Lithuanian Fund for Nature

Project LIFE05NAT/LT/000094 "Protection of European pond turtle and threatened amphibians in the North European lowlands"

ACTION A.8. Genetic investigations

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Genetic Examinations

1. Introduction

In genetic studies on *Emys orbicularis* the phylogeny and phylogeography was investigated with the help of the mitochondrial nucleotide sequences of the cytochrome b gene analysed by DNA sequencing and RNA heteroduplex analysis. 20 different haplotypes with distinct geographical ranges were found out (LENK et al. 1999). In the northern range of the species distribution the nominate subspecies *Emys orbicularis orbicularis* exists. For the *Emys orbicularis orbicularis* populations in the project areas in Lithuania, Poland and Germany two haplotypes were described: in East-Poland and Lithuania the specific haplotype I a and in East-Germany and West-Poland (east and west of the River Oder) the specific haplotype II b (LENK et al. 1999). Within the EU-Life-Project European pond turtles from different project areas were genetically examined. The aim of the genetic analysis was to evaluate if the project populations are autochthonous or not as indication for the creation of population management measures. In case of occurrences of allochthonous animals, accordant measures for eliminating allochthonous genes in the populations would be needed to apply.

2. Method

Turtles in all three project countries were genetically investigated. For these purposes a gentle and quickly executable field method was used to retrieve DNA. The animal's head is fixed between thumb and index-finger. With a thin but blunt metal spatula the animal is encouraged to open its beak and after that a cotton stick is inserted into the oral cavity. Under light pressure and with a slight rotary movement the cotton stick is moved along the palate, the tongue and the labial angles of the animal.
The mucosal-sample was conserved in 75 % alcohol. Around 90 % of all samples contained sufficient DNA for a later amplification through a Polymerase-chain reaction.
The foundation for this is provided by the analysis of a 1074 base pair long fragment of the mitochondrial cytochrom b-gene.
All samples were evaluated by Ms. Anke Müller in the genetic laboratory of the Museum of Zoology in Dresden (Senkenberg Naturhistorische Sammlungen).

3. Results and conclusions

3.1 Lithuania

Results

Table 1: Results of genetic analysis of the *Emys orbicularis* mucous membrane (Lithuania)

Analysis no.	Subspecies	Popu-lation	Turtle no.	Age	Sex	Haplo-type	Sample: no., date, person
5788	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5789	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5790	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5791	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5792	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5793	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5794	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5795	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5796	*Emys o. orbicularis*	L03				Ia	July 2008, J. Sidaravicius
5797	*Emys o. orbicularis*	L03		juv, 5 years		Ia	July 2008, J. Sidaravicius
5767	*Emys o. orbicularis*	L04	JUOD-5/6	ad, old	f	Ia	JOUD 1, July 2008, M. Meeske
5768	*Emys o. orbicularis*	L04	JUOD-32	ad, old	m	Ia	JOUD 2, July 2008, M. Meeske
5769	*Emys o. orbicularis*	L04	JUOD-11?	ad, younger	f	Ia	JOUD 3, July 2008, M. Meeske
5770	*Emys o. orbicularis*	L04	JUOD-12	ad, young	f	Ia	JOUD 4, July 2008, M. Meeske
5771	*Emys o. orbicularis*	L04	JUOD-8	ad, younger	f	Ia	JOUD 5, July 2008, M. Meeske
5772	*Emys o. orbicularis*	L05	KUC-11	ad, old	f	no result	KUC 1, July 2008, M. Meeske
5773	*Emys o. orbicularis*	L05	KUC-53	ad, old	m	Ia	KUC 2, July 2008, M. Meeske
5774	*Emys o. orbicularis*	L05	KUC-18	ad, old	f	Ia	KUC 3+, July 2008, M. Meeske
5775	*Emys o. orbicularis*	L05	KUC-18	ad, old	f	Ia	KUC 3-, July 2008, M. Meeske
5776	*Emys o. orbicularis*	L07	BES-9	sad	f?	Ia	BES 0, July 2008, M. Meeske
5777	*Emys o. orbicularis*	L07	BES-7	ad, younger	f	Ia	BES 1, July 2008, M. Meeske
5778	*Emys o. orbicularis*	L07	BES-6	ad, old	f	Ia	BES 2, July 2008, M. Meeske
5779	*Emys o. orbicularis*	L07	BES-8	ad, old	f	Ia	BES 3, July 2008, M. Meeske
5780	*Emys o. orbicularis*	L07	BES-2	ad, young	f	Ia	BES 4, July 2008, M. Meeske
5781	*Emys o. orbicularis*	L07	BES-11	ad, old	f	Ia	BES 5, July 2008, M. Meeske
5782	*Emys o. orbicularis*	L07	BES-5	ad, young	f	Ia	BES 6, July 2008, M. Meeske
5783	*Emys o. orbicularis*	L07	BES-13	ad, very old	m	Ia	BES 7, July 2008, M. Meeske
5784	*Emys o. orbicularis*	L07	BES-16	ad, younger	m	Ia	BES 8, July 2008, M. Meeske
5785	*Emys o. orbicularis*	L07	BES-17	ad, older	f	Ia	BES 9, July 2008, M. Meeske
5786	*Emys o. orbicularis*	L07	BES-18	ad, young	m	Ia	BES 10, July 2008, M. Meeske
5787	*Emys o. orbicularis*	L07	BES-20	ad, old	f	Ia	BES without no., July 2008, M. Meeske
5822	*Emys o. orbicularis*	L06		ad	f	no result	July 2009, J. Sidaravicius

