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Contributed Paper

Use of genetic, climatic, and microbiological data to inform reintroduction of a regionally extinct butterfly

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Abstract: *Species reintroductions are increasingly used as means of mitigating biodiversity loss. Besides habitat quality at the site targeted for reintroduction, the choice of source population can be critical for success. The butterfly* Melanargia russiae *(Esper's marbled white) was extirpated from Hungary over 100 years ago, and a reintroduction program has recently been approved. We used museum specimens of this butterfly, mitochondrial DNA data (mtDNA), endosymbiont screening, and climatic-similarity analyses to determine which extant populations should be used for its reintroduction. The species displayed 2 main mtDNA lineages across its range: 1 restricted to Iberia and southern France (Iberian lineage) and another found throughout the rest of its range (Eurasian lineage). These 2 lineages possessed highly divergent wsp alleles of the bacterial endosymbiont* Wolbachia*. The century-old Hungarian specimens represented an endemic haplotype belonging to the Eurasian lineage, differing by one mutation from the Balkan and eastern European populations. The Hungarian populations of* M. russiae *occurred in areas with a colder and drier climate relative to most sites with extant known populations. Our results suggest the populations used for reintroduction to Hungary should belong to the Eurasian lineage, preferably from eastern Ukraine (genetically close and living in areas with the highest climatic similarity). Materials stored in museum collections can provide unique opportunities to document historical genetic diversity and help direct conservation.*

Keywords: COI, century-old DNA, Hungary, *Melanargia russiae*, species reintroduction, *Wolbachia*

Uso de Datos Genéticos, Climáticos y Microbiológicos para Informar la Reintroducción de una Mariposa Extinta Regionalmente

Resumen: *Las reintroducciones de especies se usan cada vez más como medio de mitigación de la pérdida de la biodiversidad. Además de la calidad del hábitat en el sitio seleccionado para la reintroducción, la elección de la población fuente puede ser crítica para el éxito. La mariposa* Melanargia russiae *(medioluto montañesa) se extinguió en Hungría hace más de 100 años y recientemente se ha aprobado un programa de reintroducción. Utilizamos especímenes de museo de esta mariposa, datos de ADN mitocondrial (ADNmt), el estudio de la presencia de endosimbiontes y el análisis de similitudes climáticas para determinar cuál es la población existente que debería usarse para la reintroducción. La especie exhibe dos linajes principales de ADNmt a lo largo de su extensión geográfica: uno restringido a Iberia y al sur de Francia (linaje ibérico) y el otro hallado a lo largo del resto de su extensión (linaje euroasiático). Estos dos linajes presentan alelos wsp altamente divergentes del endosimbionte bacteriano* Wolbachia*. Los antiguos especímenes húngaros*

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representan un haplotipo endémico que pertenece al linaje euroasiático, diferenciándose de la población balcánica y de la del este de Europa por una mutación. Las poblaciones húngaras de M. russiae *estuvieron presentes en áreas con un clima más frío y más seco en relación con la mayoría de los sitios con poblaciones existentes conocidas. Nuestros resultados sugieren que las poblaciones que se usen para la reintroducción en Hungría deberían pertenecer al linaje euroasiático, de preferencia del este de Ucrania (genéticamente más cercanas y de áreas con la similitud climática más alta). Los materiales almacenados en las colecciones de museos pueden proporcionar oportunidades únicas para documentar la diversidad genética histórica y pueden ayudar a dirigir la conservación.*

Palabras Clave: ADN con un siglo de antigüedad, COI, Hungría, *Melanargia russiae*, reintroducción de especies, *Wolbachia*

摘要: *物种重引入是减缓生物多样性丧失越来越常用的一种方式。除了重引入位点的生境质量外, 源种群的选择对于重引入的成功与否也至关重要。俄罗斯白眼蝶 (Melanargia russiae) 在一百多年前在匈牙利灭绝, 最近该物种的重引入项目获得了批准。我们用这种蝴蝶的博物馆标本、线粒体 DNA (mtDNA) 数据, 内共生体筛选和气候相似性分析来确定适用于该物种重引入的现存种群。这个物种在其分布区内有两个主要的 mtDNA 谱系:一个仅分布于伊比利亚半岛和法国南部 (伊比利亚支系), 另一个则广布在剩余的分布区 (欧亚支系) 。这两个支系有高度歧化的细菌内共生体—沃尔巴克氏体 (Wolbachia) wsp 等位基因。匈牙利的标本表现为欧亚支系的一个特有单倍型, 与巴尔干半岛和欧洲东部的种群有一个突变的差异。此外, M. russiae 的匈牙利种群分布的地区相比于已知的现存种群的大部分分布区更为寒冷干燥。我们的结果表明, 应该选择欧亚支系的种群用于重引入, 并且最好是来自乌克兰东部的 (遗传相近且分布区的气候最相似) 。博物馆馆藏的材料也为记录历史上的遗传多样性和帮助指导保护提供了独特的机会。* **【翻译: 胡怡思; 审校: 魏辅文】**

关键词: COI 基因, 百年历史的 DNA, 匈牙利, 俄罗斯白眼蝶 (*Melanargia russiae*), 物种重引入, 沃尔巴克氏体 (*Wolbachia*)

Introduction

Species are the basis of biodiversity measurement and conservation measures (Gaston & Spicer 2003). The growing use of genetic data in biodiversity research, partially fueled by widely applied approaches such as DNA barcoding (Hebert et al. 2003), has led to the discovery of a new layer of biodiversity in the form of cryptic species (species merged in traditional taxonomy due to their morphological similarity) (Bickford et al. 2007; Pfenninger & Schwenk 2007). These species seem to encompass a large fraction of the alpha and beta diversity at the continental level (Dincă et al. 2015; Vodă et al. 2015), but their morphological similarity complicates monitoring and conservation programs.

