

1. An experimental design for assessing the genetic diversity of colonial waterbirds from the Danube Delta Biosphere Reserve (Romania)

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Abstract: The extensive wetland complex of the Danube Delta provides internationally important stopover sites and breeding sites for millions of migratory birds. Worldwide, natural wetlands are facing an accelerated decline due to the increased urbanization and conversion of open spaces to agriculture. The Danube Delta is no exception, being subject to anthropogenic factors that affect this wildlife hotspot, in spite of considerable conservation efforts. Despite numerous studies focused on Danube Delta waterbird particularities, knowledge of them is limited and highly fragmented. To provide a framework for assessing colonial waterbird populations from the Danube Delta, we developed a comprehensive experimental design to answer existing questions regarding genetic diversity, genetic discontinuities and the degree of genetic differentiation. This paper describes a study which overlaps landscape genetics principles and a small genetic survey in order to provide a feasible framework for studying colonial waterbirds from the Danube Delta Biosphere Reserve.

Keywords: waterbirds, Danube Delta Biosphere Reserve, experimental design, genetic diversity, COX1.

INTRODUCTION

The Old World birds share one of the most important migratory routes in the world, the Palearctic-African migratory pathway that connects Europe, Asia and Africa (Newton, 2010). Across Europe, all migratory species share a common strategy, which includes several stopover sites along their routes for feeding and rest, essential to individuals' survival. The extensive wetland complex of the Danube Delta Biosphere Reserve provides an internationally important stopover site for millions of birds, belonging to over 200 different species, travelling annually to and from Northern Eurasia and Africa (Newton, 2010; Sándor et al., 2014). In addition, due to its positioning and large surface, the Danube Delta Biosphere Reserve hosts a great number of breeding bird species. Worldwide, natural wetlands are facing an accelerated decline due to increased urbanization and conversion of open spaces to agriculture (Czech and Parsons, 2002). The Danube Delta is no exception, being subject to anthropogenic factors that affect this wildlife hotspot, despite considerable conservation efforts. Excessive exploitation of natural resources, pollution, different human activities, together with natural hazards, lead to the loss of natural habitats, enhancing pressure on native populations (Primack, 2002). Therefore, small habitats and especially isolated ones, can exhibit a discontinuity in the presence of one or more species. Fragmentation generally decreases individuals' connectivity and increases the distance between remaining habitat parts. In the case of threatened and endangered avian species, the risk of extinction due to the effects of fragmentation is considerably higher, because they usually exist at a low densities (Johnson, 2001; Lindsay *et al.*, 2008; Saunders *et al.*, 1991). Small isolated fragments of habitat are characterized by low reproductive success of males and minimum dispersal of offspring (Rappole *et al.*, 2003). A low degree of dispersion may lead to a much narrower distribution and thus to reduced abundance (Villard *et al.*, 1999). Habitat fragmentation, reduced population size and gene flow could lead to increased loss of genetic diversity within populations and an increase in the degree of genetic differentiation between populations (Frankham, 1995). In addition, the genetic diversity is an important factor, being directly involved in individual fitness and disease resistance. The susceptibility to various diseases of a certain population is highly dependent on its level of genetic diversity and its degree of genetic differentiation compared to other

populations. Numerous studies focused on the Danube Delta Biosphere Reserve have been conducted to approach waterbird particularities such as: habitat use (Platteeuw *et al.*, 2004), status (Schogolev *et al.*, 2005), breeding success (Green, 1992; Sandor *et al.*, 2015; Schogolev *et al.*, 2005) and disease outbreaks (Feare, 2007; Gaidet *et al.*, 2008; Gilbert, 2006; Ward *et al.*, 2008). However, the degree of knowledge is limited and highly fragmented, mainly due to the type of approach, hence a new kind of approach is required. A candidate solution could be landscape genetics, a multidisciplinary approach that combines spatial analyses, ecology and population genetics. Landscape genetics provides a framework for testing the relative influence of landscape and environmental features on gene flow, genetic discontinuities (Guillot *et al.*, 2005), genetic population structure (Holderegger and Wagner, 2006; 2008; Manel *et al.*, 2003), population connectivity, and even potential hotspots of disease outbreaks (Manel and Holderegger, 2013).

To provide a framework for assessing colonial waterbird populations from the Danube Delta, we developed a comprehensive experimental design that is able to answer existing questions regarding genetic diversity, genetic discontinuities and the degree of genetic differentiation. This paper describes a study which overlaps landscape genetics principles and a small genetic survey in order to provide a paradigm framework for studying colonial waterbirds from the Danube Delta.