All checked animals in the project areas Veisiejai Regional Park/ Petroškai L03, Meteliai Regional Park L04/ Juodobale herpetological reserve, Kučiuliškė Herpetological Reserve L05 and Bestraigiškė Forest District L07 were identified as autochthonous by reference to their mitochondrial haplotype Ia (tab. 1). Thus, the results confirmed that until now all populations are genetically pure, autochthonous. The only sample from Stračiūnai Herpetological Reserve L06 could not be successfully analysed, but in this area the same haplotype Ia can be assumed.

Conclusions

Until now, most of the Lithuanian populations of *Emys orbicularis* live outside from protected areas. In fact, several populations still occur in distances between 2 up to 5 km to the next neighbouring population where individual exchanges could exist. But also the populations inside protected areas are still in critical situation due to the smaller sizes of protected areas and populations. Generally, the current situation of the Lithuanian turtle populations shows in most of the cases distinct decreasing habitat and population sizes and increasing distances

between them, because many local populations became already extinct. Reasons for this are the big loss of water bodies and suitable nesting sites, general habitat destruction, high nest predation by invasive predators, different kinds of human disturbances, etc.. The declining number and sizes of populations will lead to inbreeding and the loss of further populations in the future. Thereby, individual exchange will be more complicated and reduced. Today, most of the local populations are so small and not able to survive without any exchange with other local populations. Consequently, most of these local populations will become extinct within the next 20 years without any protection measures.

Different conservation activities have to be carried out to save the last Lithuanian populations of *Emys orbicularis*. First of all, the extinction of local populations has to be stopped and the habitats of the populations have to be improved and enlarged. Furthermore, corridors between the local populations have to be created to support connections and regular individual exchange among them. Investigations and a regular monitoring for determining potential inbreeding and its consequences e.g. inbreeding depression have to be conducted e.g. to collect data according to fitness parameters e.g. indications for decreasing fertility, reproduction rate, resistances to diseases, etc.. Very small populations can be supported with the release of reared juveniles in the first 10-20 years to accelerate the population growth. The eggs of these juveniles should be taken from the releasing population or of one of the neighbouring populations (see also 3.3). In case of presumed or already ascertained inbreeding, the prophylactic exchange of young animals between the populations can be recommended as it is already done as protective measure in East-Germany. Furthermore, accompanying measures like genetic investigations should be done for control of not examined populations e.g. if they are polluted by allochthonous animals. A later important aim should be to analyse the population status and a potential inbreeding in the turtle populations when suitable methods of genetic analysis will be developed for it e.g. the microsatellite analysis. Finally, all conservation actions have to be regularly adapted to the actual situation of the Lithuanian turtle populations.

