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The African-Iberian connection in Odonata: mtDNA and ncDNA based phylogeography of *Aeshna cyanea* (Müller, 1764) (Odonata: Aeshnidae) in Western Palaearctic

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Abstract. We explore the phylogeography and inter-population relationships of the Southern Hawker dragonfly, *Aeshna cyanea* (Müller) in the Western Palaearctic region based on 603 bp Cytochrome Oxidase Subunit 1 (COI) mtDNA and 732 bp Internal Transcribed Spacer region (Internal Transcribed Spacer 1, 5.8S ribosomal RNA gene and Internal Transcribed Spacer 2, ITS region) ncDNA with an increased sampling from Europe compared to a previous study. Both DNA fragments recover a remarkable and compatible pattern: the recently described *Aeshna vercanica* Schneider et al. is the sister group of *A. cyanea*, which in turn comprises three distinct populations. These populations are: a population in the Caucasus region; a North African population; and a European population. When analysed alone, the ITS fragment recovered *A. vercanica* and the Caucasus *A. cyanea* population as separate units, but the North African and European *A. cyanea* populations were recovered as intermixed. F_{ST} population genetic analyses of COI data revealed high degrees of isolation between all populations as all inter-population values were between 0.818 (North Africa – Europe) and 0.944 (Europe – *A. vercanica*). Average pairwise distance in COI (uncorrected *p*) between populations followed this pattern and was lowest between Europe and North Africa and highest between North Africa and *A. vercanica*, and between Europe and *A. vercanica*. Within population pairwise distance values were approximately an order of magnitude lower. Pairwise distance values between populations for the ITS region were much lower than for COI, but followed the same pattern. Our results therefore support the full species status for *A. vercanica*, and clearly indicate that the current Western European *A. cyanea* population originated from a North African galcial refugium and dispersed to Europe (the Iberian Peninsula) prior to the Holsteinian interglacial period. While the North African and European populations likely remained in contact initially, the European population was probably isolated in the Iberian Peninsula during the Holsteinian interglacial period, and subsequently spread throughout Europe in late Pleistocene – early Holocene.

Key words. Odonata, *Aeshna*, phylogeography, refugia, glacial cycles.

1. Introduction

Current European biogeography is strongly affected by the Quaternary glacial cycles, with dramatic climate fluctuations over the past two million years having left complex patterns of range expansions and contractions, as well as speciation and extinction events (e.g. HEWITT 1999; SCHMITT 2007). The past couple of decades have seen a surge in species and population level biogeographical studies of the European flora and fauna (e.g. HEWITT 1999, 2004; SCHMITT 2007; NORMAND et al. 2011; SIMONSEN & HUEMER 2014; HUSEMANN et al. 2014). The emerging picture is a mixture of patterns where some studies reveal patterns that confirm the presence of major Mediterranean glacial refugia in the Iberian, Italian and Balkan peninsulas (see DE LATTIN 1964, 1967; HEWITT 2004 for general patterns) in plants (VON CRÄUTLEIN et al., 2019), vertebrates (URSENBACHER et al. 2006; JOGER et al. 2007; NEBEL et al. 2015; MARZAHN et al. 2016) and hexapods (VILA et al. 2005; LOUY et al. 2013; HINOJOSA et al. 2017) – some of these studies have demonstrated several Mediterranean refugia in the same species. However, a number of studies show markedly different patterns, which include patterns indicating northern glacial refugia (e.g. SCHMITT et al. 2007; GRATTON et al. 2008; HAMMAOUTI et al. 2009; SLOVÁK et al. 2012; BESOLD & SCHMITT 2014;

Simonsen & Huemer 2014; Daneck et al. 2015; Paućulova et al. 2016; Windmaisser et al. 2016; Quinzin et al. 2017). Others indicate the presence of refugia in eastern or southeast Western Palaearctic (Sadeghi et al. 2010; Sztencel-Jabłonka et al. 2015; Kajtoch et al. 2016; Kamp et al. 2019), while some studies indicate a rapid post glacial expansion across the entire Palaearctic (Bernard et al. 2011) or even Holarctic (Nebel et al. 2015; Kohli et al. 2018) regions from eastern Siberian refugia. Until recently, the role North African refugia may have played in Western Palaearctic Quaternary biogeography has been somewhat overlooked, not least the routes through which Europe has been colonised from such North African refugia (Habel et al. 2001; Dapporto et al. 2001; Husemann et al. 2014; el Mokhefi et al. 2016; Lefebvre et al. 2016). Understanding these patterns and their timing remains crucial not only for understanding current European biogeography, but also for predicting the effects of current and future climatic changes on biodiversity.

Insects are often a focus group for zoological phylogeography because of their significant biological diversity, fast generation time and large population sizes – which mean they often respond quickly to changes – and general ease of access. Indeed, many studies on European phylogeography and speciation are based on insect groups (several of the studies mentioned above as well as others), mostly Lepidoptera (butterflies and moths), and Orthoptera (grasshoppers and crickets). Another insect order of considerable interest for phylogeography and diversification studies are Odonata (dragonflies and damselflies): they are ecologically very diverse, but the individual species are often strongly tied to specific ecological conditions, and all have highly complex life cycles with aquatic juvenile stages and terrestrial adults. This means that they link the terrestrial and aquatic environments, and are doubly sensitive to environmental and climatic changes – they react to changes in both environments and not just one of them (e.g. Bybee et al. 2016). Several studies have indeed focused on phylogeography and/or species delimitation of Western Palaearctic Odonata. Artiss (2004) found strong genetic separation between Nearctic and Palaearctic *Libellula quadrimaculata* Linneaus, 1758, but only differentiation between Japan and the remaining Palaearctic – not within the Palaearctic region in general. Kohli et al. (2018) found divergence in the circumpolar arctic *Somatochlora salbergi* Trybom, 1889 between Japan (Hokkaido) and the rest of the populations, but otherwise no variation. Similarly, Bernard et al. (2011) found very limited divergence across the Palaearctic region in *Nehalennia speciosa* (Charpentier, 1840). In contrast to this, Hinojosa et al. (2017) found a clear pattern in *Sympetrum vulgatum* (Linneaus, 1758), which is divided into three geographical groups: Iberian Peninsula; Europe; and Caucasus. The closely related damselflies *Coenagrion puella* (Linneaus, 1758) and *C. pulchellum* (Vander Linden, 1825) share intermixed COI haplotypes in Europe (Freeland & Conrad 2002), and cannot be separated based on mtDNA. However, they are well separated based on nuclear microsatellite markers (Lowe et al. 2008), demonstrating clearly the risks of relying on mitochondrial genes only.

