891	0.084	0.727	0.585	0.091
4° C *A. ramb.* (I)	0.755	0.584	-0.238	0.933	0.270	-0.191	0.505	0.762	0.318	0.621	0.689	0.318
RT *A. ramb.* (I)				0.893	0.422	0.092	0.932	0.200	0.086	0.779	0.570	0.017
27° C *A. ramb.* (I)	0.905	-0.074	-0.263	0.929	0.330	0.083	0.966	-0.078	0.048	0.829	0.468	-0.088
4° C *A. brunn.* (I)	0.847	-0.490	0.010	0.960	0.001	-0.267	0.917	-0.292	-0.071	0.881	-0.408	-0.140
RT *A. brunn.* (I)				0.959	-0.068	-0.261	0.907	-0.254	-0.099	0.896	-0.363	-0.119
27° C *A. brunn.* (I)	0.960	-0.124	0.117	0.907	-0.244	-0.328	0.968	-0.181	0.072	0.919	-0.331	0.087
4° C *A. brunn.* (M)	0.933	-0.277	-0.033	0.948	-0.289	0.076	0.908	-0.313	0.099	0.850	-0.501	-0.013
RT A. brunn. (M)				0.888	-0.298	0.305	0.925	-0.181	0.228	0.867	-0.433	0.214
27° C A. brunn. (M)	0.920	-0.003	-0.274	0.921	-0.364	-0.074	0.945	-0.139	0.206	0.878	-0.306	0.313

	A. ramb. (IP) *vs.* *A. brun.* 27° C P<0. 001			All spots			pooled *A. ramb.* *vs.* pooled *A. brun.* 4° C P<0. 001			pooled *A. ramb.* *vs.* pooled *A. brun.* 27° C P<0. 001		
	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3
4° C *A. ramb.* (M)	0.926	0.106	0.330	0.797	0.127	0.551	0.892	0.401	0.069	0.926	0.106	0.330
RT *A. ramb.* (M)	0.981	0.174	0.057	0.805	0.185	0.391	0.941	0.297	-0.143	0.981	0.174	0.057
27° C *A. ramb.* (M)	0.871	0.426	0.175	0.845	0.343	0.127	0.860	0.449	-0.043	0.871	0.426	0.175
4° C *A. ramb.* (I)	0.890	0.360	-0.235	0.742	0.602	-0.195	0.588	0.760	0.219	0.890	0.360	-0.235
RT *A. ramb.* (I)	0.971	0.125	-0.156	0.910	0.239	-0.174	0.923	0.305	-0.158	0.971	0.125	-0.156
27° C *A. ramb.* (I)	0.983	0.010	-0.118	0.918	0.020	-0.183	0.978	0.014	-0.167	0.983	0.010	-0.118
4° C *A. brunn.* (I)	0.957	-0.218	-0.025	0.864	-0.465	0.022	0.929	-0.350	-0.087	0.957	-0.218	-0.025
RT *A. brunn.* (I)	0.963	-0.092	0.016	0.866	-0.443	0.092	0.932	-0.323	-0.074	0.963	-0.092	0.016
27° C *A. brunn.* (I)	0.988	-0.112	0.043	0.952	-0.135	0.068	0.957	-0.265	0.056	0.988	-0.112	0.043
4° C *A. brunn.* (M)	0.896	-0.344	0.135	0.936	-0.266	-0.081	0.923	-0.371	0.017	0.896	-0.344	0.135
RT A. brunn. (M)	0.965	-0.232	-0.005	0.920	-0.104	-0.247	0.928	-0.321	0.174	0.965	-0.232	-0.005
27° C A. brunn. (M)	0.947	-0.161	-0.197	0.919	0.038	-0.273	0.935	-0.262	0.225	0.947	-0.161	-0.197

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	All spots no RT treatment			*A. ramb.* (MOR) *vs.* rest 4° C P<0. 001			*A. ramb.* (MOR) *vs.* rest 27° C P<0. 001			*A. ramb.* (IP) *vs. A. brun.* 4° C P<0. 001		
	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3
4° C *A. ramb.* (M)	0.324	0.135	0.735	0.266	-0.175	0.562	0.255	0.215	-0.601	0.299	0.081	-0.361
RT *A. ramb.* (M)				0.310	-0.046	0.100	0.279	0.191	-0.531	0.303	0.194	-0.478
27° C *A. ramb.* (M)	0.351	0.407	0.233	0.269	0.487	0.142	0.124	0.645	0.102	0.251	0.377	0.133
4° C *A. ramb.* (I)	0.304	0.640	-0.326	0.293	0.270	-0.243	0.172	0.551	0.386	0.214	0.444	0.462
RT *A. ramb.* (I)				0.280	0.422	0.117	0.318	0.145	0.104	0.269	0.367	0.024
27° C *A. ramb.* (I)	0.365	-0.081	-0.360	0.292	0.331	0.106	0.330	-0.056	0.058	0.286	0.302	-0.129
4° C *A. brun.* (I)	0.342	-0.537	0.014	0.301	0.001	-0.339	0.313	-0.211	-0.086	0.304	-0.263	-0.204
RT *A. brun.* (I)				0.301	-0.068	-0.333	0.310	-0.184	-0.120	0.309	-0.234	-0.173
27° C *A. brun.* (I)	0.387	-0.136	0.161	0.284	-0.245	-0.418	0.330	-0.131	0.087	0.317	-0.213	0.127
4° C *A. brun.* (M)	0.376	-0.304	-0.045	0.297	-0.289	0.097	0.310	-0.227	0.120	0.293	-0.323	-0.019
RT *A. brun.* (M)				0.279	-0.298	0.389	0.316	-0.131	0.277	0.299	-0.279	0.312
27° C *A. brun.* (M)	0.371	-0.003	-0.376	0.289	-0.364	-0.094	0.323	-0.100	0.250	0.303	-0.197	0.456

	A. ramb. (IP) *vs. A. brun.* 27° C P<0. 001			All spots All treatments			pooled *A. ramb. vs.* pooled *A. brun.* 4° C P<0. 001			pooled *A. ramb. vs.* pooled *A. brun.* 27° C P<0. 001		
	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3	comp. 1	comp. 2	comp. 3
4° C *A. ramb.* (M)	0.283	0.134	0.607	0.263	0.121	0.645	0.285	0.305	0.146	0.283	0.134	0.607
RT *A. ramb.* (M)	0.299	0.218	0.104	0.266	0.177	0.458	0.300	0.226	-0.301	0.299	0.218	0.104
27° C *A. ramb.* (M)	0.266	0.535	0.321	0.279	0.327	0.149	0.275	0.341	-0.091	0.266	0.535	0.321
4° C *A. ramb.* (I)	0.272	0.452	-0.431	0.245	0.574	-0.228	0.188	0.578	0.461	0.272	0.452	-0.431
RT *A. ramb.* (I)	0.297	0.156	-0.287	0.300	0.228	-0.204	0.295	0.232	-0.334	0.297	0.156	-0.287
27° C *A. ramb.* (I)	0.300	0.012	-0.217	0.303	0.019	-0.214	0.312	0.011	-0.352	0.300	0.012	-0.217
4° C *A. brun.* (I)	0.292	-0.274	-0.047	0.285	-0.443	0.026	0.297	-0.266	-0.184	0.292	-0.274	-0.047
RT *A. brun.* (I)	0.294	-0.115	0.029	0.286	-0.422	0.108	0.298	-0.246	-0.155	0.294	-0.115	0.029
27° C *A. brun.* (I)	0.302	-0.140	0.078	0.314	-0.128	0.079	0.305	-0.202	0.117	0.302	-0.140	0.078
4° C *A. brun.* (M)	0.273	-0.432	0.249	0.309	-0.253	-0.095	0.295	-0.282	0.037	0.273	-0.432	0.249
RT *A. brun.* (M)	0.295	-0.291	-0.010	0.304	-0.099	-0.289	0.296	-0.244	0.367	0.295	-0.291	-0.010
27° C *A. brun.* (M)	0.289	-0.202	-0.363	0.303	0.036	-0.319	0.298	-0.199	0.474	0.289	-0.202	-0.363

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		comp. 1	comp. 2	comp. 3	comp. 4	comp. 5	comp. 6	comp. 7	comp. 8	comp. 9	comp. 10	comp. 11	comp. 12
All spots with no RT	1	-0.324	0.135	0.735	0.282	-0.502	0.065	-0.022	-0.016				
	2	-0.351	0.407	0.233	-0.218	0.613	0.300	-0.210	-0.316				
	3	-0.304	0.640	-0.326	0.128	-0.111	-0.577	-0.102	0.140				
	4	-0.365	-0.081	-0.360	0.673	0.115	0.470	0.063	0.200				
	5	-0.342	-0.537	0.014	0.233	0.184	-0.500	-0.141	-0.486				
	6	-0.387	-0.136	0.161	-0.241	0.248	-0.174	0.717	0.376				
	7	-0.376	-0.304	-0.045	-0.376	-0.099	0.049	-0.596	0.504				
	8	-0.371	-0.003	-0.376	-0.388	-0.492	0.263	0.226	-0.456				
A. ramb. (M) *vs.* rest 4° C P<0. 001	1	-0.266	0.175	0.562	0.650	0.134	0.031	-0.028	0.084	0.246	0.207	0.103	-0.136
	2	-0.310	0.046	0.100	0.149	-0.182	0.495	0.184	-0.432	-0.195	-0.497	-0.022	0.293
	3	-0.269	-0.487	0.142	-0.018	-0.594	-0.013	-0.477	0.061	-0.002	0.206	-0.163	0.132
	4	-0.293	-0.270	-0.243	0.310	0.029	-0.494	0.048	-0.200	-0.392	-0.209	0.165	-0.419
	5	-0.280	-0.422	0.117	-0.086	0.552	-0.080	0.009	0.418	-0.027	-0.253	-0.025	0.413
	6	-0.292	-0.331	0.106	-0.325	-0.110	0.148	0.662	-0.008	0.244	0.243	0.112	-0.292
	7	-0.301	-0.001	-0.339	-0.125	0.227	0.099	-0.403	-0.327	0.549	-0.018	0.380	-0.032
	8	-0.301	0.068	-0.333	0.115	0.294	0.124	0.033	-0.193	-0.113	0.454	-0.648	0.040
	9	-0.284	0.245	-0.418	0.131	-0.229	0.282	-0.003	0.661	0.045	-0.207	-0.019	-0.230
	10	-0.297	0.289	0.097	-0.278	0.063	0.107	-0.119	0.070	-0.565	0.392	0.471	0.109
	11	-0.279	0.298	0.389	-0.471	0.071	-0.164	-0.218	-0.041	0.018	-0.312	-0.366	-0.384
	12	-0.289	0.364	-0.094	-0.024	-0.281	-0.582	0.268	-0.006	0.228	0.010	-0.010	0.482
A. ramb. (M) *vs.* rest 27° C P<0. 001	1	-0.255	-0.215	0.601	0.501	-0.026	0.009	0.506	-0.035	0.121	-0.054	-0.002	0.016
	2	-0.279	-0.191	0.531	-0.159	-0.092	0.299	-0.690	-0.017	-0.044	0.037	0.024	-0.019
	3	-0.124	-0.645	-0.102	-0.091	-0.219	-0.673	-0.091	0.181	-0.072	0.026	-0.055	-0.013
	4	-0.172	-0.551	-0.386	-0.135	-0.135	0.626	0.245	-0.111	0.025	0.057	-0.113	0.003
	5	-0.318	-0.145	-0.104	-0.125	0.598	-0.108	0.001	-0.300	0.052	-0.226	0.580	0.048
	6	-0.330	0.056	-0.058	0.052	0.493	-0.123	-0.049	-0.223	-0.130	0.303	-0.676	-0.075
	7	-0.313	0.211	0.086	-0.404	-0.187	-0.066	0.258	0.032	0.076	0.278	0.164	-0.685
	8	-0.310	0.184	0.120	-0.534	-0.048	-0.041	0.217	0.224	0.195	-0.421	-0.276	0.422
	9	-0.330	0.131	-0.087	0.120	0.020	0.104	0.088	0.466	-0.744	0.083	0.188	0.145
	10	-0.310	0.227	-0.120	0.068	-0.495	-0.147	-0.018	-0.590	-0.072	0.227	0.121	0.384
	11	-0.316	0.131	-0.277	0.364	-0.200	0.000	-0.208	-0.044	0.012	-0.645	-0.149	-0.387

