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## Genetic homogeneity of the Sedgling *Nehalennia speciosa* (Odonata: Coenagrionidae) indicates a single Würm glacial refugium and trans-Palaeartic postglacial expansion

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### Abstract

The phylogeographic structures of taiga species often support the hypothesis of East Palaeartic refugia for these taxa, but the phylogeographic structures of northern temperate and southern boreal bog species are still poorly understood. Therefore, we analysed the genetic diversity and differentiation of a stenotopic damselfly, *Nehalennia speciosa*, across its trans-Palaeartic range by means of sequencing two mitochondrial gene fragments, 16S rRNA-ND1 and cytochrome *c* oxidase II. Only four single nucleotide polymorphisms were detected over the 1130 sequenced nucleotides. This low genetic diversity and differentiation and thus the lack of phylogeographic structure imply postglacial expansion from a single Würm Ice Age refugium, most likely located in the Far East of Asia, i.e. Manchurian refugium. From here, the species could have colonized large parts of the Palaeartics, including Europe, during the postglacial.

**Key words:** Phylogeography – range expansion – genetic diversity – genetic differentiation – mtDNA sequences – dragonfly

### Introduction

The analysis of distribution patterns as well as their origin and dynamics is one of the central aspects in biogeographical research (e.g. Reinig 1937; de Lattin 1967). In this context, the interest has focused either on species with very small ranges, e.g. endemics of mountain systems (Holdhaus 1954; Varga 1975), or on taxa with very large ranges (e.g. Reinig 1937; Varga 1977). An example of the latter has been the vast trans-Palaeartic distribution type including boreal and temperate species. First biogeographical interpretations, e.g. by de Lattin (1964), explained the genesis of the majority of these distribution patterns by exclusively postglacial expansion from East Asian Würm glacial refugia to the West, i.e. Europe, so that these species have to be considered as pure East Palaeartic elements. These core areas are represented by several putative refugia, for example the Mongolian and the Manchurian one (e.g. de Lattin 1967; Dévai 1976), of which one or several could have served as glacial retreats for these species.

However, shortly after the establishment of this relatively simplistic concept, it was disputed by many biogeographers questioning this exclusive East Palaeartic origin. These authors argued for a polycentric origin of postglacial expansion, also including West Palaeartic Würm Ice Age refugia (e.g. Varga 1975, 1977; Aspöck et al. 1976; Aspöck 1979; Malicky et al. 1983). Since then, many important advances in biogeographical research have taken place, with genetic analyses representing a milestone (Hewitt 1996, 1999). Recent genetic studies strongly support this pronounced polycentricity and hence survival in glacial refugia, both in the West and in the East Palaeartic (e.g. Uimaniemi et al. 2000; Schmitt and Seitz 2001; Hundertmark et al. 2002; Goropashnaya et al. 2004). In other cases, some species formerly believed to be

‘Siberian’ elements are highly likely to be West Palaeartic elements surviving the last Ice Age exclusively in extra-Mediterranean refugia including the ice-free corridor in Central Europe (e.g. Babik et al. 2004; Ursenbacher et al. 2006). Yet, most of the genetic studies of trans-Palaeartic taiga species have supported de Lattin’s (1964) assumption, i.e. exclusive survival in East Palaeartic Würm Ice Age refugia (e.g. Zink et al. 2002; Goropashnaya et al. 2004; Oshida et al. 2005; Fedorov et al. 2008; Saitoh et al. 2010).

