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ORIGINAL ARTICLE

Tests of multiple molecular markers for the identification of Great Spotted and Syrian Woodpeckers and their hybrids

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Abstract Great Spotted and Syrian Woodpeckers (*Dendrocopos major* and *D. syriacus*) are known to hybridize in nature; however, the extent of this phenomenon is not known due to difficulties in hybrid detection based on plumage analyses. Here, we tested five markers (one mitochondrial and four nuclear) and a set of six microsatellite loci for the identification of these two Woodpeckers and their hybrids. Sequencing of DNA from 26 individuals of both Woodpeckers from different parts of their ranges: one allopatric (*D. major*; Norway) and two sympatric (Poland and Bulgaria) showed that both species can be clearly separated based on all sequence markers. The highest number of fixed nucleotide sites were found in the mtDNA control region and intron 5 of the transforming growth factor. Analyses of microsatellite data distinguished the two species, but all loci showed a large number of common alleles and their utility in identifying hybrids is therefore doubtful. According to the DNA sequence analyses, 2 out of 18 specimens within the sympatric range in Poland were identified as possible hybrids, most probably paternal backcrosses. Moreover, both hybrids are from synantropic populations (settled in cities), whereas none of the *D. major* sampled in forests and in its allopatric range (Norway) showed signs of an intermixed genotype. Further research on hybridization and introgression in woodpeckers is undoubtedly needed and could be useful for understanding ecological and ethological interactions among these species, particularly for *D. syriacus*, which is relatively rare in Europe.

Keywords Mitochondrial DNA · Microsatellites · Hybridization · Picidae · *Dendrocopos major* · *Dendrocopos syriacus*

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Zusammenfassung

Multiple molekulare Marker zur Identifizierung des Buntspechts, des Blutspechts und ihrer Hybriden

Buntspechte und die Blutspechte (*Dendrocopos major* und *D. syriacus*) sind dafür bekannt, in der Natur zu hybridisieren, aber das Ausmaß dieses Phänomens ist aufgrund von Schwierigkeiten bei der Erkennung von Hybriden auf der Basis von Gefiederanalysen nicht bekannt. Wir testeten fünf verschiedene molekulare Marker (einen mitochondrialen und vier nukleare) und einen Satz von sechs Mikrosatelliten-Loci für die Identifizierung dieser beiden Spechtarten und ihren Hybriden. Die Sequenzierung der DNA von 26 Individuen beider Arten aus verschiedenen Regionen ihres Verbreitungsgebietes, eine allopatrische (*D. major* - Norwegen) und zwei sympatrische (Polen und Bulgarien) zeigten auf, dass beide Arten auf der Basis von allen Sequenzmarkern eindeutig identifiziert werden können. Die größte Anzahl fester Nukleotid-Stellen wurde in der mtDNA-Kodierungsregion und im Intron 5 des transformierenden Wachstumsfaktors gefunden. Die Daten aus der Mikrosatellitenanalyse ermöglichen es zwar, die beiden Arten zu unterscheiden, aber alle Loci zeigten eine große Anzahl von gemeinsamen Allelen, wodurch ihr Nutzen bei der Identifizierung von Hybriden zweifelhaft ist. Die DNA-Sequenzanalyse zeigte, dass 2 von 18 Proben aus dem sympatrischen Areal in Polen als mögliche Hybride identifiziert wurden und wahrscheinlich aus väterlichen Rückkreuzungen stammen. Darüber hinaus sind beide Hybriden aus städtischen Populationen, während keiner der *D. major* in Wäldern und in seinem allopatrischen Verbreitungsgebiet (Norwegen) Anzeichen eines vermischten Genotyps zeigte. Weitere Untersuchungen der Hybridisierung und Introgression bei Spechten sind zweifellos notwendig und hilfreich für das Verständnis der ökologischen und ethologischen Interaktionen zwischen diesen Arten, insbesondere für *D. syriacus*, welcher in Europa relativ selten vorkommt.

Introduction

Hybridization is caused by incomplete or ineffective reproductive isolation mechanisms. Most examples of interspecific breeding concern closely related species pairs or allospecies within superspecies groups (e.g., Randler 2002). These species are likely to be genetically compatible and often have similar life histories and behavior that enables mating. Hybridization occurs often in situations related with range and abundance shifts. One possibility is when two species came into contact at the borders of their ranges. The second scenario is when one of the two species expands its range (naturally or via introduction) and is much less common than the local relative in the new area. The third example may happen when at least one of the species seriously declines in part of its range. Hence, in all these situations, the pressure of breeding needs may force an individual of one species (less common) to accept an individual of the other species as a mate, resulting in hybrid offspring (Hubbs 1955; Randler 2002; Aliabadian and Nijman 2007). However, there are also examples of continuing hybridization between two species which become similarly common in some areas (e.g., Great Spotted *Dendrocopos major* and Syrian Woodpeckers *D. syriacus*; Gorman 1997).