In addition to species, intraspecific genetic variation is a fundamental parameter in biodiversity conservation (Fraser & Bernatchez 2001). Genetic diversity increases the ability of individuals or populations to use local resources and to tolerate environmental stress (e.g., Saccheri et al. 1998; Bijlsma & Loeschcke 2012). Extinction is typically preceded by the erosion of genetic diversity, which can cause the loss of useful traits (e.g., resistance to parasites, tolerance to climatic extremes) (Menzies et al. 2012; Willoughby et al. 2015). Thus, genetic diversity is a key element in determining the responses of organisms to human perturbations such as climate change, habitat fragmentation and deterioration, and biological invasions (Kolbe et al. 2004; Roger et al. 2012).

Conservation aims to prevent loss of species, their genetic diversity, and habitats (Wilson & Peter 1988). Increasingly, the drastic measure of reintroduction of species that are locally or globally extinct in the wild is being taken (e.g., Seddon et al. 2005; Weeks et al. 2011; Ewen et al. 2012). These efforts have had varying success, and most have focused on plants, mammals, and birds (Seddon et al. 2005; Bajomi et al. 2010; Godefroid et al. 2011). However, butterflies, as an intensively studied and flagship group for invertebrate conservation, have been the subject of several reintroduction programs, particularly in Europe (Schultz et al. 2008). The successful reintroduction of *Maculinea arion* to the United Kingdom has become a model for similar actions (Thomas et al. 2009).

With support from the national government and the European Union, several projects are striving to reconstruct the formerly vast marshlands and forest steppes of Hungary. In this country, these biotopes are fragmented and threatened by desertification because of extensive canalization in the last 2 centuries. These projects consider the enrichment of natural communities via the eradication of invasive species and the control and breeding of taxa important for the biodiversity of the region. One of the best known examples involves the endemic snake *Vipera ursinii rakosiensis*, whose populations were saved from extinction through well-planned breeding programs and habitat restoration (Halpern 2007; Péchy et al. 2015). Another program involves the reintroduction of the butterfly *Melanargia russiae* (Esper´s marbled white), which was extirpated from Hungary in 1913 (Bálint & Katona 2013). The Hungarian populations of this species were geographically isolated from any other known populations by at least

600 km (Kudrna et al. 2015) and were emblematic of the distinctive fauna of the Pannonian steppe (Frivaldszky 1865). The extirpation of the butterfly was perceived as a great loss by Hungarian naturalists because the swarming of the species was an impressive sight that attracted local and foreign entomologists (Fountaine 1898). In 2016, a century after the species' extirpation, a plan focusing on the reintroduction of *M. russiae* in Hungary was approved by the nature conservation authorities. The plan recognized the crucial need to identify the genetic background of the species and to compare the floristic assemblages in the habitats involved.

In Europe *M. russiae* has a fragmented distribution. It occurs in 4 areas where its populations are usually assigned to different subspecies: Iberia and southern France (subspecies *cleanthe*); Italy (subspecies *japygia*); Balkans (subspecies *japygia* or the nominotypical one, depending on author); and eastern Europe, Ukraine, and European Russia (usually assigned to the nominotypical subspecies). The range of the last group extends into Transcaucasia and reaches western Siberia and eastern Kazakhstan (Tolman & Lewington 2008; Tshikolovets 2011). Hence, a key question regarding the reintroduction of *M. russiae* in Hungary is the determination of the parental stock. It is currently unclear whether the extinct population was a Pannonian endemic (as suggested by Abafi-Aigner 1904, 1907) or whether the Pannonian Plain was recently colonized by *M. russiae* from the Italian peninsula, the Balkans, or the Russian steppes. For each of these scenarios there are several examples in the history of the Carpathian Basin biota (Forró 2007).

We analyzed mtDNA from >100-year-old museum specimens of the extinct Hungarian population and compared the results with a comprehensive genetic data set that includes representatives from across the distribution of *M. russiae*. This analysis made it possible for us to ascertain the extant populations that are most genetically similar to the extirpated Hungarian populations. We then assessed which areas with extant populations are climatically more similar to the Hungarian sites targeted for reintroduction. Finally, we screened specimens for the presence of the widespread bacterial endosymbiont *Wolbachia*. Such a procedure, to our knowledge, has rarely been done for European butterflies prior to reintroduction, although *Wolbachia* could greatly influence the dynamics of natural populations of insects (Werren et al. 2008), potentially jeopardizing the success of reintroductions.