While the phylogeography of a number of forest and forest-steppe species with trans-Palaeartic distribution patterns has been relatively well understood, tyrphophilic and tyrphobiontic species are mostly unexplored in this matter. Thus, there is a strong need for the studies of species widespread over the taiga bogs and bogs in biomes south of the taiga belt. It remains unknown whether such species show polycentric patterns, and it is unclear whether such elements could have survived ice age conditions in the West Palaeartics. To unravel these questions, we analysed the phylogeography of the Sedgling, *Nehalennia speciosa* (Charpentier, 1840), the smallest European dragonfly. Its geographic range extends from France and Belgium (5°E) to Japan (145°E) and has strongly varying latitudinal limits, with extremes between 36.3° and 61.6°N and the main distribution between 47° and 60°N in Europe, 52° and 60°N in W Asia and 40° and 51°N in the Far East of Asia (Bernard and Wildermuth 2005). The species’ current range covers the southern boreal taiga zone and the northern temperate zone. In the latter, the populations are affiliated with extrazonal boreal habitats, i.e. *Sphagnum* bogs and fens and water bodies bordered by them (Bernard and Wildermuth 2005). *Nehalennia speciosa* is a highly stenoeccious species restricted to a specific combination of habitat conditions, providing appropriate texture and microclimate, such as (1) low depth of water, (2) abundance of submerged vegetation (parts of helophytes, *Sphagnum*, other mosses, *Utricularia*), (3) emergent ‘lawn-like’, fairly dense formations of strictly defined narrow-leaved plants (mostly selected *Carex* spp., the most important being *C. limosa* Linnaeus and *C. lasiocarpa*

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Ehrhart) with admixtures of some broad-leaved plants and (4) water quality with fairly low pH and low conductivity, hardness and trophy (Bernard and Wildermuth 2005). The species has been believed to express an extremely low dispersal capacity. However, there is some evidence for colonization of new localities and long-distance dispersal (Bernard and Wildermuth 2005; Bernard and Buczyński 2008). All these characteristics make the sedgling a suitable model species for the study of the phylogeography of boreal bog species.

## Materials and Methods

### Sample stations

Individuals from 12 localities all over the natural range of *N. speciosa* were collected (Table 1, Fig. 1). Five of these localities are in Europe, one in Bavaria (Germany), three in Poland and one in Lithuania. The remaining seven localities are in Asia, two of them in the West Siberian Lowland, one in the Amur Province, one in the Russian Far East and three in Japan (one on Hokkaido and two on Honshu).

### DNA analyses

All European individuals were frozen in liquid nitrogen. Asian samples were stored in absolute ethanol. DNA was extracted from head and thorax of each individual (1–3 individuals per site) using

Table 2. Name, location, sequence and length of primers used for amplification

Name	Location	Sequence	Length (bp)	References
ND S	NDI	5' TAG AAT TAG AAG ATC AAC CAG C 3'	22	Pashley and Ke 1992
ND II 16S rRNA		5' ACA TGA TCT GAG TTC AAA CCG G 3'	22	Vogler and DeSalle 1993
COIIa	COII	5' ATG GCA GAT TAG TGC AAT GG 3'	19	Liu and Beckenbach 1992
COIIb	COII	5' GTT TAA GAG ACC AGT ACT TG 3'	19	Liu and Beckenbach 1992

COII, cytochrome *c* oxidase II.

the DNEasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. PCR amplification was carried out in a Biometra Tgradient cycler using a 20- $\mu$ l reaction volume consisting of 8  $\mu$ l of 5Prime PCR HotMasterMix, 9  $\mu$ l of water, 0.5  $\mu$ l of each primer and 2  $\mu$ l of the 1 : 100 diluted DNA extract. We amplified and sequenced a 512-bp fragment of the mtDNA fragment 16S rRNA-ND1 (Table 2) and a 618-bp fragment of the mtDNA gene cytochrome *c* oxidase II (COII; Table 2). The PCR conditions were as recommended by the manufacturer, with

Table 1. Regions, sampling localities, their abbreviations, geographic positions of sampling sites, number of analysed individuals per gene 16S rRNA-ND1 and COII as well as detected haplotypes in *Nehalennia speciosa* over its entire trans-Palaeartic distribution