Interspecific breeding and hybrids are hard to detect and study, but such situations are relatively common within the order of birds (Aves), as almost 20 % of bird species can hybridize (Panov 1989; Grant and Grant 1992; Randler 2002; McCarthy 2006). Among woodpeckers (family Picidae), several species pairs are known to hybridize (Short 1982; Randler 2002). Most examples concern strictly American genera: *Campephilus*, *Sphyrapicus*, *Melanerpes*, *Celeus*, *Centurus*, *Veniliornis*, and *Picumnus* (Selander and Giller 1959; Johnson and Johnson 1985; Seneviratne et al. 2012; Fuchs et al. 2013). Examples are also known within African *Campethera* woodpeckers (Short 1982), *Picus* (*viridis/canus*) in Eurasia (Beuch 2012), *Dryocopus* (*schulzi/linneatus*) in South America (Madroño Nieto and Pearman 1992), and among a few *Picoides* species in North America (Miller 1955; Short 1982). Within the genus *Dendrocopos*, the most often hybridizing woodpecker is probably the Great Spotted Woodpecker as it can mate and breed with: Syrian Woodpecker (Winkler 1971; Skakuj and Stawarczyk 1994; Gorman 1997; Dudzik and Polakowski 2011), Sind Woodpecker *D. assimilis* (Short 1982), and White-backed Woodpecker *D. leucotos* (Laine 1993). Hybrids between *D. syriacus* and *D. assimilis* are also known (Short 1982). *Dendrocopos major* and *D. syriacus* are considered as sister species, and they differ in some morphological (plumage), behavioral (e.g., calls), and ecological (habitat preferences) characteristics. *D. major* is the most common woodpecker in Eurasia, and its range covers almost the whole temperate zone. *Dendrocopos syriacus* originally inhabited only south-west Asia. However, since the end of the nineteenth century, it has expanded into the Balkans (Reister 1894; Kohl 1954), and in the second half of the twentieth century, it has reached central (Keve 1955) and eastern Europe (Marisova 1964) as far north as Poland (Ciosek and Tomiałojć 1982). This species is still relatively rare in Europe (BirdLife International 2012) and is protected under the Bird Directive of European Union (2009/147/EC). In central Europe, *D. major* and *D. syriacus* are sympatric (supplementary Fig. 1), but they rarely breed in

the same areas. *Dendrocopos major* is generally a forest-dwelling species (e.g., Michalek and Miettinen 2003), whereas *D. syriacus* inhabits parks, gardens, and scattered riparian forests (Michalczuk and Michalczuk 2011; Kajtoch 2012; Ciach and Fröhlich 2013). Hybrids of these two woodpeckers were first noted in Hungary (Keve 1955; Gorman 1997). During the last 3 decades (1980–2010), 17 observations of mixed pairs or hybrids were documented in Poland (Dudzik and Polakowski 2011); however, this count is probably highly underestimated as several such examples have been noted in Poland in recent years (http://clanga.com/). These data suggest that hybridization between the species may not be a rare phenomenon and that hybrid individuals should be present in sympatric populations. Winkler (1971) and Short (1982) suggested a moving hybrid zone between these two species in Europe while *D. syriacus* expands into central Europe, but the interbreeding tended to decline rapidly once *D. syriacus* became established. This is probably a misconception, as hybrids are being found in central Europe in areas with abundant *D. syriacus* populations (Gorman 1997). Identification of hybrids of *D. major* and *D. syriacus* was previously described based on plumage differences (Skakuj and Stawarczyk 1994; Gorman 2004; Dudzik and Polakowski 2011, see also supplementary Fig. 2). However, these studies did not include identification of possible backcrosses and did not show any information about hybrid frequency in mixed populations. Hybrids cannot always be detected and identified using just morphological features (Senn and Pemberton 2009). Morphological variables can allow for the identification of first-generation (F1) hybrids but, on the other hand, backcrosses (hybrids in further generations) are often indistinguishable from one of the parent species (Senn and Pemberton 2009).