Methods

Data Set and DNA Sequencing and Analyses

The data set included 101 cytochrome *c* oxidase subunit I (COI) sequences of *M. russiae* representative of the range of the species, including three from specimens of the extinct Hungarian population (collected in 1912), the latter stored in the Hungarian Natural History Museum, Budapest (HNHM) (Supporting Information). The 47 COI sequences specifically generated for this study were obtained using standard protocols, as were the combinations of primers designed to facilitate sequence recovery from old specimens (Supporting Information). All COI sequences are publicly available in GenBank (Supporting Information) and in the data set DS-MELARUSS (https://doi.org/10.5883/DS-MELARUSS) from the Barcode of Life Data System (http://www.boldsystems.org/).

We collapsed the 101 COI sequences to 43 unique haplotypes with TCS version 1.21 (Clement et al. 2000). We used the same program to construct a maximum parsimony haplotype network that had a 93% connection limit. We inferred phylogenetic relationships with Bayesian inference (BI). We used the 43 haplotypes of *M. russiae* and 3 outgroup sequences from *M. galathea*, *M. leda*, and *M. ines* (Nazari et al. 2010). We ran both BI analyses and the estimation of node ages in BEAST version 1.8.0 (Drummond & Rambaut 2007).

Wolbachia Infection Analyses

Of 47 specimens of *M. russiae* surveyed for the presence of the bacterial endosymbiont, 37 were reliably assessed for presence or absence of *Wolbachia* (Supporting Information). We sequenced the coxA PCR products of 16 specimens and the wsp PCR products of 14 specimens. Sequences obtained during the screening are available in GenBank (Supporting Information) and in the data set DS-MELARUSS (https://doi.org/10.5883/DS-MELARUSS) from the Barcode of Life Data System (http://www.boldsystems.org/).

Mapping Genetic Structure

To examine spatial patterns of genetic differentiation, we performed a principal coordinates analysis (PCoA) based on uncorrected pairwise distances (p distances) of the COI sequences. The resulting configuration was projected in red-green-blue (RGB) space, and the resulting colors were plotted on the map in the R package recluster (Dapporto et al. 2014*b*).

Climatic Analyses

We downloaded the 19 climatic layers from WorldClim (http://www.worldclim.org/, version 2.0 1970–2000) at a resolution of 5 minutes. Then we applied a principal component analysis (PCA) to the 19 layers using the princomp R function and retained the components (principal component layers) with an eigenvalue >1. For each principal component layer, we calculated the mean values among the cells of the area targeted for the reintroduction. Finally, we calculated the Euclidean distance

between the mean values of the target area and the values in the principal component layers of each 5 × 5 minute cell. We used the log-transformed layer of Euclidean distances to map climatic difference in the target area.

Results

DNA Results

The Bayesian analysis recovered *Melanargia transcaspica* as a well-supported sister clade to *M. russiae*. *M. transcaspica* has often been regarded as a subspecies of *M. russiae* but was recently proposed as a distinct species based on genetic and morphological (male genitalia) differences (Nazari et al. 2010). *M. russiae* possessed 2 well-supported lineages: one restricted to the Iberian Peninsula and southern France (hereafter Iberian) and the second included all other specimens analyzed, ranging from Italy in the west to central Asia in the east (hereafter Eurasian). Within this latter lineage, a well-supported clade was formed by specimens from Sicily (Sicilian lineage) (Fig. 1). The century-old specimens of *M. russiae* from Hungary fell within the Eurasian lineage and clustered, with relatively good support, with haplotypes from eastern Turkey, Greece, Albania, and European Russia (Fig. 1). The 3 main COI lineages of *M. russiae* were also apparent when genetic diversity was plotted geographically (Fig. 2a, b).

The COI haplotype network (Fig. 3) showed that *M. transcaspica* was differentiated by at least 13 mutations (out of 658 bp) from *M. russiae*. Within the latter species, the Iberian haplogroup differed by a minimum of seven mutations from the Eurasian haplogroup. Geographic structure was also present within the Eurasian lineage, although levels of differentiation were lower. Italy was represented by endemic haplotypes, with the exception of a haplotype shared with a specimen from eastern Kazakhstan, and Sicilian haplotypes were linked to the Italian haplogroup but differed from them by three mutations. Most Asian haplotypes clustered, and the same was true for the Balkan haplotypes (although this haplogroup also included specimens from eastern Turkey and European Russia) (Fig. 3). The 3 Hungarian specimens represented a unique (now apparently extinct) haplotype (h17) that differed by 1 mutation from specimens from Albania, Greece, and European Russia (h3) (Fig. 3). This mutation involved a change between adenine and guanine (A-G) and is likely not an artifact caused by DNA deamination in old samples.