MATERIALS AND METHODS

Study design

Importance of study design. Usually, population genetic studies rely on an opportunistic sampling process (from known localities or highly accessible areas), that often fails to illustrate the true spatial dependency (Storfer *et al.*, 2007), offering a misleading lack of spatial imprint or even erroneous model inferences (Fortin and Dale, 2005; Legendre *et al.*, 2002). Thus, in terms spatial distribution, sampling points should follow a continuous distribution, rather than a discrete one. Therefore, each study requires its own blueprint, in terms of spatial distribution of sampling points and sampled individuals' thresholds, to sample the variable(s) of interest within the scale of spatial dependency (Coulon *et al.*, 2004). Also, an important factor that ensures a successful outcome is the study scale (sampling size, spatial or even temporal dimensions), that should be estimated through pilot data or data from similar organisms.

In our case, the study design should be developed to be large enough to fulfill its aim with a high degree of confidence. But first of all, we must specify a proper aim: **Genetic diversity assessment and the correlation between colony susceptibility to disease outbreaks and populations' gene pool richness.** To fulfill this aim, several milestones must be achieved, such as: a large sample scattered across the entire Danube Delta, colony dispersal and immigration proportions, discontinuous distribution of colonies, genetic variance within colonies and also the disease outbreak incidence background. Due to the large study area (the entire Danube Delta), degree of complexity, ethics, involved human resources and cost effectiveness reasons, a comprehensive experimental design is more than necessary.

Incorporating landscape data. A right approach to this kind of study requires two distinct types of data. The first one is landscape data, a geographic framework, which includes satellite maps with proper resolutions, field surveys, field collections and habitat characteristics, while the second one is represented by multilocus genetic data (both nuclear and mitochondrial). As previously noted by Storfer *et al.* (2007), the scale at which data are collected should match the scale of the study questions and hypotheses.

Sampling. Sampling design is one of the most important components in a study blueprint providing or removing the experiment success. Therefore, the sampling design is a matter of proper questioning of the problem to increase the likelihood of highlighting the involved traits. There is no recipe for a successful sampling design, due to its unique purpose to respond to a specific aim. Despite that, developing a sampling design is not a completely blind step, there are some pre-designed sampling models that should provide a proper starting point. Storfer *et al.* (2007) describes 8 sampling approaches, grouped into two classes, random and systematic, based on the method involved.

For our study aim, neither one these 8 models fit well enough, therefore we developed a mixed model, stratified - hierarchical – random-start sampling. This new model takes 3 of the pre-designed models and puts them in action layered to different zoom scales. The stratified model is applied to the macroscale, dividing the study area into 6 sectors (Figure 1A) based on waterbody geographic distribution, and should provide a convenient area coverage. The hierarchical model is a broad- to fine-scale compromise and it is applied to each sector. Due to the colonies discrete distribution and nested distribution of waterbird individuals in the colony, theoretically each colony should be sampled to ensure a continuous sampling. In practice, this is quite impossible due to the cost and human

resources involved and equally for animal ethics reasons. A solution to that is to reduce the number of sampled colonies in a way that keeps the continuous distribution of the sampling points through points distance variability (Figure 1B). The last model, random-start sampling is applied within each colony to ensure a good estimation of colony diversity. Overall, this mixed sampling model provides an effective and feasible solution to a problem that at first glance seems impossible to solve.