Nowadays, advances in molecular techniques allow for species and hybrid identification based on DNA analyses. The utility of mitochondrial DNA, most often used in phylogenetics and phylogeographic studies and also for species barcoding (Hebert et al. 2003), is limited for hybrid identification because mtDNA alone cannot identify hybrid origin and DNA introgression, as it shows only maternal inheritance (Wilson et al. 1985), and cannot be used to identify male-mediated gene flow. However, it can be used to identify the maternal species in F1 hybrids (McDevitt et al. 2009). More useful, however, is nuclear DNA because hybrids and their backcrosses carry this from both ancestral species, and hybridization and introgression can be detected using nuclear markers (Avise and Ball 1990; Weins and Servedio 2000). The utility of nuclear markers for identification of species and their hybrids has been shown for introns (e.g., Pacheco et al. 2002; Nadachowska and Babik 2009), single nucleotide polymorphisms (SNP; e.g., Väli et al. 2010; Hohenlohe et al. 2011), and microsatellites (e.g., Gay et al. 2007; Väli et al. 2010). A combined set of nuclear and mitochondrial markers may be the best option for species and their hybrids identification, especially in birds, when hybridizing species diverged recently and share a high proportion of alleles (e.g., Gay et al. 2007; Väli et al. 2010).

There is high disproportion between genetic studies on *D. major* and *D. syriacus*, as the phylogeography of only *D. major* has been described (but only based on mtDNA; Zink et al. 2002; Garcia-del-Rey et al. 2007; McDevitt et al. 2011; Perktas and Quintero 2013), whereas nothing is known about the population genetics of *D. syriacus*. In addition, microsatellites have not been used for population studies on either of these two species thus far. This lack of knowledge about the genetics of *D. syriacus* and molecular differences with *D. major* has hindered the identification of their hybrids and backcrosses.

The main aim of this research was to test the utility of several molecular markers (one mitochondrial, four nuclear introns, and a set of microsatellite loci) for the identification of *D. major* and *D. syriacus* and their possible hybrids. As the extent of hybridization between these two species is not known, it seems important to establish a method to indentify their hybrids. This will ultimately help in understanding the mechanisms of their evolutionary, ecological, and ethological interactions.

Methods

Sampling

Samples of woodpecker tissues were collected from other research and ringing projects, and were taken from specimens delivered to museum collections (Museum and Institute of Zoology Polish Academy of Science and Museum of Natural History in Institute of Systematics and Evolution of Animals PAS), during the last 5 years (2009–2013). Details are presented in Table 1. Several individuals of *D. major* and *D. syriacus* from different localities were gathered, as well as other woodpecker species as outgroups (all other European *Dendrocopos* species and the Three-toed Woodpecker *Picoides tridactylus*). Sampling was designed to collect specimens of *D. major* from sympatric (Poland) and allopatric (Norway) populations. *D. syriacus* was collected from a sympatric population on the verge of species expansion (Poland) and from the core of its range in Europe (Bulgaria) where it is parapatric with *D. major*. Two specimens of possible hybrid origin (according to plumage characteristics) were used in the analyses. One young bird found dead in Krakow city in 2009, whose plumage was most *syriacus*-like but some characters suggested hybrid origin; however, its body

Table 1 Woodpecker samples used in study, localization of sampling sites and source of tissue used for DNA extraction

Fenotype	Specimen symbol	Locality	Country	Tissue	No of specimens
D. major	NO1-4	Ostfold vicinity	Norway	Feather	4
D. major	MA	Mazury District	Poland	Feather	1
D. major	KR1	Krakow city	Poland	Muscle	1
D. major	SM	Sudeten Mts.	Poland	Feather	1
D. major	SL	Slonsk vicinity	Poland	Feather	1
D. major	CA	Carpathian Mts.	Poland	Feather	1
D. major	NP	Niepołomice Forest	Poland	Muscle	1
D. major	RA1-2	Radom city	Poland	Feather	2
D. major	WA1[a]	Warsaw city	Poland	Feather	1
D. syriacus	KR3[a], KR4	Krakow city	Poland	Muscle	2
D. syriacus	WA2	Warsaw city	Poland	Muscle	1
D. syriacus	ZA1-5	Zamosc vicinity	Poland	Feather	5
D. syriacus	GM	Warsaw vicinity	Poland	Egg shell	1
D. syriacus	BU1-4	Kalimok station	Bulgaria	Blood spot, feather	4
D. leucotos		Carpathian Mts.	Poland	Muscle	1
D. minor		Krakow vicinity	Poland	Feather	1
D. medius		Krakow vicinity	Poland	Feather	1
P. tridactylus		Carpathian Mts.	Poland	Feather	1

[a] Birds with morphological characteristics suggestive of hybrid origin

was in an advanced stage of decomposition and not all characters could be determined (Wójcik J.D.). The second bird (young male) was caught in Warsaw city (2012) and its plumage was generally like *D. major* but some features suggested hybrid origin (Elas M.). Tissues were either preserved in ethanol (muscle) or absorptive paper (blood spots) in a freezer, or kept dry in plastic bags (feathers, egg shells).