Wolbachia Infection Patterns

Of the 37 specimens screened for the endosymbiont *Wolbachia*, 54% (20 specimens) were infected (Fig. 4, Supporting Information). Infection rates were high for Italian and Balkan population samples (91% Italy; 72% Balkans). Within the Iberian Peninsula, 28% of the specimens were infected by *Wolbachia*, and all (5 specimens) originated from northeastern Spain (Catalonia) (Fig. 4, Supporting Information). The single specimen analyzed from European Russia (Moscow region) was not infected. All infected specimens possessed the same coxA allele (coxA 14) (Fig. 4). According to the *Wolbachia* MLST Database (Baldo et al. 2006), this allele is widespread among insects; it has been detected in various butterfly and moth species and in Coleoptera, Hymenoptera, Diptera, and Hemiptera. However, wsp clearly differed between the infected specimens from Catalonia (allele wsp 694) and the Italian and Balkan specimens (allele wsp 61) (Fig. 4, Supporting Information). No detailed information has been found regarding taxa bearing allele wsp 694, whereas wsp 61 has been reported in a few species of butterflies from Russia, Japan, and the United States, as well as in 1 species of Hemiptera from Serbia and 1 species of Trombidiformes from China. The 2 wsp alleles were highly differentiated, displaying a nucleotide p distance of 17.5%.

Climatic Analyses

The 4 principal component layers with eigenvalues >1 (Supporting Information) where linked to summer precipitation, overall temperature, overall precipitation, and precipitation seasonality. The Hungarian area targeted for the reintroduction of *M. russiae* has a substantially lower level of summer precipitation and of overall temperature compared with areas where the species currently occurs (Supporting Information). The resulting map of climatic similarity (Fig. 2c) revealed that high climatic resemblances with areas where *M. russiae* occurs are only found at sites in eastern Ukraine (Donetsk and Lugansk regions) (Fig. 2c) (Demyanenko 2013; Martynov & Plushtsch 2013; Kudrna et al. 2015).

Discussion

Genetic Patterns Within *M. russiae*

In general, the genetic structure we found within *M. russiae* was consistent with results of previous studies (Nazari et al. 2010), but the increased resolution of the current data set clarified some patterns and revealed new ones. Specifically, we confirmed the differentiation of *M. russiae* from *M. transcaspica*, a taxon recently raised to species rank (Nazari et al. 2010) (Figs. 1, 3). Their level of sequence divergence suggests a split of these species roughly 1.8 million years ago (mya) (95% CI, 1.0–2.8) (Fig. 1). The 3 commonly recognized subspecies of *M. russiae* did not entirely match the COI data. The Iberian lineage apparently matched the distribution of subspecies *cleanthe*, but subspecies *japygia* and *russiae*