Region	Locality	Abbreviation	Geographic position	Individuals analysed 16S rRNA-ND1/COII	Haplotypes (16S rRNA-ND1/COII)
Europe/Germany	Kollerfilz	KOL	47°47'N 12°03'E	2/1	B/A
Europe/Poland	Dury	DUR	53°38'N 18°21'E	2/1	A/A
Europe/Poland	Laniewo	LAN	54°05'N 20°23'E	3/2	A/A
Europe/Poland	Broduszurki	BRO	49°49'N 22°21'E	2/2	A/A
Europe/Lithuania	Purviniskiai	PUR	55°01'N 25°38'E	2/1	A/A
Asia/Russia/W Siberia	Bakcharskoe Bog	BAK	56°48'N 82°51'E	1/1	B/C
Asia/Russia/W Siberia	Iksinskoe Bog	IKS	56°50'N 83°16'E	2/2	B/D
Asia/Russia/Amur Province	Blagoveshchensk	BLA	50°23'N 127°40'E	1/0	B/-
Asia/Russia/Far East	Sokolovskaya Bay	SOK	42°54'N 133°53'E	2/2	B/B
Asia/Japan/Hokkaido	Onnenai	ONN	43°10'N 144°20'E	1/0	B/-
Asia/Japan/Honshu	Menokoshi	MEN	40°58'N 141°14'E	2/1	B/B
Asia/Japan/Honshu	Kamegaoka	KAM	40°53'N 140°19'E	3/3	B/B

COII, cytochrome *c* oxidase II.

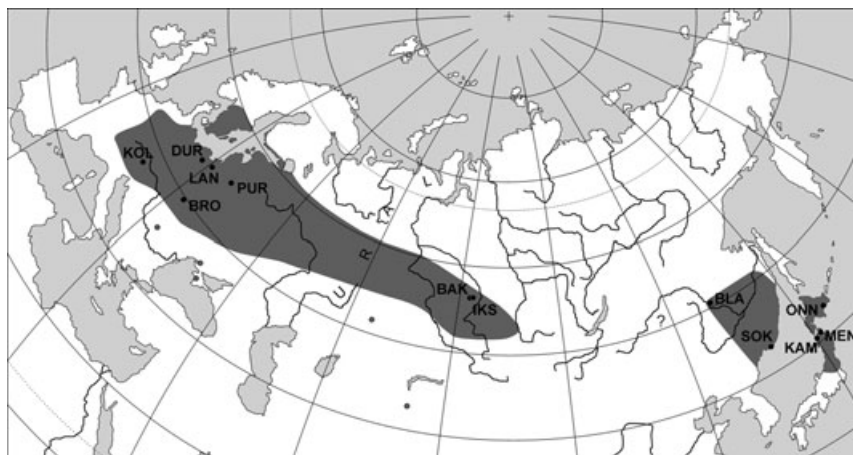


Fig. 1. Location of 12 sampling sites of *Nehalennia speciosa* in Eurasia. For abbreviations of localities, see Table 1. The species' extent of occurrence, based on historical and current data and some extrapolation of them, is given as a shaded area. Distant isolates are distinguished as small circles. An unlocalizable and highly doubtful record in Southern Transbaikalia is marked by a question mark

annealing temperature of 50°C for 16S rRNA-ND1 and 47°C for COII.

The sequencing reaction was carried out using the DYEnamic ET terminator cycle sequencing kit (GE Healthcare, Chalfont St Giles, the United Kingdom) for sequencing reactions run on a MegaBACE 1000 automated sequencer (GE Healthcare). DNA sequences were corrected and aligned by eye as no indels occurred. We excluded ambiguous data from the beginnings and ends of the fragments in the analyses. An unrooted minimum spanning haplotype network based on both genes and statistical parsimony was calculated using TCS 1.18 (Clement et al. 2000; default settings). To calculate genetic distances to other *Nehalennia* species, we used previously published sequences of the COII gene (Iserbyt et al. 2010; Genbank accession numbers GQ256050, GQ256053-66, GQ256068-78).