Recently, Schneider et al. (2015) studied the *Aeshna cyanea* (Müller, 1764) complex and described a new species, *A. vercanica* Schneider et al., 2015, based on distinctive specimens from the Hyrcanian Forest region in northwest Iran and southeast Azerbaijan (Fig. 1). When they analysed molecular differences in both mitochondrial and nuclear genetic markers between *A. vercanica* and *A. cyanea*, they discovered a distinctive pattern in the latter with clear distinction between populations in the Caucasus, North Africa, and Europe. As their focus was on the description of the new species, *A. vercanica*, they did not explore the pattern in *A. cyanea* further, but noted that the African population may be a glacial relict (Schneider et al. 2015). *Aeshna cyanea* is a distinctive, versatile and mobile species, which occurs widespread in Western Europe from Gibraltar and Sicily to northern England, southern Scandinavia and southern Finland. It is absent from Ireland, and rare and localised in Scotland. While it is apparently absent from south eastern Italy and southern Balkan, it probably occurs throughout the rest of Eastern Europe to the Ural and Caucasus mountains, although the exact distribution is poorly known. It is absent from most of Anatolia, the Middle East, and north eastern Africa, but there are rare and localised populations in north western Africa (Fig. 1; Askew 2004; Schneider et al. 2015; Kalkman & Kitanova 2015). Here we augment Schneider et al.'s (2015) data set with specimens from northern and Eastern Europe to explore the phylogenetic and phylogenetic patterns, population genetics, and molecular divergence times in the *A. cyanea* complex in relation to Quaternary glacial cycles in the Western Palaearctic.

2. Material and Methods

2.1. Material

We sampled sixteen specimens of *Aeshna cyanea* from Europe. These samples were augmented with Genbank sequences from a further six European specimens, three North African specimens, nine specimens from the Caucasus region and three *A. vercanica* specimens from Iran/Azerbaijan. All Genbank sequences were included in Schneider et al.'s (2015) initial study of *A. cyanea/vercanica*. A single specimen of each of the species *Aehsna juncea* (Linneaus, 1758) and *Aeshna mixta* Latreille, 1805 were used as outgroups. The full data set thus comprise 39 specimens. All specimen data is given in Table 1. All voucher specimens sequenced for this study are deposited at the Natural History Museum Aarhus. The distribution and sample localities map in Fig. 1 was constructed by exporting a blank map from DIVA-GIS 7.5.0.0 using the global country boundaries dataset provided on the

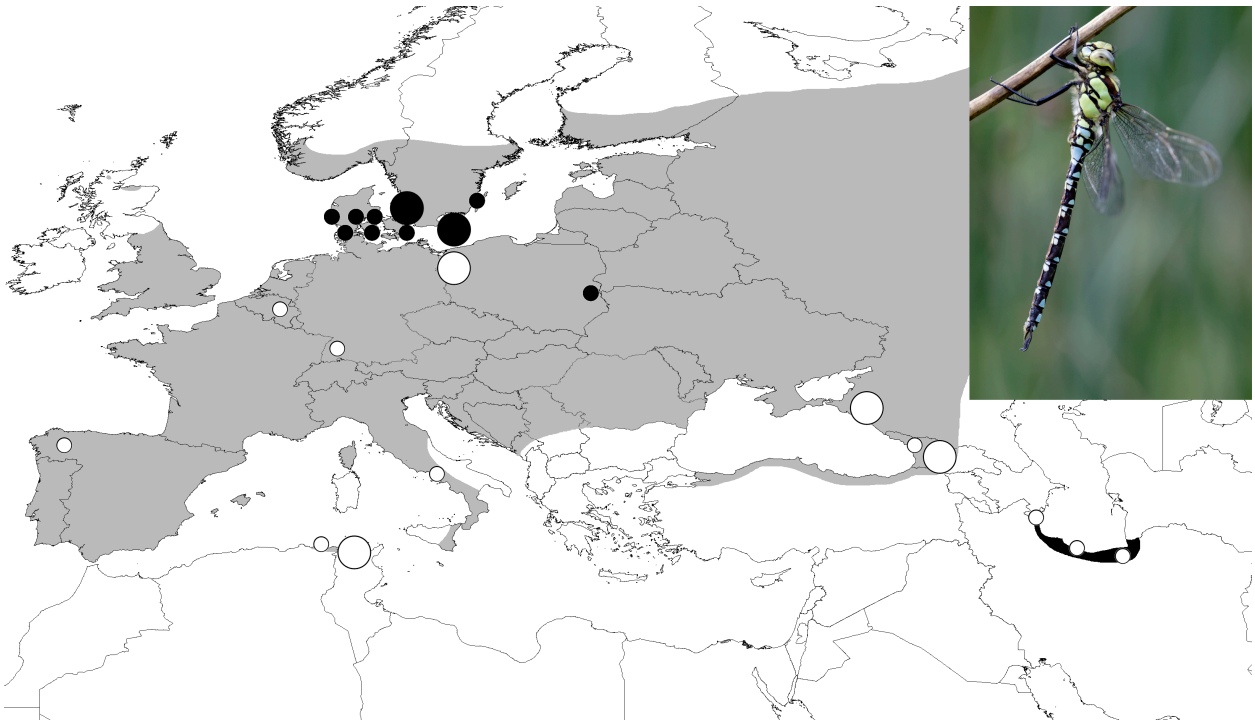

Fig. 1. Geographic range and sampling sites for *Aeshna cyanea* (inserted photo) and *A. vercanica*. Distribution of *A. cyanea* is shown in grey, while distribution of *A. vercanica* is shown in black. Open circles indicate sampling sites in Schneider et al. (2015), while filled circles indicate our sampling sites. Small circles indicate a single specimen, while large circles indicate several specimens. Modified from Schneider et al. (2015).

program's website (Hijmans et al. 2012). Species distributions (based on Schneider et al. 2015; Kalkman & Kitanova 2015), and sample localities (based on Schneider et al. 2015 and our sample data) were added manually in Adobe Photoshop CS2.