	12	-0.323	0.100	-0.250	0.280	0.042	0.029	-0.177	0.442	0.597	0.345	0.123	0.158
A. ramb. (I) *vs. A. brun.*	1	-0.299	-0.081	-0.361	0.652	-0.256	0.048	0.108	0.477	0.193	0.057	0.032	-0.033
4° C P<0. 001	2	-0.303	-0.194	-0.478	0.174	0.012	-0.143	0.032	-0.638	-0.425	-0.007	0.050	0.006
	3	-0.251	-0.377	0.133	0.117	0.788	0.334	-0.033	0.151	-0.070	-0.047	-0.010	0.008
	4	-0.214	-0.444	0.462	0.106	-0.065	-0.578	0.218	-0.157	0.314	-0.041	-0.068	-0.128
	5	-0.269	-0.367	0.024	-0.383	-0.269	0.129	-0.012	0.130	-0.019	0.334	0.435	0.488
	6	-0.286	-0.302	-0.129	-0.422	-0.309	0.256	-0.103	0.131	-0.041	-0.248	-0.475	-0.394
	7	-0.304	0.263	-0.204	-0.273	0.157	-0.215	0.170	0.133	0.161	-0.660	0.373	0.063
	8	-0.309	0.234	-0.173	-0.196	0.238	-0.427	-0.497	0.160	0.087	0.454	-0.023	-0.237
	9	-0.317	0.213	0.127	0.133	-0.078	0.361	-0.338	-0.452	0.565	-0.064	-0.075	0.190
	10	-0.293	0.323	-0.019	-0.142	0.119	-0.023	0.572	0.021	-0.013	0.252	-0.518	0.339
	11	-0.299	0.279	0.312	0.010	-0.093	0.280	0.316	-0.095	-0.158	0.224	0.388	-0.560
	12	-0.303	0.197	0.456	0.212	-0.177	-0.109	-0.333	0.165	-0.546	-0.244	-0.116	0.253
A. ramb. (I) *vs. A. brun.*	1	-0.283	-0.134	-0.607	0.233	-0.274	-0.249	0.317	-0.364	0.149	-0.035	0.004	-0.293
27° C P<0. 001	2	-0.299	-0.218	-0.104	0.106	0.009	-0.215	-0.042	0.155	-0.751	0.397	0.090	0.197
	3	-0.266	-0.535	-0.321	-0.305	-0.139	0.405	-0.364	0.285	0.160	-0.139	-0.047	0.058
	4	-0.272	-0.452	0.431	-0.270	0.297	0.109	0.448	-0.284	0.007	-0.017	0.234	-0.163
	5	-0.297	-0.156	0.287	0.243	0.179	-0.220	-0.248	-0.028	0.085	0.002	-0.739	-0.224
	6	-0.300	-0.012	0.217	0.153	-0.139	-0.470	0.084	0.559	0.227	-0.348	0.323	0.054
	7	-0.292	0.274	0.047	0.305	-0.052	0.556	0.324	0.379	0.089	0.344	-0.041	-0.236
	8	-0.294	0.115	-0.029	0.501	0.355	0.259	-0.351	-0.309	-0.039	-0.315	0.331	0.164
	9	-0.302	0.140	-0.078	-0.162	0.170	-0.149	0.035	-0.122	0.473	0.444	-0.043	0.606
	10	-0.273	0.432	-0.249	-0.486	0.430	-0.150	-0.160	0.108	-0.090	-0.032	0.079	-0.425
	11	-0.295	0.291	0.010	-0.222	-0.203	0.158	0.322	-0.081	-0.296	-0.503	-0.359	0.364
	12	-0.289	0.202	0.363	-0.174	-0.618	0.015	-0.371	-0.311	-0.002	0.178	0.190	-0.171
All spots	1	-0.263	0.121	-0.645	0.141	-0.151	-0.086	0.617	0.066	0.251	0.051	-0.006	0.009
	2	-0.266	0.177	-0.458	-0.527	-0.342	0.026	-0.482	-0.068	-0.221	-0.073	0.014	-0.027
	3	-0.279	0.327	-0.149	0.470	0.325	0.440	-0.349	-0.254	0.197	-0.128	-0.170	0.056
	4	-0.245	0.574	0.228	0.069	0.087	-0.643	-0.091	-0.038	0.010	0.342	-0.031	-0.077
	5	-0.300	0.228	0.204	-0.299	0.330	0.034	0.331	-0.092	-0.209	-0.514	0.337	0.277
	6	-0.303	0.019	0.214	-0.456	0.215	0.432	0.209	0.108	0.135	0.425	-0.247	-0.326
	7	-0.285	-0.443	-0.026	-0.167	0.202	-0.246	-0.157	-0.044	0.317	0.143	-0.220	0.630

	8	-0.286	-0.422	-0.108	0.040	0.311	-0.330	-0.120	-0.061	0.076	-0.310	0.055	-0.631
	9	-0.314	-0.128	-0.079	0.295	0.133	0.055	-0.062	0.628	-0.583	0.146	-0.011	0.106
	10	-0.309	-0.253	0.095	0.188	-0.207	0.129	0.024	-0.471	-0.149	0.409	0.569	0.006
	11	-0.304	-0.099	0.289	0.144	-0.451	-0.029	0.194	-0.276	-0.258	-0.244	-0.591	-0.017
	12	-0.303	0.036	0.319	0.080	-0.437	0.085	-0.149	0.454	0.503	-0.220	0.266	-0.019
pooled *A. ramb. vs.* pooled *A. brun.*	1	-0.285	-0.305	0.146	0.421	-0.159	0.691	-0.177	-0.180	0.138	-0.199	0.046	0.004
4° C P<0. 001	2	-0.300	-0.226	-0.301	0.029	-0.152	0.200	0.287	0.362	-0.298	0.603	-0.180	0.070
	3	-0.275	-0.341	-0.091	0.604	0.139	-0.621	0.018	-0.139	0.073	0.032	0.053	-0.025
	4	-0.188	-0.578	0.461	-0.440	-0.268	-0.219	0.016	0.240	0.033	-0.207	-0.058	0.000
	5	-0.295	-0.232	-0.334	-0.445	0.258	0.050	-0.010	-0.523	-0.218	-0.019	0.382	0.114
	6	-0.312	-0.011	-0.352	-0.188	0.415	0.074	-0.030	0.246	0.491	-0.220	-0.380	-0.266
	7	-0.297	0.266	-0.184	-0.042	-0.336	-0.123	-0.140	0.105	0.318	-0.087	0.066	0.730
	8	-0.298	0.246	-0.155	-0.090	-0.554	-0.157	-0.378	-0.219	-0.116	0.053	-0.146	-0.508
	9	-0.305	0.202	0.117	0.102	0.305	-0.012	-0.331	0.365	-0.625	-0.325	-0.006	0.094
	10	-0.295	0.282	0.037	0.057	-0.144	0.016	0.618	0.179	0.048	-0.264	0.495	-0.278
	11	-0.296	0.244	0.367	-0.023	0.095	-0.013	0.403	-0.446	-0.106	-0.005	-0.557	0.160
	12	-0.298	0.199	0.474	-0.080	0.280	0.011	-0.261	0.050	0.280	0.564	0.296	-0.086
pooled *A. ramb. vs.* pooled *A. brun.*	1	-0.283	-0.134	-0.607	0.233	-0.274	-0.249	0.317	-0.364	0.149	-0.035	0.004	-0.293
27° C P<0. 001	2	-0.299	-0.218	-0.104	0.106	0.009	-0.215	-0.042	0.155	-0.751	0.397	0.090	0.197
	3	-0.266	-0.535	-0.321	-0.305	-0.139	0.405	-0.364	0.285	0.160	-0.139	-0.047	0.058
	4	-0.272	-0.452	0.431	-0.270	0.297	0.109	0.448	-0.284	0.007	-0.017	0.234	-0.163
	5	-0.297	-0.156	0.287	0.243	0.179	-0.220	-0.248	-0.028	0.085	0.002	-0.739	-0.224
	6	-0.300	-0.012	0.217	0.153	-0.139	-0.470	0.084	0.559	0.227	-0.348	0.323	0.054
	7	-0.292	0.274	0.047	0.305	-0.052	0.556	0.324	0.379	0.089	0.344	-0.041	-0.236
	8	-0.294	0.115	-0.029	0.501	0.355	0.259	-0.351	-0.309	-0.039	-0.315	0.331	0.164
	9	-0.302	0.140	-0.078	-0.162	0.170	-0.149	0.035	-0.122	0.473	0.444	-0.043	0.606
	10	-0.273	0.432	-0.249	-0.486	0.430	-0.150	-0.160	0.108	-0.090	-0.032	0.079	-0.425
	11	-0.295	0.291	0.010	-0.222	-0.203	0.158	0.322	-0.081	-0.296	-0.503	-0.359	0.364
	12	-0.289	0.202	0.363	-0.174	-0.618	0.015	-0.371	-0.311	-0.002	0.178	0.190	-0.171

Table S5. Proteins identified in the selected spots.

No	Spot	Spot. No.	Prot. No.	Mascot Id.	selection criteria	fingerprint fragments	Mascot score	EST Agabus contig No.	Acc. No. NCBI/ uiniprot best match	Ref.
1	1	610	1	Enolase	only protein identified in the spot	LAMQEFMILPTGATSFSE AMK, YDLDFK, RIQTAIDK, KACNCLLLK,	249	5790	XM_001653700	18
2	2	705	1	Acyl-CoA dehydrogenase/oxidase	only protein identified in the spot	AFTGFIVER, ETPGLTPGRK, GITFEDVR, ENVLIGEGAGFK, IYQIYEGTSQIQR	303	152	XM_965413.3	24
3	3	956	1	Myosin heavy chain1	structural protein, highly abundant	IEDEIAKLEEK, AQEAFEKEEK, KELEVLNSK, LEGEKGSMGEVQEK, SDLEQQLAETQDR, NQLFQQK, KLEQEISGSK, DIEDLELSVQK, NLNDEIAHQDELINK, TAEELQAAEDK, LEQTLDELEDSLER, ELEQTIQR, DKEISSLTAK, LEDEQSLVGK, IEELEEEVEAER	-	7281	XM_008198304	
4		956	2	paramyosin	structural protein, highly abundant	LSAELAQFR, QTSIEIEQLNSR, VQALTAECEDIR, SAEMQFEESLTR, IEAELTTLAGDYDEVTK, SLEIEVK, LEEVEANAIVGGK, QLQESEGASQQTVTR	-	1399	XM_965626.3	
5		956	3	Actin-related, muscle specific	structural protein, highly abundant	DLTDYLMK, GYSFTTTAER, GYSFTTTAER, SYELPDGQVITIGNER, CDVDIRK,	-	1320	L12254	

					DLYANTVLSGGTTMYPGIADR, EITALAPSTMK, IIAPPERK				
6	956	4	Enolase	present in other spots	EGLEVTQQNEVDDFMIK, FGLDATAVGDEGGFAPNIQENK, EALNLIVDAIEK, IEIGMDIAASEFYK, ACNCLLLK, VNQIGTVSEAIK	371	5790	XM_001653700	18
7	956	5	electron transfer flavoprotein subunit alpha	variable houskeeping genes	VLVAEGEAFK, LDVSPVSDVIGIK, LLYDLADK, AAVDAGFVANDLQVGQTGK	278	80	refXM_964213.3	5
8	956	6	beta-1 tubulin	structural protein, highly abundant	FPGQLNADLR, LAVNMVPFPR, ALTVPELTQQMFDAK, YLTVAAVFR, EVDEQMLNIQNK	-	4617	EU373305	
9	956	7	phosphoglucomutase	only present in this spot	ANSEYDLGAAFDGDGDR, EMFEVPTGWK, FIVQPEIK, TADNFSYVDPIDK, ILFEDGSR	233	65	refXM_961301.3	23
10	956	8	long-chain specific acyl-CoA dehydrogenase, mitochondrial-like	no known relation to thermal biology	ETLQMLVDPTSK, GILLVGTPEQK	95	1133	XM_003707539	
11	956	9	ATP synthase subunit beta	present in several spots	VVDLLAPYAK, IGLFGGAGVGK	70	164	gbBT127859	7,19
12	956	10	fructose 1,6-bisphosphate aldolase	present in several spots	VTETVLAAVYK	46	5054	XM_002000231	21
13	956	11	HSP70		MTSTLQQSSLK	44	4593	gbKF986612	12,30