## Results

Twenty-four DNA sequences were obtained for 16S rRNA-ND1 representing all sampling sites, while 16 sequences were obtained for COII from 10 sampling sites. The obtained sequences (GenBank accession numbers JF746931 to JF746970) showed a very low level of variability among all studied individuals reflected in only four variable nucleotide sites in 1130 sequenced base pairs (i.e. 0.35%). 16S rRNA-ND1 alignments revealed only one single nucleotide polymorphism (SNP) with populations from north-eastern Central Europe representing type A and the ones from Bavaria and Asia type B (Table 1). In COII, three SNPs were detected. One mutation separated European (type A) from Asian individuals (types B, C and D). Type B was the sole haplotype in COII of the Far East Asian individuals. In the West Siberian individuals, two further SNPs occurred: type C in the sample from the Bakcharskoe Bog and type D in the sample from the Iksinskoe Bog (Table 1). In total, only five very similar haplotypes were recognized combining these two genes, i.e. one in Poland and Lithuania, one in Bavaria, one in East Asia and two additional ones in West Siberia (Fig. 2).

The genetic distances to the other *Nehalennia* species were rather high, with p-distances of 0.123 between *N. speciosa* and *N. irene* (Hagen, 1861) and 0.103 between *N. speciosa* and *N. gracilis* Morse, 1895.

## Discussion

### Genetic diversity and differentiation

In comparison with other animal species (e.g. Hochkirch and Görzig 2009), genetic diversity within *N. speciosa* is extremely low and varies little all over the species' range. This pattern differs strongly from what is known in species that survived the glacial period in typical Mediterranean refugia (reviewed in Schmitt 2007) or in comparable areas of the southern parts of North America (reviewed in Hewitt 2004). Species with taiga

belt distribution patterns frequently show lower genetic diversity within species and populations than the former biogeographical groups (e.g. Uimaniemi et al. 2000; Hundertmark et al. 2002; Zink et al. 2002; Drovetski et al. 2004; Goropashnaya et al. 2004; Oshida et al. 2005; Fedorov et al. 2008; Saitoh et al. 2010). However, in all cases known to the authors, this genetic diversity is considerably higher than in *N. speciosa*. Furthermore, genetic diversity in *N. speciosa* is notably lower than in the two North American congeners, *N. irene* and *N. gracilis* (Iserbyt et al. 2010). Genetic distances between the three *Nehalennia* species are rather high, suggesting that speciation took place long before the Pleistocene. Furthermore, not all *Nehalennia* species have been sequenced yet. The next relative of *N. speciosa* might be *N. integricolis* Calvert, 1913 (Belyshev and Haritonov 1981). Yet, the genus *Nehalennia* as a whole seems to be genetically quite conservative, i.e. rather invariable (compare with other genera of dragonflies: Turgeon and McPeck 2002; Turgeon et al. 2005; Rach et al. 2008).

This even more applies to the differentiation among populations as all samples of *N. speciosa* are very similar, without any clear phylogeographic signal. This feature is mostly shared with the North American congeneric *N. irene*, but not with *N. gracilis*, of which three well-distinguished phylogeographically informative haplotype groups have been recognized in eastern Canada.

Comparing with other taxonomic groups (e.g. amphibians and reptiles), Mediterranean taxa often show very pronounced genetic splits among different refugia, even on relatively small geographical scale within one Mediterranean peninsula (reviews: Gómez and Lunt 2007; Schmitt 2007). Similar patterns are also exhibited by high mountain species of Europe (Schmitt 2009) or by those living on ancient volcanic islands (Hochkirch and Husemann 2008). However, phylogeographic structures, in general, are much less pronounced in taiga species. Most of them only show some phylogeographic signal in the south-eastern parts of the Eurasian boreal and adjoining cold-temperate region (e.g. Oshida et al. 2005; Fedorov et al. 2008; Saitoh et al. 2010), but none of them shows such a low degree of differentiation as *N. speciosa*.