2.2. Methods

2.2.1. Laboratory procedures

Following Schneider et al. (2015) we targeted the barcode region of the mitochondrial gene COI (Hebert et al. 2003), and the full Internal Transcribed Spacer region (ITS region) in the nuclear genome. This allow us to utilise the sequences published by Schneider et al. (2015) and build directly on their work. COI has been widely used in species delineation and phylogeograpic studies in insects (e.g. Gratton et al. 2008; Simonsen et al. 2008; Hammaouti et al. 2009; Huemer 2011; Mutanen et al. 2012; Simonsen & Huemer 2014), although the use of a single, mitochondrial gene for such studies has been criticised (e.g. Dupois et al. 2012; Kondandaramaiah et al. 2013; Simonsen et al. 2019). The nuclear ITS region, which comprises the genes ITS1, 5.8S, and ITS2 has been used less frequently, but shows considerable inter- and intra-specific variation in insects (e.g. Weekers et al. 2001; Jödicke et al. 2003; Chobanov et al. 2016). As haplotype network analyses are sensitive to missing data, we trimmed the data sets to make sure that there was no missing data. The aligned combined data set is available as Supplementary Material S1.

DNA was extracted at Aarhus University (AU), Denmark using either E.Z.N.A. Tissue DNA Kit (Omega BIO-TEK) or DNeasy Blood & Tissue Kit (Qiagen). The E.Z.N.A. Tissue DNA Kit protocol was followed with some modifications: Samples were incubated at 42° C for 18–23 hours during lysis, steps 5 and 6 in the protocol were skipped, and samples were incubated with Elution Buffer for 5–10 min at 70° C and eluted once in 200 µl. The DNeasy Blood & Tissue Kit protocol was followed with some modifications: Samples were incubated at 42° C for 20 hours during lysis, elution buffer AE was heated to 60° C prior to elution, samples were incubated with buffer AE for 10 min at 60° C and eluted once in 100 µl. The lower lysis temperature compared to the 55–56° C in the manufacturers' protocols, follows Krosch & Cranston (2012), who demonstrated that a lower lysis temperature is suitable for longer lysis times.

We used the following PCR protocol for COI: 95° C, 2 min; then 35–45 cycles of 95° C, 30 s; 45° C, 30 s; 72° C, 1 min and a final extension of 72° C for 5 min using the primers OdoF2 (with universal tail): ***TGTAAAACGACGGCCAGT***TTTCTACAAAYCAY AARGATATTGG (tail in boldface italics); and OdoR3 (with universal tail): ***CAGGAAACAGCTATGAC***TAAA CYTCTGGRTGRCCAAARAATCA (tail in boldface italics). We used the same PCR protocol for ITS using the primers VRAIN2F (with universal tail): ***TGTAAAACGA CGGCCAGT***CTTTGTACACACCGCCCGTCGCT (tail

Table 1. Specimens used in this study with geo-groups, localities, voucher designations, and Genbank accession numbers provided.

Genus	Species	Geogroup	Country	Region	Voucher	Reference	Genbank# COI	Genbank # ITS
Aeshna	*juncea*	-	Denmark	West Jutland	ENT-DNA-249	New	MN847868	MN993616
Aeshna	*mixta*	-	Denmark	East Jutland	ENT-DNA-172	New	MN939165	MN993632
Aeshna	*vercanica*	-	Iran	—	ve1	Schneider et al. 2015	KU180302	KU180365
Aeshna	*vercanica*	-	Iran	—	ve2	Schneider et al. 2015	KU180303	KU180368
Aeshna	*vercanica*	-	Azerbaijan	Lankaran	ve3	Schneider et al. 2015	KU180322	KU180361
Aeshna	*cyanea*	Caucasus	Armenia	—	c18	Schneider et al. 2015	KU180315	KU180369
Aeshna	*cyanea*	Caucasus	Armenia	—	c2	Schneider et al. 2015	KU180316	KU180370
Aeshna	*cyanea*	Caucasus	Armenia	—	c5	Schneider et al. 2015	KU180319	KU180371
Aeshna	*cyanea*	Caucasus	Armenia	—	c12	Schneider et al. 2015	KU180321	KU180372
Aeshna	*cyanea*	Caucasus	Georgia	—	c17	Schneider et al. 2015	KU180314	KU180375
Aeshna	*cyanea*	Caucasus	Russia	Mostovsky, Caucasus	c15	Schneider et al. 2015	KU180312	KU180380
Aeshna	*cyanea*	Caucasus	Russia	Mostovsky, Caucasus	c16	Schneider et al. 2015	KU180313	KU180381
Aeshna	*cyanea*	Caucasus	Russia	Mostovsky, Caucasus	c1	Schneider et al. 2015	KU180317	KU180382
Aeshna	*cyanea*	Caucasus	Russia	Mostovsky, Caucasus	c4	Schneider et al. 2015	KU180318	KU180383
Aeshna	*cyanea*	North Africa	Algeria	—	c14	Schneider et al. 2015	KU180311	KU180374
Aeshna	*cyanea*	North Africa	Tunesia	—	c13	Schneider et al. 2015	KU180310	KU180385
Aeshna	*cyanea*	North Africa	Tunesia	—	c6	Schneider et al. 2015	KU180320	KU180386
Aeshna	*cyanea*	Europe	Belgium	—	c7	Schneider et al. 2015	KU180304	KU180373
Aeshna	*cyanea*	Europe	Germany	Baden-Württemberg	c10	Schneider et al. 2015	KU180307	KU180376
Aeshna	*cyanea*	Europe	Italy	—	c8	Schneider et al. 2015	KU180305	KU180377
Aeshna	*cyanea*	Europe	Poland	West Pomerania	c9	Schneider et al. 2015	KU180306	KU180378
Aeshna	*cyanea*	Europe	Poland	West Pomerania	c11	Schneider et al. 2015	KU180308	KU180379
Aeshna	*cyanea*	Europe	Spain	—	c3	Schneider et al. 2015	KU180309	KU180384
Aeshna	*cyanea*	Europe	Poland	Biala Podlaska	ENT-DNA-1131	New	MN939093	MN963665
Aeshna	*cyanea*	Europe	Sweden	Öland	ENT-DNA-989	New	MN939088	MN963656
Aeshna	*cyanea*	Europe	Denmark	Bornholm	ENT-DNA-135	New	MN939079	MN963667
Aeshna	*cyanea*	Europe	Denmark	Bornholm	ENT-DNA-136	New	MN939080	MN963658
Aeshna	*cyanea*	Europe	Denmark	Bornholm	ENT-DNA-344	New	MN939085	MN963666
Aeshna	*cyanea*	Europe	Denmark	Bornholm	ENT-DNA-345	New	MN939086	MN963668
Aeshna	*cyanea*	Europe	Denmark	Bornholm	ENT-DNA-346	New	MN939087	MN963662
Aeshna	*cyanea*	Europe	Denmark	Northeast Zealand	ENT-DNA-347	New	MN939090	MN963670
Aeshna	*cyanea*	Europe	Denmark	Northeast Zealand	ENT-DNA-348	New	MN939091	MN963663
Aeshna	*cyanea*	Europe	Denmark	Northeast Zealand	ENT-DNA-349	New	MN939092	MN963664
Aeshna	*cyanea*	Europe	Denmark	Møn	ENT-DNA-342	New	MN939083	MN963669
Aeshna	*cyanea*	Europe	Denmark	Fyn	ENT-DNA-332	New	MN939089	MN963660
Aeshna	*cyanea*	Europe	Denmark	East Jutland	ENT-DNA-112	New	MN939078	MN963657
Aeshna	*cyanea*	Europe	Denmark	East Jutland	ENT-DNA-343	New	MN939084	MN963661
Aeshna	*cyanea*	Europe	Denmark	South Jutland	ENT-DNA-176	New	MN939082	MN963659
Aeshna	*cyanea*	Europe	Denmark	West jutland	ENT-DNA-175	New	MN939081	MN963655