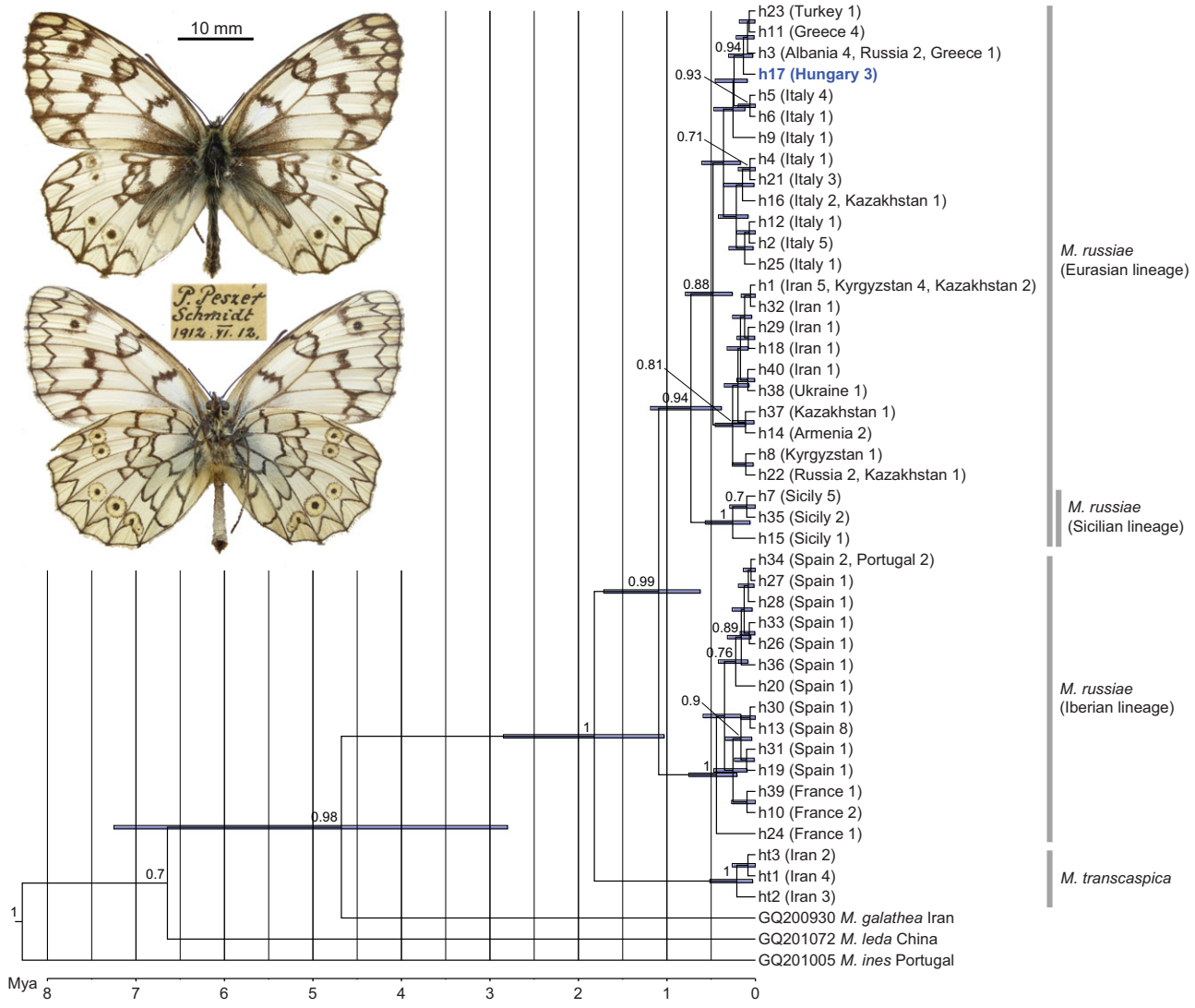

Figure 1. Bayesian ultrametric tree for M. russiae *and* M. transcaspica *based on cytochrome c oxidase subunit 1 sequences (number above recovered nodes, Bayesian posterior probabilities >0.7; node bars, 95% highest posterior density for age estimations; grey vertical bars, main lineages of* Melanargia russiae *and* M. transcaspica *[recently separated from* M. russiae*; Nazari et al. 2010]; in parentheses, number of specimens and country of occurrence for each haplotype of* M. russiae *and* M. transcaspica*; haplotype codes defined in Supporting Information; illustration, upperside [above] and underside [below] of a specimen (RVcoll14O397) from the extinct Hungarian population that was DNA barcoded). Photo by G. Katona.*

did not show a clear differentiation with respect to COI sequences (all assigned to the Eurasian lineage). From Italy to eastern Kazakhstan, only the Sicilian populations stood out as a slightly divergent lineage (Figs. 1–3).

The diversification of *M. russiae* mtDNA started about 1.1 mya (95% CI, 0.6–1.7), when the Iberian mtDNA lineage split from the Eurasian lineage. The Sicilian populations separated from the other Eurasian populations about 0.7 mya (95% CI, 0.4–1.2). Genetic differentiation of the Iberian populations occurs in several other species of butterflies and highlights the genetic diversity on the Iberian Peninsula (e.g., Dapporto et al. 2011, 2014*a*; Dincă et al. 2015). The island of Sicily is also known for its unusually high number of endemic, intraspecific genetic lineages, despite its proximity (3 km) to mainland Italy (Vodă et al. 2015, 2016). It has been hypothesized that such checkered distributions are caused by a combination of factors, such as reproductive interference, reduced dispersal, density-dependent phenomena, and differences in climatic niches (Vodă et al. 2015). A recent study on *Melanargia* showed that some taxa of this genus differentiated at the species level possess very low divergence at COI (Habel et al. 2017). From this perspective, in-depth analyses of multiple