14	4	991	1	tropomyosin-1	structural protein, highly abundant	AVTNAESEVATQNR, KLQQIEEDMEKSEER, LTEASQAADESFR, SQQDEERMDQLTNQLK, LLAEDADGKSDEVSR, IMELEEELK, SLEVSEEKANQR	109	2153	XM_962035.2	
15		991	2	myosin heavy chain mucsle-like	structural protein, highly abundant	LAEAEETIESLNQK, NYSTELFR, SQLELSQVR, IQEKEEEFENTRK, ALDSMQASLEAEAK	242	1590	XM_004524701	
16		991	3	Actin-related, muscle specific	structural protein, highly abundant	DLTDYLMK, GYSFTTTAER	61	1320	L12254	
17	5	1042	1	glycogen phosphorylase	present in other spots	AFTGFIVER, ETPGLTPGRK, GITFEDVR, ENVLIGEGAGFK, IYQIYEGTSQIQR	62	-	gi170037076	22
18		1042	3	ATP-binding cassette transporter	only present in this spot	ALLDVIAR	41	-	gi157131642	27
19		1042	4	Enolase	present in other spots	IEIGMDIAASEFYK	32	5790	XM_001653700	18
20	6	1140	1	myosin-light-chain-2	structural protein, highly abundant	MAQSGGSDDDDVVVNAFK, TFDNEGVIDGEK, ECDDAFDAMIIDDK	217	1219	AK382826	
21		1140	2	Actin-related, muscle specific	structural protein, highly abundant	DLTDYLMK, GYSFTTTAER, CDVDIRK, DLYANTVLSGGTTMYPGIADR, IIAPPERK	236	1320	L12254	
22		1140	3	tropomyosin-1	structural protein, highly abundant	AVTNAESEVATQNR, LTEASQAADESFR, MDQLTNQLK, LLAEDADGKSDEVSR, LAFVEDELEVAEDR, IMELEEELK, SLEVSEEKANQR	509	2153	XM_962035.2	
23		1140	4	translationally controlled tumor	only present in this spot	DIITGDEMFSDSYK, LVDEVVYEVTGK,	199	1439	dbjAK401040	9,20

				protein		SYTLYLK, LEENAPDQVEVFK				
24		1140	5	ATP synthase subunit beta	present in several spots	VALVYGQMNEPPGAR, IPSAVGYQPTLATDMGTMQER	117	164	gbBT127859	7,19
25		1140	6	14-3-3 protein zeta	only present in this spot	VISSIEQK, YLAEVATGDTR	101	8852	efXM_008202503	28
26		1140	7	guanine nucleotide-binding protein subunit beta-1	no known relation to thermal biology	TFVSGACDASAK	76	5082	refXM_965038.3	
27		1140	8	farnesoic acid o-methyltransferase-like protein mRNA	no known relation to thermal biology	WSGGNISAGR, ASFQGGLIPGK	75	4775	BT126452	
28	7	1233	1	Actin-related, muscle specific	structural protein, highly abundant	DLTDYLMK, GYSFTTTAER, CDVDIRK, DLYANTVLSGGTTMYPGIADR	168	1320	L12254	
29		1233	2	tropomyosin-1	structural protein, highly abundant	AVTNAESEVATQNR, LTEASQAADESFR, LLAEDADGKSDEVSR, SLEVSEEKANQR	391	2153	XM_962035.2	
30		1233	3	tropomyosin-2	structural protein, highly abundant	LAEASQAADESER, IVELEEELR, SLEVSEEK	226	3151	NM_001099807	
31	8	1366	1	Actin	structural protein, highly abundant	AGFAGDDAPR, DSYVGDEAQSKR, VAPEEHPILLTEAPLNPK, DLTDYLMK, SYELPDGQVLTIGNER, DLYANTVLSGGTTMYPGIADR, EITALAPSTIK	460	1320	gi113215	
32		1366	2	glycogen phosphorylase	present in other spots	YEYGIFAQK, SPVDFNLK, VLYPNDNFFEGK	88	-	gi170037076	22
33		1366	3	kettin	structural protein, highly abundant	EGEPVILNAR	44	-	gi30230467	
34		1366	4	Arginine Kinase	only present in this spot	GTFFPLTGMSK	40	5595	dbjAK402526	8

35		1366	5	sarco(endo)plasmic reticulum-type calcium ATPase	no known relation to thermal biology	VGEATETALIVLAEK	36	184	gi4191598	
36		1366	6	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	short fingerprint fragment	YEAPLINAR	35	1833	XM_006889119	19
37	9	1380	1	myosin heavy chain mucsle-like	structural protein, highly abundant	QLSKANAEAQIWR, AKYESEGVAR, LKVDDLAAELDASQKECR, ECRNYSTELFR, NYSTELFR, LKGAYEEGQEQLEAVR, GAYEEGQEQLEAVR, ENKNLADEVK, DLLDQIGEGGR, SQLELSQVR, IQEKEEEFENTRK, YQQQLKDTQAALEEEQR, RANALQNELEESR, QAEQELGDAHEQLNELSAQNASISAAKR, LADELRAEQDHAQTQEK, KALETQIKDLQVR, ALETQIKDLQVR, VRELENELDGEQRR, ELSFQAEEDRKNHER, AQQELEEAEERADLSEQAIAK	155	1590	XM_004524701	

38		1380	2	Myosin heavy chain1	structural protein, highly abundant	VKPLLNVTR, IEDEIAKLEEK, AQEAFEKEEK, ELEVLNSK, TDLLGRLEGEK, LEGEKGSMGEVQEK, SDLEQQLAETQDR, KKLEQEISGSK, DIEDLELSVQKSEQDK, NLNDEIAHQDELINK, LTQEAVADLER, NKKELEQTIQR, ELEQTIQRK, DKEISSLTAK, LEDEQSLVGK, IEELEEEVEAER, DLEESNIQHEGTLANLR	1024	7281	XM_008198304	
39	10	1387	1	putative translation elongation factor 2?	no known relation to thermal biology	VNFTVDEIR, STLTDSLVSK, QFAEMYAEK	110	-	gi52630939	
40		1387	2	glyceraldehyde 3-phosphate dehydrogenase GAPDH	no known relation to thermal biology	GAGQNIIPASTGAAK	56	659/5362	gbAY588063	
41		1387	3	kettin	structural protein, highly abundant	EGEPVILNAR	47	-	gi30230467	
42		1387	4	Actin A3	structural protein, highly abundant	DSYVGDEAQSKR	39	-	gi5751	
43	11	1392	1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	short fingerprint fragment	YEAPLINAR	41	1833	gi156406590	19
44		1392	2	glycogen phosphorylase	present in other spots	YEYGIFAQK, SPVDFNLK	67	-	gi170037076	22
45		1392	3	sarco(endo)plasmic reticulum-type calcium ATPase	no known relation to thermal biology	NILFSGTNVAAGK	49	-/184	gi4191598	
46		1392	5	projectin	structural protein, highly	DDGGTPIEYYQVEK	42	-	gi29134779	

					abundant				
47		1392	6	NADH dehydrogenase (ubiquinone) Fe-S protein 1 isoform 1	short fingerprint fragment, no match in the EST library	FASEVAGVDDLGTTGR	53	-	gi72133227	19
48		1392	7	ATP synthase subunit beta	present in several spots	VVDLLAPYAK	40	164	gbBT127859	7,19
49		1392	8	HSP 60		GYISPYFINTSK	40	-	gi4680247	3,4,6, 29,30
50	12	1423	1	Acyl-CoA dehydrogenase/oxidase		AFTGFIVER, ETPGLTPGRK, GITFEDVR, ENVLIGEGAGFK	111	152	XM_965413.3	24
51		1423	2	Actin A3	structural protein, highly abundant	AGFAGDDAPR, DSYVGDEAQSKR	60	515	XM_003401265	
52		1423	3	Vitellogenin	weak relationship with thermal biology	YTIQSSVTTNK	54	-	gi2522237	11
53	13	1430	1	Actin-related, muscle specific	structural protein, highly abundant	AGFAGDDAPR, GYSFTTTAER	92	1320	L12254	
54		1430	2	similar to Neural conserved at 73EF CG11661-PF	only present in this spot	SSPYCTDVAR, LSGQDVER	84	-	gi66517287	17
55		1430	3	pro-phenol oxidase subunit 2	short fingerprint fragment, no match in the EST library	LDSLVSSR	56	-	gi86515394	10,13
56		1430	4	filamin-B-like	no known relation to thermal biology	EAVPVTEVGSQCK	47	4391	XM_003692762	
57	14	1495	1	Myosin heavy chain1	structural protein, highly abundant	LSLQVVQR, AQEAFEKEEK, ELEVLNSK, NQLFQQK, DIEDLELSVQK, ELEQTIQR	283	7281	XM_008198304	

58	1495	2	glyceraldehyde 3-phosphate dehydrogenase	no known relation to thermal biology	AEDGHLVVNGHK, IAVFSER, DPSNIPWGK, AGAEYVVESTGVFTTVEK, VVSNASCTTNCLAPLAK, GAGQNIIPASTGAAK, VIPSLNGK, LTGMAFR, VPTPNVSVVDLTVR, GASYDDIK, IKEASEGEMK, LISWYDNEYGYSNR	934	659	gbAY588063	
59	1495	3	L-lactate dehydrogenase	selected in other spots	DELLCPVAPPVTSSGNK, INASTDYAVTAGSK, LCVVTAGAR, FIIPQLMK, VIGSGTNLDSSR, FLMSQR, LTEHELAK, SATVMDEVQK	499	112	refXM_008193228	3,14
60	1495	4	transferrin	only present in this spot	YYAVAVVK, APLYALIK, MSEYEVDEK, MCSVCAGNIDSNDSNPQETK, CLATGNGDVAFVK, SGDFELLCPK, NMIVTSNAK, SAADIDEIR	440	6382	D7EIU3	15
61	1495	5	long-chain specific acyl-CoA dehydrogenase, mitochondrial-like	no known relation to thermal biology	ETLQMLVDPTSK, FFEEVNDPAK, GILLVGTPEQK, VTNGTFAAFCLTEPSSGSDAGSIK, VTAFIVDR, AFGGVTSGPPENK, FGMAGALSGTMR, IDSYGGIQEK	421	1133	XM_003707539	
62	1495	6	Aldose reductase	variable houskeeping genes	SKPGEVTQAVK, EDLFITSK, EDGELFPIK, SIGVSNFNEEQLER, NISITAYSPLGSPDR, TPAQVLLR, IIVPLCDAER	341	5074	XM_964433.3	25
63	1495	7	troponin T skeletal muscle	structural protein, highly abundant	GPNFTITK, DNLSSNLSSAQLER	117	5692	XM_008203244	

64		1495	8	fructose 1,6-bisphosphate aldolase	present in several spots	DDNGTPFVELLR, NTPSYQAILENANVLAR, YASICQMNR, VTETVLAAVYK, ALQASVLQAWGAK, AGQDELMKR	282	5054	XM_002000231	21
65	15	1804	1	Acyl-CoA dehydrogenase/oxidase	selected in other spots	AFTGFIVER, ETPGLTPGRK, GITFEDVR, ENVLIGEGAGFK	211	152	gi170043822	24
66		1804	2	ATP carrier protein 2	variable houskeeping genes	TAVAPIER, GNMANVIR	60	5925	XM_001606623.3	26
67		1804	3	Actinin-type, actin-binding	present in other spots	QTDNSLAGVQK	56	8964/1102	gi91080533	2
68		1804	4	L-lactate dehydrogenase		LCVVTAGAR	44	112	refXM_008193228	3
69	16	2021	1	Actin-related	structural protein, highly abundant	AGFAGDDAPR, DSYVGDEAQSKR, DLTDYLMK, EITALAPSTIK	232	1320	gi113215	
70		2021	2	Actinin-type, actin-binding	only protein identified in the spot with relation to thermal biology	FAIQDISVEEMTAK, LMEEYER, QTDNSLAGVQK	136	8964/1102	gi91080533	2
71		2021	3	P-type, ATPase, SERCA	no known relation to thermal biology	NILFSGTNVAAGK, IGVFTEEEDTTGK	69	184	gi4191598	

