The European Zoological Journal

ISSN: (Print) 2475-0263 (Online) Journal homepage: https://www.tandfonline.com/loi/tizo21

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To cite this article: E. Boscari, B. Koese, M. Palazzini Cerquetella, R. Fabbri & A. Grapputo (2020) Analyses of rare collection samples as conservation tool for the last known Italian population of *Graphoderus bilineatus* (Insecta: Coleoptera), The European Zoological Journal, 87:1, 131-137, DOI: 10.1080/24750263.2020.1732487

To link to this article: https://doi.org/10.1080/24750263.2020.1732487

Published online: 28 Feb 2020.

The European Zoological Journal, 2020, 131–137
Vol. 87, No. 1, https://doi.org/10.1080/24750263.2020.1732487

Analyses of rare collection samples as conservation tool for the last known Italian population of *Graphoderus bilineatus* (Insecta: Coleoptera)

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(Received 28 November 2019; accepted 14 February 2020)

Abstract
Graphoderus bilineatus is a predacious diving beetle, widely distributed across Europe. Its poor dispersal ability and the fragmentation and deterioration of its habitats have been indicated as the major causes of decline. In several western European countries, the species is extinct, justifying its inclusion as "vulnerable" in the IUCN red list. Aiming for the conservation of the last known population of *G. bilineatus* in the northern Italian region of Emilia Romagna, at the lake Pratignano, we surveyed its genetic diversity at the mitochondrial COI gene and compared it to that of other European populations. Two fixed COI haplotypes were found in the Italian and Austrian populations, respectively. Both haplotypes were unique among the European populations surveyed, suggesting these populations suffered a bottleneck and geographic isolation. Populations in western Europe showed lower genetic diversity and higher degree of differentiation than eastern populations. The uniqueness of Pratignano haplotype makes it difficult to choose a source population from which to transfer animals for a possible restocking. Selection of the source population should be based mainly on ecological considerations, but at the same time ensuring a good genetic diversity to maximize the adaptive potential.

Keywords: *Conservation, Dytiscidae, mt DNA, haplotype diversity, COI*

Introduction

Freshwater habitats are threatened at an unprecedented level by the expansion of human activities. Although freshwater covers less than one percent of Earth's surface, it holds a large percentage of all known species. Among macroinvertebrates and beetles, diving beetles (Dytiscidae) represent one of the major aquatic radiations, inhabiting almost all inland waterbodies, where they often play key ecological roles as the only predators (Foster & Bilton 2014). Many Dytiscidae species declined, particularly in the western Palearctic, with urbanisation, habitat loss and pollution as the main possible causes (Foster & Bilton 2014). The most vulnerable species are those with restricted dispersal abilities which limit their responses to track habitat changes. For these species, ensuring landscape connectivity is critical to avoid decline and local extinction (Kokko & López-Sepulcre 2006; Iversen et al. 2017; Hansen et al. 2018).

Graphoderus bilineatus (de Geer, 1774) is a predacious water beetle of about 14–16 mm. The beetle usually has a univoltine life cycle, but bivoltine in the most southern populations, e.g. Italy. Adult females lay eggs in spring and early summer, the larvae are aquatic whereas the pupal stage is terrestrial (Nilsson & Holmen 1995). Adults are able to fly but the species dispersal capacity is considered poor (Lundkvist et al. 2002; Kehl & Dettner 2007; Iversen et al. 2017). *G. bilineatus* is widely distributed across most of Europe, west to east from France to Siberia and north to south from Scandinavia to northern Italy, but its distribution is fragmented, it is rare and declining, particularly throughout its western range where it is considered extinct in Belgium (Scheers