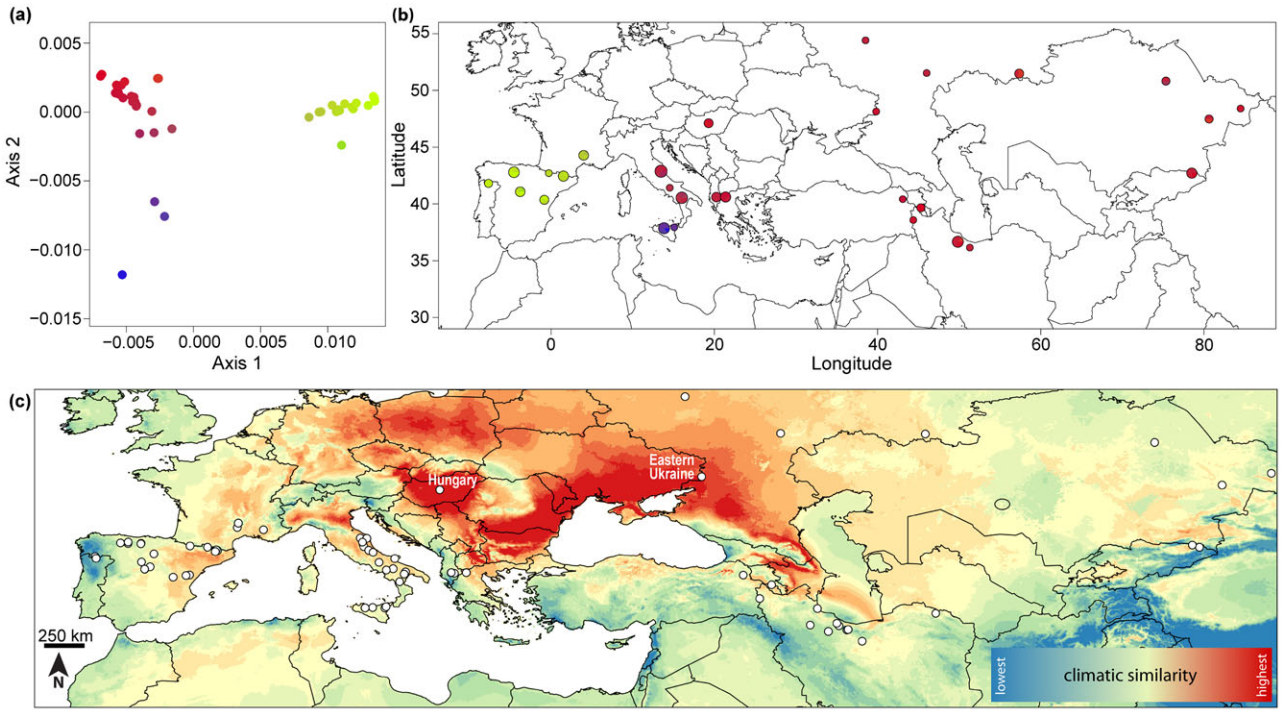

Figure 2. (a) Principal coordinates analysis projection of genetic distances among M. russiae *specimens (dots) in the bidimensional red-green-blue (RGB) color space; (b) individual values in (a) plotted on a map of western Palaearctic; and (c) climatic similarity between the western Palaearctic and the Hungarian area targeted for the reintroduction of* M. russiae *(dots, sites where DNA of* M. russiae *originated). The degree of shading is directly proportional to climatic similarity with the Hungarian site. Populations in eastern Ukraine inhabit areas climatically most similar to central Hungary.*

genetic and morphological markers are needed to test the potential existence of cryptic species within *M. russiae*.

Genetic Affinities of The Extinct Hungarian Population

Historical specimens from the HNHM provided a unique opportunity to document the genetic features of an extinct, highly isolated population that has sometimes been viewed as a distinct subspecies (Abafi-Aigner 1904, 1907). Our results indicate that the Hungarian population sample belonged to the Eurasian COI lineage of *M. russiae* (Fig. 1), but the 3 specimens had a haplotype not sampled elsewhere (h17) that was differentiated by 1 mutation from the nearest haplotype (h3) (Fig. 3). These results provide an example of how the extinction of a local population has led to loss of genetic variation. Such loss may be particularly relevant in a genus in which small levels of COI diversification can correspond to high overall diversification (Habel et al. 2017). These findings also emphasize the value of museum collections which, in combination with continuously advancing DNA techniques, can reveal past genetic diversity with important implications for fields such as phylogeography, taxonomy, and conservation (Strutzenberger et al. 2012; Prosser et al. 2015; Miller et al. 2016).

Wolbachia Infection in *M. russiae*

The bacterial endosymbiont *Wolbachia* was widespread among specimens of *M. russiae* (Fig. 4 & Supporting Information). These bacteria often manipulate the reproductive system of their hosts to enhance their spread in the infected population. Their strategies can involve male killing, feminization of genetic males, and cytoplasmic incompatibility. The latter occurs when sperm from infected males cannot produce viable offspring with eggs of females that are not infected by the same *Wolbachia* strain (Werren et al. 2008; Russell et al. 2012). *Wolbachia* infection can rapidly spread into a population, and because of maternal inheritance, can cause a selective sweep that leads to the fixation of the mitochondrial haplotype of the infected specimens. A series of studies report a correlation between patterns of *Wolbachia* infection and mtDNA structure (Gompert et al. 2006; Nice et al. 2009; Ritter et al. 2013), and a recent study on *Spialia* butterflies has pinpointed the role *Wolbachia* likely played in the formation of a cryptic species endemic to Iberia (Hernández-Roldán et al. 2016).

In the case of *M. russiae*, the correspondence between mtDNA genetic structure and patterns of *Wolbachia* infection is only partial, so it is difficult to determine whether *Wolbachia* played a role in the genetic