in boldface italics); and VRAIN2R (with universal tail): ***CAGGAAACAGCTATGAC***TTTCACTCGCCGTTA CTAAGGGAATC (tail in boldface italics). The COI primers were developed for this study, while the ITS region primers were from Félix et al. (2001). All samples were sequenced at Macrogen Europe using the Sanger Method. Contigs and consensus sequences were obtained using DNA Baser Sequence Assembler v5.8.0 (Heracle Biosoft 2018). We checked the identity of all sequences using BLAST on GenBank and/or BOLD (Barcode Of Life Data base) Identification System.

2.2.2. Phylogenetic analysis and molecular dating

We analysed the combined COI + ITS data set in MrBayes v3.2.5 (Ronquist et al. 2012). The data set was divided into two partitions (COI and ITS region), for each partition we allowed the program to assess the best model for evolution (nst = mixed) with a gamma distribution. The analysis was run for 100 million generations with sampling every 10,000 generations, and the first 50% were discarded as burnin. We allowed Mr Bayes to access the best model rather than using a dedicated software package for this purpose following the recommendation in the Mr Bayes v3.2.5 manual (Ronquist et al. 2012). To avoid potential over-parameterisation, we did not partition the COI dataset into codon partitions.

We ran molecular dating analyses in BEAST v2.6 in conjunction with BEAUti v2.6 (Bouckaert et al. 2019). As in MrBayes the data was partitioned into COI and the ITS region, while the substitution model for both partitions was set to GTR with a gamma distribution based on the model assessed by MrBayes, and the tree prior set to the Yule Model; all other priors were left as default. We first ran an exploratory analysis in BEAST for 100 million generations with sampling every 10,000 generation to compare the result to the result obtained from the analysis in MrBayes. We then fixed the topology in BEAST to correct the major difference between the analyses (i.e. including the Spanish specimen in *A. cyanea* Europe – see Results), and set the clock to Relaxed Exponential with the clock rate left to 1.0, and the age of their last common ancestor was set to 1.5 million years (based on an uncorrected *p* COI divergence rates of 0.036 – see Results) with a sigma value of 1.0. The new analysis was run for 10 million generations. To test the stability of the result we reran the latter analysis twice with the age of the last common ancestor of *A. vercanica* + *A. cyanea* set to 1.0 million years and 2.0 million years, respectively. In the two latter analyses the sigma values were set to 0.5 and 1.5, respectively. In all BEAST analyses we constructed a consensus tree in TreeAnnotator 2.6.0 (part of the BEAST package) discarding the first 50% of the trees as burnin. The log files from MrBayes and BEAST were examined in Tracer 1.6.0 (part of the BEAST package), and trees were analysed in FigTree 1.4.2 (part of the BEAST package).

2.2.3. Phylogeographic analyses, genetic diversity and population genetics

Prior to running the phylogeographic and population genetic analyses, we removed outgroups, otherwise the data sets were identical to the data sets used for phylogenetic analyses. We constructed median-joining haplotype networks (Bandelt et al. 1999) for the individual COI and ITS data sets, and for the combined data set in Network v5.0.1.1 (available at: fluxus-engineering.com).

We separated the samples into populations to explore genetic diversity and population genetic patterns. We treated *A. vercanica* (3 specimens) as a single population, whereas *A. cyanea* was split into three population geo-groups based on geography: *A. cyanea* Caucasus (9 specimens), *A. cyanea* North Africa (3), and *A. cyanea* Europe (22) (Table 1). We calculated the average uncorrected *p* distance for each gene individually between populations and within populations in MEGA 7 (Kumar et al. 2016). The uncorrected *p* distance has been used in a wide range of studies, and employing it here allows for direct comparison to earlier results. The within and between population values for COI and the ITS region are shown in Tables 2 and 3, respectively. To assess how isolated the populations are we calculated the F_{ST} value between populations based on the COI data set in Arlequin 3.5.2.2 (Excoffier et al. 2005) with 10,000 permutations to test for statistical significance. F_{ST} values are shown in Table 4.

3. Results

3.1. Phylogenetics and phylogeography

The 39 taxa data set for the phylogenetic analyses was comprised by 603 bp COI, and 733 bp ITS region including gaps. The 37 taxa data set for the haplotype network analyses was comprised by 603 bp COI and 702 bp ITS. The difference in the ITS data set length corresponds to gaps in the alignment that includes outgroup taxa, whereas without outgroups, the alignment had no gaps. 16 different haplotypes were identified in the ingroup in the COI data set, and eleven haplotypes were identified in the ingroup in the ITS region data set.