DNA sequence analyses

DNA extraction was performed either using Nucleospin Tissue kit (Macherey–Nagel, Düren, Germany) or, for older samples not directly preserved and those of small starting amounts, using Sherlock AX DNA isolation kit (A & A Biotechnology, Gdynia, Poland). Sequences of three woodpecker autosomal introns: beta-fibrinogen gene (intron 7) (BF7), myoglobin gene (intron 2) (MG2), transforming growth factor (intron 5) (TGF5), and one Z-linked intron—brahma gene (intron 15) (BR15) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and used for designing primers using PRIMER 3 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers were located within regions conserved in the *Dendrocopos* genus. Primers for mitochondrial control region (CR) were as in McDevitt et al. (2011). Characteristics of primers used in this study are presented in supplementary table 1. Amplification was done using Qiagen PCR Core Kit (Hilden, Germany). The cycling profile for the PCR was: 95 °C for 4 min, 35 cycles of 95 °C for 30 s, 54 °C for 1 min, 72 °C for 2 min, and a final extension period of 72 °C for 10 min. After purification, PCR fragments (NucleoSpin Extract II; Macherey–Nagel) were sequenced using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and an ABI 3100 Automated Capillary DNA Sequencer. All sequences were deposited in GenBank (accession numbers KF445345–KF445387a).

Sequences were checked and aligned using BioEdit v.7.0.5.2 (Hall 1999) and ClustalX (Thompson et al. 1997). Mitochondrial haplotypes were identified and standard genetic indices such as number of polymorphic and segregating sites, number of haplotypes, haplotype diversity, and nucleotide diversity for *D. major* and *D. syriacus* were computed using the program DnaSP v.5 (Librado and Rozas 2009). Mitochondrial and TGF5 haplotype networks for *D. major* and *D. syriacus* samples were constructed using the Median-Joining network method (Bandelt et al. 1999) in the Network 4.6.1.0. software (http://www.fluxus-engineering.com/). Nuclear genotypes as well as nuclear polymorphic and segregating sites were counted manually, while alleles numbers were not estimated as within nuclear introns were found heterozygous nucleotide positions and alleles could not be determined precisely without cloning. Simple Neighbor-Joining (NJ) phylogenetic trees were constructed separately on mtDNA and concentrated nuclear DNA sequences using MEGA v.5 (Tamura et al. 2011). To estimate utility of particular markers for assignment of individuals to *D. major* or *D. syriacus*,

numbers of discriminating sites (in which different nucleotides were fixed in each of these two species) were determined.

Microsatellite analyses

Five microsatellite loci developed for *D. leucotos* (Ellegren et al. 1999) were chosen according to previous cross-species amplification success in both *D. major* or *D. syriacus*: Dlu1, Dlu3, Dlu4, Dlu5, and Dlu6 (Rutkowski et al. 2006). Moreover, three loci developed for *D. medius* (Vila et al. 2008) were chosen on the basis of cross-species amplification efficiency in *D. major* (Rutkowski, unpublished): DMC111, DMC115, and DMC118. All these eight loci were preliminary tested and all gave PCR products for both *D. major* or *D. syriacus*. However, DMC118 also gave many other additional products and there were problems with Dlu4 genotyping (due to possible amplification of duplicated locus). These two loci were excluded from further analyses. Six loci were amplified in two multiplexes using fluorescent labeling primers and Qiagen multiplex PCR master mix (Qiagen). The cycling scheme was as follows: 94 °C for 15 s followed by 40 cycles of 94 °C for 20 s, 55 °C for 90 s, and 72 °C for 30 s; the final extension was at 72 °C for 10 min. PCR products were electrophoresed on an ABI 3130xl Genetic Analyser with GeneScan 500 LIZ size standard (Applied Biosystems). Allele sizes were determined using GeneMapper software (Applied Biosystems).

Number of alleles, allelic richness, and the observed and expected heterozygosities were calculated with Arlequin 3.5 and FSTAT (Goudet 2002; Excoffier and Lischer 2010). Tests of departures from Hardy–Weinberg equilibrium, and tests of linkage disequilibria were performed using GENEPOP (Rousset 2008).