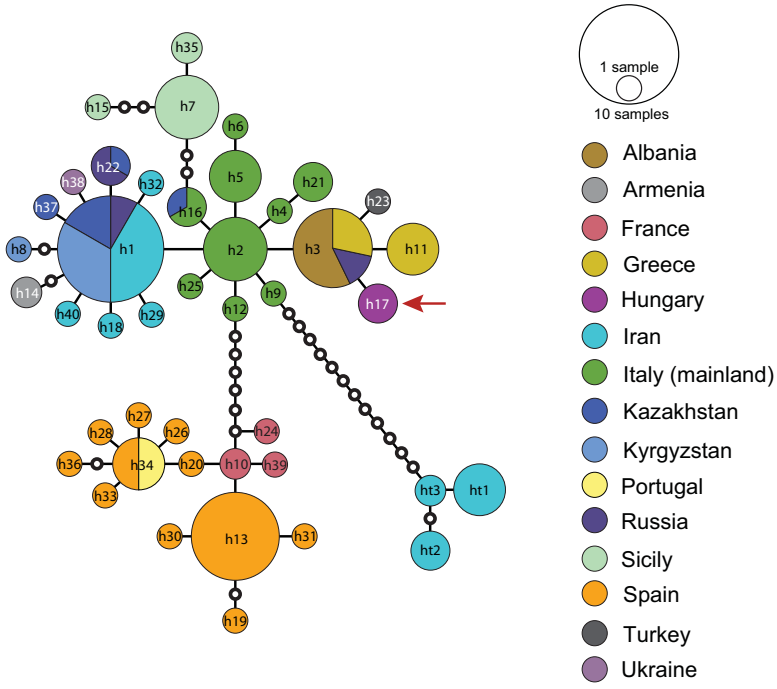

Figure 3. Maximum parsimony haplotype network based on cytochrome c oxidase subunit 1 sequences (658 bp) of M. russiae *(h haplotypes) and* M. transcaspica *(ht haplotypes) (circles, scaled to relative frequency of each haplotype in the data set; branches represent 1 point mutational step; small black circles, unsampled haplotypes; arrow, Hungarian haplotype [h17]).*

differentiation between the Iberian and Eurasian lineages or whether long-term geographic isolation was the dominant factor. Butterflies of the Eurasian mtDNA lineage bear wsp allele 61, which was also detected in the Sicilian populations, indicating that the colonizers of this island were already infected. In contrast, individuals belonging to the Iberian lineage possess the highly divergent wsp allele 694 (Fig. 4 & Supporting Information). Interestingly, within the Iberian lineage, infection seems restricted to northeastern Spain (Catalonia) because all 5 individuals from this area were infected, whereas the 13 from Iberia (Portugal and Spain, excluding Catalonia) were all uninfected. Three hypotheses might explain this pattern: Catalan specimens became infected through horizontal transmission (e.g., Ahmed et al. 2016) and infection has not yet spread to the other Iberian populations; infection outside Catalonia failed due to inefficient transmission (e.g., Hurst et al. 2001); or the 13 specimens from outside Catalonia were false negatives; that is, they were actually infected, but we failed to detect it. This latter hypothesis seems unlikely because the uninfected individuals were tested for COI amplification to confirm the quality of the DNA extractions (Supporting Information). The lack of infection of Iberian specimens outside Catalonia may also be due to the role of the Ebro River as a dispersal barrier, a pattern reported in other organisms (e.g., O´Regan 2008; Alda & Doadrio 2014).

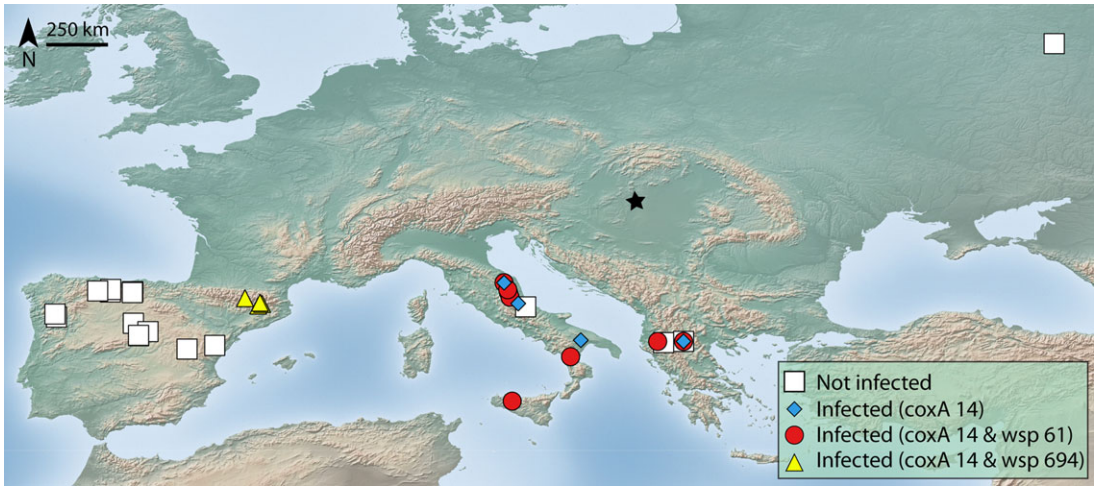

Figure 4. Pattern of bacterial Wolbachia *infection in* M. russiae *(star, location of the extinct Hungarian population for which the* Wolbachia *assessment was not successful).*

Unfortunately, infection status could not be reliably established for the century-old Hungarian specimens. However, given their mitochondrial genetic similarity to other specimens in the Eurasian lineage (Fig. 3), which were heavily infected with *Wolbachia* (Fig. 4 & Supporting Information), it is quite likely that the Hungarian population was infected, presumably with the strain now present in Italy and the Balkans. For the purpose of reintroducing *M. russiae* in Hungary, our results indicate the importance of not mixing specimens from the Iberian and Eurasian lineages because this might lead to cytoplasmic incompatibility (Werren et al. 2008).

Although precise data are lacking, we did not notice any obvious bias in sex ratios in the infected populations of the Iberian or Eurasian lineages of *M. russiae*, which suggests *Wolbachia* are not killing male hosts, but the potential for cytoplasmic incompatibility cannot be excluded without additional study.