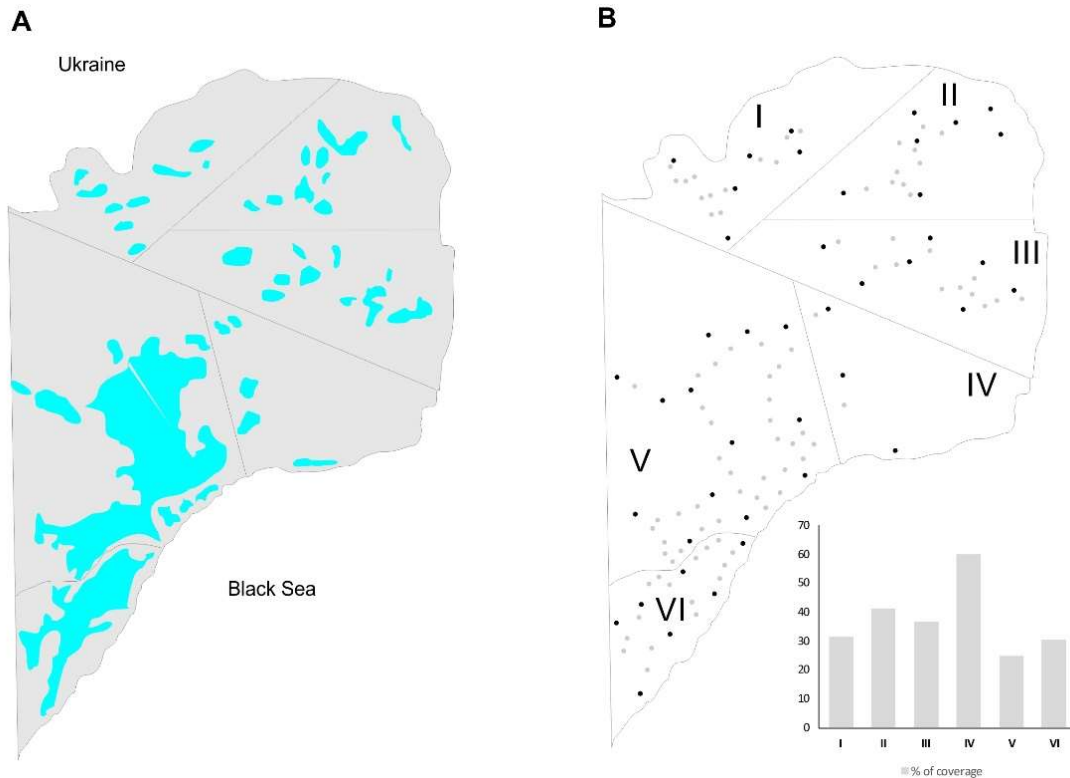


Figure 1 The sampling design for assessing the genetic diversity of colonial waterbirds from the Danube Delta. A- the main waterbody distribution across the Danube Delta Biosphere Reserve; B- The representation of the stratified - hierarchical – random-start sampling mixed model.

Molecular markers. Fast evolving parts, such as mitochondrial sequences (mtDNA) or nuclear introns, are suitable for elucidating young evolutionary relationships (for example between species), whereas older relationships are better analyzed with more conservative genes, like nuclear exons (Lin and Danforth, 2004). The most commonly used genes in bird phylogeny and population studies have been: recombination activating genes *RAG-1* (Barker *et al.*, 2002; Ericson and Johansson, 2003; Irestedt *et al.*, 2001), and *RAG-2* (Barker *et al.*, 2004; Beresford *et al.*, 2005), myoglobin (Ericson and Johansson, 2003; Irestedt *et al.*, 2002) and proto-oncogene *c-Myc* (Ericson *et al.*, 2000; Irestedt *et al.*, 2002). Different authors (Moeller and Townsend, 2011; Susko and Roger, 2012) acknowledge the necessity of studies concerning the phylogenetic information and experimental design in molecular assessments. In accordance with all these, a suitable combination of molecular markers should be chosen. The best choice for our purpose is a combination of mitochondrial ones (cytochrome oxidase subunit I or cytochrome b) and nuclear ones. The most promising candidate for a nuclear marker suitable to our study should be *MHC I* (major histocompatibility complex class I), being able to maximize the nuclear informativeness with aspects of genetic diversity, phylogeny and even diseases background or parasitism.

Pilot study

To increase the project feasibility, we conducted a pilot study to identify the optimal DNA source and mitochondrial marker to describe the phylogenetic, population and ecological aspects of colonial waterbirds from the Danube Delta.

During the summer of 2016 around 45 tissue samples were collected from 11 locations scattered across the entire Danube Delta, belonging to 11 species. For ethical reasons the least invasive sampling methods were used to collect 4 types of tissue (3 kinds of feathers, skin fragments, eggs and egg shells). All tissue samples were subjected to genomic DNA extraction using NucleoSpin® Food kit (Macherey-Nagel GmbH & Co. KG, DE) in accordance with manufacturer specifications. The total DNA was eluted in 50 µl Elution Buffer CE and then spectrophotometrically quantified.

The PCR was carried out to partially amplify the mitochondrial cytochrome oxidase subunit I gene (COX, ~600 bp) using the BirdF1-Ixmod and BirdR1-Ixmod primer set previously described by Päckert et al. (2014). The sequencing was done using the Dye-terminator method through a commercial service company (Macrogen Inc., NL). The COX sequences were aligned using the multiple-alignment ClustalW algorithm (Thompson et al., 1994) implemented in MEGA 7 software (Kumar et al., 2016). Genetic diversity was assessed using haplotypes and genetic diversity indices (H_d - haplotype diversity, K - average number of differences, H_e - expected heterozygosity for polymorphic loci, π - nucleotide diversity) in ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). To illustrate the phylogenetic relationships within individuals, the Maximum Likelihood method was applied using RAxML v.8.1 software (Stamatakis, 2014).