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2015) and the United Kingdom (Foster 1996). The species is also elusive even in stable and consistent populations, which makes it very difficult to determine its conservation status. The species is included as vulnerable in the International Union for Conservation of Nature (IUCN) Red List (Foster 1996), and it is protected in most European countries, following the Annexes II and IV of the Habitats Directive 92/43/EEC. In Italy the species is rare and declining in its original distribution. Historically 15 sites were known for the presence of the species, in Piedmont, Lombardy, Trentino-South Tirol, Emilia-Romagna and Tuscany regions. In the last 40 years, surveys of *G. bilineatus* in Trentino-South Tirol and Tuscany were not successful (Nardi et al. 2015). Both Piedmont and Lombardy local authorities failed to find beetles in 2016–2017. In 2019, the Lombardy region, on request from the Environmental Ministry to update the status of Natura 2000 sites, declared the species absent from these sites (Cristina Barbieri, personal communication). In Emilia-Romagna, the species was present at four sites: Mezzolara (Bologna), two around Ravenna and within the Natura 2000 site IT4040001 at lake Pratignano (Nardi et al. 2015; "https://ambiente.regione.emilia-romagna.it/it/parchi-natura2000/sistema-regionale/fauna/fauna-minore/invertebrati/insetti/schedario/ditisco-a-due-fasce", last accessed 31.01.2020). During the last monitoring campaign (2016–2017), it was only present at lake Pratignano, where it is also declining. The lake lies at around 1300 m a.s.l. It is an isolated water body of about 0.55 km^2 and up to 4.5 m depth, surrounded by meadows and beech tree. It was heavily disturbed by human activities in the past. The lake surface is shrinking due to progressive advance of vegetation and reduced winter precipitations in the past years.

The LIFE "EREMITA" project aims to improve the status of habitats and species of Community interest of Natura 2000 sites in Emilia-Romagna region. As a part of this project, we have investigated the genetic diversity and degree of isolation of the population of *G. bilineatus* in lake Pratignano, the only recently confirmed population in Italy. We use the COI mitochondrial gene to compare Pratignano haplotypes and genetic diversity to other populations across Europe to evaluate suitable populations for restocking this Italian site.

Materials and methods

Sampling

The conservation status of *G. bilineatus* makes it arduous to obtain large numbers of samples. The Emilia-Romagna authorities provided 29 samples of *G. bilineatus* from three countries: Hungary (HUN), Lithuania (LTU) and Croatia (HRV), and nine samples from lake Pratignano, Italy (ITA), collected about 10 years ago. Most samples consisted of whole beetles stored in either 75% or 95% ethanol, with a subset consisting of a single leg or part of a leg, either conserved in ethanol 95% or dried. Details of each sample are reported in Table I.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from each sample using the Qiagen DNeasy Blood and Tissue kit. When the entire beetle was available only a part of it, usually the thorax, was used for the DNA extraction. We followed the kit protocol with the following modifications: i) the part of the body used for extraction broken mechanically to facilitate tissue digestion, ii) the lysis phase elongated up to 3 hours to increase the amount of tissue digested, iii) the elution volume reduced to only 50 µl to obtain a higher concentration of extracted DNA.

To avoid contamination of dried samples with DNA from alcohol-stored samples, extraction of

Table I. Samples of *Graphoderus bilineatus*.

Country	Locality	Year of Collection	Lat.	Lon.	N. of individuals	Type of biological sample	Conservation
Hungary (HUN)	*Drava*	2010-2014	46.1156 N	17.2046 E	*5*	*Whole*	*EtOh70*
	Bodrog	2007	48.2568 N	21.4948 E	*5*	*Whole*	*EtOh70*
	Danube	2017	46.2081 N	18.8847 E	*1*	*Two legs*	*EtOh95*
Lithuania (LTU)		2017	55.8428 N	24.4585 E	*2*	*Whole*	*EtOh75*
Croatia (HRV)	*National park Kopacki*	2010-2012	45.6099 N	18.801 E	*5*	*Whole*	*EtOh75*
	River Drava, Dsijek	2010-2012	45.585 N	18.6364 E	*5*	*Whole*	*EtOh75*
	Lonnjsko Poye Natural Park	2010-2012	45.2907 N	16.8075 E	*6*	*Whole*	*EtOh75*
Italy (ITA)	*Lake Pratignano*	2009	44.1761 N	10.8185 E	*2*	*Whole*	*EtOh75*
					3	*One leg*	*EtOh95*
					4	*One1 leg*	*Dried*

dried legs was conducted in a separate building with dedicated instruments and consumables.

