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Impact of the Carpathians on the genetic structure of the spruce bark beetle *Ips typographus*

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Abstract The Carpathians are a range of mountains forming an arc roughly 1,500 km long across Central and Eastern Europe. They are an important area for biodiversity and belong to one of the major refuges of the last ice ages for many organisms. The forests of the Carpathians are dominated by spruce, which have suffered continuous outbreaks of the eight spined spruce bark beetle, *Ips typographus*, in recent decades. The phylogeography of this spruce pest is well documented, however, little is known on small scale, i.e., the Carpathians. Here we applied a mitochondrial marker and studied the genetic variation and structure of Carpathian populations and compared data with published one from other European populations. Twelve haplotypes were characterized and 42 % of those were not detected in other European populations. Despite a slight genetic structure, differences were observed in the haplotype distribution and diversity between the Western/Southern Carpathians and the Eastern Carpathians reflecting at least two potential refugial areas for *I. typographus* within the Carpathian mountain system. Further data show that the Eastern Beskidian Mountains of the Carpathians could act as barrier for several European haplotypes. This small-scale analysis reveals that the Carpathians have been an important glacial and post-glacial refuge for *I. typographus*. This information is important for a preventive and reactive forest management.

Keywords *Ips typographus* · Glacial refugia · Dispersal · Gene flow · COI

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Introduction

The eight spined spruce bark beetle *Ips typographus* L. (Coleoptera, Scolytinae) belongs to the widest known European scolytids in forestry, from an economic standpoint. It is considered as a monophagous species, usually colonizing weakened Norway spruce (*Picea abies* Karst.) in Eurasia. Females bore breeding galleries in the inner bark where they lay eggs in lateral niches. The complete larval development and adult maturation take place on the same host. The life cycle is completed in 7–11 weeks and produces one to three generations per year depending on altitude and temperature (Wermelinger 2004). In a "balanced ecosystem," the level of *I. typographus* populations is low because suitable hosts are scarce and widely dispersed in the landscape (Stenseth and Kirkendall 1989). However, after "exceptional climatic events" (i.e., storms, severe drought, or branch breakage under snow) the spruce bark beetles rapidly reach high population densities, which may result in dramatic mortalities of spruce trees.

Due to its striking ecological and economic damage in European spruce forests (Grégoire and Evans 2004; Jakus et al. 2011), *I. typographus* is the target of numerous research projects on ecological, genetic, and phytosanitary

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aspects (Lieutier et al. 2004). All of these studies are essential to infer ecological characteristics useful to control its population outbreaks. Molecular tools are increasingly used in population genetic studies of forest pests since a better understanding of population dynamics and its driving forces is crucial for establishing management strategies. As a result, mitochondrial and nuclear markers have been applied to analyze the phylogeography scheme and the evolutionary history of *I. typographus* populations in Europe (Avtzis et al. 2012). However, the recent discovery of cryptic numts in *I. typographus* (Bertheau et al. 2011) and the reanalysis of key European populations with a wider sampling size (Bertheau et al. 2013) improved our knowledge in phylogeography. Overall, these two articles showed the detection of three major haplotypes, one widely represented in North and Central Europe, one in the south of Europe and one across Europe (Fig. 1c). Due to high gene flow, a low population differentiation was observed. Further, *I. typographus* appeared to have a relatively recent expansion event dating back to the Holocene (ca. 7–15 kya) from a single late-Pleistocene glacial refuge. Owing to its monophagy, *I. typographus* was necessarily restricted in one of the refugial areas of its host *P. abies* during the last glacial maximum (Bertheau et al. 2013).

The Carpathians are the broadest mountain range in Europe (Pecskay et al. 2006) and represent the largest unmanaged old-growth forests of Central Europe (Gurung et al. 2009). The Carpathians are divided into the Western Carpathians with the Tatra Mountains and parts of the Beskids, the Eastern Carpathians with the High Beskids and Galicia, and the Southern Carpathians with the Transylvanian Alps (Ruffini et al. 2006). The Carpathians are important for biodiversity and belong to one of the major refuges for many organisms during the last glacial periods (e.g., plants: King and Ferris 1998; Magri et al. 2006; Mraz et al. 2007; Ronikier et al. 2008; Tollefsrud et al. 2008 or e.g., animals: Schmitt and Seitz 2001; Jaarola and Searle 2002; Babik et al. 2005; Sommer and Benecke 2005; Kotlik et al. 2006; Ursenbacher et al. 2006; Mardulyn et al. 2009). The Norway spruce, *P. abies*, survived the last glaciations in the Carpathians (Schmidt-Vogt 1977; Tollefsrud et al. 2008), where it is still the dominant tree species.

