

GENETIC TECHNIQUES FOR DESIGNATION OF MAIN FLYWAYS OF THE WOODPIGEON (*COLUMBA PALUMBUS*) IN EUROPE AS A TOOL FOR CONTROL AND PREVENTION OF PATHOGENIC DISEASES

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Abstract. The population genetic structure of the Woodpigeon (*Columba palumbus*) in Europe was studied analyzing the distribution of different D-loop haplotypes among bird samples, collected in various breeding and wintering sites of the species (in Russia, Belarus, Lithuania, Sweden, Hungary, France, Spain and Portugal). Totally 89 different haplotypes ascertained as belonging to 5 haplo-groups were identified after examination of partial D-loop sequences consisting of 359 bp mtDNR fragments derived from 360 samples. The results of this study indicate that the Eastern Atlantic Flyway is used by Woodpigeons breeding in NW Russia, Belarus, Lithuania, Sweden and wintering mainly in the Iberian Peninsula. A distinct population genetic structure is characteristic of Woodpigeons breeding in Hungary and using the Mediterranean Flyway. The designation of main flyways of Woodpigeons in Europe is necessary for the control and prevention of potential spread of pathogenic diseases and parasites, as this species is the most important game bird in Europe.

Keywords: Woodpigeon, flyways, mtDNA D-loop, transmission of diseases, Europe.

KARVELIO KERŠULIO (*COLUMBA PALUMBUS*) MIGRACIJOS KELIŲ EUROPOJE TYRIMAI PATOGENINIŲ LIGŲ PREVENCIJAI IR KONTROLEI TAIKANT GENETINIUS METODUS

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Santrauka. Mitochondrinės DNR D-kilpos sekų analizė panaudota karvelio keršulio (*Columba palumbus*) migracijos kelių tyrimams Europoje. Karvelis keršulis (daugelio patogeninių ligų ir parazitų pernešėjas) yra labiausiai medžiojama paukščių rūšis Europoje, todėl nustatyti šių paukščių migracinius kelius būtina patogeninių ligų kontrolei ir prevencijai. Ištyrus 360 karvelio keršulio mėginių (surinktų Rusijoje, Baltarusijoje, Lietuvoje, Švedijoje, Vengrijoje, Prancūzijoje, Ispanijoje ir Portugalijoje), nustatyti 89 skirtingi haplotipai, priskirti penkioms haplogrupėms. Genetiniais metodais nustatyta, kad dauguma Baltijos regione perinčių karvelių keršulių žiemoja Ispanijoje ir Portugalijoje. Jų migracijos kelias iš perėjimo vietų į žiemojimo vietas driekiasi Baltijos ir Šiaurės jūrų pakrantėmis. Skirtingas migracijos kelias būdingas karveliams keršuliams, perintiems Vengrijoje. Šie paukščiai žiemoja vakarinėse Viduržemio jūros pakrantėse. Karveliai keršuliai yra potencialūs įvairių patogenų pernešėjai. Atsiradus patogeninių ligų židiniams įvairiuose Europos regionuose, prieš numatant būtinausias prevencines priemones, labai svarbu įvertinti karvelių keršulių migracijos kelius.

Raktažodžiai: karvelis keršulis, migracijos keliai, mitochondrinė DNR, ligų pernešimas, Europa.

Introduction. The Woodpigeon *Columba palumbus* is a Palearctic species, breeding in all countries of Europe (Saari, 1997). The total European breeding population of the Woodpigeon is estimated at about 8.000.000–15.000.000 individuals and is increasing (BirdLife International, 2004). Populations breeding in Southern

and Western Europe are mainly partially migratory or sedentary, while Eastern European and Fennoscandian breeders are completely migratory (Rouxel, Czajkowski, 2004). There is a large mixing between populations using different flyways across longitudinal ranges of Europe.