The low genetic diversity and differentiation of *N. speciosa* have been recently confirmed by (1) results obtained for two other genes (COI and ITS1) from four localities in Russia and Japan (Suvorov 2011) and (2) results of a regional allozyme study restricted to Poland and Lithuania (Bernard and Schmitt 2010). Furthermore, *N. speciosa* is also morphologically uniform, and no subspecific division has been recognized all over its trans-Palaearctic range (Belyshev and Haritonov 1981; R. Bernard, unpublished data). Thus, a most recent common ancestor (MRCA) for living populations of this species must have existed short time ago, most likely not earlier than the last Ice Age.

### Biogeographical implications

The genus *Nehalennia* consists of six species, three of which are restricted to the Nearctic, one to Florida and one to Central and South America (Westfall and May 2006; Tsuda 2000). As *N. speciosa* appears to be cladistically nested within the American taxa, the Palaearctic species is arguably of Nearctic origin. An American antecedent of it, possibly a common ancestor shared with *N. integricolis* (Belyshev and Haritonov 1981), might have colonized north-eastern Asia via Beringia in the Pleistocene, starting a process of allopatric speciation.

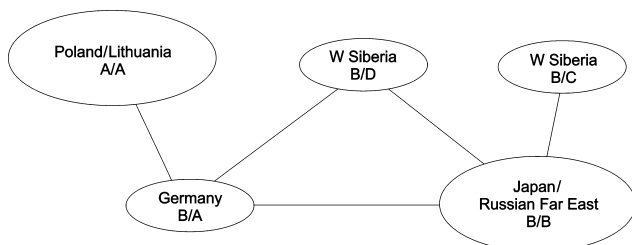


Fig. 2. Mitochondrial haplotype network based on 16 individuals covering the complete range of *Nehalennia speciosa*



The low diversity and differentiation of current *N. speciosa* strongly contradicts polycentricity of the species – suggested by Belyshev and Haritonov (1981) – at least during the last Ice Age. Our interpretation is particularly supported by the contrasting patterns found in the North American congener *N. gracilis*. This species shows a significant phylogeographic structure strongly supporting polycentricity (Iserby et al. 2010). Therefore, we argue that *N. speciosa* was restricted to just one refuge area during the last Ice Age. Possibly, bottlenecks during this refugial stage might have been responsible for the strong genetic poverty of the species and therefore favour a geographically restricted area of this refugium. Additionally, this genetic poverty partly might have also been the result of a fairly recent (i.e. late Pleistocene) arrival of the ancestor of modern *N. speciosa* to Eurasia from North America.

The most probable location of the species' Würm refugium is in the Far East of Russia with adjoining areas in China and Korea (i.e. the Manchurian refugium sensu de Lattin 1967). This assumption is supported by (1) the relative geographical proximity of this area to Beringia, the assumed way of entrance to Eurasia, (2) the current absence of the species in other putative refuge areas in Asia (Bernard and Wildermuth 2005), (3) the assumed unsuitable conditions in the West Palaearctics during the latest glacial period and (4) the general fact that the expansion of West Palaearctic Odonata species has reached at most as far east as western or central Siberia (Kosterin 2005). However, because of the low genetic differentiation all over the trans-Palaearctic distribution of *N. speciosa* and missing genetic diversity centres in the East, we cannot exclude the possibility of a larger distribution of this species during the latest glacial period, maybe even extending as far west as the ice-free corridor between the northern and the southern (i.e. mountain) ice-shields in Europe. The pattern of periglacial occurrence in Europe is postulated by Brockhaus (2007a,b) for some other dragonfly species – including trans-Palaearctic ones – both cold-stenothermal and more eurythermal.