72	17	2390	1	myosin heavy chain mucsle-like	structural protein, highly abundant	YESEGVAR, AEELEEAKR, LAEAEETIESLNQK, ATAIANAAEKK, LKVDDLAAELDASQKECR , NYSTELFR, DLLDQIGEGGR, SQLELSQVR, IQEKEEEFENTRK, ALDSMQASLEAEAK, DTQAALEEEQR, EALGISER, ANALQNELEESR, TLLEQADR, QAEQELGDAHEQLNELSAQNASISAAK, LADELRAEQDHAQTQEK, KALETQIK, LDEAEANALKGGKK, ELENELDGEQR, MQDLVDKLQQK, QIEEAEEIAALNLAK, ADLSEQAIAK	1858	1590	XM_004524701	
73		2390	2	Actin	structural protein, highly abundant	DLTDYLMK, GYSFTTTAER, SYELPDGQVITIGNER	147	1320	L12254	
74		2390	3	paramyosin	structural protein, highly abundant	LSAELAQFR, QTSIEIEQLNSR, QLQVTLDQLGISQR	183	1399	XM_965626.3	
75		2390	4	V-type proton ATPase catalytic subunit A isoform 2-like	no known relation to thermal biology	YGYVFAVSGPVVTAEK, VGYYELVGEIIR, TASWEFNPLTIK, TVISQALSK, YSNSDVIVYVGCGER, LAEMPADSGYPAYLGAR	203	3853	XM_004533325	
76		2390	5	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	variable houskeeping genes	YEAPLLNAR, QATYVNTEGR, AQQTLVAVTAPGLAR	154	1833	XM_006889119	19

77		2390	6	HSP 60	only present in this spot	LASGVALLK, VNDALNATR, AAVEEGIVPGGGTALLR, NAGVDGSVVVAK	141	1252	KC556801	3,4,6, 29,30
78	18	2589	1	myosin heavy chain mucsle-like	structural protein, highly abundant	ANAEAQIWR, AEELEEAKR, LAEAEETIESLNQK, LATEVEDLQLEVDR, SQLELSQVR, IQEKEEEFENTRK, ALDSMQASLEAEAK, DTQAALEEEQR, EALGISER, ANALQNELEESR, TLLEQADR, LDEAEANALKGGK, QIEEAEEIAALNLAK	979	1590	XM_004524701	
79		2589	2	Actin	structural protein, highly abundant	DLTDYLMK, GYSFTTTAER, SYELPDGQVITIGNER, CDVDIRK, DLYANTVLSGGTTMYPGIADR, EITALAPSTMK, IKIIAPPER, IIAPPERK, QEYDESGPSIVHR	546	1320	L12254	
80		2589	3	paramyosin	structural protein, highly abundant	LSAELAQFR, LIEKDEEIEVIR, QTSIEIEQLNSR	198	1399	XM_965626.3	
81		2589	4	fructose 1,6-bisphosphate aldolase	present in several spots	DDNGTPFVELLR, YASICQMNR, VTETVLAAVYK, ALQASVLQAWGAK	255	5054	XM_002000231	21
82		2589	5	ATP synthase subunit beta	present in several spots	TIAMDGTEGLVR, IPVGAETLGR, VVDLLAPYAK, IGLFGGAGVGK	165	164	gbBT127859	7,19
83		2589	6	HSP70		SDIGEVLLVGGMTR	56	4593	gbKF986612	12,30

84		2589	8	tubulin alpha 1 chain like	structural protein, highly abundant	DVNAAIATIK	41	229	XM_005178656	
85		2589	9	long-chain specific acyl-CoA dehydrogenase, mitochondrial-like	no known relation to thermal biology	GILLVGTPEQK	40	1133	XM_003707539	
86	19	2914	1	glycogen phosphorylase	only protein identified in the spot	YEYGIFAQK, SPVDFNLK	66	-	gi170037076	22
87	20	2944	1	Acyl-CoA dehydrogenase/oxidase	only protein identified in the spot	ETPGLTPGRK, ENVLIGEGAGFK	63	152	gi170043822	24
88	21	2961	1	triosephosphate isomerase (tpi gene)	only protein identified in the spot	GAFTGEISPAMLK, VAHALAEGLK	116	9743	embAJ496728	
89	22	3046	1	ATPase, F1 complex, alpha subunit	only non-structural protein identified in the spot	ADLEETGR, VLSIGDGIAR, VVDALGNPIDGK, AVDSLVPIGR, ELIIGDR	264	874	refXM_961241.3	16
90		3046	2	Actin related proteins	structural protein, highly abundant	AGFAGDDAPR, DSYVGDEAQSKR, GYSFTTTAER, IIAPPERK	227	1320	gi5751	
91		3046	3	glyceraldehyde-3-phosphate dehydrogenase	no known relation to thermal biology	GAGQNIIPASTGAAK, LTGMAFR	72	5263	gi1945477	

GENERAL DISCUSSION

This thesis aimed to understand the mechanisms underlying the evolution of differences in range size between sister species. For this purpose we chose the *Agabus brunneus* complex, for which we have studied the evolution of their response to naturally experienced temperature stress the background of their evolutionary history, testing for their climatic niche divergence, integrating morphology, thermal tolerance and the comparison of their protein expression profiles to study the evolution of their response to temperature.

With that intention we set several objectives at the beginning of the thesis. In Chapter 1, our main objective was to understand the role of thermal niche differences in shaping geographical expansion and speciation processes within the *A. brunneus* complex. How ecology and genetics interact to cause the evolution of barriers to gene flow is now more used in the analysis of speciation genetics (Via 2001), and the study of present niche and its evolution might be very informative. In our case, the niche divergence using climatic variables has been central to the diagnosis of its present and past evolution. Even though their recent diversification, and the fact that two out of the three species had broadly overlapping ranges, we were able to characterize them as ecologically different and detect the importance of the resistance to low temperatures for one of the species, *A. brunneus*, as an innovation that allowed its range expansion.

The diversification of some aquatic beetles in the west Mediterranean area is known to have occurred during the Pleistocene and late Miocene (Ribera and Vogler 2004). The *A. brunneus* complex diverged in the last 0.5 MY, a very recent diversification that highlights the speciation in this region not only as result of the Pleistocene refugia but also in more recent times. This brief lapse of time does not allow recovering reciprocal monophyly between the markers used. The study of organisms in an early stage of speciation gives a unique opportunity to understand some of the determining processes (Schluter 2001, Via 2001). However, it might be also difficult to amplify markers that present enough resolution for recent speciation. In our particular case only two genes among those tried allowed distinguishing part of the evolutionary history of the species complex (cox1 and H3, mitochondrial and nuclear genes respectively). While the nuclear marker separated the three species in agreement with morphology, physiological tolerance to thermal limits and climatic niche divergence, mitochondrial markers recovered a paraphyletic *A. ramblae* ancestral to *A. brunneus* and *A. rufulus*, and might be the result of an incomplete lineage sorting derived from the recent evolution of the group (Funk & Omland 2003). This was a surprising result as the three species can be unambiguously separated by the morphology of the aedeagus, a character generally used in the identification of species of Coleoptera and were thought to be well-differentiated species.

In Chapter 2 our main interest was to assess the possibility of comparing the overall protein expression of wild populations subjected to different temperature treatments. We found out that experiments performed on two populations of the same species (*A. ramblae*) showed a similar amount of variability at different levels: technical replica, biological replica, temperature treatments between populations and the recovery of a 'thermal' signature that allowed to recover the temperature treatments used for a group of proteins. Working with natural populations is of great interest, but the uncontrolled underlying variability must be accounted for previous to further interpretation of the results. With this work we showed the feasibility and reproducibility of these kind of studies with non-model organisms under no previous control, and highlight their potential to address evolutionary and ecological questions.

In Chapter 3 our aim was to trace changes in protein expression through the speciation processes within the *A. brunneus* complex, and to relate these changes with the evolution of phenotypic traits known to differ between species (morphology, climatic niche, thermal tolerance). Although the species of the complex have been well characterized for those traits and they are clearly differentiable, we obtain a substantial amount of proteins that do vary between *A. ramblae* from Morocco and the other populations when subjected at 27° C, related mainly to energy metabolism. *Agabus brunneus*, that diversified presumable from *A. ramblae* populations within the Iberian peninsula, acquired at some point the capacity to tolerate lower temperatures, and this feature is though to be related with its geographic expansion. When comparing *A. ramblae* population from Iberia to *A. brunneus* populations, we found a similar amount of proteins varying than in the previous comparison, but this time in the 4° C treatment, and with proteins related with thermal stress. These findings highlight the role of metabolic and stress proteins on the diversification history of the group, and constitute a first step for being able to employ proteomics to unravel the intricate factors leading to speciation in the *Agabus brunneus* complex.

The use of proteomics is not free of limitations and possible pitfalls (Biron *et al.* 2006a, Knudsen and Chalkley 2011). The statistical support depends on the availability of a sufficient quantity of individuals in every studied population, what might be difficult to achieve when working with natural populations. Depending of the group of study this could be an important constraint. Regarding the possible problems of sensitivity of the gels, moderately abundant proteins may not be seen if the signal is obscured by a nearby spot representing a highly abundant protein. Very hydrophobic proteins may do not enter the gel, there is no resolution for very acidic or basic proteins and scarce proteins may not be detected at all. For the correct identification of the spots selected, the databases rely on previously known protein sequences that belong to model species. In our study we build an EST library with one the species, and we were therefore able to check the reliability of the identifications of proteins, helping us to make decisions on the possible identity of the proteins involved in the observed patterns.

Despite all these difficulties, the possibilities opened by the use of environmental population proteomics are immense, as protein expression is intimately related to the performance of individuals in nature and there is no a priori selection of the proteins that might be discovered with variable expression in response to environmental variables (Cieslak and Ribera 2009; Tomanek, 2010).

This work can be considered a first glimpse on the variability that may be encountered even in closely related species, and the possibilities of population proteomics when combined with other techniques that broad the understanding of the implication of the proteins identified. Others methods that could be used in addition to those employed in this thesis are real time PCR of proteins under the same temperature treatments, changes observed in same conditions at the level of transcriptome and functional analysis of the spots identified. Regarding the thermal tolerance and capacities of species, we have by now information on the critical thermal ranges and intermediate symptoms reacting to ramping temperature on one side (Calosi *et al.* 2008a, Hidalgo-Galiana *et al.* 2014), and on the other the response at proteomic level facing temperatures that are experiencing on field. But the curve of response of species to temperature also be studied at other points by looking, for example, at the capacity of rapid cold hardening, measuring with better techniques the variability of temperature that the body experienced as temperature varies or by studying the response of the species to longer term environment related experiments, to complete the mosaic of responses that this group of species has developed.

Another remaining question is the determination of the sequence of events in the geographical expansion of the studied species where physiological changes that allowed the species to expand range previous to the geographic expansion?, or did they acquire them as local adaptations after they expanded their range? The response might be related t regulatory pathways that are hard to track, but the combination of other techniques and disciplines as mentioned before might elucidate the implied mechanisms.

GENERAL CONCLUSSIONS

1. The *A. brunneus* complex diversified ca. 0.6-0.25 Ma, most likely in the south of the Iberian peninsula after the colonization of *A. ramblae* from north Morocco. Whilst insular populations (*A. ramblae* in the Balearic Islands and *A. rufulus* in Corsica and Sardinia) did not apparently differentiate substantially in either morphology or ecology, continental *A. brunneus* evolved the most distinctive morphology within the complex, as well as wider tolerance to cold habitats, something that seems to have facilitated range expansion.

2. From a reduced potential distribution during the LIG, *A. brunneus* and *A. ramblae* appear to have expanded their ranges during the last glacial (0.03-0.01 Ma) (*A. brunneus* to a much wider area), covering most of their LGM potential rages in the western Mediterranean.

3. This expansion was accompanied by a population expansion however, both species have not occupied areas beyond their LGM potential distribution except for some isolated populations.

4. In Sardinia, the Balearic Islands and possibly Tunisia, secondary contact between species of the complex has resulted in introgression, with some specimens showing discordance between mitochondrial haplotypes typical of *A. brunneus* and nuclear sequences and morphology typical of *A. rufulus* or *A. ramblae* respectively.

5. It is possible to conduct proteomic studies on wild populations of non-model organisms to obtain physiologically relevant data with relatively less noise.

6. The reproducibility and uniformity of for two distinct populations of a species of water beetle (*Agabus ramblae*) suggest that the experimental setup allowed the detection of a common stress-related response to temperatures at the extremes of the range they experience in their natural environment.

7. The protein expression patterns of a complex of closely related species can be associated to parallel changes in their ecology and thermal tolerance, contributing to understand their evolutionary history and their geographical distributions.

8. The first of the transitions, the colonisation of the Iberian peninsula by North African populations of Agabus ramblae during the Middle Pleistocene, was accompanied by a change in the response to high temperatures in many proteins related to energy metabolism although no substantial morphological change or differences in the lower thermal limit are found.