RESULTS AND DISCUSSION

Pilot study results

DNA was successfully isolated from 45 samples regardless of tissue type. Despite this, the quantity and quality of DNA varied greatly depending on the tissue type used (Figure 2). The best results in terms of DNA quantity and quality were obtained from flight feathers (remiges), but for ethical reasons covert feathers (tectrices) seem to be the optimal DNA source for this study (tectrices taken from a live individual do not affect its flight), and used in the next steps. Regarding the amplification step, 3 mitochondrial regions were tested: *cytochrome b* gene, *COX1* gene and *D-loop*. After several optimizations and tests on different waterbird species, the *COX1* gene was chosen as the most suitable mitochondrial marker, due to the wide range of species and genetic informativeness reviewed from literature.

DNA sequences of approximately 600 bp were successfully generated using a *COX1* primer set, 10 sequences from 6 different species. After alignment with all sequences available on GenBank of each sequenced species (6 alignments corresponding to each analyzed species), an overlapping region of 552 bp was available across all species for the *COX1* region (a phylogenetic dataset of 100 sequences: Polymorphic sites = 179; Parsimony informative sites = 173). To confirm the genetic informativeness of the *COX1* gene reviewed from literature, a phylogenetic and population study was conducted. The markers ability to properly describe the relationships between both closely and distantly related taxa was checked using phylogenetic reconstruction. The inferred ML tree showed good delimitation at all the main phylogenetic levels of the tree: species, family and order (Figure 3).

To assess the *COX1* gene accuracy and resolution in discriminating individuals, a population study was performed for each species. The present data reveal an outstanding ability for observing genetic diversity levels (Table 1). Nucleotide diversity per site had moderate values (~0.002) in all species except *Pelecanus crispus* where the value was significantly higher (~0.007). Overall, the moderate nucleotide diversity values are an indicator of a suitable molecular marker, having enough variability to show the spatial genetic imprint and slight variation within a population (Fixation index, population genetic structure and its degree of expected heterozygosity).

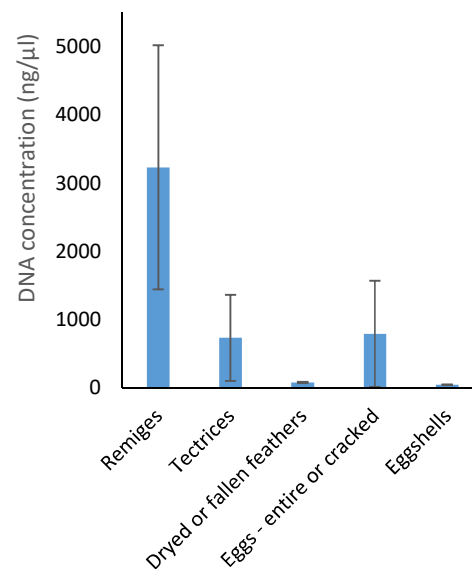


Figure 2 The average (and standard deviation) DNA amount isolated from 4 types of tissue

In addition, for each analyzed species at least one new unique haplotype, specific to Danube Delta populations, was observed. This situation highlights the necessity and the value of further landscape genetic studies on colonial waterbirds from the Danube Delta.