Despite our attempts to amplify both the mitochondrial COI and the nuclear gene CAD, only the COI gave products of amplification due to imperfect storage conditions of most samples. Therefore, the mitochondrial DNA COI was the only genetic marker useful for the population genetic analysis. In insects, the nucleotide variability of the COI is different in different regions of the gene, therefore we attempted the PCR amplification of the entire mitochondrial COI gene with the universal primers COI_LCO1490_For and TL2-N-3014-Rev already described (see Table II). However, we obtained the amplification of the whole COI only for four samples, since most were highly degraded. Therefore, we opted for the PCR amplification of short fragments (reducing the size of the amplification products up to 200 bp and up to six amplified regions) using several sets of primers that amplified overlapping regions of the gene that could be later assembled to obtain the full COI sequence. The list of all the primers and combinations are shown in Table II. The location of the primers on the COI is shown in Figure 1.

PCR amplifications were carried out in a final volume of 20 µL containing: 1× PCR buffer B (Solis BioDyne), about 100 ng genomic DNA, 0.25 µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U FIRE®Pol DNA polymerase (Solis BioDyne). PCR conditions were as follows: (i) initial denaturation at 94°C for 2 min; (ii) 36 cycles of denaturation at 94°C for 30 s, annealing ranging from 51°C to 55°C (depending on the T_m of the primer pair) for 45 s, and extension at 72°C for 1 min (the extension was 30 s longer for 1500 bp amplicons); and (iii) a final extension at 72°C for 5 min. PCR products were purified with Exo1-SAP and sent for sequencing to Eurofins Genomics (Germany). Sanger sequencing was conducted in both directions using the same primers used for the PCR amplification.

Considering the small number of samples that could be analysed, we added 28 other partial sequences of COI of *G. bilineatus* obtained by Koese and colleagues (unpublished data) from several locations in Germany, the Netherland, Austria, Russia and Sweden. Only the portion in 3′ end of COI (741 bp) in common between the two datasets was used for the analysis.

Sequences are available at Genbank under the accession numbers MN517557 - MN517607.

Geneious ver. 8.1 (Biomatters) was used to assemble and align sequences. The following diversity indexes were calculated using DNASP (Librado & Rozas 2009): nucleotide diversity (π), haplotype diversity (h) and the average number of nucleotide differences (k). Haplotype genealogies were constructed with the TCS method (Clement et al. 2000) implemented in PopArt software (Leigh & Bryant 2015).

Results and discussion

We obtained the full COI sequence (1386 bp) from 23 out of 38 samples (61%) and for further four samples

Table II. Sequences of primers used to amplify the COI gene of *Graphoderus bilineatus* and primer combinations used to amplify short overlapping subregions from degraded samples; *Primers specifically developed for *G. bilineatus* in this work.

#	Name of the primer	Primer sequence	Used combinations	Reference
1	COI_LCO1490_For	GGTCAACAAATCATAAAGATATTGG	1-12; 1–10; 1-7	Folmer et al. 1994
2	COI_C1-J-1718_For	GGAGGATTTGGTAATTGATTAGTTCC	2-8; 2-7	Simon et al. 1994
3	COI_Gb_C1-J-1859_For	GGGACAGGATGAACAGTTTATCCTCC	3-10; 3–9; 3–8	*
4	COI_C1-J-2183_For	CAACATTTATTTTGATTTTTTGG	4-12; 4-9	Simon et al. 1994
5	COI_Gb_2269_For	TGCCATATTAGCTATTGGAC	5-11	*
6	COI_Gb_2634_For	CAATAGGGGCTGTATTCGCA	6-12	*
7	COI_Gb_1918_Rev	TACAGAAGCTCCTCCATGGG		*
8	COI_C1-N-2191_Rev	CCTGGTAAAATTAAAATATAAACTTC		Simon et al. 1994
9	COI_C1-N-2329_Rev	ACTGTAAATATATGATGTGCTCA		Simon et al. 1994
10	COI_HCO2198_Rev	TAAACTTCAGGGTGACCAAAAAATCA		Folmer et al. 1994
11	COI_Gb_2700_Rev	GTGATTCCTGTAAATAAAGGAAATC		*
12	TL2-N-3014-Rev	TCCAATGCACTAATCTGCCATATTA		Simon et al. 1994