While the phylogeography of *I. typographus* has been relatively well investigated in Europe, there are still gaps on small scale, i.e., in the Carpathians, to determine precisely its origin. These studies included only one or two Carpathians populations (Stauffer et al. 1999; Sallé et al. 2007; Bertheau et al. 2013) preventing an exact identification of locations of this refugial area. Here we aimed to study the genetic variation and structure of *I. typographus* populations in the Carpathians. In order to obtain detailed information about the genetic diversity of this particular refugial area, we have chosen the mitochondrial COI marker. These findings were compared to the data recently published by Bertheau et al. (2013). Besides getting a picture about the genetic structure at a small spatial scale, we aimed to unravel the amount of glacial refugia in the Carpathians and the barriers of the Carpathian mountain ranges for *I. typographus* during and after the last glaciations event.

Materials and methods

Beetles sampling

Ips typographus adults were collected in the Carpathian region in 2009 and 2010 from fallen or standing *P. abies* trees. Nineteen populations were sampled in the Eastern and Southern Carpathians, with a particular effort in the Western Carpathians (Table 1; Fig. 1a). Only one individual per mother gallery was collected to prevent the sampling of siblings. Samples were immediately placed in absolute ethanol after collection and stored at −20 °C.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from the whole body using either the DNeasy Blood and Tissue Kit (Qiagen) or the NucleoSpin® Tissue (Macherey-Nagel) following manufacturers' protocols. DNA was eluted in 100 µl of the elution solution provided in the kit and stored at −20 °C. An 875-bp fragment of COI was amplified by polymerase chain reaction (PCR) using the sense primer described by Juan et al. (1995) and the antisense primer UEA10 (Lunt et al. 1996). Reactions were carried out in 25 µl total volumes containing 30–50 ng DNA, 1× PCR buffer, 1 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 µM of each primer. PCR was performed with an initial denaturation step at 94 °C for 3 min followed by 33 cycles of 94 °C for 60 s, 45 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were purified either using the QIAquick PCR purification Kit (QIAGEN) or externally by Macrogen service Inc. (Seoul, Korea), and the sequencing was performed externally by Macrogen. The number of individuals sequenced per population is shown in Table 2. Sequences were edited using the program Chromas 1.45 (McCarthy 1996), corrected manually by eye and aligned using ClustalW (Thompson et al. 1994) as implemented in Bioedit (Hall 1999). The COI haplotypes observed in this study were compared to those obtained by Stauffer et al. (1999) and Bertheau et al. (2011, 2013) (Genbank accession numbers: ITU82589, AF036150–AF036156, JN133853–JN133881).

Due to the detection of cryptic nuclear copies of mtDNA (numts) in *I. typographus* (Bertheau et al. 2011), the 190 sequences obtained were carefully checked for ambiguous

sites. Twelve sequences, mainly from Romanian populations, indicated or corresponded to numt coamplification: 11 matched with ItNumt1 (JN133882) and one with ItNumt4 (JN133885) defined by Bertheau et al. (2011). Consequently, these numt sequences were removed from the dataset. All the other haplotypes gave clear, unambiguous sequence chromatograms, and no indicator of pseudogenes was observed (Zhang and Hewitt 1996).

Phylogenetic reconstructions and haplotype network estimation

Maximum likelihood (ML) and maximum parsimony (MP) tree reconstructions were produced using Paup*4b10 (Swofford 2002). The ML trees were generated using the HKY model (Hasegawa et al. 1985) with unequal base frequencies (freqA = 0.3053; freqC = 0.1938; freqT = 0.3728; freqG = 0.1280), the model that best fits the data under the hierarchical likelihood-ratio test (hLRT) criterion determined with Modeltest v3.7 (Posada and Crandall 1998). MP tree reconstruction used a heuristic search method with 1,000 random-addition sequence replicates and explored tree space by tree bisection and reconnection branch swapping. The robustness of the trees was assessed by 1,000 bootstrap replicates. *I. typographus* ssp. *japonicus* (AF036157) was used as outgroup. The likelihood-ratio test LRT (Felsenstein 1988) supported a molecular clock model ($\chi^2 = 4.04$, df = 10, $P < 0.05$). To infer the relationships between haplotypes, a haplotype network using TCS version 1.21 (Clement et al. 2000) was performed.