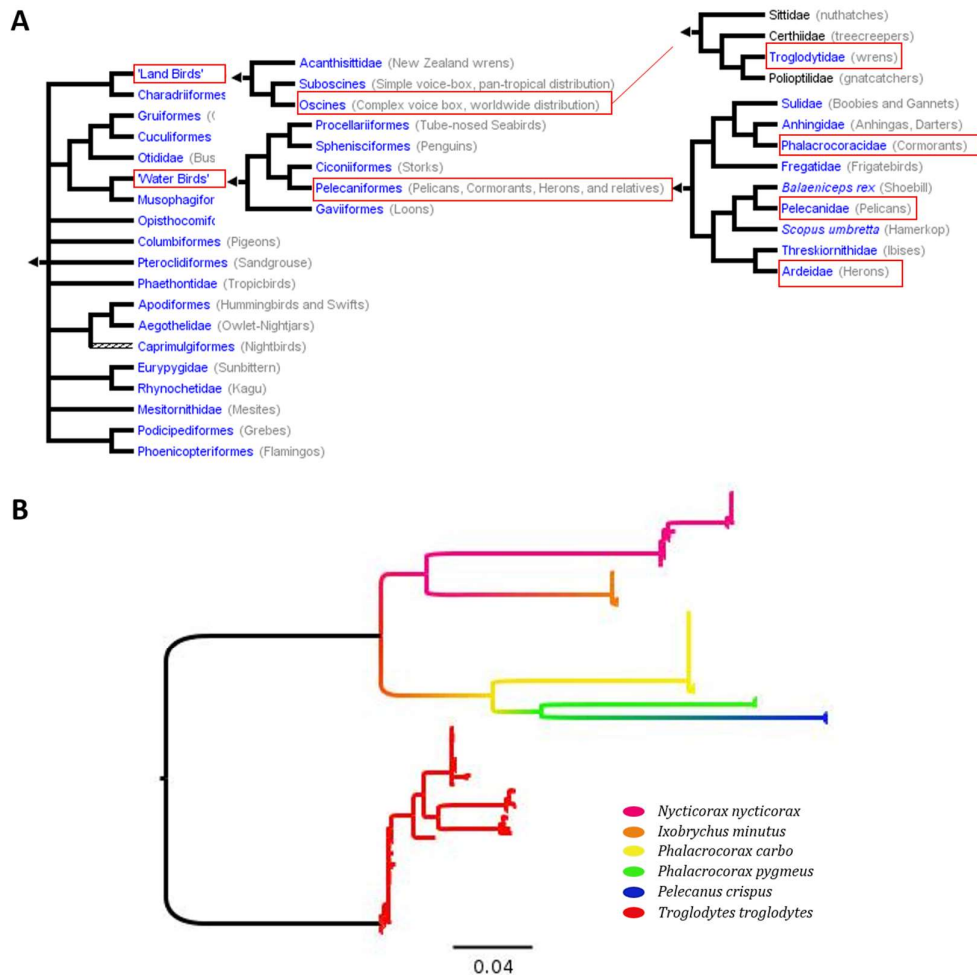


Figure 3 The taxonomic classification of the analyzed samples. A- The sample membership to higher taxa to the superorder level (Neoaves) according to Tree of Life - Neoaves (David, *et al.*, 2008); B- Phylogenetic relationships reconstruction through Maximum Likelihood method in RAxML.

Table 1 Genetic diversity among analyzed samples

Species	N	Nh	DD-Nh	π	H_d	FST	K	k	H_e
<i>Pelecanus crispus</i>	3	1	1 (1)	0.0074	0.6667	NA	7	NA	0.6666
<i>Phalacrocorax pygmeus</i>	3	2	1 (2)	0.0022	0.6667	NA	2	NA	0.6666
<i>Phalacrocorax carbo</i> *	20	4	2 (4)	0.0014	NA	0.6141	NA	0.95	NA
<i>Nycticorax nycticorax</i> *	18	9	1 (1)	0.0027	NA	0.9299	NA	17.44	NA
<i>Ixobrychus minutus</i> *	9	6	1 (1)	0.0037	NA	0.8751	NA	1.416667	NA

* - interpopulational study; N – number of sequences; Nh – number of haplotypes; DD-Nh – number unique haplotypes found just in Danube Delta, the number from the brackets represent the total number of sample from Danube Delta; π - nucleotide diversity; FST - Fixation Index, AMOVA; H_d – haplotype diversity; K –haplotype mean distance; k – mean distance between populations; H_e - expected heterozygosity.

Expected outcomes of the experimental design

The scale of the experimental design and the used tool will allow us to analyze and draw the spatial distribution of the genetic diversity and the phylogenetic relationships between colonial waterbirds

from the Danube Delta Biosphere Reserve. Also, aspects such as genetic discontinuities and degree of genetic differentiation between colonies will be revealed through this design. The expected results have great implications in conservation management, ecological and ethological studies. Furthermore, by overlapping the landscape, genetic diversity and disease outbreaks background, a correlation between colony susceptibility to disease outbreaks and populations' gene pool richness could be drawn. Also, with additional molecular markers, such as *MHC I*, it will be possible to identify current and future hotspots of disease outbreaks, with potential applicability in wildlife conservation and even public health.

CONCLUSIONS

To the best of our knowledge, this is the first experimental design of landscape genetics for colonial waterbirds from the Danube Delta. The design outline provides a paradigm framework for studying bird landscape genetics, being the first one of its kind in Romania. Also, this paper highlights the importance of study design and a proper sampling model. The design's feasibility was confirmed by the results of the pilot study, suggesting outcomes with high applicability.

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