The designation of main flyways of Woodpigeons in

Europe (connecting their breeding, stop-over and wintering sites) is necessary for the control and prevention of potential spread of various diseases and parasites, as this species is the most important game bird in Europe, with about 9 million birds annually hunted (Purroy, Rodero, 1986; Rouxel, Czajkowski, 2004). Woodpigeons carry certain pathogens potentially dangerous to humans and poultry. The highly pathogenic avian influenza H5 and H7 viruses and a highly pathogenic Newcastle disease virus strain were found in pigeons (Capua, Alexander, 2009). Viral infections caused by these viruses can cause serious consequences for the poultry industry and can be directly transmitted to humans (Munster et al., 2007, Lvov et al., 2010). Ornithosis, caused by the infectious agent *Chlamydophila psittaci*, is a disease commonly associated with pigeons, as well as the disease organisms causing histoplasmosis and cryptococcosis (Hubalek, 2004). The non-pathogenic species of *Sarcocystis* parasites (*Sarcocystis columbae*) were found in Woodpigeons in Lithuania (Prakas et al., 2011). As millions of Woodpigeons are annually used as game dish in many countries of Europe, pathogenic viruses and parasites can potentially get into the human environment.

We have applied sequencing of variable fragments of mtDNA as one of the most suitable techniques for investigation of intra-specific genetic variability and population genetic structure of different populations of the Woodpigeon. The aim of this study was to identify the main flyways of the Woodpigeon populations in Europe based on D-loop sequences analysis in order to use the obtained results as a tool for control and prevention of pathogenic diseases, transmitted by these birds.

Material and methods

The genetic study based on D-loop sequences analysis of 360 Woodpigeons collected in eight European countries (in Russia, Belarus, Lithuania, Sweden, Hungary, France, Spain and Portugal) in 2004–2009 was performed to determine the main flyways of different migratory populations of the species. The following samples of migratory Woodpigeons (including breeding birds and individuals sampled in their migratory stop-over sites) were analyzed: 85 samples from the Kaliningrad region of Russia; 12 - from Lithuania; 40 - from Belarus, 30 - from Sweden; 23 - from Hungary and 15 - from France. 45 samples from Portugal and 51 samples from Spain collected during winter period probably included representatives of both local resident birds and migrants from other regions of Europe. 59 samples from sedentary birds were collected in four sampling sites of Spain during the breeding season.

DNA extraction from ethanol preserved tissue samples and from blood samples followed by amplification and sequencing of partial D-loop sequences using primer pair PAL-2 (Butkauskas et al., 2008) was implemented in this study. DNA was extracted using universal and rapid salt-extraction of genomic DNA for PCR-based analysis (Aljanabi, Martinez, 1997) after small amount (about 50 mg) of tissue sample was digested with proteinase K. Initially, a *Columbidae* species-specific primer pair PAL-

2 (5′–3′ forward primer sequence CATATTCATGACCCCCATACG, 5′–3′ reverse primer sequence GGCCTGAAGCTAGTCGTGAT) was constructed using the software “Primer 3”. The designation of primers was based on identification of conservative fragments detected after alignment of the homological sequences of the D-loop of *Columba livia* (Acc. No. AB222242 in Seki, 2006), *Columba janthina* (Acc. No. AB222222 in Seki, 2006), *Columba junoniae* (Acc. No. AJ784894 in Lopez, 2004) and *Columba bollii* (Acc. No. AJ784893 in Lopez, 2004). PCR was performed using the Eppendorf Mastercycle gradient PCR machine. PCR reaction contained 250 ng of template DNA, 0.75 µl 1 U Taq Polymerase (MBI Fermentas), 2.5 µl 1xPCR buffer (MBI Fermentas), 2.5 µl MgCl₂ at 2.5 mM concentration, 2.5 µl 2mM dNTP, 0.1 µM forward and reverse primers PAL-2 in a final volume of 25 µl. Amplification was performed with 35 cycles of the following steps: 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s, proceeded by 2 min at 95 °C and followed by 5 min at 72 °C. The quality and concentration of PCR products were checked in 1.5% agarose gel and amplificate was purified by calf intestine alkaline phosphatase (CIAP, Fermentas) and FastAP™ thermosensitive alkaline phosphatase (ExoI, Fermentas) added into tubes with 5 µl of amplificate and heated for 15 min at 37 °C and then for 15 min at 85 °C. The amplified fragments were sequenced by the ABI Prism 377 automatic sequencer using the same primers PAL-2 labelled with fluorescent dye (IRD800; li-Cor). Sequencing results were analysed using CLC Free Workbench version 4.0.1 (CLC bio A/S) and the identification of different haplotypes was carried out manually after the alignment of all determined sequences of Woodpigeons with the reference sequence of *Columba livia* (Acc. No. AB2222420 in Seki, 2006).