Population genetic parameters and analyses of population structure

Total gene diversity H_T, average within-population diversity H_S, haplotype diversity H_d, mean number of pairwise differences, and nucleotide diversity π of the Carpathian *I. typographus* populations were calculated using Arlequin 3.11 (Excoffier et al. 2005). Allelic richness r was computed using the rarefaction method proposed by Petit et al. (1998) using the software Contrib. Occurrence of a significant phylogeographic structure was assessed by testing if G_{st} (coefficient of genetic variation over all populations) was significantly smaller than N_{st} (equivalent coefficient taking into account the similarities between haplotypes) by the use of 1,000 permutations in the program Permut (see Pons and Petit 1996). Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to partition the molecular variance into different hierarchical levels using Arlequin. Samples were grouped according to the three main Carpathian regions: Western versus Eastern versus Southern Carpathians (see Table 1).

Table 1 Characteristics of *Ips typographus* sampling sites

Country	Site	Code	Elevation (m)	Latitude	Longitude	Collector
Slovakia	Tichá dolina valley	W1	1,300	49°22′N	19°97′E	
Slovakia	Mengusovská dolina valley	W2	1,460	49°13′N	20°07′E	
Slovakia	Pod nechcerkom	W3	1,200	49°17′N	19°97′E	
Slovakia	Hrebienok	W4	1,290	49°15′N	20°22′E	
Slovakia	Velická dolina valley	W5	1,360	49°14′N	20°17′E	
Slovakia	Bielovodská dolina valley	W6	1,110	49°23′N	20°1′E	
Slovakia	Liptovský Ján	W7	654	49°05′N	19°68′E	
Slovakia	Brezno	W8	510	48°82′N	19°67′E	
Slovakia	Sklené	W9	879	48°76′N	18°86′E	
Slovakia	Blatnica	W10	608	48°95′N	18°95′E	
Slovakia	Široká dolina valley	W11	1,160	49°24′N	20°14′E	
Slovakia	Ždiar	W12	914	49°28′N	20°24′E	
Poland	Morskie oko	W13	1,446	49°20′N	20°08′E	
Romania	Vaser-Maramures	E1	750	47°47′N	24°41′E	V. Mihalciuc
Romania	Tomnatec-Cluj	E2	1,200	46°32′N	23°19′E	V. Mihalciuc
Romania	Padis-Oradea	E3	1,400	46°47′N	22°85′E	V. Mihalciuc
Romania	Garcin-Brasov	S1	750	45°35′N	25°42′E	V. Mihalciuc
Romania	Gradiste-Hunedoara	S2	1,400	45°37′N	23°21′E	V. Mihalciuc
Romania	Comandau-Covasna	S3	1,150	45°76′N	26°28′E	V. Mihalciuc

Only non-author collectors' names are listed

W Western Carpathians, *E* Eastern Carpathians, *S* Southern Carpathians

Results

Sequence alignment and haplotypes reconstruction

DNA from 178 individuals of *I. typographus* from the 19 Carpathian populations were successfully amplified and sequenced. The final alignment of the COI sequences comprised 558-bp, with a total of 12 polymorphic nucleotides of which one was parsimony informative. All mutations were transitions and on the third codon position not affecting the amino acids. Twelve different haplotypes were identified and after alignment with the *I. typographus* haplotypes detected by Bertheau et al. (2011, 2013), 42 % new haplotypes coded It30 to It34 could be characterized (Table 2; Fig. 1). They are available from GenBank under accession numbers KF011239 to KF011243. Two haplotypes corresponded to HTI and HTII defined by Stauffer et al. (1999) and five matched with the haplotypes It1, It5, It7, It18, and It19 defined by Bertheau et al. (2011, 2013). HTI was the most common haplotype with 102 individuals, followed by It1, It19, and HTII with 33, 17, and 14 individuals, respectively (Table 2; Fig. 1a). The haplotypes It5 and It18 shared three individuals, and six haplotypes (It7 and It30–It34) were found only once. The geographic distribution of the 12 haplotypes is shown in Fig. 1a. The widespread HTI was detected in all the populations while HTII was restricted to the Western Carpathians. The haplotypes It1 and It19 were mainly found in the Western Carpathians despite their presence in the Southern Carpathians. The haplotypes It7, It30, It32–34 were observed specifically in the Western Carpathians whereas It18 and It31 were present only in the Eastern and Southern Carpathians (Table 2; Fig. 1a).