The analysis in MrBayes (Supplementary Figure S2) recovered the '*A. cyanea*-group' (*A. vercanica* + *A. cyanea*) as monophyletic with strong support (pp = 1), just as *A. vercanica* and *A. cyanea* are recovered as reciprocally monophyletic with strong support (pp = 1 and 0.996, respectively). *Aeshna cyanea* Caucasus is not recovered as monophyletic, but forms as paraphyletic grade at the base of *A. cyanea*. *Aeshna cyanea* Europe and *A. cyanea* North Africa are recovered as reciprocally monophyletic sister groups in a well-supported western clade (pp = 0.9998), but support for a monophyletic *A. cyanea* Europe group is very low (pp = 0.52), while support for a monophyletic *A. cyanea* North Africa group is strong (pp = 1). The

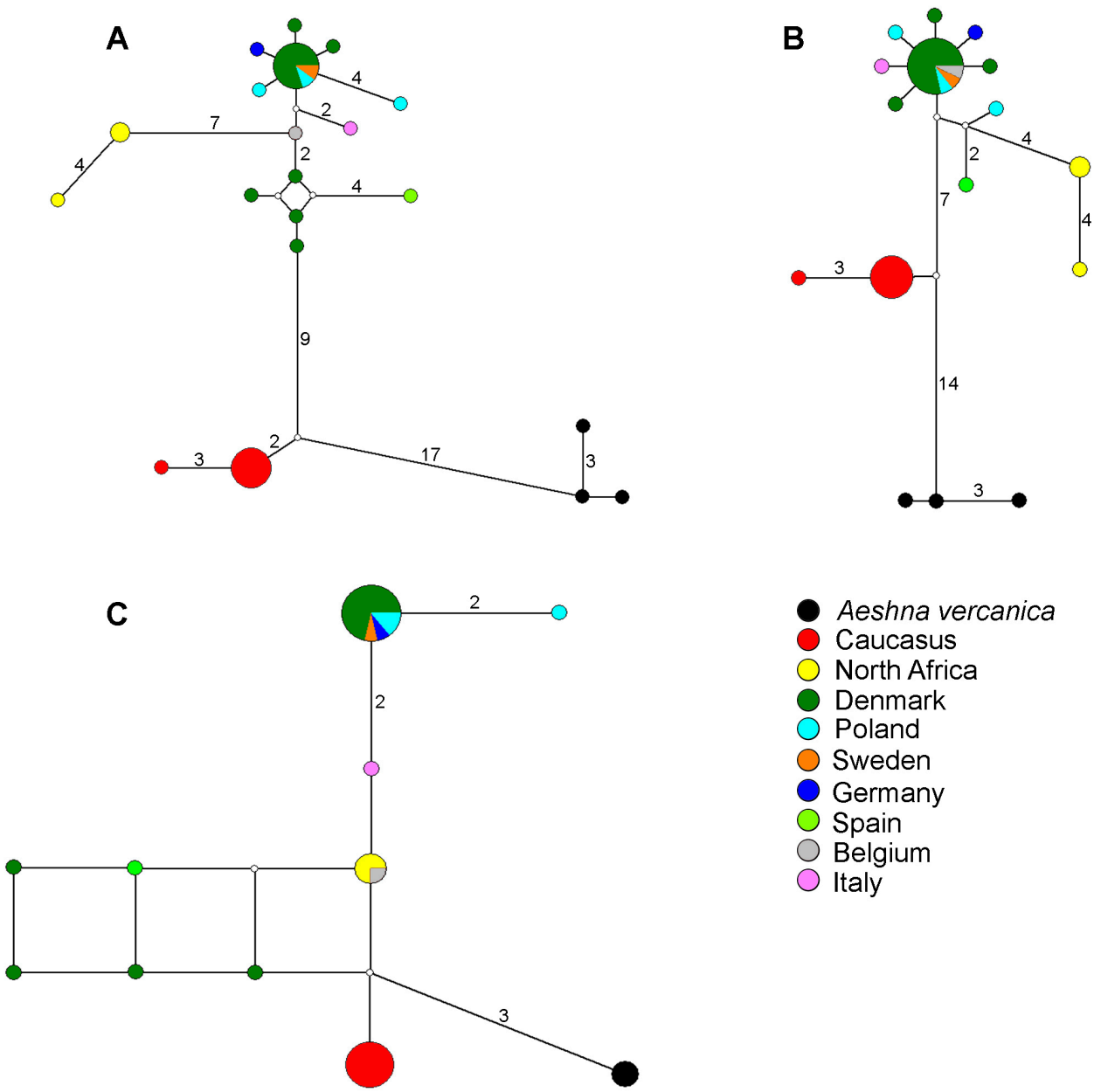

Fig. 2. Median-joining haplotype networks of *Aeshna cyanea* and *A. vercanica*. Numbers next to a branch indicate number of mutations. **A**: COI + ITS region; **B**: COI; **C**: ITS region.

unconstrained analysis in BEAST (Supplementary Figure S3) returned an overall similar result with two important differences: 1) *A. cyanea* Caucasus is recovered as monophyletic with strong support (pp=0.9892) and comprise the sister group of the remaining *A. cyanea*; 2) The single Spanish specimen is removed from *A. cyanea* Europe and is placed as sister to *A. cyanea* North Africa. However, the support for this placement is poor (pp= 0.6589). The support for the monophyly of the remaining European specimens remains poor (pp=0.6709).

The median-joining haplotype networks display the same overall pattern (Fig. 2). In the network based on the combined data set (Fig. 2A) *A. vercanica* is connected to the remaining network by a very long branch that connects to a median vector. *Aeshna cyanea* Caucasus connects to the same median vector by a short branch, which also connects via a long branch to an overall group comprising the North African and European specimens. *Aeshna cyanea* North Africa connects directly by a long branch to the complex *A. cyanea* Europe. The network based on the COI data set (Fig. 2B) shows the same general relationships, with the only difference being that *A. cyanea* North Africa via a medium length branch are connected to a median vector they share with the Spanish and one Polish specimen. This group is connected to the remaining European specimens. The network based on the ITS region data set (Fig. 2C) is somewhat different, but overall compatible to the other analyses; while *A. vercanica* and *A. cyanea* Caucasus are still separated from the remaining specimens, the European and North African specimens are not separated as the North African specimens and a single Belgian specimen share a unique

Table 2. Average uncorrected *p* values for COI within and between geo-groups of *A. cyanea*, and *A. vercanica*.