The haplotypic network based on D-loop sequences consisting of 359 bp mtDNR fragments using Phylogenetic Network Analysis Software (Network version 4.500 available at www.fluxus-engineering.com) was constructed and the frequency distribution of representatives of different haplogroups in selected sampling sites representing different breeding, migratory, sedentary and wintering populations of the Woodpigeon in Europe was analyzed.

Results. 89 different haplotypes were identified among 360 analyzed samples of Woodpigeons and ascertained as belonging to five haplogroups: “A”, “B”, “C”, “D” and “G”. A complex haplotypic network derived from sequences of D-loop of Woodpigeons collected in different European countries was constructed (Fig. 1).

The results of the current study confirmed our previous observations (Butkauskas et al., 2008) that some less frequent unique haplotypes were detected exclusively among Woodpigeons breeding in the Iberian Peninsula or in the Baltic Region (Fig. 1). It may be suggested that part of Woodpigeons breeding in the Baltic Region and Belarus are short distance migrants, while most birds breeding in this region migrate to the wintering areas located in Portugal and Spain.

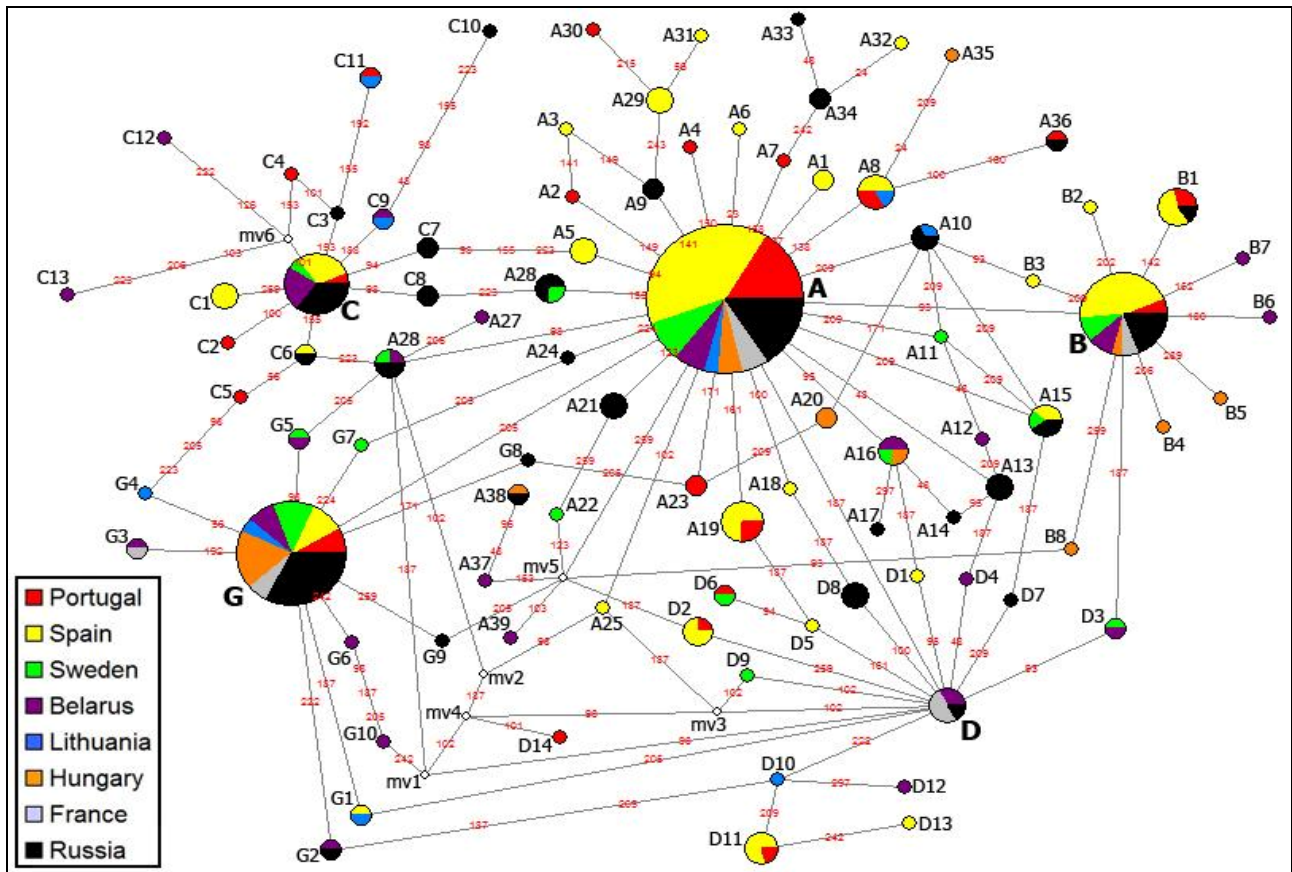


Figure 1. Unrooted statistical parsimony haplotypic network: circle area is proportional to haplotype frequency; small circles (positions mv*) indicate inferred intermediate haplotypes

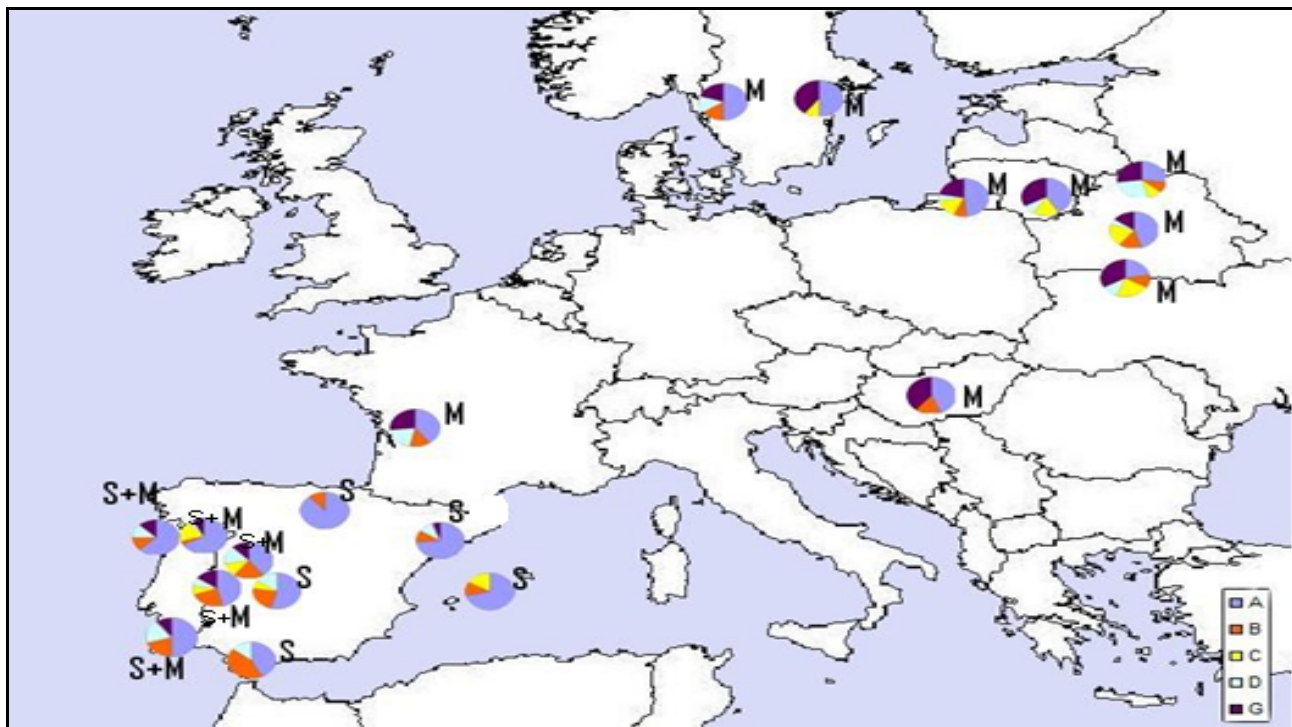


Figure 2. Frequency distribution of representatives of five different haplogroups “A”, “B”, “C”, “D” and “G” (shown in different colours) among migratory (M), sedentary (S) and mixed populations including resident and migratory birds (S+M) of the Woodpigeon in Europe

The different frequencies of representatives of certain haplogroups detected among 19 study areas in Europe enabled to identify the genetic population structure characteristic of different sedentary and migratory populations of the Woodpigeon and to designate their main flyways (Fig. 2).