Hybridization was tested between species using a Bayesian admixture analysis approach implemented in STRUCTURE v.2.2 (Pritchard et al. 2000; Vaha and Primmer 2006; Sanz et al. 2009) to obtain individual genetic assignment to either *D. major* or *D. syriacus* based on the six microsatellite loci. We assumed the presence of two genetic clusters/species ($K = 2$; McDevitt et al. 2009; Senn and Pemberton 2009). STRUCTURE was run with 10 independent runs using 500,000 iterations, with a burn-in period of 100,000 iterations. A threshold for hybrid identification was not initially assigned as this can depend on the allele frequency differences between species, and there is also the risk of misidentifying 'pure' individuals as hybrids due to ancestral polymorphism (Senn and Pemberton 2009). This threshold is crucial, and there is always a trade-off between assignment efficiency and accuracy. The selection of this threshold has varied between 0.01 and 0.2, depending on the hybridization study (McDevitt et al. 2009; Senn and Pemberton 2009; Frantz et al. 2013). In addition, a principal component analysis (PCA) was performed using the *adegenet* (Jombart 2008) and *ade4* (Dray and Dufour 2007) packages in R v.2.12.1 (R Development Core Team 2010). Unlike the previous methods, it does not assume HWE or linkage disequilibrium.

Results

Sequence markers

The alignment of the control region for all studied woodpeckers revealed that there were no indels present within the *D. major*, *D. syriacus*, and *D. leucotos* group, but some indels were observed between this group and other species. For the nuclear introns, there were small or large indels differentiating the *D. major–syriacus–leucotos* group from other species, most large indels differentiating *D. medius* and *P. tridactylus* from other *Dendrocopos* species. Standard mtDNA and nucDNA genetic indices calculated for *D. major* and *D. syriacus* are presented in supplementary table 2. *Dendrocopos major* is much more diverse than *D. syriacus*. Simple NJ trees (supplementary fig. 3) showed that there is uncertainty in woodpecker phylogeny. First, the position of outer taxa (*D. minor*, D. *medius*, and *P. tridactylus*) is different with respect to mtDNA and nuclear DNA. Only some nodes have statistical support. In both trees, *D. major*, *D. syriacus*, and *D. leucotos* form a monophyletic cluster (100 % support). However, their position is different depending on the marker type. According to mtDNA *D. major* is a sister species to *D. leucotos* (86 % support), whereas, according to nucDNA, *D. major* is a sister species to *D. syriacus* (but only with 61 % support). The haplotype network of mtDNA, and,

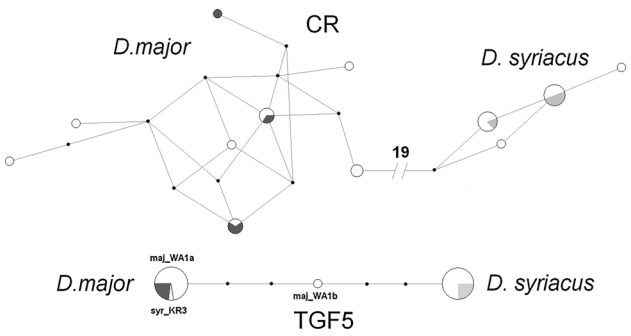

Fig. 1 Haplotype networks of mitochondrial DNA (control region, *CR*) and transforming growth factor (intron 5, *TGF5*) constructed for two *Dendrocopos* species. *White* samples from Poland, *dark gray* Norway, *light gray* Bulgaria, *black dots* missing haplotypes, *numbers* number of mutations in longer branches, *syr_KR3 D. syriacus* which showed *D. major* TGF5, *maj_WA1a&b* two alleles found in *D. major* WA1

Table 2 Polymorphic sites within five DNA markers compared among *Dendrocopos major* and *D. syriacus* samples