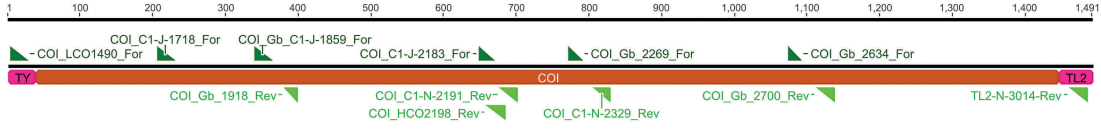

Figure 1. The location of the primers used to amplify the COI gene of *Graphoderus bilineatus* samples.

only the 3′ end of the gene (741 bp), three from Croatia and one from Hungary. For 11 samples, including the four dry legs from lake Pratignano, the legs from Hungary and six samples of beetles conserved in ethanol of which three from Hungary and three from Croatia, no amplification attempt yielded any reliable product. The 23 samples produced a total of 11 haplotypes containing 18 polymorphic sites and an average number of nucleotide differences k = 3.24. The total haplotype diversity (Hd) was 0.91 (±SD 0.034) and the nucleotide diversity (π_i) 0.00234 (±SD 0.00022). Contrary to all other populations, the Italian population showed no diversity, with only one haplotype at the COI gene as shown by the haplotype network representing the shared alleles between colour coded populations (Figure 2(a)). The inclusion of the partial COI from Koese and colleagues allowed us to also use the four samples from Croatia and Hungary, for which we did not obtained the full sequence of COI, bringing the total dataset at 55