A specific structure of haplotypes was characteristic of certain sedentary populations of the Woodpigeon breeding in different areas of Spain. Representatives of haplogroup "A" were the most common among sedentary populations from the Iberian Peninsula, while more variable spectrum of haplogroups with significantly higher distribution of representatives of haplogroup "G" was characteristic of migratory Woodpigeon populations. The genetic structure of Woodpigeon populations represented by sedentary and migratory birds collected in their main wintering grounds located in Spain and Portugal was similar to that found among breeding birds sampled in the Baltic Region and Belarus. These findings indicate that migrants from Northern and Eastern Europe form a substantial part among all Woodpigeons wintering in the Iberian Peninsula. A distinct population genetic structure was characteristic of Woodpigeons breeding in Hungary and using the Mediterranean Flyway.

Discussion and conclusions. Up to now, migratory routes of Woodpigeons in Europe were mainly designated using available ringing recoveries of the species (Saari, 1979; Cramp (ed.), 1985; Švažas, 2001; Rouxel, Czajkowski, 2004). However this method is restricted due to poor recovery rates in Eastern and Northern Europe and biased due to differences of hunting activities in various regions of Europe. The hydrogen isotope analysis of feathers from Woodpigeons hunted in France and the Iberian Peninsula as a forensic tool to estimate their natal origins was recently used (Hobson et al., 2009) and it was estimated that about 50 % of the five million Woodpigeons harvested annually in France were resident or from nearby countries to the east. However, isotopes distribution is broad, thus migratory pattern evaluation has a low resolution and can not exactly locate the origin of individuals. The monitoring of migratory routes of individual Woodpigeons using satellite tracking is increasingly used (Cohou, 2011), but this method is limited to individual birds and is not applicable on population level.

The genetic analysis has provided an important tool for designation of main flyways of the Woodpigeon in Europe on the population level. This information can be effectively used for control and prevention of pathogenic diseases transmitted by this species. In some abundant and widely distributed bird species one major continuous meta-population can predominate in their distribution range (Puigcerver et al., 2007), while in other species sub-populations with different genetic structure can be identified in different parts of their range (Sruoga et al., 2005; Tubelyte et al., 2011).

The results of this study confirmed the importance of wintering grounds located in the Iberian Peninsula and of the Baltic Sea–North Sea Flyway for Woodpigeons of Northern and Eastern European origin. The key wintering

grounds of Woodpigeons in Europe are located in the Iberian Peninsula, with more than four million birds annually counted in the key wintering sites (Bea et al., 2003). These results coincide with available data on migratory routes of woodpigeons, based on the ringing recoveries. Available ringing recoveries indicate the flyway of Woodpigeons of Lithuanian origin, stretching along the coasts of the Baltic and North Seas to Atlantic coastal regions of Southwest France and further to Spain (Švažas, 2001). A similar migratory pattern is characteristic of Latvian breeders, with most recoveries received from wintering grounds located in Southwest France, Portugal and Spain (Viksne, 2001). The Eastern Atlantic flyway was also defined for Woodpigeons breeding in Fennoscandia and NW Russia (Saari, 1979; 1984; Cramp (ed.), 1985; Noskov et al., 2002; Grishanov et al., 2008).

A different migratory route is characteristic to Woodpigeons of Hungarian origin, with all recoveries received from the West Mediterranean region (Italy and Southern France, particularly from Corsica and Sardinia) (Bankovics, 2001; Faragó, 2009). Such flyway is also characteristic of birds from the Czech Republic and Southern Germany (Cramp (ed.), 1985; Rouxel, Czajkowski, 2004).

The main flyways of Woodpigeons in Europe designated by means of the mtDNA analysis methods were similar to those earlier identified by means of other genetic techniques (polymorphic proteins and random amplified polymorphic DNA analysis) in our previous studies (Sruoga et al., 2005; Butkauskas et al., 2008).

The results of this study can be effectively used for control and prevention of the potential spread of dangerous diseases and parasites, transmitted by migratory populations of Woodpigeons in Europe. The knowledge of main flyways of the most important game bird in Europe is necessary for early detection of highly pathogenic diseases in case of their outbreaks in various regions of Europe.

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