Specimen	mtDNA control region	Beta-fibrinogen i.7	Myoglobin i.2	Transforming growth factor i.5	Brahma protein i.15
maj_NO1	CGACCACCGTTCTAAGCTGTCAGTAGAGG	TATGACGGGACCACA	GYCMCTCGG	CGCA	CC
maj_NO2	.....G.............T.C.....	....	R........	....	..
maj_NO3	.....G.............C.....	...R.....R..G..	....	....	.T
maj_NO4	.....G.............T.C.....	...R.....R..G..	....	....	.T
maj_MA	.....G.............T.C.....	Y..R..R...Y.RY.	R........	....	.T
maj_KR1	....TG...........A..C.....	Y.GA..R..RYYRY.	A.Y......	....	..
maj_SM	.....G.............C.....	C.GA..A...T.GT.	R........	....	.T
maj_SL	.....G.............C.....	C.GA..A...T.GT.	R........	....	..
maj_CA	.....G..........C...T.....	..GA..A...T.GTC	A...Y....	....	..
maj_NF	....TG...........A..C.....	C.GA..A...T.GT.	A.Y......	....	.T
maj_RA1	.....G....C......CA..C.....	....	A........	....	.T
maj_RA2	.....G.......G...C...CAC.....	.M.......RY.R..	A........	....	.T
maj_WA1	.....G..........C.C.C.C.....	.....YR..RY.RY.	R........	Y.Y.	YT
syr_KR3	TACAT.TTT..TC.GAT.ACTT..GATAA	..GARTARK.T.GT.	..Y..G.C.	....	..
syr_KR4	TACATGTTT..TC.GAT.ACTT..GATAA	..GARTA...T.GT.	.......CK	TATG	TT
syr_ZA1	TACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......CK	TATG	TT
syr_ZA2	.ACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......C.	TATG	T.
syr_ZA3	.ACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......C.	TATG	T.
syr_ZA4	TACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......C.	TATG	TT
syr_ZA5	.ACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......CK	TATG	TT
syr_GM	TACAT.TTT..TC.GAT.ACTTA.GATAA	..GARTA...T.GT.	.......CK	TATG	TT
syr_WA2	.ACAT.TTT..TC.GAT.ACTT..GATAA	..GARTA...T.GT.	.......CK	TATG	TT
syr_BU1	TACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......C.	TATG	TT
syr_BU2	TACAT.TTT..TC.GAT.ACTT..GATAA	..GA.TA...T.GT.	.......C.	TATG	TT
syr_BU3	.ACAT.TTT..TC.GAT.ACTT..GATAA	..KATTA...T.GT.	......MCK	TATG	TT
syr_BU4	TACAT.TTT..TC.GAT.ACTT..GATAA	.WGA.TA...T.GT.	.......CK	TATG	TT
Dns	20	1	1	4	1

Dns number of discriminating (fixed) nucleotide sites

separately, TGF5 constructed for *D. major* and *D. syriacus*, showed that both species belong to separate clades (Fig. 1). *Dendrocopos major* was more diverse (8 haplotypes found in 13 individuals) than *D. syriacus* (3 haplotypes in 13 individuals). In *D. major* and *D. syriacus*, there are no clear differentiations between individuals from different areas (for *D. major*, two haplotypes were shared between Norwegian and Polish populations, and, similarly for *D. syriacus*, two haplotypes were found in both Bulgaria and Poland). There were also some polymorphic nucleotide sites of the nuclear introns within populations of *D. major* and *D. syriacus*, but these differences were not related to geographic origin.

The most important finding of sequence marker analyses is the determination of their utility for *D. major* and *D. syriacus* discrimination. The best marker for this purpose is undoubtedly the control region, as within 806 bp there are 20 nucleotide positions that discriminate these two species (Table 2). Among nuclear markers, the best is TGF5 as it has four discriminating sites. The other markers have only one discriminating site each. BF7 and MG2 are highly polymorphic; however, most polymorphic sites are not fixed between species and, moreover, many of these sites are heterozygous, whereas in TGF5 and BR15 heterozygous sites are rare. BR15 was the least variable marker.

Microsatellites

There were 4–7 alleles per locus in *D. major* and 3–5 in *D. syriacus* (supplementary table 3). Linkage disequilibrium was not detected in any of the studied species, whereas departures from Hardy–Weinberg equilibrium were detected in three loci in *D. major* and a single locus in *D. syriacus* (supplementary table 3), but these departures likely reflect discrepancies due to the sampling design. Standard genetic indices are presented in Table 2. According to these values, *D. major* is much more diverse than *D. syriacus*. Both species were separated by the Bayesian