11. In the second transition, between *A. brunneus* and *A. ramblae*, the phenotypic changes were paralleled by changes in the protein expression of several stress-related proteins when exposed to low temperatures.

12. Ecological and physiological changes between *A. brunneus* and Iberian *A. ramblae* may have evolved in close geographical proximity, resulting in the formation of two genetically isolated species through a process of reinforcement.

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APPENDIX

Other publications during the period of the PhD

Molecular Phylogenetics and Evolution 59 (2011) 377–385

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

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Late Miocene diversification of the genus *Hydrochus* (Coleoptera, Hydrochidae) in the west Mediterranean area

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ABSTRACT

We provide a reconstruction of the phylogenetic relationships, the geographical and temporal origin, and the mode of diversification of the Mediterranean species of the aquatic beetle family Hydrochidae (Coleoptera, Hydrophiloidea). A total of ca. 3 KB of sequence data of three mitochondrial and two nuclear genes were used to reconstruct the phylogeny of 62 specimens of 21 species of *Hydrochus*, including all western Mediterranean species but one. We estimated the times of divergence using Bayesian methods and an evolutionary rate of 0.0115 substitutions/site/MY, and used an ultrametric calibrated tree to construct a Lineage Through Time (LTT) plot to test alternative models of diversification. A well resolved, well supported phylogeny showed that all western Mediterranean *Hydrochus* formed a clade, sister to a group including species with a central and eastern European distribution. The origin of the western Mediterranean clade was estimated to be at ca. 13MY, and the speciation events took place between this time and the end of the Messinian, at about 5.3MY. The LTT plot best fitted a model with a shift in the rate of diversification at ca. 8 MY, with a single speciation event (originating two Iberian endemics) subsequent to this period. We conclude that most of the western Mediterranean species of Hydrochidae, including the Ibero-Maghrebian endemics, are ancient elements likely to have remained in the same geographical area since their Miocene origin. Our results add to a growing body of evidence showing the importance of Mediterranean long-term, Tertiary refugia as both cradles and museums of diversity.

1. Introduction

The Mediterranean region is one of the world hotspots of biodiversity (Médail and Quézel, 1999; Myers et al., 2000), with a complex geological history and a rich mosaic of habitats favouring diversification (Blondel and Aronson, 1999). For many Mediterranean groups of organisms the peninsulas (Iberia, Balkans and Turkey) contain the highest diversity, with a substantial part of these species forming species radiations of restricted distributions in each of these areas (e.g. Oosterbroek and Arntzen, 1992; Crivelli and Maitland, 1995; Petit et al., 2003; Sanmartín, 2003). Many insect groups, and among them Coleoptera, follow this general pattern (see e.g. Jäch, 1993; Fery and Brancucci, 1997; Fery and Hosseinie, 1998; Löbl and Smetana, 2004 for some aquatic families), but there is a general lack of data of the origin of these species in what refers both to their temporal and geographical origin. Recent work on aquatic Coleoptera established the recent (Pleistocene) origin of most Iberian endemics of one of the families (Dytiscidae), which are in general vicariant species with widespread European distributions (Ribera, 2003; Ribera and Vogler, 2004; Ribera and Faille, 2010). Only in some cases (e.g. genus *Deronectes*) there was an older diversification within the Iberian peninsula. In a different family (Hydraenidae), although some groups of narrow range endemics show also a predominantly Pleistocene origin (e.g. *Haenydra* lineage, Ribera et al., 2011), others seem to have an older Origin, such as the *Ochthebius* (*Enicocerus*) *exsculptus* species group, with two Late Miocene Iberian endemic species (Ribera et al., 2010a). The idiosyncratic origin of different groups with similar distributions suggests that until the phylogeny and biogeography of a wide range of Mediterranean groups is investigated it would not be possible to draw general conclusions about the origin and assemblage of the Mediterranean fauna.

Among aquatic Coleoptera, one group with a predominantly Mediterranean distribution of which there is virtually no phylogenetic or biogeographic information is the family Hydrochidae (Hydrophiloidea) (Hansen, 2004). Hydrochidae includes only one accepted genus, *Hydrochus*, with a worldwide distribution and about 180 described species (Hansen, 1999; Short and Hebauer, 2006). All *Hydrochus* are aquatic, living in stagnant or slowly flowing water (Jäch, 1998). In the west Mediterranean (Iberian peninsula, Morocco and south France), the genus *Hydrochus* is represented by 12 species, 7 of them endemic to the area. In the east Mediterranean (the Balkans, Turkey, the Near East and Iran) there are six widespread species and only two described endemics from Iran and Turkey

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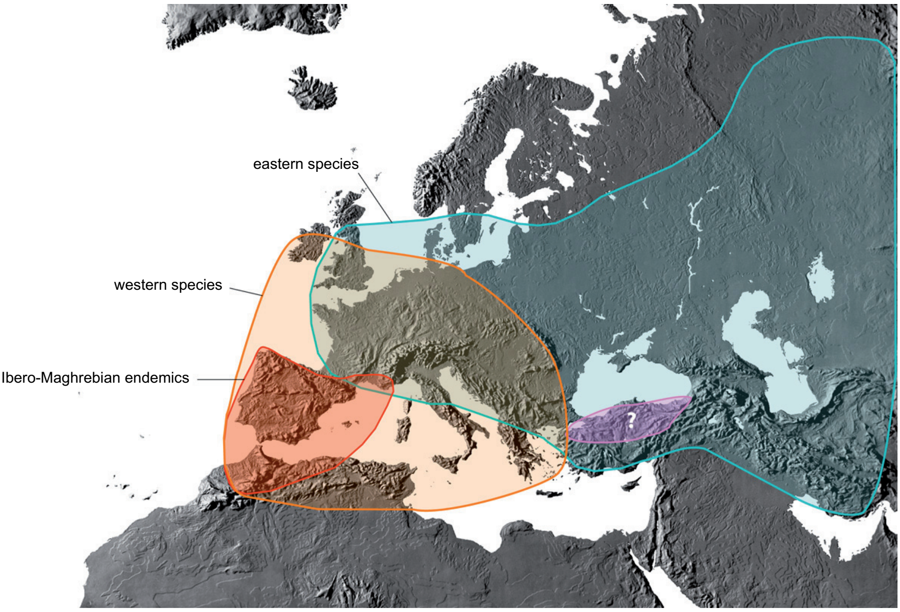

Fig. 1. Main distribution types among the species of west Palaearctic *Hydrochus*. Ibero-Maghrebian endemics include *H. aljibensis*, *H. angusi*, *H. ibericus*, *H. interruptus*, *H. nooreinus*, *H. smaragdineus*, *H. tariqui* and the non-sampled *H. obtusicollis*; western species include *H. angustatus*, *H. flavipennis*, *H. grandicollis* and *H. nitidicollis*; and eastern species *H. crenatus*, *H. elongatus* and the non-sampled *H. farsicus*, *H. ignicollis* and *H. nodulifer*. With a question mark, uncertain distribution of *H. roberti*. See Fig. 2 for the phylogenetic relationship of the species, Appendix A for the detailed data of the studied material, and Appendix D for the detailed distributions.

(Hansen, 2004; Hidalgo-Galiana et al., 2010, Fig. 1 see Appendices D and E for the detailed distribution of the studied species), although some recent records and unpublished information from the collections of the Naturhistorisches Museums in Wien suggest that there could be several undescribed species in the area.

We provide here a reconstruction of the phylogenetic relationships, the age and the geographical origin of the western Palaearctic species of *Hydrochus* to understand their diversification and the current patterns of diversity. We use molecular data (mitochondrial and nuclear) of 16 of the 20 species present in the area, and use molecular-clock methods to estimate the age of most of the known Ibero-Maghrebian endemics.

2. Materials and methods

2.1. Background on the taxonomy of the group and taxon sampling

We include data of 16 out of 20 known species of western Palaearctic *Hydrochus* (Hansen, 2004; Appendix E), with the exception of (1) *Hydrochus obtusicollis* (Fairmaire), with a restricted distribution in north Morocco (Bennas et al., 2007), (2) *Hydrochus ignicollis* Motschulsky, with a wide European distribution, (3) *Hydrochus nodulifer* Reitter known from the Caucasus and Iran and (4) *Hydrochus farsicus* Hidalgo-Galiana, Jäch & Ribera from Iran (Hansen, 2004; Hidalgo-Galiana et al., 2010) (Appendices D and E). There are several possibly undescribed species in Turkey apparently closely related to the *Hydrochus elongatus* group (which includes *H. ignicollis* and *Hydrochus crenatus* (Fabricius)) (M.A. Jäch, personal communication, 2009). The recent record of *Hydrochus ibericus* Valladares, Díaz & Delgado from this area (Mart et al., 2009) corresponds to *H. farsicus* (U. Incekara, personal communication, 2010).

For some of the species we studied more than one specimen (Appendix A) to test for the monophyly of the currently recognised species and to detect possible intraspecific variability (specially in the case of islands or geographically isolated regions). We included as outgroups several species of *Hydrochus* from other regions of the world (Appendix A). Trees were rooted with sequences of other families of Hydrophiloidea clearly outside Hydrochidae (Bernhard et al., 2009), obtained from GenBank (Appendix A).

2.2. DNA extraction and sequencing

Specimens were killed and preserved in absolute ethanol in the field. We employed for DNA isolation a standard phenol–chloroform non-destructive extraction (voucher specimens MNCN-AH1 to MNCN-AH36) or "Charge Switch gDNA Tissue Kits" (Invitrogen, Carlsbad, USA) (voucher specimens MNCN-AH37 to MNCN-AH70, see Appendix A), following the instructions of the manufacturer. Typically only males were sequenced, and the male genitalia (or aedeagus, used for species identification) examined and preserved previous to the extraction to ensure a correct identification. Voucher specimens and DNA aliquots are deposited in the Museo Nacional de Ciencias Naturales (MNCN, Madrid) and the Institut de Biologia Evolutiva (IBE, Barcelona) (Appendix A).

Five gene fragments were amplified: three mitochondrial markers, the 3' end of the subunit 1 of the Cytochrome Oxidase (*cox1*), an internal fragment of Cytochrome b (*cob*) (both protein coding) and 12S rRNA (*rrnS*); and two fragments of nuclear ribosomal genes, the 5' end of 18s rRNA (*SSU*) and an internal fragment of 28s rRNA (*LSU*). For each fragment both forward and reverse sequences were obtained (see Table 1 for the primers used). In some specimens the *cox1* fragment was amplified using internal primers to obtain two fragments of around 400 bp each (Table 1).

General PCR cycling conditions used for DNA amplification were: 3 min at 96 °C, [30s at 94 °C, (30s- 1 min) at 47–50 °C (depending on the annealing temperatures of primer pair used),

Table 1
List of primers used for amplification and sequencing.

Gene	Primer	Sequence	Reference
cox1	Jerry (5′)	5′ CAACATTTATTTTGATTTTTTGG	Simon et al. (1994)
	Pat (3′)	5′ TCCAATGCACTAATCTGCCATATTA	Simon et al. (1994)
	Chy1 (5′)	5′ T(A/T)GTAGCCCA(T/C)TTTCATTA(T/C)GT	Ribera et al., 2010b
	Tom1 (3′)	5′ AC(A/G)TAATGAAA(A/G)TGGGCTAC(T/A)A	Ribera et al., 2010b
cob	CB3 (5′)	5′ GAGGAGCAACTGTAATTACTAA	Barraclough et al. (1999)
	CB4 (3′)	5′ AAAAGAAA(AG)TATCATTCAGGTTGAAT	Barraclough et al. (1999)
rrnS	12Sai (5′)	5′ AAACTAGGATTAGATACCCTATTAT	Simon et al. (1994)
	12Sbi (3′)	5′ AAGAGCGACGGGCGATGTGT	Simon et al. (1994)
SSU	18S 5′ (5′)	5′ GACAACCTGGTTGATCCTGCCAGT	Shull et al. (2001)
	18S b5.0	5′ TAACCGCAACAACAACTTTAAT	Shull et al. (2001)
LSU	ka (5′)	5′ ACACGGACCAAGGAGTCTAGCATG	Monaghan et al. (2007)
	kb (3′)	5′ CGTCCTGCTGTCTTAAGTTAC	Monaghan et al. (2007)

(50s- 1 min) at 72 °C] (repeated for 35–40 cycles), and 10 min at 72 °C. Sequencing was performed by the Sanger method in an external facility. The products obtained were purified by a standard ethanol precipitation. Sequencing errors/ambiguities were edited using the Sequencher 4.7 software package (Gene Codes Corporation, Ann Arbor, USA). New sequences were deposited in GenBank with accession numbers HM569373-HM569596 (Appendix A).