Phylogenetic trees and haplotype network

Both ML and MP phylogenetic analyses yielded congruent trees with low significant pattern among the 12 haplotypes, except the group gathered It19, It30, and It32 supported by a bootstrap value of 64 (Fig. 2). Because of the low level of variation among sequences, bootstrap values were low and few nodes being supported slightly above the 50 % threshold. The haplotype network reflected the same patterns as the phylogenetic trees. The four main haplotypes HTI, It1, It19, and HTII were closely related with only one mutation step difference (Fig. 1b). HTI appears to be the source of all haplotypes though It30 and It33 diverged from It19.

Population genetic parameters and analyses of population structure

The proportion of the various haplotypes yielded a total haplotype diversity H_T of 0.62 while the average within-population diversity H_S was 0.56. However, nucleotide diversity π and mean number of pairwise differences MNPD

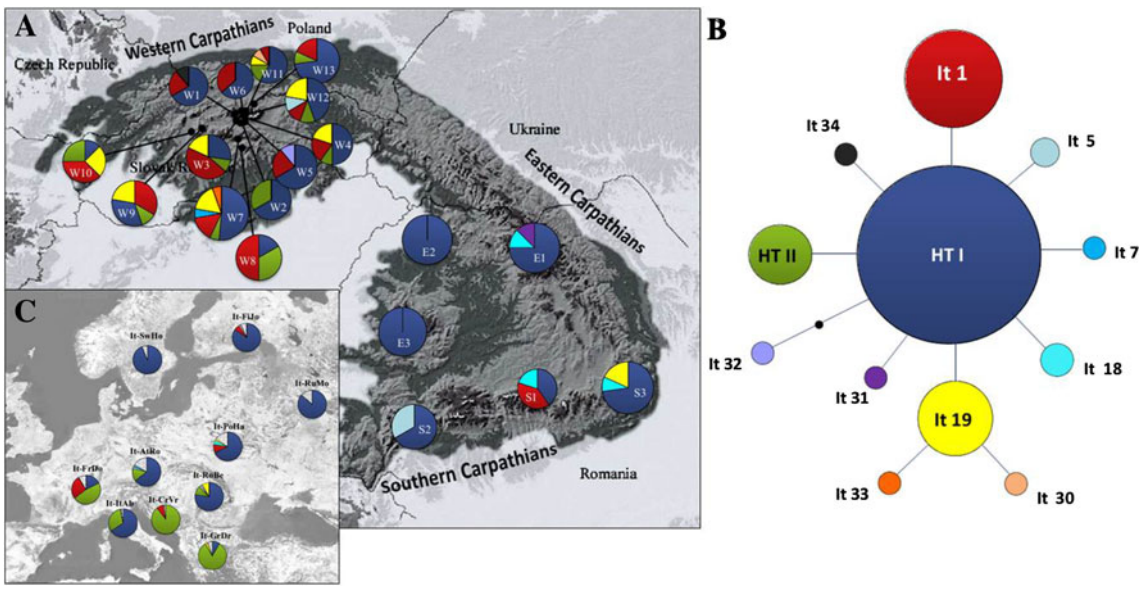

Fig. 1 Haplotype distribution and haplotype network of 178 Carpathians *Ips typographus* COI sequences. Haplotypes HTI–HTII and It1–It19 are those identical to Stauffer et al. (1999) and Bertheau et al. (2011, 2013), respectively, while newly identified ones are coded It30–It34. **a** Geographic distribution of the haplotypes among the 19 sampled populations. Sites names and precise coordinates are given in Table 1. **b** Haplotype network of the 12 haplotypes detected in Carpathians *I. typographus*. Each *line* corresponds to a mutational step and each *empty circle* to a missing intermediate. Haplotype frequencies are represented by the size of the circle. **c** Geographical distribution of the mitochondrial COI haplotypes among the European populations from Bertheau et al. (2013). Only haplotypes found in this present study are represented