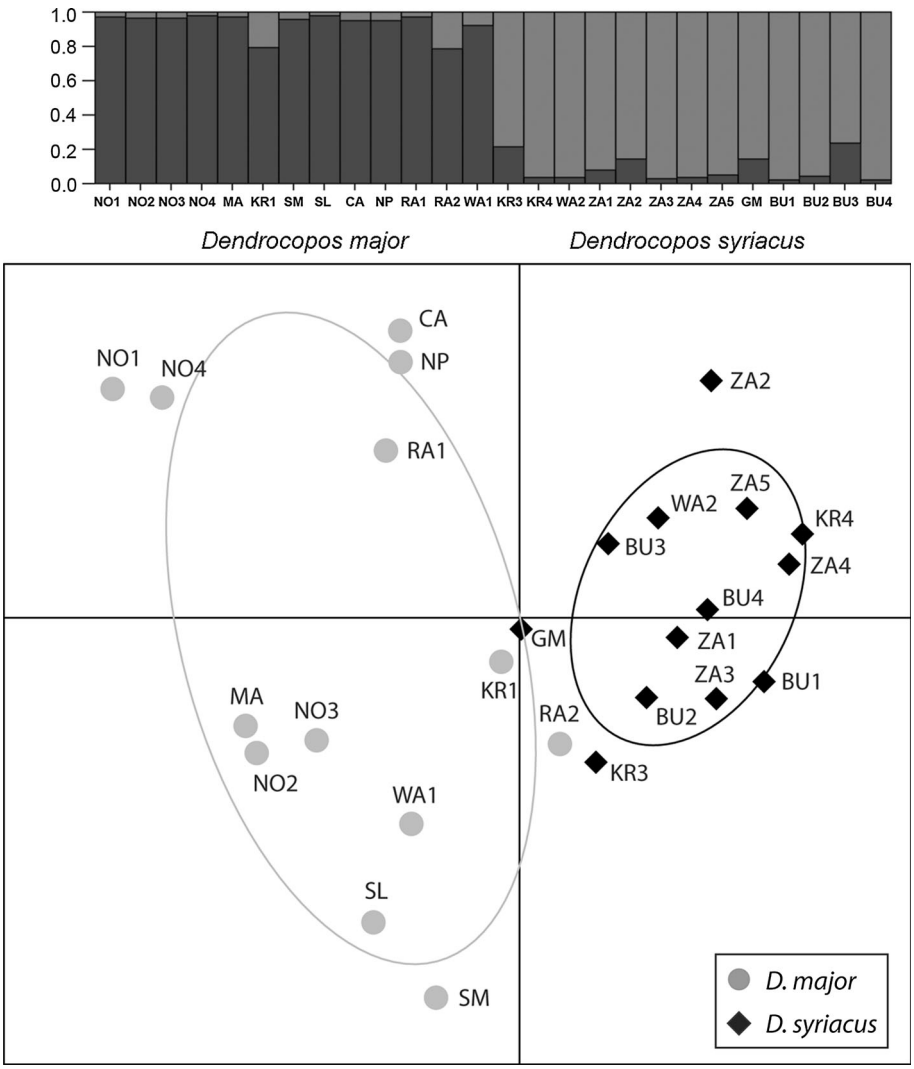

Fig. 2 Microsatellite results. *Upper* structure results with a *K* value of 2 for all studied individuals of *Dendrocopos major* (*dark gray*) and *D. syriacus* (*light gray*). *Lower* principal component analysis results for microsatellite genotyping of all studied individuals of *Dendrocopos major* (*gray*) and *D. syriacus* (*black*). *Symbols* correspond to individuals (see Table 1)

analysis in STRUCTURE (Fig. 2), but this was less clear with the PCA (Fig. 2).

Hybrid detection

The data from multiple markers suggests that none of the studied woodpecker individuals was an F1 hybrid. There are two individuals where it is probable that the individual in question is a hybrid according to the DNA sequence analyses. One is *D. syriacus* from Krakow city (KR3), which has "syriacus" CR, but "major" BR15 and TGF5, and the other two nuclear introns (BF7 and MG2) are highly heterozygous. The second bird is *D. major* from Warsaw (WA1), which has "major" CR, whereas according to the nuclear introns, it is highly heterozygous and seems to be intermediate according to TGF5 and BR15. These are the same two individuals that were also considered to be hybrids according to their phenotypes (as described in "Sampling"). The results of the microsatellite analyses were less clear. The allele ranges for all six loci overlapped (supplementary table 3) and neither the Bayesian clustering nor the PCA could confidently assign an individual as being of hybrid origin (Fig. 2). Two *D. major* (KR1 and RA2) and four *D. syriacus* (KR3, ZA2, GM, BU3) have more than 10 % of their 'genome' assigned to the other species (Fig. 2), with KR1, RA2, KR3, and BU3 having over 20 % assignment to the other species. According to the PCA, four individuals—two *D. major* (KR1 and RA2) and two *D. syriacus* (KR3 and GM)—seemed to be somewhat intermediate between the two species (Fig. 2). *Dendrocopos major* individual WA1 did not appear to be intermediate to the microsatellite analyses (Fig 2).

Discussion

The genetic analyses support the close relationship between *D. major* and *D. syriacus*—species which hybridize in nature. These two species belong to a

superspecies group which includes Asian species: the White-winged Woodpecker *D. leucopterus*, Sind Woodpecker *D. assimilis*, and Himalayan Woodpecker *D. himalayensis* (Gorman 2004). Presented phylogenetic trees suggest that *D. major* is closely related with *D. syriacus* (sister species according to nuclear DNA) but also with *D. leucotos* (according to mtDNA; supplementary fig. 3). These three species form a monophyletic clade, but their genetic relationships are not congruent with respect to mtDNA and nuclear DNA (supplementary fig. 3; see also Weibel and Moore 2002; Moore et al. 2006; Rutkowski et al. 2007; Fuchs et al. 2013).