2.3. Phylogenetic analyses

We aligned length-variable fragments with MAFFT 5.8 on-line version (Katoh et al., 2002), shown to perform better than alternative pair-wise alignment methods (Golubchik et al., 2007), using the G-INS-i algorithm and default values for the rest of parameters.

We used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) to estimate the topology and node support. We included a combined data matrix partitioned according to two different criteria: (1) the five genes (*cox1*, *cob*, *rrnS*, *LSU* and *SSU*), and (2) a partition by codon position on the combined *cox1* and *cob* fragments, plus the mitochondrial ribosomal gene (*rrnS*) and the two nuclear ribosomal genes combined (*LSU* plus *SSU*) (i.e. a total of five partitions for each criteria). For each partition we implemented the available evolutionary model with the closest match to that selected by ModelTest 3.6 (Posada and Crandall, 1998), using Akaike weights as selection criteria. MrBayes ran 7.5×10^6 or 40×10^6 generations for the gene and codon partition respectively, using default values and saving trees each 1000 generations. "Burn-in" values were estimated by plotting the standard deviation of the split frequencies between two simultaneous runs and visually checking for convergence. The two partition strategies were compared using Bayes factors (BF) (Kass and Raftery, 1995), as computed in Tracer 1.5 (Drummond and Rambaut, 2007) using 1000 replicates. A partition was considered significantly better when the ln(BF) had an increase of 10 or more for each additional parameter (p) (i.e. a PM factor = $\Delta \ln BF/\Delta p > 10$; Pagel and Meade, 2004; Miller et al., 2009).

We also used Maximum Likelihood as implemented in the online version of RAxML 7.0.3 (which includes an estimation of bootstrap node support, Stamatakis et al., 2008), using GTR + G as the evolutionary model, estimated independently for each of the gene partitions. To check for possible incongruence between mitochondrial and nuclear genes we run two separate analyses in RAxML with the respective sequences, using the same conditions as for the combined dataset.

2.4. Estimation of the ages of divergence

Molecular dating was carried out with Beast v1.4.7 (Drummond and Rambaut, 2007). Beast generates ultrametric rooted trees, incorporating a time-scale if a calibration point or an *a priori* rate are specified. We excluded the multiple specimens of the same species and constrained all well supported nodes according to the results of the previous phylogenetic analyses, and employed an uncorrelated lognormal relaxed clock and a global GTR + I + G evolutionary model (Drummond and Rambaut, 2007). As there is no fossil record or an unambiguous biogeographic event that could be used to calibrate the tree we used an estimated rate of 0.0115 substitutions/site/MY for the combined mitochondrial genes. This rate is the standard pair-wise difference of 2.3% per MY (Brower, 1994), which could be different for *Hydrochus*, but agrees with estimations based on biogeographic events in some related Coleoptera groups for a mix of protein-coding and ribosomal mitochondrial genes (0.010 substitutions/site/MY, Ribera et al., 2010b; 0.013 substitutions/site/MY, Papadopoulou et al., 2010). As this rate applies to the combined mitochondrial sequence, we build a matrix excluding the nuclear genes and analysed it as a single partition. The prior rate was set as a normal distribution with average 0.0115 substitutions/site/MY and a standard deviation of 0.0005. We set a Yule speciation process (a pure birth process, with a uniform probability of speciation) as a tree prior, and made two independent runs with the same settings and combined the results after deletion of 10% of the generations as burnin with Tracer 1.5 and other applications of the Beast package (Drummond and Rambaut, 2007).

2.5. Rate and mode of diversification

We estimated the rate of diversification using the log-lineage through time approach (LTT) (Harvey et al., 1994; Nee et al., 1994). We used Genie (Pybus and Rambaut, 2002) to compile the LTT plot using the ultrametric tree obtained in Beast. LTTs represent graphically the time elapsed between successive branching events (Barraclough and Nee, 2001). The ultrametric tree contains information on the number of lineages and the molecular distance of every lineage to the root (the relative time of each node from the root node).

We used the γ-statistic (Pybus and Harvey, 2000) for measuring the relative timing of the diversification, i.e. whether there is a constant diversification through the tree, or the interior nodes are closer to the tips or to the root than expected under a pure birth process. The γ-values of complete reconstructed phylogenies follow a standard normal distribution. If $\gamma < 0$, the internal nodes can be said to be closer to its root than expected under a pure birth process, and vice versa (Pybus and Harvey, 2000). To test the significance of the γ-statistic we generated a null distribution of 10,000 random simulations using a pure birth process including the known missing taxa, and tested the observed γ-statistic against it (Pybus and Harvey, 2000). We also found the number of missing taxa that would be necessary to render the observed γ-statistic non-significant.

Table 2
Length of the sequenced fragments, with maximum and minimum length before and after alignment and number of informative characters, evolutionary model selected by ModelTest for the different partitions, and model implemented in MrBayes.

partition	max.	min.	aligned	informative	optimal model	implemented model
cox1	826	826	826	334	GTR+I+G	GTR+I+G
cob	358	358	358	152	Tim+I+G	GTR+I+G
rrnS	358	349	367	143	TVM+G	GTR+G
LSU	597	584	599	26	GTR+I	GTR+I
SSU	602	600	604	14	Trnef+I	GTR+I
1st codon	394	394	394	114	GTR+I+G	GTR+I+G
2nd codon	394	394	394	34	GTR+I+G	GTR+I+G
3rd codon	396	396	396	338	GTR+I+G	GTR+I+G
LSU+SSU	1190	1184	1203	40	GTR+I+G	GTR+I+G
Total	2730	750*	2754	669	GTR+I+G	GTR+I+G

* Specimen MNCN-AH4, with incomplete cox1 sequence only (see Appendix A). The rest of measures are given only for genes with the complete sequence.

We tested the adequacy of our data to different diversification models with likelihood methods. The models tested were a pure birth (Yule), a birth–death with constant diversification rate, two models with variable diversification rates (logarithmic and exponential), and a pure birth model with a shift in the diversification rate (Table 3). We checked the significance of the result with a function that generates a null distribution of the statistic and returns the probability of the observed AIC (Akaike Information Criterion) for constancy of diversification rates (as in Rabosky, 2006). All diversification tests were done using the R libraries 'ape' (Paradis et al., 2004) and 'laser' (Rabosky, 2006).

Table 3
Models of diversification tested. Models tested: pure birth (Yule), constant rate without extinction; birth–death (bd), constant rate with extinction; DDL, density-dependent variable rate (logarithmic); DDX, density-dependent variable rate (exponential); yule2rate (y2r), pure birth with a shift in diversification. r1, estimated speciation rate (first parameter in all models); 2nd, second parameter (extinction rate in the bd model, carrying capacity (k) in DDL, density-dependent parameter (x) in DDX, second speciation rate in y2r); 3rd, third parameter (time of shift in diversification in y2r); AIC, Akaike Information Criterion; dAIC, delta-AIC, difference in AIC scores between the model and the overall best-fit model. (a) Models when the variation within *H. grandicollis* was included, (b) models when the variation within *H. grandicollis* was not included (see Text).

Model	r1	2nd	3rd	AIC	dAIC
(a) H. grandicollis included					
Pure birth	0.120			33.66	6.33
Birth–death	0.120	0		35.66	8.33
DDL	0.340	16.945		30.08	2.75
DDX	1.036	0.991		30.47	3.14
Yule 2 rate	0.369	0.063	7.87	27.34	0
(b) H. grandicollis not included					
Pure birth	0.096			35.60	16.08
Birth–death	0.096	0		37.60	18.08
DDL	0.726	12.239		19.52	0
DDX	1.515	1.317		29.54	10.02
Yule 2 rate	0.369	0.032	7.87	24.57	5.05

3. Results

3.1. Phylogenetic analyses

The final matrix included 66 specimens of 25 recognised species (21 of them in the genus Hydrochus, 15 of them in the ingroup W Palearctic clade) (Appendix A). There were no length differences in the protein coding genes among the studied specimens, and among the ribosomal genes length differences were mostly in the *LSU* gene (Table 2). For the combined matrix, ModelTest selected GTR + I + G as the best evolutionary model. Of the different models selected for the individual partitions some are not implemented in MrBayes, and thus we selected the most similar one with an equal or lower number of parameters (Table 2). For the partition by genes the two independent runs converged at ca. 4×10^6 generations (used as the "burn-in"), reaching a standard deviation of the split frequencies of ca. 0.006. For the partition by codons plus the nuclear and mitochondrial ribosomal genes the two runs converged at ca. 25×10^6 generations, reaching a standard deviation of the split frequencies of ca. 0.002. The runs of both partitions had enough ESS (Effective Sample Size) and a convergence diagnostic in MrBayes (PSRF, potential scale reduction factor) close to one (Ronquist and Huelsenbeck, 2003), indicating a good convergence of the MCMC chains.

The topology obtained with the two partition schemes in MrBayes was identical for the Mediterranean species, and differed only in the relative position of the species of the *Hydrochus brevis* group and the clade with the American and Australian species, which was poorly supported in both cases (Bayesian posterior probability, Bpp = 0.88 and 0.55 for the genes and codon partitions respectively, Fig. 2). The Bayes factors favoured the partition by codons, with a difference in lnBY of more than 500 units for two additional parameters (Table 3) (i.e., PM >>10).

Differences between the topologies of the two reconstruction methods used (Maximum Likelihood and Bayesian Analysis) were minimal, and affecting only three nodes: the placement of the *Hydrochus angusi* Valladares and *H. ibericus* clade (sister to the rest of clade B in RAxML, see below and Fig. 2), and the position of the Australian species and the species of the *H. brevis* group (Fig. 2). In all cases these ambiguities affected poorly supported nodes, and the two alternative topologies for the only ambiguous node among the Mediterranean species did not affect any of the results.

3.2. Phylogeny of the Mediterranean species of Hydrochus

The monophyly of the genus *Hydrochus* was strongly supported (Bayesian posterior probability, Bpp $\geq$ 0.99, ML boostrap, MLb = 100%, Fig. 2), with a basal split within the sampled species of the genus separating four main well-supported lineages: (1) the central and northern European species of the *H. brevis* group; (2) the single species from Australia; (3) the American species; and (4) all remaining Palaearctic species sister to a South African species. This Palaearctic-South African clade was very well supported (Bpp = 1.0, MLb = 100%, Fig. 2, see Appendix A for the localities of the specimens). The resolution among these four lineages was poorly supported.

Within the main Palaearctic lineage, all species of *Hydrochus* with a distribution centred in the western Mediterranean region were included in a well supported clade (Bpp = 1.0, MLb = 86%, Fig. 2), sister to two species with a mostly central and northern European distribution (*H. crenatus* and *H. elongatus* (Schaller)). This west Mediterranean clade had two well-supported lineages with overlapping geographical distributions (clades A and B in Fig. 2), including both Ibero-Maghrebian endemics (*Hydrochus aljibensis* Castro & Delgado and *Hydrochus interruptus* Heyden in lineage A, and *Hydrochus tariqi* Ribera, Hernando & Aguilera, *H. nooreinus*