Table 2 Within-population haplotypes and diversity indices

Code	*N*	#HT	HT	$H_d \pm$ SD	*r* [5]	MNPD ± SD	$\pi \pm$ SD
W1	9	3	HTI(6), It1(2), It34(1)	0.51 ± 0.16	0.833	0.56 ± 0.49	0.0010 ± 0.0010
W2	6	2	HTI(4), HTII(2)	0.53 ± 0.17	1	0.53 ± 0.51	0.0010 ± 0.0011
W3	11	4	It1(5), HTI(3), It19(2), HTII(1)	0.75 ± 0.10	2.048	1.05 ± 0.75	0.0019 ± 0.0015
W4	10	4	HTI(5), It1(2), It19(2), HTII(1)	0.73 ± 0.12	2.052	0.91 ± 0.69	0.0016 ± 0.0014
W5	9	3	HTI(6), It1(2), It32(1)	0.56 ± 0.17	1.389	0.83 ± 0.65	0.0015 ± 0.0013
W6	11	2	HTI(7), It1(4)	0.51 ± 0.10	0.955	0.51 ± 0.46	0.0009 ± 0.0009
W7	21	6	HTI(11), It19(4), It1(3), HTII(1), It7(1), It33(1)	0.70 ± 0.09	1.811	0.92 ± 0.66	0.0017 ± 0.0013
W8	6	3	It1(3), HTII(2), HTI(1),	0.73 ± 0.16	1.833	1.13 ± 0.85	0.0020 ± 0.0018
W9	9	4	HTI(3), It1(3), It19(2), HTII(1)	0.81 ± 0.09	2.294	1.11 ± 0.80	0.0020 ± 0.0016
W10	8	4	It1(3), HTII(2), It19(2), HTI(1),	0.82 ± 0.10	2.393	1.39 ± 0.95	0.0025 ± 0.0019
W11	12	5	HTI(7), HTII(2), It1(1), It19(1), It30(1)	0.67 ± 0.14	1.931	0.94 ± 0.69	0.0017 ± 0.0014
W12	9	5	HTI(4), It19(2), HTII(1), It1(1), It5(1)	0.81 ± 0.12	2.492	1.06 ± 0.77	0.0019 ± 0.0016
W13	11	3	HTI(8), It1(2), HTII(1)	0.47 ± 0.16	1.182	0.51 ± 0.46	0.0009 ± 0.0009
E1	8	3	HTI(6), It18(1), It31(1)	0.46 ± 0.20	1.25	0.50 ± 0.47	0.0009 ± 0.0010
E2	9	1	HTI(9)	0.00 ± 0.00	0	0.00 ± 0.00	0.0000 ± 0.0000
E3	7	1	HTI(7)	0.00 ± 0.00	0	0.00 ± 0.00	0.0000 ± 0.0000
S1	5	3	HTI(2), It1(2), It18(1)	0.80 ± 0.16	2	1.00 ± 0.80	0.0018 ± 0.0017
S2	6	2	HTI(4), It5(2)	0.53 ± 0.17	1	0.53 ± 0.51	0.0010 ± 0.0011
S3	11	3	HTI(8), It19(2), It18(1)	0.47 ± 0.16	1.182	0.51 ± 0.46	0.0009 ± 0.0009
Total	178	12		0.62 ± 0.08		2.20 ± 1.27	0.0039 ± 0.0022

Codes for populations are in Table 1. Numbers in bracket after haplotype name is the number of individuals with that haplotype

N number of sequenced individuals, *HT* number of haplotypes, H_d haplotype diversity, *r* allelic richness after rarefaction to five, *MNPD* mean number pairwise differences and its standard, π nucleotide diversity

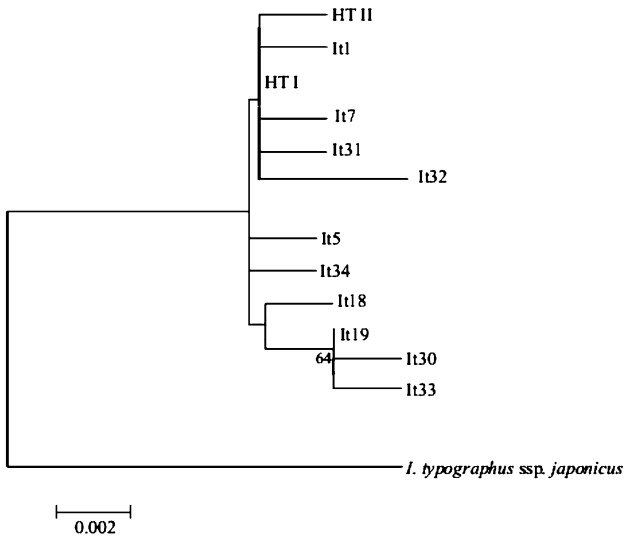

Fig. 2 Maximum likelihood tree of the 12 haplotypes of *Ips typographus* based on the HKY model (Hasegawa et al. 1985). Only bootstrap support (1,000 replicates) higher than 50 % is shown. *I. typographus* ssp. *japonicus* (AF036157) was used as outgroup