Dendrocopos major and *D. syriacus* are clearly separated according to the mitochondrial CR (Fig. 2). Within c. 800 bp of CR, 20 nucleotide positions are fixed between these two species. In the studied samples, there were no haplotypes shared between both species. These two species also have genetic differences in all of the studied nuclear introns (both autosomal and Z-linked) but most of polymorphic nucleotide positions are not fixed and both species share many alleles. The exception is TGF5. In BF7, MG2, and BR15, only a single nucleotide position was fixed between these two species, whereas in TGF5, there were four such fixed positions. Regardless of the number of fixed nucleotide positions, all these introns allow for species identification. They also can help in hybrid identification as hybrid individuals (at least F1) should be heterozygous in these fixed nucleotide positions. Indeed, two of the presumed hybrids (KR3 with *D. syriacus*-like phenotype and WA1 with *D. major*-like phenotype) show either mixed genotypes or high heterozygosity in some of the nuclear introns. However, any of presumed hybrids did not give clear picture of hybrid origin. This can perhaps be interpreted as evidence that these individuals are rather backcrosses as opposed to F1 hybrids. This explanation fits with their morphological characteristics, which were not so straightforward as in the hybrids described by Dudzik and Polakowski (2011). It is important to note that there was no evidence for maternal backcrossing as all woodpeckers with *D. major* phenotype had *D. major* mtDNA and the same was found in *D. syriacus*. Field observations suggest that hybridization of these two woodpeckers is mostly unidirectional—almost all mixed pairs observed in Poland consist of a male with a *D. major* phenotype and a female with a *D. syriacus* phenotype (Dudzik and Polakowski 2011; Michalczuk, Kajtoch, and Malczyk, unpublished data). Moreover, the inference of hybrid status for the two individuals implies that there is no evidence of nuclear introgression in any of the other individuals in the dataset.

Dendrocopos major and *D. syriacus* can be distinguished based on the set of six microsatellite loci used in this work (Fig. 2). All these loci showed a high frequency of common alleles in both species, which is likely a result of ancestral polymorphism. This may limit the utility of these microsatellites for hybrid identification (Senn and Pemberton 2009). Several individuals showed evidence of admixed genotypes in both the Bayesian analysis in STRUCTURE and the PCA, but not conclusively so. No individuals showed a genotype that could be clearly attributed to a hybrid and the differences observed may reflect normal changes in allele frequencies among the individual species. Therefore, this suite of microsatellites is unlikely to identify potential hybrids between the species. More microsatellite loci (preferably loci which have no overlapping alleles between parent species; Senn and Pemberton 2009) are needed to find a set of microsatellites which could be used for hybrid detection (Vaha and Primmer 2006). The development and characterization of large number of polymorphic microsatellites is possible and efficient via next-generation sequencing techniques (Abdelkrim et al. 2009; Gardner et al. 2011; Kajtoch et al. 2012).

Conclusions

The data presented here show that it is possible to discriminate between *D. syriacus* and *D. major* using molecular markers. The best option would be to use two or three sequence markers: CR (whole), TGF5, and BR5 to include a Z-linked locus (these introns could be either sequenced fully or just their SNPs). On the other hand, the six microsatellite loci used in this study did not have enough power to reliably identify hybrids. However, a larger set of loci could be useful for hybrid detection (Vaha and Primmer 2006; Senn and Pemberton 2009).

Two potentially hybrid individuals were identified from the sympatric range of both species in Poland, which is the present boundary of *D. syriacus*' range (according to both phenotype and genotype). It is important to note that both of these potential hybrids are from large cities (Krakow and Warsaw), whereas none of the *D. major* sampled in forests and in Norway (far from *D. syriacus* range) showed signs of an intermixed genotype. At the front of *D. syriacus* expansion, woodpeckers may have been expected to hybridize more often (Skakuj and Stawarczyk 1994). However, hybrids were found regularly in areas where *D. syriacus* was established in stable and abundant populations like in Hungary (Gorman 1997), so the range and frequency of hybridization may be more substantial.

This work should be treated as an important contribution for further studies. The tested set of different genetic markers could be used for studies on phylogeography, population genetics, and demography of *D. syriacus* and *D. major*. However, the most interesting topic is the estimation of hybridization and introgression between these two

species. It is essential to verify how frequent hybrids (and backcrosses) are between *D. major* and *D. syriacus* populations in different parts of their sympatric and allopatric ranges, as such knowledge could be important for understanding ecological and ethological interactions among them. In addition, they could prove useful for the management and conservation of *D. syriacus*.

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