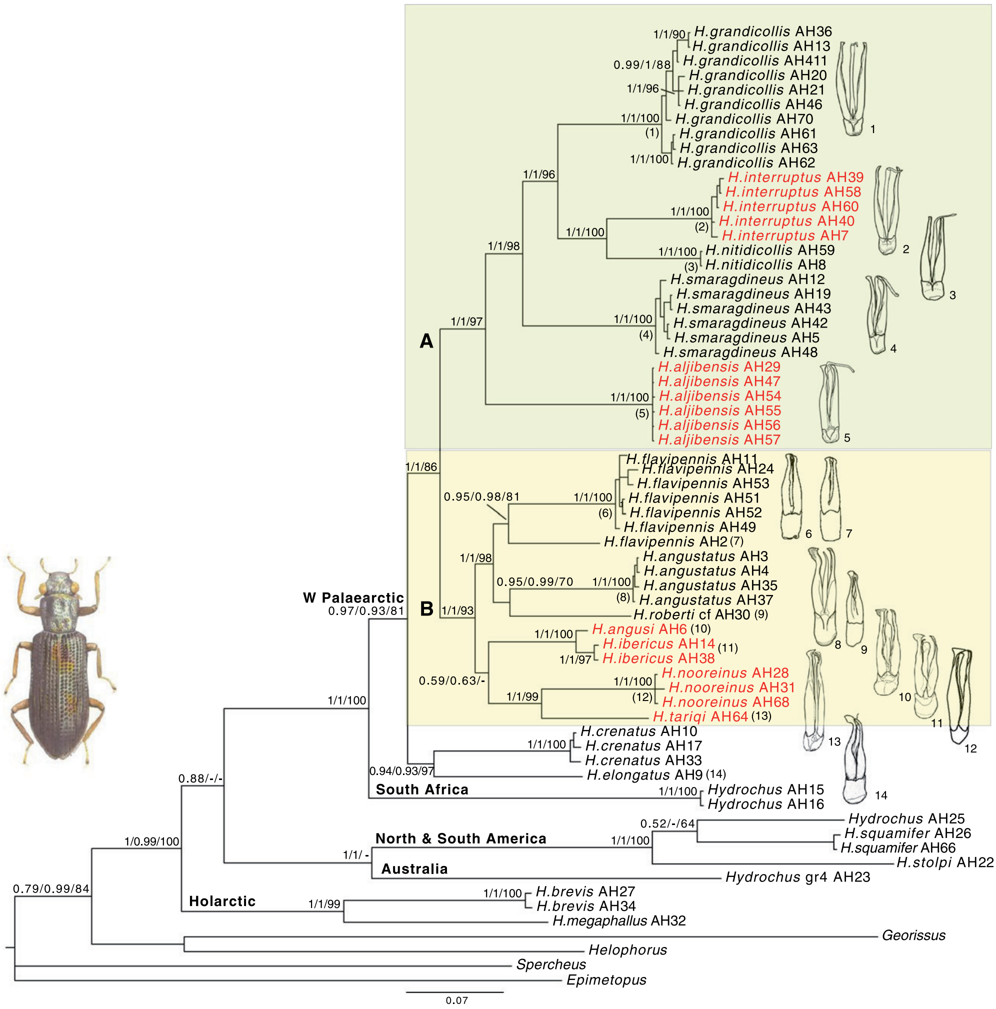

Fig. 2. Phylogram obtained with MrBayes with the combined nuclear and mitochondrial sequence and a partition by gene. Numbers above the branches, Bayesian posterior probabilities of the partition by gene/Bayesian posterior probabilities of the partition by codon/Bootstrap support values in RAxML. In red, Ibero-Maghrebian endemics. Outline drawings, male genitalia (numbers correspond to those below branches), not at the same scale. Habitus, *Hydrochus tariqi* (from Ribera et al., 1999). See Appendix A for the detailed precedence of the sequenced specimens and Fig. 1 for the general distribution of the species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Berge Henegouwen & Sáinz-Cantero, *H. angusi* and *H. ibericus* in clade B), as well as widely distributed species (Fig. 1; see Appendix D for the detailed distribution of the species). In clade B there were three well supported nodes with poorly supported relationships between them, two including Ibero-Maghrebian endemics and the third a group of species including the widely distributed *Hydrochus angustatus* Germar, a specimen provisionally identified as *Hydrochus roberti* Shatrovskij from Turkey (previously known only from the Caucasus, Shatrovskij, 1993) and the *Hydrochus flavipennis* complex. All species in clade A are present in the Iberian peninsula and the Maghreb, with two endemics of this area forming a basal paraphyletic series (*Hydrochus aljibensis* and *Hydrochus smaragdineus* Fairmaire). There are two species extending their distribution towards central and northern Europe and/or the eastern Mediterranean (*Hydrochus nitidicollis* Mulsant and *Hydrochus grandicollis* Kiesenwetter), together with the Iberian *H. interruptus*, nested within them (Figs. 1 and 2; Appendix D).

The ML analyses using only the nuclear genes recovered some of the nodes (the main Palaearctic clade, the west Mediterranean clade) but contributed little to the resolution of the west Mediterranean species on their own (Appendix B). The ML tree obtained with the mitochondrial sequence was very similar to that obtained with the combined sequence (Appendix C), except for the basal relationships among the western Palaearctic lineage.

All currently recognised species were monophyletic with strong support and generally low intraspecific variation, with the only exception of *H. flavipennis* Küster, with two lineages with a very deep divergence supported exclusively by the mitochondrial genes

(Fig. 2 and Appendix B). One of these lineages included specimens from central Spain, and the second specimens from Morocco, Spain and Tunisia (Appendix A). *H. grandicollis* presented also a strong geographical structure, with good support for the respective monophyly of specimens from Morocco, Spain and Sicily (Figs. 1 and 2; Appendix A). The variation within other species (e.g. *H. interruptus*, *H. smaragdineus*, Fig. 2) had no obvious geographical structure.

Our results are in good correspondence with informal groupings made according to morphology, and in particular to the structure and shape of the male genital organ (the aedeagus), traditionally used for species identification in the genus (e.g. Angus, 1976; see Fig. 2). Although without a formal analyses it is not possible to obtain firm conclusions, the strong asymmetry of the parameres of the male genitalia and the presence of a flagellum at the apex of the median lobe seems to be the plesiomorphic condition for the main Palaearctic clade, not present in the species of the *H. brevis* group and all the non-Palaearctic species. The European species of the *H. brevis* group (i.e. *H. brevis* (Herbst) and *Hydrochus megaphallus* Berge Henegouwen) form a very distinct lineage, most likely including some Nearctic species with a very similar external morphology and structure of the aedeagus (see e.g. Smetana, 1988). Within the main Palearctic clade, the species of the *H. elongatus* group maintain the plesiomorphic condition (flagellum plus asymmetry of the parameres), but share a characteristic apical expansion of the left paramere (Angus, 1976; Hansen, 1987). Species of clade B maintain the strong asymmetry, but the flagellum is lost or very reduced. Species of clade A seem to have secondarily developed more symmetrical parameres, and developed a longer flagellum (see the outline of the male genitalia of the studied species in Fig. 2).

There are only four recognised western Palaearctic species not included in our phylogeny. Of these, only *H. obtusicollis* occurs in the western Mediterranean: it is a rare species endemic to north Morocco likely to be related to *H. angustatus* according to the morphology of the aedeagus (Bennas et al., 2007). The aedeagus of two eastern European species (*H. ignicollis*, *H. nodulifer*) is clearly similar to that of *H. elongatus* (Hansen, 1987; Shatrovskij, 1993). Finally, the aedeagus of the Iranian *H. farsicus* and that of some of the undescribed species from Turkey in the collections of the NMW (M.A. Jäch, personal communication, 2009), although with less clear affinities, share some characters of the species in the *H. elongatus* group (Hidalgo-Galiana et al., 2010).

3.3. Rate of diversification and molecular dating

Using a standard mitochondrial rate (2.3% per MY) in Beast the split between the western Mediterranean clade and the species of the *H. elongatus* group was estimated to have occurred around Mid Miocene (ca. 14MY, Fig. 3). The diversification of the western Mediterranean clade was dated at ca. 13MY, and the speciation events took place between this time and the end of the Messinian, at about 5.3MY, with the only exception of the separation between *H. ibericus* and *H. angusi*, estimated to have originated during the lower Pleistocene (ca. 1.5MY, Fig. 3).

We restricted the analyses of diversification to the clade including the western Mediterranean species (i.e. nodes A and B in Fig. 2). We included the two lineages of *H. flavipennis*, which most likely represent distinct species estimated to have originated at more than 8 MY. Due to the uncertainty in the taxonomic status of the geographical variants within what is currently known as *H. grandicollis*, we did two set of analyses, one including four specimens, one from each of the four main geographical areas (Fig. 3, Appendix A), and another with a single specimen, i.e. not considering the geographic variation. The LTT plot (Fig. 4), reflecting the temporal pattern of diversification, showed a steady initial increase in lineages, a plateau (stasis), and a final increase mainly corresponding to haplotype diversification within *H. grandicollis*. The γ-statistic rejected the null hypothesis of a constant birth

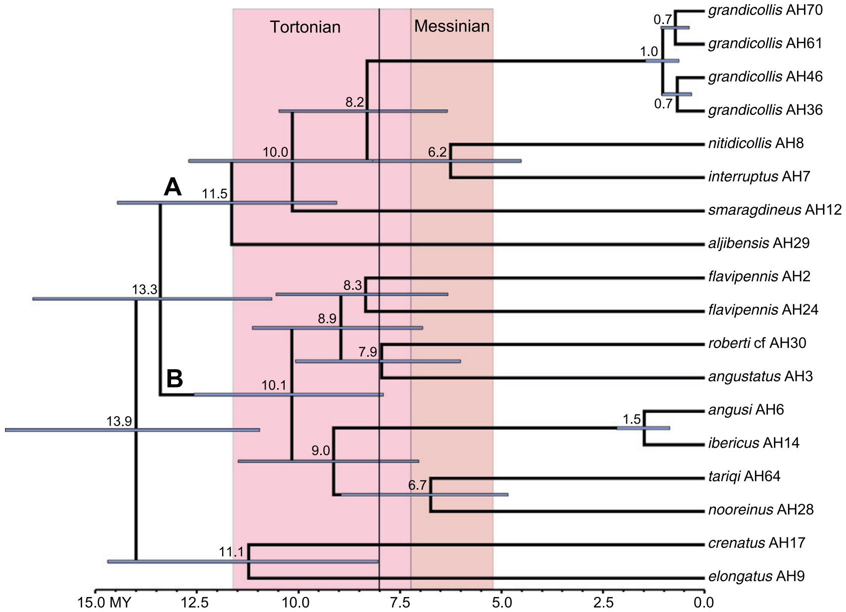

Fig. 3. Ultrametric tree obtained with Beast, using the mitochondrial sequence only and calibrated with a rate of 0.0115 substitutions/site/MY. Numbers in nodes, estimated age (MY); node bars, 95% confidence intervals of the age estimate. The vertical line at ca. 8 MY marks the estimated inflexion in the speciation rate.

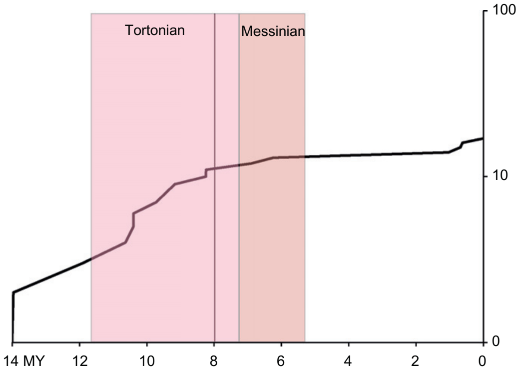

Fig. 4. Lineage Through Time plot (LTT) obtained from the tree in Fig. 3. The vertical line at ca. 8 MY marks the estimated inflexion in the speciation rate in the Yule 2 rate model (see text and Table 3). Vertical axis, logarithm of the number of lineages.

and death model, with the nodes significantly shifted towards the origin both when the variation within *H. grandicollis* was included ($\gamma = -1.84$, $p < 0.02$) or excluded ($\gamma = -3.26$, $p < 0.0001$). For both tests we considered only one missing species (*H. obtusicollis*, Appendix E), but estimated how many species would be necessary to render the γ-statistic not significant. When the variation within *H. grandicollis* was included, the number of missing species had to be increased from 1 to 10 (i.e. from 6% of missing species to 62%), and when not included, to 120 (i.e. from 8% to >900%).

The model selected in the test of diversification was in both cases a rate variable model, logistic (DDL) when the variation within *H. grandicollis* was not included, and a pure birth with a shift in the rate of diversification (yule2rate) when included (Table 3). For the logistic model the estimated carrying capacity was ca. 13 species (parameter $k = 12.24$), and the shift in the diversification rate for the Yule 2 rate model was estimated to have occurred at around 8 MY (Table 3). In both cases the best constant rate model was a pure birth, but they were significantly worst than the best variable rate models, as measured with the null distribution of the differences in the Akaike information criteria (dAICrc, $p < 0.0005$ and $p < 0.02$ when not including and including variation within *H. grandicollis* respectively).

4. Discussion

4.1. Hydrochus phylogeny

We obtained a robust phylogeny for the western Palaearctic species of *Hydrochus*, with very similar results for the two methods used and strong support for most internal nodes. All species currently found in the Iberian peninsula and Morocco formed a monophyletic clade sister to the species of the *H. elongatus* group, with a distribution centred in the eastern Mediterranean with extensions to central and northern Europe in some cases (Hansen, 1999; Fig. 1; Appendices D and E). *Hydrochus roberti*, so far only recorded from the Caucasus and Turkey, would be the only species of the "western clade" not present in the Iberian peninsula or Morocco. However, from our results it is clear that the name *H. flavipennis* has been used for what it is actually a complex of species with uncertain distributions. This complexity is apparent from the variety of morphologies of the aedeagus found as Quaternary fossils in Britain (Angus, 1976), strongly suggesting the presence of several species.