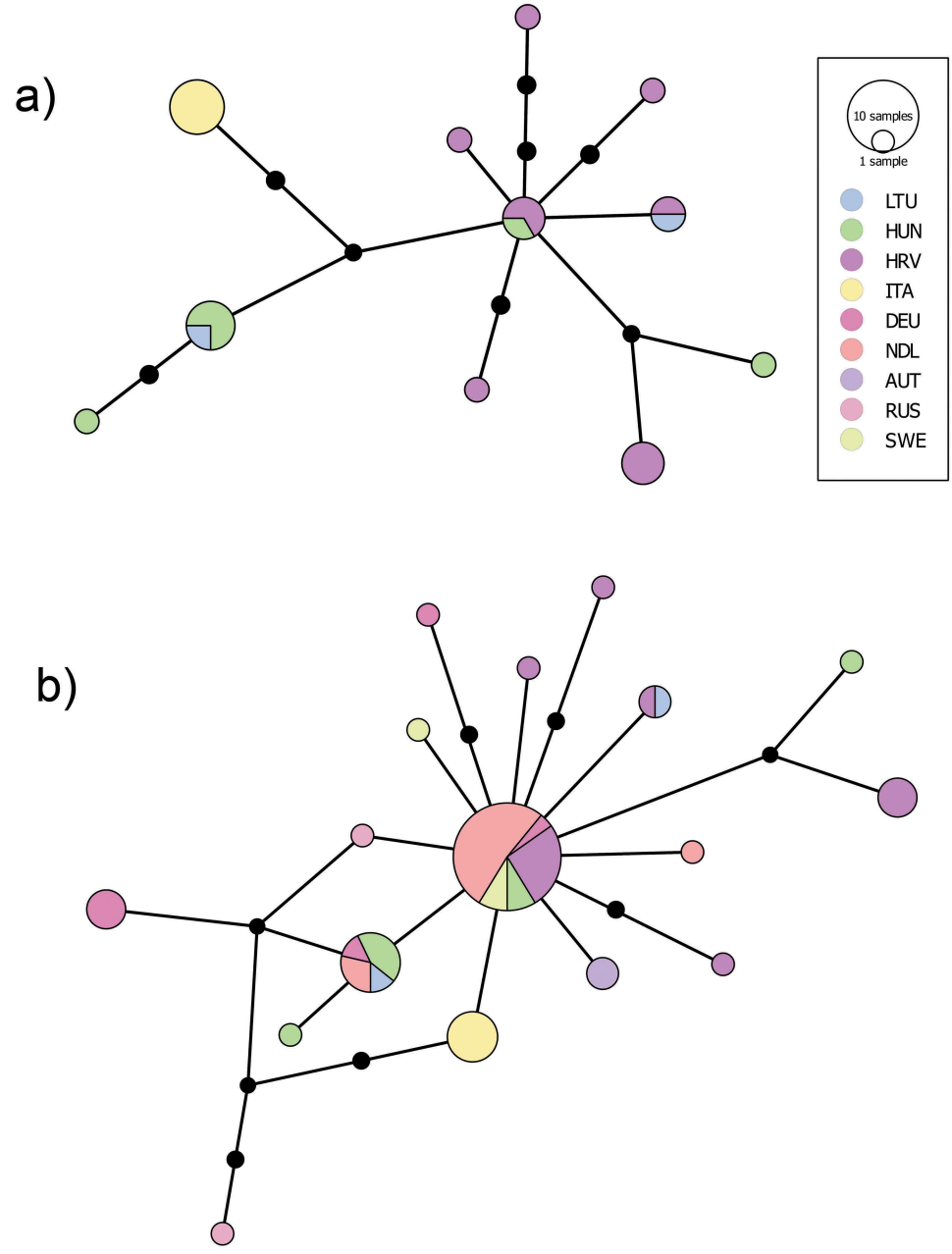

Figure 2. Haplotype network obtained with TCS from (a) the full COI for four populations from different countries of *Graphoderus bilineatus* and (b) the partial (3′end) COI sequences from nine countries of *G. bilineatus*.

samples of *G. bilineatus*. Despite the increasing number of samples and the reduction of nucleotide sites, the uniqueness of the lake Pratignano haplotype was confirmed (Figure 2(b)). This new dataset comprised 17 different haplotypes with 21 polymorphic sites and an average number of nucleotide differences (k) of 1.678. Total haplotype diversity was Hd = 0.803 (±SD 0.049) and the nucleotide diversity π_i = 0.0023 (±SD 0.00031). The diversity indexes per population for both data sets are shown in Table III.

This survey of genetic diversity at the mitochondrial COI gene of European populations of *G. bilineatus* has showed that the Pratignano population has a single mitochondrial COI haplotype, present only in this population and not in any of the analysed European populations (Figure 2(a) and (b)). The lack of diversity and individuality of lake Pratignano haplotype may suggest that this population suffered a bottleneck and geographic isolation, but samples from geographically close populations, such as in France and Switzerland, should be investigated to confirm the distinctiveness of Pratignano. Other unique haplotypes have also been found in Austria and in some distinct populations within countries (e.g. The Netherlands) but in those cases, no more than two individuals were sampled. Other populations have shown various degrees of diversity including those with a very low number of sampled individuals as Lithuania and Russia. Population genetic differentiation was highest for Italian and Austrian populations when compared to the other European populations as shown in Table IV. These results showed that most genetic variation in this species is contained primarily in the Eastern/North-Eastern part of its European range, where the species is more abundant. A negative and significant Tajima's D (−2.00561, $P < 0.05$) indicate either a recent population expansion or purifying selection in COI of *G. bilineatus*. This coupled with the relatively low diversity and lack of genetic structure in the haplotype network (Figure 2), suggest a historical connectivity of lakes, river systems and wetlands throughout central Europe that have allowed the dispersion of this erratic species across Europe (Iversen et al. 2013). On the contrary, in Western Europe, where the species has been declining, with episode of local extinction (Foster 1996), the sampled populations generally showed lower genetic diversity. The Netherlands also showed low genetic diversity despite the sampling effort being the highest among all sites, which most likely reflects the effects of habitat fragmentation and loss as well as pollution of the water system. Indeed, the removal of pollutants from water system has favoured the recovery of *G. bilineatus* in the Netherlands populations (Cuppen et al. 2006; Foster & Bilton 2014). Spatial isolation and local habitat conditions have been shown to alter intraspecific dispersal properties as a consequence of either local habitat connectivity (Hanski et al. 2004) or at the front edge of the expanding distribution (Thomas et al. 2001).

For a successful reintroduction, analyses of neutral loci and markers under selection should be coupled in order to assess the local adaptation of populations.

Table III. Diversity indexes for each population estimated using the entire COI sequences when available and on the partial 3′end COI which includes samples provided by B.K.