Nevertheless, if our preferred (first) hypothesis holds true, this philopatric habitat specialist, with its seemingly low dispersal capacity at the local scale (Reinhardt 1994; Schmidt and Sternberg 1999; Bernard and Wildermuth 2005), must have been able to colonize most of the Palaearctics from a single – most probably this eastern – refugium during the short postglacial period. This might be explained by two mutually not exclusive hypotheses: (1) the habitat of *N. speciosa* was so widespread and interconnected in northern Asia that fairly low environmental resistance allowed a quick expansion throughout the Palaearctics and (2) wind drift in aerial plankton (Bernard and Wildermuth 2005) – and thus also occasional long-distance dispersal – of this small size species has occurred with some frequency. The postglacial or slightly earlier expansion also included Japan, which shares identical haplotypes (this study) and morphological features (R. Bernard, unpublished data) with continental Asia. In contrast, Japanese populations of several other dragonfly species are morphologically differentiated from populations in continental Asia (Sugimura et al. 2001), thus supporting the hypothesis of their longer presence in Japan and hence glacial survival on these islands in the Japanese arboreal refugium (cf. de Lattin 1967).

The current distribution of *N. speciosa* shows a large gap in Asia between the West Siberian Lowland and the Amur province (Fig. 1). This gap might be narrower if the record of the species in the mountains of South Transbaikalia is accepted.

However, *N. speciosa* is only generally given in a table without any data or known explanation (Haritonova 1990), and thus, its occurrence in this region is treated as erroneously recorded or highly doubtful. Belyshev and Haritonov (1981) explain this disjunct distribution as a consequence of different Würm refugia and postglacial expansion. Our genetic data strongly contradict this assumption, especially as nearly identical haplotypes exist on both sides of this gap. Therefore, *N. speciosa* most probably reached West Siberia from the East. A transgression of vast southern areas of eastern and central Siberia is hardly likely due to the barrier function of the high mountain systems and the rarity of suitable habitats over most of this region. Consequently, this part of Siberia might have been circumvented. The only possible way is the northern bypass, i.e. through the Central Siberian Plateau and possibly even northern Siberia, probably during the Holocene Climatic Optimum. Subsequently, the climatic cooling after this optimum might have caused the extinction of the species at these higher latitudes. According to this hypothetical reconstruction, the range disjunction is at most a few thousand years old which corresponds with the age of Siberian species' disjunctions generally suggested by Kosterin (2005). After having reached the West Siberian Lowland, further westwards expansion of *N. speciosa* must have occurred reaching as far west as eastern France and Belgium (Bernard and Wildermuth 2005).

### Outlook

Our results open the more general question whether trans-Palaearctic bog and acidic fen species share this genetic and biogeographical pattern. The biology of *N. speciosa* is quite specific (Bernard and Wildermuth 2005), and the species represents just one single example. However, our results are a good starting point for future large-scale genetic analyses at the trans-continental level to test the generality of the patterns presented in this study.

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### Zusammenfassung

*Genetische Homogenität der Zwerglibelle Nehalennia speciosa (Odonata: Coenagrionidae) deutet auf ein einziges Würm-Eiszeit-Refugialgebiet und transpaläarktische postglaziale Ausbreitung hin*

Die phylogeographischen Strukturen von Arten der Taiga unterstützen oft eine Ausbreitung aus ostpaläarktischen Refugien dieser Taxa, jedoch sind die genetischen Strukturen von nördlich-temperaten und südlich-borealen Moorarten bislang kaum untersucht. Daher untersuchen wir die genetische Diversität und Differenzierung der stenotopen Kleinlibelle *Nehalennia speciosa* über ihr gesamtes transpaläarktisches Verbreitungsgebiet mittels mtDNA Sequenzierung von zwei Genen, 16S rRNA-ND1 und COII. Nur vier SNPs wurden in insgesamt 1130 sequenzierten Nukleotiden festgestellt. Diese niedrige

genetische Diversität und Differenzierung und dadurch bedingte fehlende phylogeographische Struktur impliziert postglaziale Expansion aus einem einzigen würmglazialen Refugium, welches sich vermutlich im Fernen Osten Asien befand, also das Mandshurische Refugium repräsentiert. Von hieraus besiedelte die Art große Teile der Paläarktis unter Einschluss von Europa während des Postglazials.

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