COI, average	Europe	North Africa	Caucasus	*A. vercanica*
Europe	0.002			
North Africa	0.013	0.004		
Caucasus	0.016	0.021	0.001	
A. vercanica	0.039	0.044	0.028	0.004

Table 3. Average uncorrected *p* values for the ITS region within and between geo-groups of *A. cyanea*, and *A. vercanica*.

ITS, average	Europe	North Africa	Caucasus	*A. vercanica*
Europe	0.002			
North Africa	0.002	0.000		
Caucasus	0.005	0.003	0.000	
A. vercanica	0.008	0.006	0.006	0.000

Table 4. Pairwise F_{ST} values between geo-groups of *A. cyanea*, and *A. vercanica*. Numbers in boldface are statistically significant.

Fst, pairwise	Europe	North Africa	Caucasus
North Africa	**0.81849**		
Caucasus	**0.89558**	**0.91336**	
A. vercanica	**0.94409**	0.90000	**0.93460**

haplotype. The combined *A. cyanea* Europe and *A. cyanea* North Africa group remain separated from *A. vercanica* and the *A. cyanea* Caucasus group.

3.2. Genetic diversity

The uncorrected *p* distance for COI (Table 2) within geo-groups is very low and ranged from 0.001 to 0.004. The same distance between populations was approximated an order of magnitude higher ranging from 0.013 to 0.044, but there were considerable differences between the different populations. *A. cyanea* Europe and *A. cyanea* North Africa were most similar with a value of 0.013, while the distance between *A. cyanea* Europe and *A. cyanea* Caucasus, and *A. cyanea* North Africa and *A. cyanea* Caucasus were 0.016, and 0.021, respectively. Not surprisingly, *A. vercanica* and the various *A. cyanea* showed the greatest differences. However, the distance values varied from 0.028 between *A. vercanica* and *A. cyanea* Caucasus, to 0.039 and 0.044 between *A. vercanica* and *A. cyanea* North Africa, and *A. vercanica* and *A. cyanea* Europe, respectively. The average uncorrected *p* distance between *A. vercanica* and all *A. cyanea* was 0.036. The uncorrected *p* distance for the ITS region (Table 3) within populations was 0 for all populations except *A. cyanea* Europe, where it was 0.002. The uncorrected *p* distance between populations was low between all populations, but lower between *A. cyanea* populations (0.002–0.005) than between *A. vercanica* and the different *A. cyanea* populations (0.006–0.008). The pairwise F_{ST} values (Table 4) are high (0.81849–0.94409) between all populations. F_{ST} values range from 0.0 to 1.0 and indicates how isolated two populations are from each other with 0.0 indicating no isolation with free interbreeding, and 1.0 indicating full isolation and no interbreeding (e.g. Meirmans & Hedrick 2011). Our results thus indicate that all the populations display a high degree of isolation, with *A. cyanea* Europe and *A. cyanea* North Africa with an F_{ST} value of 0.82 being least isolated, while the other population pairs, including *A. vercanica* and the different *A. cyanea* populations, show a higher and similar degree of isolation ($F_{ST} \approx 0.90–0.94$).

3.3. Divergence times

As outlined above we used an enforced topology in the divergence time analysis in BEAST (Fig. 3). *Aeshna cyanea* Caucasus is non-monophyletic in the MrBayes analysis, but monophyletic with strong support in the BEAST analysis. The group also appear united in all the haplotype analyses, and the average uncorrected *p* value between the specimens in the group is the lowest of all the groups. We therefore allowed it to remain monophyletic in the divergence time analysis without any constrains. However, we enforced monophyly of all European specimens including the single specimen from Spain. The Spanish specimen grouped with the North African specimens in the unconstrained BEAST analysis, but it was nested within the European specimens in the MrBayes analysis (albeit with weak support in both analyses). It also appears closer associated with the European specimens in all three haplotype network analyses. As there are no fossil records that can be assigned to the *A. cyanea* group, and no phylogeny of *Aeshna* with divergence times has been published, we rely on COI divergence to calibrate the dating analysis in BEAST. Following Brower's (1994) estimate of an average COI evolution rate of 2% pr. million year, we set the split between