Reintroduction of *M. russiae* in Hungary

A review of British and American conservation efforts for threatened butterfly species (Schultz et al. 2008) shows that reintroductions are often desirable. Although there are many variables that can affect the success of a species reintroduction, habitat availability and quality are critical for butterflies, as is an appropriate choice of a source population (Wynhoff 1998; Kuussaari et al. 2015).

The 2 sites targeted for the reintroduction of *M. russiae* in Hungary are forest-steppes south of the town of Dabas (about 50 km outside Budapest). Both sites are protected as part of the Natura2000 network. One of these sites represents the best known historical area of occurrence of *M. russiae* in Hungary, and it is where the species was last observed in 1913 (Bálint & Katona 2013). The other site is slightly south of the original site. The species was once common in the area but likely became extinct due to the drainage of wetlands combined with changes in forest management (Bálint & Katona 2013). In the last few years, efforts have been made to change the canal system in the targeted sites to retain water in the area. This action has increased the diversity of the plant communities, creating biotopes similar to those occupied by *M. russiae* in Kazakhstan (A. Máté, personal communication). The reestablishment of *Coenonympha oedippus* (a species that also disappeared from the sites with *M. russiae*) from a nearby stock has led to an expanding population of this butterfly (A. Máté and Zs. Bálint, personal communication).

The larvae of *M. russiae* feed on various species of Poaceae, most often *Brachypodium pinnatum*, *B. sylvaticum*, *Stipa* sp., *Aegilops geniculata*, and *Poa* sp. (e.g., Lafranchis 2007; Tolman & Lewington 2008; Tshikolovets 2011). In the Pannonian region *Poa annua* was recorded as the main larval host plant (Frohawk & Rothschild 1912*a*, 1912*b*). In the above-mentioned sites, all the mentioned Poaceae genera are still abundant, suggesting no shortage of larval food plant for *M. russiae* (Molnár & Kun 2000; A. Máté, personal communication).

Our genetic data showed that specimens from the Balkans and European Russia (all belonging to the Eurasian lineage of the species) are genetically closest (1 COI mutation) to the extinct Hungarian population (Fig. 3 & Supporting Information). Furthermore, excluding the Sicilian specimens, levels of mtDNA genetic differentiation within the Eurasian lineage of *M. russiae* were low because the specimens from Armenia and Kyrgyzstan that were genetically most distant from the Hungarian haplotype differed by only five mutations. The habitats occupied by *M. russiae* on the Ukrainian and Russian steppes are probably the most similar to the Pannonian steppes. For example, all the sites we sampled were at low elevations (under 250 m asl), and eastern Ukraine represents the area inhabited by *M. russiae* that is climatically most similar to the Hungarian area targeted for the reintroduction of the species (Fig. 2c). However, *M. russiae* appears to have considerably reduced its range in Ukraine (Kudrna et al. 2015), suggesting its general decline outside the Mediterranean region. Causes of the reduction are not well understood.

Detected patterns of *Wolbachia* infection suggest that using any given population from the Eurasian lineage should not present a problem. Selecting the same *Wolbachia* strain as the spatially nearest population may be critical in case of a potential future establishment of gene-flow between the reintroduced and neighboring populations. This is a plausible hypothesis in the long term if one considers climatically induced distribution shifts in taxa with high dispersal (Parmesan & Yohe 2003; Chen et al. 2011; Breed et al. 2013), which could bring into contact populations that are currently isolated and that could be infected by different *Wolbachia* strains.

M. russiae's distribution is currently fragmented, but some populations can be abundant like in most occupied sites in Italy (V. Dincă, R. Vodă, and L. Dapporto, personal observation) and in the Balkans and Kazakhstan (A. Máté & B. Benedek, personal communication). It is desirable to sample individuals for reintroduction from such large populations because the impact on the source population will be small and there is a higher probability of incorporating more genetic variability in the founder population, increasing its adaptability and hence reducing its extinction risk.

Overall, our genetic and climatic data suggest that populations from the Eurasian lineage, preferably from eastern Ukraine, represent a suitable source for the Hungarian sites targeted for reintroduction. A good alternative could be populations from European Russia and even from the Balkans (Albania, Greece). These populations, although inhabiting areas with a climate different from the Hungarian sites, are genetically most similar to the extinct Hungarian haplotype. If climate changes

rapidly, such populations might even become best suited for reintroduction to Hungary (Weeks et al. 2011), but a detailed assessment of expected climate evolution at the Hungarian sites would be necessary to determine whether these populations are preferred over Ukrainian populations.

Specimens of the Iberian lineage should not be used as source (or later of restocking) due to their genetic differentiation with respect to the Eurasian lineage and because the 2 lineages were infected by highly divergent wsp alleles, suggesting the presence of different *Wolbachia* strains.

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Supporting Information

Supplementary methods, samples used in the study including locality data, GenBank accession numbers and results of *Wolbachia* screening (Appendix S1), primers used in the study (Appendix S2), and images of the former habitat of *M. russiae* in Hungary (Appendix S3) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

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