	Entire COI						Partial (3′ end) COI					
Population	N	S	H	Hd (±SD)	K (±SD)	π_i (±SD)	N	S	H	Hd (±SD)	K (±SD)	π_i (±SD)
Hungary (HUN)	6	6	4	0.80 (±0.172)	2.40 (±1.51)	0.00173 (±0.0013)	7	4	4	0.81 (±0.13)	1.429 (±0.985)	0.00193 (±0.00152)
Lithuania (LTU)	2	2	2	1.00 (±0.5)	3.0 (±2.449)	0.00216 (±0.0025)	2	2	2	1.00 (±0.5)	2.0 (±1.732)	0.0027 (±0.00331)
Croatia (HRV)	10	11	7	0.91 (±0.077)	2.73 (±1.581)	0.00197 (±0.0013)	13	8	6	0.769 (±0.103)	1.692 (1.057)	0.00228 (±0.0016)
Italy (ITA)	5	0	1	0.00	0	0	5	0	1	0.00	0	0
Partial sequences provided by BK												
Germany (DEU)							6	5	4	0.8 (±0.172)	2.4 (±1.51)	0.00324 (±0.00235)
Netherlands (NLD)							15	2	3	0.362 (±0.145)	0.381 (±0.381)	0.00051 (±0.00058)
Austria (AUT)							2	0	1	0.00	0.00	0.00
Russia (RUS)							2	6	2	1.00 (±0.5)	6.00 (±4.583)	0.00810 (±0.00875)
Sweden (SWE)							3	1	2	0.667 (±0.314)	0.667 (±0.667)	0.00090 (±0.00112)

N = number of sequences; S = number of segregating sites; H = number of haplotypes; Hd = Haplotype diversity; K = Average number of differences; π_i = Nucleotide diversity.

Table IV. Pairwise population F_{ST}.

	AUT	DEU	NDL	RUS	SWE	LTU	HRV	HUN	ITA
1	0								
2	0.4	0							
3	0.72158	0.4472	0						
4	0.25	−0.05882	0.6086	0					
5	0.67568	0.25	0.10714	0.19512	0				
6	0.5	0.02326	0.33868	−0.14286	0.11765	0			
7	0.30681	0.32392	0.08654	0.34338	−0.04316	0.03925	0		
8	0.44809	0.15247	0.20996	0.23718	0.14151	−0.12609	0.15198	0	
9	1	0.56835	0.76834	0.47368	0.82558	0.77273	0.43224	0.59144	0

Although divergence at the COI may not reflect divergence in other regions of the genome showing signals of population local adaptation, it can serve as a guide to assess genetic diversity and population genetic similarity (Avise 1996). For the purpose of restocking, the national and international conservation guidelines (ISPRA, 2007, IUCN/SSC, 2013) state that the source population should not be significantly differentiated from the native population. On the contrary, in case of reintroductions, the ethics are less stringent, since there is no longer an autochthonous gene pool to be safeguarded. In this case, the criteria for choosing the source population must be based both on the results of the genetic investigations and on ecological considerations. If confirmed, the uniqueness of the Pratignano haplotype makes it difficult to choose a source population from which to transfer animals intended for restocking due to the lack of another genetically similar European population. In such case, the selection of the source population should be based mainly on ecological considerations such as sustainability of animal removal from the chosen source populations, similarity of abiotic ecological parameters and compatibility of ecological communities, which could reflect similar local adaptations. At the same time, ensuring good genetic diversity to maximize the adaptive potential of the reintroduced population is suggested. Since the rarity of *G. bilineatus* has been attributed to high turnover rate and anthropogenic destruction of freshwater habitats in Western Europe (Foster 1996; Foster & Bilton 2014) and since its dispersal ability is restricted (Iversen et al. 2017), conservation of this diving beetle should be aided by integrating habitat maintenance or restoration with landscape connectivity (Hansen et al. 2018; Knoblauch & Gander 2019).

Acknowledgements

We would like to thank Alexandra Glavaschi, for improving the presentation of the manuscript, Leonardo Congiu for assistance in coordinating contacts with the Emilia-Romagna Regional authority and the LIFE EREMITA – Coordinated actions to preserve residual and isolated populations of forest and freshwater insects in Emilia-Romagna, project n. LIFE 14 NAT/IT/000209 for funding. The authors have no conflict of interest to declare.

Funding

European Commission under grant LIFE Programme Project n. LIFE14 NAT/IT000209.

Disclosure statement

No potential conflict of interest was reported by the authors.

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