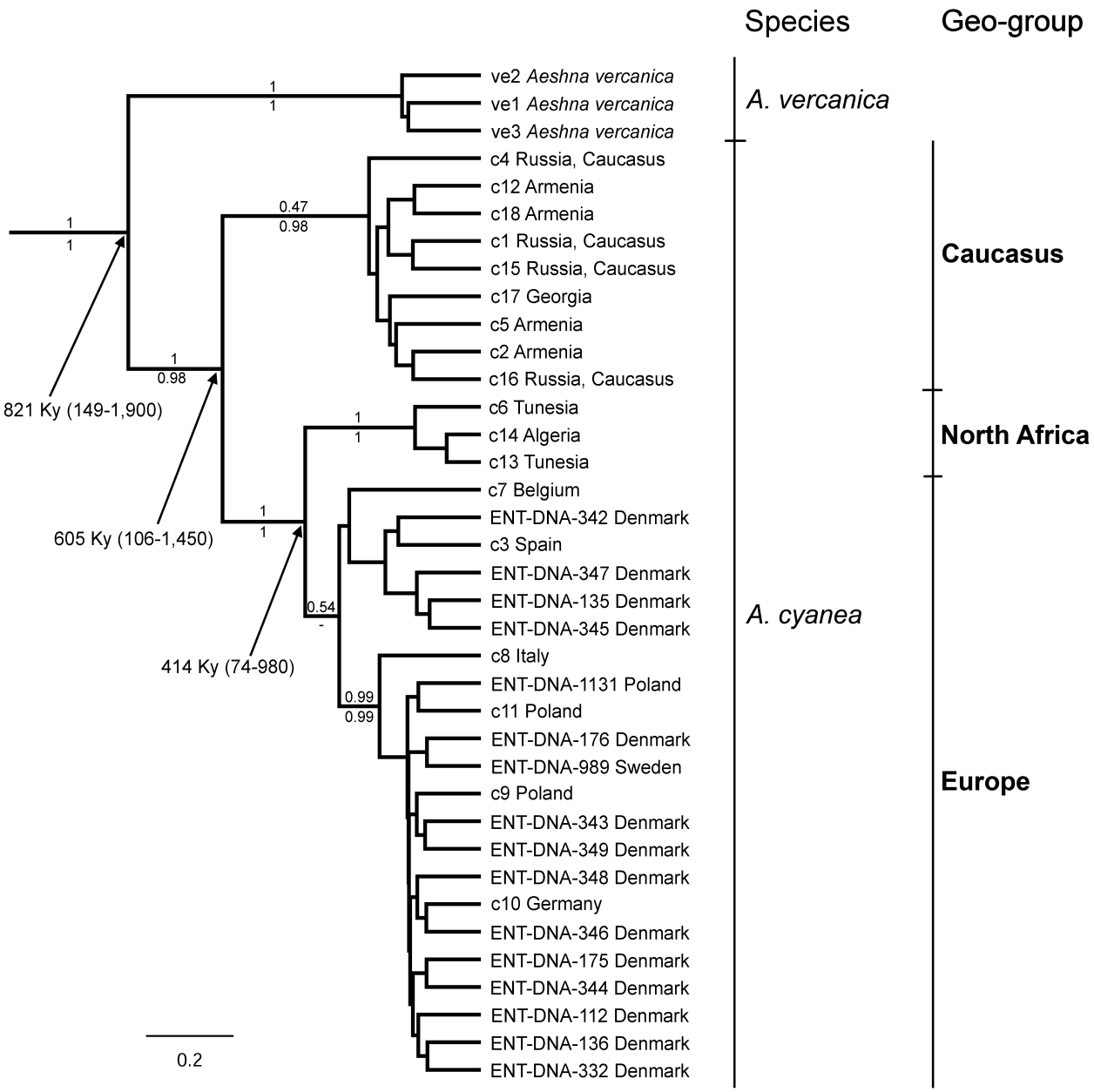

Fig. 3. Timed phylogram from the 10 million generation constrained BEAST analysis. Timing of the major splits discussed in the text are indicated by arrows. Numbers above a branch are posterior probabilities from Mr Bayes, while numbers below a branch are posterior probabilities from BEAST.

A. vercanica and *A. cyanea* (uncorrected $p = 0.036$) to 1.5 million years +/– 1.0 million years, with settings otherwise as explained above. The resulting tree with the dating values for the major splits is shown in Fig. 3. The split between *A. cyanea* and *A. vercanica* is estimated to be *ca* 0.82 mya (0.15–1.90 mya); the split between *A. cyanea* Caucasus, and *A. cyanea* North Africa + *A. cyanea* Europe is estimated to be *ca* 0.61 mya (0.106–1.45 mya), while the split between *A. cyanea* North Africa and *A. cyanea* Europe is estimated to be *ca* 0.41 mya (0.07–0.98 mya). The divergence times were only slightly higher in the analysis where the split between *A. vercanica* and *A. cyanea* was set to 2.0 million years, and slightly lower when it was set to 1.0 million years (results not shown).

4. Discussion

Our results confirm the conclusion by SCHNEIDER et al. (2015) that *A. vercanica* and *A. cyanea* form two distinct and well-separated clades. While SCHNEIDER et al. (2015) were not able to resolve the relationship between *A. vercanica*, *A. cyanea* Caucasus, and *A. cyanea* North Africa + *A. cyanea* Europe, our results clearly place *A. vercanica* as the sister taxon of *A. cyanea*. The very high pairwise F_{ST} values (Table 4) between *A. vercanica* and the three *A. cyanea* geo-groups ($F_{ST} \geq 0.9$) indicating virtually no interbreeding between *A. vercanica* and *A. cyanea* also support the full species status of the former. Interestingly the lowest pairwise F_{ST} value (0.9) is

between *A. vercanica* and *A. cyanea* North Africa, and this is also the only pairwise F_{ST} value that is not statistically significant. Given the considerable geographical and phylogenetic distance between the two groups, we consider this most likely an analytical abnormality, possibly due to the low sample size of the two populations. We also confirm Schneider et al.'s (2015) initial observations that *A. cyanea* is divided into three distinct and geographically separated sub-clades: one in Caucasus; one in northwest Africa; and one in Europe. Although our Bayesian analysis in MrBayes (Supplementary Figure S2) fail to recover *A. cyanea* Caucasus as monophyletic (a result similar to Schneider et al. (2015)), the Bayesian analysis in BEAST (Supplementary Figure S3), and all haplotype network analyses (Fig. 2A–C) recover the group as monophyletic, and distinct from the other *A. cyanea* geo-groups. Schneider et al. (2015) considered the Caucasus group to be a loosely related group, but our results – especially the very low internal uncorrected *p* distance (Tables 2–3), and the high F_{ST} values (Table 4) compared to other groups – indicate that the group is both well-defined and isolated. Schneider et al. (2015) considered *A. cyanea* North Africa and *A. cyanea* Europe to comprise a single subclade related to the western Mediterranean refugium. Although our results confirm that the two groups are closely related and form a distinct clade, there are also clear indications that they form two separate groups. Our Bayesian analyses have difficulties placing the Spanish specimen clearly in one of the groups with the unconstrained BEAST analysis placing it with the North African group (Supplementary Figure S3), albeit with very low support (pp≈0.65), and MrBayes placing it with the European group (Supplementary Figure S2) with very low support (pp≈0.52). However, the haplotype networks do place it with the other European specimens (Fig. 2A–C), and while the pairwise F_{ST} value between *A. cyanea* North Africa and *A. cyanea* Europe (including Spain) is the lowest in the analysis, it is still very high (0.81849) and indicate a high degree of isolation between the groups (Table 4).

It is interesting to compare out results to Hinojosa et al.'s (2017) study of another dragonfly, *Sympetrum vulgatum*. This species is widespread in the Palearctic region and divided into three subspecies: *S. vulgatum decoloratum* in Caucasus and Anatolia; *S. vulgatum ibericum* in the Iberian Peninsula; and *S. vulgatum vulgatum* in the rest of Europe (Hinojosa et al. 2017). Based on COI and the ITS region they found that the three subspecies are separated, but that the genetic divergence in COI is low (0.1–0.4%), while there was no difference in the ITS region. Interestingly, they found that *S. vulgatum vulgatum* from Europe and *S. vulgatum decoloratum* from Caucasus and Anatolia were more similar to each other (COI divergence = 0.1%) than either were to *S. vulgatum ibericum* from Spain (COI divergence = 0.3–0.4%). Their results are in other words more or less the opposite of ours. It is also worth noting that the three well-defined subspecies in *S. vulgatum* display an ***inter***-subspecific variation in COI that is directly comparable to the ***intra***-population variation we find in the *A. cyanea* group.