were generally low (Table 2). The indices of population structure G_{st} and N_{st} were 0.092 and 0.031, respectively, and did not differ significantly from each other, indicating a weak phylogeographic structure. The within-population diversity indices are summarized in Table 2.

For the grouping option, AMOVA showed that only the components of variance among groups and within populations were significant. A low amount of the variability was found between the three regions (Western vs. Eastern vs. Southern Carpathians, 5.04 %, $P < 0.01$). Although this structure was significant, the AMOVA analysis showed that most of the diversity was found within population (92.96 %, $P < 0.05$).

Discussion

To date, no study has investigated genetic structure and genetic diversity of *I. typographus* populations on a small scale, especially in key areas with fundamental and applied science interests. Here, we sampled *I. typographus* populations in the spruce forests of the Carpathians, where 19 populations were screened with the mitochondrial COI marker. While differences were observed in the haplotype distribution and diversity between the Western/Southern Carpathians (Tatra Mountains/Transylvania Alps) and Eastern Carpathians (Beskids Mountains), our results in general reflect those obtained on the European scale.

One finding of this study was the high number of haplotypes observed in this area compared to the previous study of Bertheau et al. (2013). The haplotype HTI, the most widely found in Europe, was also the main haplotype in the Carpathians followed by the haplotypes It1, It19, and HTII. However, the haplotype HTII was only the fourth most commonly detected haplotype in the Carpathians with 7.8 % of individuals, whereas it was the second commonly represented on the European scale (Fig. 1c). Furthermore, while the total genetic diversity is similar to that obtained in Europe, a difference was observed between the Western and Eastern Carpathians. The Western (Tatra Mountains) was richer in haplotypes than the Eastern Carpathians, with It1 and It19 highly represented and with six specific haplotypes (HTII, It7, It30, It32–34). In the Eastern Carpathians two populations presented only one haplotype and one specific haplotype was observed (It31). The Southern Carpathians (Transylvania Alps) presented also one population with high genetic diversity and all individuals shared haplotypes found specifically either in the Tatra (It1 and It5) or Beskids Mountains (It18). These contrasting data between West, East, and South might be a result of the unbalanced sampling size in these three regions. More populations were collected in the Tatra Mountains compared to Beskids ones, which could confer higher genetic diversity and the detection of more specific haplotypes. Indeed, the specificity of the HTII in the Western Carpathians did not agree with the data of Bertheau et al. (2013). On the other hand, it is not rare to detect different genetic lineages among the Carpathian region. For example a clear genetic split between the Northwestern and the Southern Carpathians populations was observed not only in the alpine plant species, *Hypochaeris uniflora* (Mraz et al. 2007) and *Campanula alpina* (Ronikier et al. 2008) but also in the caddisfly, *Drusus discolor* (Pauls et al. 2006). Hence, Schmitt (2009) concluded, in his review, that these different genetic lineages would testify the presence of multiple glacial survival centers within the Carpathians.