There are no known species restricted to Italy or the Balkans (Appendix E), contrary to what happens with other groups with predominantly Mediterranean distributions (Myers et al., 2000; Murienne et al., 2010). The period estimated for the divergence of these two main clades (eastern and western Mediterranean) was the mid Miocene (ca. 14 MY), a time in which the Italian peninsula was mostly submerged or partly merged with what would form the Balkan and Anatolian peninsulas, and there was no land connection between the north and south sides of the Mediterranean (Dercourt et al., 1985; Bruch et al., 2007). The geographic scenario of the mid Miocene Mediterranean would thus support the hypothesis of a vicariant split between two main lineages, one in the west centred in the Iberian peninsula and the second in the east including Anatolia plus the Middle east and the Balkans, in agreement with the general pattern described by Oosterbroek and Arntzen (1992) for a diversity of groups.

4.2. Diversification of the Mediterranean Hydrochus

We included in our study all the western Mediterranean species of *Hydrochus* with the sole exception of *H. obtusicollis*. The LTT plot of this lineage can thus be considered an accurate representation of the diversification history of the current species, showing no net speciation in the west Mediterranean clade since the Messinian other than the split between *H. ibericus* and *H. angusi* and the geographical variation within *H. grandicollis*, if this is considered to be the sign of incipient speciation despite the apparent lack of morphological differences (see below). The preferred diversification model adjusted to the LTT plot reflected this fact, clearly rejecting a constant diversification in front of variable rate models. The level of missing taxa necessary to cancel this effect, as measured with the γ -statistic, is unrealistically high, with 10 species when the variation within *H. grandicollis* was considered (i.e. an increase of more than 60% of the known fauna of the genus in the west Mediterranean), and more than 100 when not included (i.e. an increase of more than 900%).

The change in diversification rate was estimated to have occurred at ca. 8MY. According to our estimations, the species diversification of the genus in the Mediterranean took place during the mid to late Miocene (ca. 13-5 MY). The shoreline reconstructions on this period based on coral deposits (see e.g. Braga et al., 2003; Jolivet et al., 2006) reflect a succession of islands of different sizes in the Ibero-Maghrebian area due to strong tectonic activity and sea level changes. This could have favoured multiple vicariant events originating most of the extant W Mediterranean species of *Hydrochus*. On the contrary, the east side of the Mediterranean formed a continuous emerged mass of land for most of the Miocene (Blondel and Aronson, 1999; Jolivet et al., 2006; Popov et al., 2006; Barrier and Vrielynck, 2008), apparently offering less opportunities for diversification, although the incomplete representation of species of this clade in our phylogeny does not allow a detailed comparison. The apparent lack of speciation since the end of the Miocene (Messinian) in the western Mediterranean clade could be associated to the decreased opportunities for vicariant isolation with the coalescence of the Baetic cordilleras and the formation of the Straits of Gibraltar (Braga et al., 2003; Jolivet et al., 2006). In any case, the age estimation establish a clear pre-Pleistocene origin for most of the extant western Palaearctic species of *Hydrochus*, including the Iberian and the Ibero-Maghrebian endemics. This will agree with age estimates of some endemics of mountains systems in central and north Iberia (e.g. *Ochthebius* subgenus *Enicocerus*, Ribera et al., 2010a), or the ancient origin of the species of some clades of other Mediterranean arthropods (e.g. Murienne et al., 2010), but is in sharp contrast with estimations for other aquatic Coleoptera (e.g. Dytiscidae, Ribera and Vogler, 2004; Ribera and Faille, 2010; or other groups of Hydraenidae, Ribera et al., 2011), where most of the endemic species are of more recent, Pleistocene origin.

In contrast to their ancient origin, the species for which enough material was studied did not show a strong geographical structure, with the exception of *H. grandicollis*, with monophyletic divergent lineages in Sicily, Morocco, the Iberian peninsula and Slovenia. There are no apparent differences in the morphology of the aedeagus among the populations of *H. grandicollis*, and the nuclear markers used were not variable enough to show differences among them, although they may be the only case of recent speciation within the clade of western Mediterranean *Hydrochus*. The case of *H. flavipennis* is likely a problem of an unrevised taxonomy, with clearly different but unrecognised species (see above). The general pattern of large inter- but relatively low intra-lineage divergence suggests the existence of short coalescent times due to reduced population size and/or a high rate of population extinction (Charlesworth, 2009). This potential high extinction rate is, however, unlikely to be the reason for the observed decrease in diversification rates in the LTT plot, which has a well defined transition point (Quental and Marshall, 2009).

We have shown the existence of an ancient element of the Mediterranean fauna, likely to have persisted in the area through the Pliocene and Pleistocene epochs. The contribution of the glacial cycles in shaping the current diversity patterns in the Mediterranean has been widely recognised (Hewitt, 2000; Petit et al., 2003; Schmitt, 2007; Médail and Diadema, 2009), but the presence of old Miocene species is of special relevance both for the possibilities they offer to help to understand the origin of the Mediterranean fauna and their intrinsic evolutionary distinctiveness. In this sense, the view of some southern Mediterranean areas as "cumulative refugia", both cradles and museums of biodiversity (Médail and Diadema, 2009; Tzedakis, 2009) seems to be fully applicable also to at least some groups of arthropods.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.01.018.

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Hydrochus farsicus sp.n. from Iran and notes on other Palearctic species of the genus (Coleoptera: Hydrophiloidea: Hydrochidae)

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The family Hydrochidae (or subfamily Hydrochinae for some authors) includes one recognised genus (*Hydrochus* Leach) with more than 200 species and a worldwide distribution (Hansen, 1999; Short & Hebauer, 2006). The West Mediterranean is among the most diverse areas for the genus in the Palearctic Region, with 12 species known from Spain and Morocco, including several endemic species described recently (Hansen, 2004). On the contrary, in the East Mediterranean and the Middle East few species are known so far, although some undescribed species from Turkey are deposited in the Naturhistorisches Museum Wien (NMW). No species of *Hydrochus* has ever been recorded from Iran (Hansen, 2004). Other groups of aquatic Coleoptera (e.g. Dytiscidae, Nilsson, 2004 or Hydraenidae, Jäch, 2004) display a more balanced distribution of species, and include a high number of Turkish or Iranian endemics. The situation in *Hydrochus* may be due to lack of knowledge, as the undescribed specimens in the NMW and some recent works (Incekara *et al.*, 2004; Mart *et al.*, 2009) may suggest, but it may be that in Turkey and the Middle East the genus *Hydrochus* is generally less speciose than in the West Mediterranean.

In this paper two species of *Hydrochus* are recorded from Iran, one of which is described as new. In addition, some taxonomic notes on other Palearctic species are provided.

***Hydrochus farsicus*, new species**

Figs 1–2

Type locality. Sepidan, Province of Fars, Iran.

Type material. *Holotype* (NMW): "2 - IRAN Fars, 13.8.1998 / 6km W Sepidan / rd. Sepidan-Yasuj / brook (Cheshmeh Saran) / leg. Elmi & Fery (# 2098)" and holotype label. Aedeagus glued on the same card. Base of aedeagus slightly damaged. *Paratypes* (NMW): Two females with the same data as holotype, plus paratype labels.

Diagnosis. The only reliable characters to identify this new species are those of the male genitalia (Fig. 2). Other putative morphological characters may be shared with still undescribed, closely related species and are thus not reliable for an unambiguous identification.

Description. Habitus as in Fig. 1. Elytra and body appendages brown, except apex of maxillary palpi and base of mandibles darker; head black; pronotum brown with central area darker; surface with light bluish or greenish metallic reflections. Ventral side evenly dark brown, except for head black. Head with deep, coarse evenly distributed punctures, intermixed with smaller punctures with short whitish setae; four small tubercles between eyes. Pronotum elongate, subcylindrical, wider anteriorly; with seven depressions, three anterior, rounder and four posterior, more elongate; lateral posterior depressions smaller, reaching posterior margin of pronotum. Punctation and pubescence as on head. Elytra subparallel-sided, slightly wider posteriorly; with 10 longitudinal striae formed by deep, regularly aligned punctures. Humeral region of elytra prominent. Tibiae with a regular dense row of setae in upper apical part; femora with evenly distributed small setae. Ventral surface covered with short, thick and dense pubescence, surface strongly microrecticulate, cells small and with a shagreen-like aspect. Medial line of ventrites, medial area of metaventrite, and two lateral areas in the metaventrite glabrous or with less dense pubescence. Ventrites with a strong transverse medial ridge, almost forming a carina.

FIGURE 1. *Hydrochus farsicus*, habitus (Holotype).

Aedeagus as in Fig. 2, 0.98 mm long, 0.3 mm wide; robust, parameres and median lobe asymmetrical: apex of left paramere with an asymmetrical triangular expansion; apex of right paramere sinuated, not expanded. Apex of median lobe expanded, poorly sclerotized, with a small flagellum.

Variation. Length 3.2–3.4 mm; maximum width 1.0–1.3 mm. Without apparent secondary sexual dimorphism.

Distribution. Only known from the type locality.

Remarks. The morphology of the aedeagus of *H. farsicus* resembles that of some Iberian species (*Hydrochus ibericus* Valladares, Díaz & Delgado and *H. angusi* Valladares), although it may also be related to the group of *H. elongatus* (Schaller), with a more eastern distribution (Hidalgo-Galiana *et al.*, in preparation). Mart *et al.* (2009) recorded *H. ibericus* from Turkey, but unfortunately they did not figure the aedeagus and did not give details of the material used for comparison.

Etymology. Named after the Iranian province of Fars, from where this species was collected.

***Hydrochus nodulifer* Reitter, 1897**

Material studied. One male (Coll. Pütz, Eisenhüttenstadt, Germany): IRAN: Prov. Gilan, Siahkal County, Elburz Mts., S-Slope, Deylaman-Barresar road, sifted, 1688 m, 36°51'07"N, 49°49'67.3"E, 07.VI.2008, leg. A. Pütz "IR08-25".

Remarks. First record for Iran. This species was described from "Elisabethpol" [= Ganja (or Ganca), Azerbaijan] (Reitter, 1897). A lectotype was designated by Shatrovskij (1993), who also figured the aedeagus. *Hydrochus nodulifer* has also been recorded from Eastern Anatolia and the Black Sea Area of Turkey (Mart *et al.*, 2009).

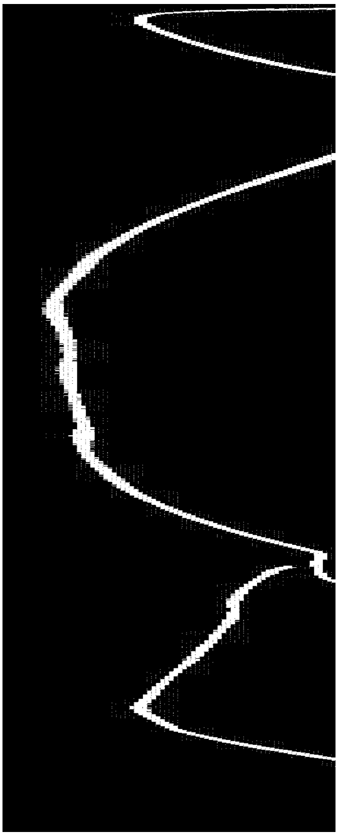

FIGURE 2. *Hydrochus farsicus*, aedeagus, dorsal view (scale bar 0.3 mm)

Hydrochus smaragdineus Fairmaire, 1879

Hydrochus angustatus bicolor Rey, 1885 **syn.n.**

Material studied. Lectotype (of *Hydrochus angustatus bicolor* Rey, 1885), male (Muséum d'Histoire Naturelle, Lyon): "Hydrochus / bicolor Rey [male symbol] / mus. Lyon. [red handwritten label]"; "Museon Den Haag / Hydrochus [male symbol] / bicolor Rey [hdw] / det. A. L. van Berge / Henegouwen 1986"; "lectotype. Van Berge / Henegouwen 1985 [red label, hdw]".

Remarks. The study of the lectotype of *H. angustatus bicolor* revealed that its aedeagus is identical to that of *H. smaragdineus* Fairmaire (in the interpretation of Valladares, 1995), and thus it has to be considered a subjective junior synonym and not a subspecies of *Hydrochus angustatus* Germar, 1824.

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