The deep split between populations in the Caucasus, and populations in Europe in general was also found by Kamp et al. (2018) in a detailed study of the Middle-Spotted Woodpecker based on several mitochondrial and nuclear markers. Several studies over the past few years have found evidence of a Pleistocene glacial refugium in Northern Africa (reviewed by Husemann et al. 2014). Ferreira et al. (2016) studied the damselfly *Coenagrion puella* throughout the Western Palaearctic. They found no pattern in Europe – including the Iberian Peninsula and Russian Caucasus, but found that North African populations were highly distinct regardless of the genetic markers used. el Mokhefi at al. (2016) also found that the pine processionary moth (*Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775)) potentially has several North African refugia. The close connection we find between North Africa and the Iberian Peninsula is supported by several previous studies. Joger et al. (2007: reptiles), Habel et al. (2011: butterflies), and Lefebvre et al. (2016: termites) show that western North African and Iberian populations are often closely related, even if they are distinct today. Similarly, Husemann et al. (2014) considered the Tunisian-Algerian-Moroccan northern regions to have comprised a single Atlanto-Mediterranean glacial refugium together with the Iberian Peninsula.

Although the divergence time analysis in BEAST is uncertain, our results do indicate the timing of the events that have shaped the current distribution of the *A. cyanea* complex in Western Palearctic. Schneider et al. (2015) suggested that the original spilt between *A. vercanica* and *A. cyanea* occurred in the Hyrcanian Forest in what is today northern Iran. Our analysis dates this split to have occurred approximately 0.82 mya, which coincides with climatic changes at the beginning of the Cromerian 'complex' in the 'North Western Europe stage' (Ehlers & Gibbard 2008, Gibbard & Cohen 2008, Houmark-Nielsen et al. 2012), and the end of the Krinitisian/Krinitsa period in the 'Russian Plain Stages' (Gibbard & Cohen 2008). During the interglacial periods in the mid-Cromerian (*ca* 0.7–0.6 mya: Gibbard & Cohen 2008, Houmark-Nielsen et al. 2012), *A. cyanea* may have spread west and southwest through Anatolia and the Middle East to North Africa, and further into the Maghreb region along the southern Mediterranean coast to Tunisia, Algeria and Morocco. During the various inter-glacial cycles in the mid-Cromerian (Ehlers & Gibbard 2008, Gibbard & Cohen 2008, Houmark-Nielsen et al. 2012), populations in the Middle East and eastern North Africa may have become extinct leading to genetic isolation and eventually the split between *A. cyanea* Caucasus and the ancestor of *A. cyanea* North Africa + Europe around 0.6 mya – perhaps further enforced by the onset of the Donian Glaciation in the 'Russian Plain Stages' (mid-Cromerian) *ca* 0.5–0.6 mya (Gibbard & Cohen 2008). Prior to the Holsteinian interglacial period (*ca* 0.4–0.39 mya, (Gibbard & Cohen 2008, Houmark-Nielsen et al. 2012)) *A. cya-*

nea probably dispersed from western North Africa to the Iberian Peninsula, and the populations on either side of the Strait of Gibraltar probably formed a loosely connected meta-population until they were finally separated *ca* 0.41 mya at the start of the Holsteinian interglacial period (GIBBARD & COHEN 2008, HOUMARK-NIELSEN et al. 2012). Finally, *A. cyanea* probably spread from the Iberian Peninsula throughout Europe following the isolation in the Iberian Peninsula. However, our data does not provide sufficient resolution to allow us to assess whether this dispersal has happened during some warming periods in the Saale Glaciation (*ca* 390–130 kya), the Eemian Interglacial (*ca* 130–115 kya), the Weichsel Glaciation (*ca* 115–12 kya), or the warming in early Holocene (*ca* 11.7 kya) (GIBBARD & COHEN 2008, HOUMARK-NIELSEN et al. 2012, HELMENS 2014). The relatively high genetic variation within *A. cyanea* Europe indicates that the European population has not been through a recent genetic bottleneck like e.g. *Sympetrum vulgatum* (HINOJOSA et al. 2017) or *Nehalennia speciosa* (BERNARD et al. 2011). The colonization of Europe from a North African-Iberian refugium may fit a general picture (e.g. HUSEMANN et al. 2014), but the scenario is not completely uncontroversial. HUSEMANN et al. (2014) also mention North Africa – Italy (via Sicily) as a common colonization route, and DAPPORTO et al. (2011) demonstrate this pattern for the butterfly *Maniola jurtina* (Linneaus, 1758). Nevertheless, the Italian specimen in our data set clearly group with northern European taxa in a separate subgroup (with strong support in the Bayesian analyses) (Figs 2A–C, 3; Supplementary Figures S2–S3), despite the fact that the current North African populations are closer to Sicily than they are to the Iberian Peninsula (Fig. 1).

Despite our reasonably clear results, we must urge some caution as *A. cyanea* occurs beyond our sampled area throughout Eastern Europe and deep into Russia to the southern Ural Mountains (KALKMAN & KITANOVA 2015). It is quite possible that populations in Russia and Ukraine are more closely related to *A. cyanea* Caucasus than to *A. cyanea* Europe. However, we do have specimens from eastern Poland and the Swedish island Öland in the Baltic Sea, which clearly belong in *A. cyanea* Europe. Some previous studies on other taxa, that have shown an East-West split in Europe, have placed specimens from Öland (TINNERT et al. 2016) and eastern Poland (SZTENCEL-JABŁONKA et al. 2015) in eastern groups clearly distinct from Western Europe.

5. Conclusions

The *Aeshna cyanea* complex appears to be an excellent model system for studying the phylogeography and responses to Quaternary climate and landscape changes of Western Palaearctic insects, and genome based studies with a broad taxon sampling of the group should be a priority.

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Authors' contributions

T.J.S. and K.O. designed the study, secured funding and collected material. M.D. carried out laboratory work, data mining and the initial analyses. T.J.S. carried out the bulk of the analyses and drafted the text. All authors contributed to the Discussion and the final version of the paper.

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File 1: Supplementary Data S1.nex – MrBayes Nexus file of the combined COI and ITS region data set)

File 2: Supplementary Figures.pdf — **Fig S2:** Tree from the 100 million generation analysis in MrBayes. — **Fig. S3:** Tree from the unconstrained 100 million generation analysis in BEAST.