Despite these differences among the Carpathians, an absence of global genetic differentiation among *I. typographus* populations appeared through the genetic analyses. Firstly, the AMOVA showed that the greatest genetic diversity was found within populations and the indices of genetic differentiation, G_{st} and N_{st}, did not differ significantly. Secondly, the haplotype network was star-shaped, typical for a recent demographic expansion, with HTI at the central position and the other haplotypes deriving from it by a maximum of three mutation events. In insect species exhibiting recent range expansion, it was not rare to find a large proportion of genetic variation within populations (Conord et al. 2006). Thus, this specific network as well as the phylogenetic analyses also reflected a weak differentiation between *I. typographus* populations. One explanation for these discrepancies, in addition to an eventual sampling bias, could be the distribution of HTI, represented at high frequency in all populations, as well as the close relationships between haplotypes, which erase the differences between the different parts of the Carpathians. Subject to confirmation using a larger sampling size, the lack of genetic differentiation and the occurrence of four common haplotypes (HTI, It1, It5, and It19) among the Carpathians suggest important gene flow which is consistent with previous studies using nuclear markers (Stauffer et al. 1999; Sallé et al. 2007). The dispersal ability of phytophagous insects is an important ecological factor influencing the population differentiation (Peterson and Denno 1998). Like other bark beetle species, *I. typographus* is known to have good dispersal capacities, from a few hundred meters to several kilometers (Botterweg 1982; Gries 1985; Weslien and Lindelow 1990), facilitating the search of suitable hosts (Lieutier 2002). This high dispersal ability might result from an adaptation to the patchy breeding resources in spruce forests. Consequently, such recurrent long-distance migration to find suitable hosts may result in low levels of genetic structure, which has been observed in non-genetic studies (Duelli et al. 1997; Faccoli and Stergulc 2004) as well. In addition to the natural dispersal capacities of this bark beetle, the strong human pressure with the wood trade could play a role in homogenizing *I. typographus* population genetic structure.

The analysis of Carpathian populations revealed new mitochondrial data to complement the phylogeography story of *I. typographus*. Here, several scenarios could be proposed regarding refugial area and colonization routes of *I. typographus* integrating haplotypes distribution and frequency from previous and current studies, as well as information related to the glacial distribution of *P. abies*. *I. typographus* has a very recent genetic impact as its expansion reaches just back to a single late-Pleistocene glacial refuge, as it is unlikely that the haplotypes would remain so closely related if they had been isolated in two or more refugia (Bertheau et al. 2013). The Carpathian Mountains, known to be one of the refugial areas of *P. abies* during the Quaternary ice ages, harbored several isolated refugia, two in the Northern Carpathians including Western Carpathians (Tatra Mountains) and the northern part of the Eastern Carpathians (Beskids Mountains) as well as one in the Southern Carpathians (Transylvania Alps) (Tollefsrud et al. 2008). The presence and significant frequencies of the main European haplotypes (HTI, HTII and It1) of *I. typographus* as well as the high genetic diversity found in the Tatra and Transylvania regions suggest that these two areas may also be glacial refugia for *I. typographus*. From there, westward and northward recolonizations took place as most of the specific Carpathians haplotypes were found in countries such as Austria, Belarus, or Finland (Bertheau et al. 2013; Fig. 1c). The important

presence and directions of post-glacial recolonization of the haplotypes HTII and It19 in the Southern part of Europe from the Carpathians could be possible through long-range dispersal during the warming periods (Forsse and Solbreck 1985). Another explanation could be that the mountain systems of the Southern Carpathians and the Eastern Balkan were linked by cool forested areas during at least the last ice age allowing massive gene flow between populations (Schmitt 2009). Nevertheless, it appears also consistent to hypothesize a Southern refuge such as the Dinaric Alps or the Southwest Bulgarian mountains for *I. typographus*, also known as refugial areas of *P. abies* (Tollefsrud et al. 2008), as a source for the post-glacial westward and northward colonizations during the last interglacial. A micro-scale study would be crucial to confirm these assumptions.

An interesting point to consider is that the haplotype HTII and related ones were neither found in the southern and eastern Carpathians populations in this study nor in the north-eastern ones from Belarus to Scandinavia and Moscow (Bertheau et al. 2013). The Carpathians and more specifically the Beskids mountains could have acted as a natural barrier for the haplotype HTII limiting its expansion further north. However, these mountains did not strictly prevent gene flow between regions since only the haplotype HTII and its derivations were stopped. The absence of these southern haplotypes seemed to be a result of genetic drift unrelated to the fitness of these individuals. Behavior assays of *I. typographus* individuals having one of these southern haplotypes with northern region temperatures would help to test this assumption.

The findings of this study clearly demonstrate that a small-scale genetic approach is crucial both to supplement results of earlier European scale phylogeographic studies on *I. typographus* population history and to provide critical information for both preventative and reactive forest management. Moreover, the observed differentiation within one mountain system and potential natural barriers for organism dispersion could help to complement the frame about the glacial refugia, since molecular studies focusing on geographical patterns of genetic structure of Carpathian populations are limiting. With better sampling and the use of nuclear markers like microsatellites (Sallé et al. 2003; Stoeckle and Kuehn 2011) or the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) we will be able to gain a clearer picture of the migration and colonization of *I. typographus* in the Carpathian area after the last ice age.

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