3.2 Poland

Results

Animals in the westpolish project areas Rybocice Pk03, Drawiny Pk04 and Miedzychod were identified as autochthonous by reference to their mitochondrial haplotype IIb (tab. 2). Exceptionally, turtles in the Drzeczkowo area Pk05 have another haplotype Ia-neu357 (tab. 2). Due to the fact that Polish conservationists released animals in different west-polish areas in previous years with different origin e.g. east-polish individuals with the haplotype Ia (see down), it is not possible to evaluate the autochthonic status of the “Drzeczkowo” population until now and to describe a possible new haplotype.

Animals inhabiting the investigated east-polish project areas (Nadrowskie bagno Reserve, Karzełek Lake and an intraforest pond near Likusy) have the same mitochondrial haplotype Ia as the Lithuanian turtles. The animals of these populations are autochthonous.

Table 2: Results of genetic analysis of the *Emys orbicularis* mucous membrane (Poland)

Analysis no.	**Subspecies**	**Population**	**Age**	**Sex**	**Haplo-type**	**Sample: no., date, person**
5798	*Emys o. orbicularis*	Pk03		m	no result	100, 25.05.2007
5799	*Emys o. orbicularis*	Pk03			no result	MK (MY?) DNA
5802	*Emys o. orbicularis*	Pk03	juv		IIb	81, 16.05.2008
5803	*Emys o. orbicularis*	Pk03	sad		no result	47, 16.05.2008
5804	*Emys o. orbicularis*	Pk03	juv		IIb	H136, 16.05.2008
5805	*Emys o. orbicularis*	Pk03	sad		IIb	2H, 16.05.2008
5806	*Emys o. orbicularis*	Pk03	juv		no result	82, 16.05.2008
5807	*Emys o. orbicularis*	Pk03	juv		IIb	83, 17.05.2008
5808	*Emys o. orbicularis*	Pk03	juv		IIb	N84, 23.05.2008
5809	*Emys o. orbicularis*	Pk03	juv		IIb	N85, 23.05.2008
5810	*Emys o. orbicularis*	Pk03	juv		IIb	N86, 23.05.2008
5811	*Emys o. orbicularis*	Pk03	juv		IIb	N87, 23.05.2008
5800	*Emys o. orbicularis*	Pk05			Ia-neu357	os nr.6
5801	*Emys o. orbicularis*	Pk05			Ia-neu357	os nr.5
5812	*Emys o. orbicularis*	Pk05			Ia-neu357	DRZ3/2009, M. Rybacki / 2009
5813	*Emys o. orbicularis*	Pk05			Ia-neu357	DRZ7/2009, M. Rybacki / 2009
5814	*Emys o. orbicularis*	Pk05			Ia-neu357	DRZ8/2009, M. Rybacki / 2009
5815	*Emys o. orbicularis*	Pk04		f	IIb	DRA2/2009, M. Rybacki / 2009
5816	*Emys o. orbicularis*	Miedzychod			IIb	Mie1/2009, M. Rybacki / 2009
5817	*Emys o. orbicularis*	Nadrowskie bagno Res.			Ia	2
5818	*Emys o. orbicularis*	Nadrowskie bagno Res.			Ia	3
5819	*Emys o. orbicularis*	Nadrowskie bagno Res.			Ia	7
5820	*Emys o. orbicularis*	Karzełek Lake by Likusy			Ia	14
5821	*Emys o. orbicularis*	Intraforest pond by Likusy			Ia	15

Conclusions

In former centuries, the number and sizes of turtle populations were much bigger in West-Poland areas than nowadays. Today, only a small number of small relict populations are known there. Furthermore, the distance between these populations is usually large so that no exchange of genes can be assumed among them over a longer period. Inbreeding can be the consequence of this increasing isolation. Only one population has a bigger size of individuals, but uncontrolled releasing activities by Polish conservationists cannot be excluded in this area. Unfortunately, juveniles from bigger east-polish populations were released in West-Poland without any official documentation and although turtles in West- and East-Poland have different haplotypes. In particular, it is necessary to examine exactly the occurrence of the “new” haplotype Ia-neu357 in the west-polish area Drzeczkowo. Hence, further collection and analyses of genetic samples are needed for clarifying the origin of the west-polish animals.

Until now, there are no indications for inbreeding in the relict populations with regard to fitness parameters like fertility, reproduction rate etc.. Nevertheless, conservation measures for the support of the small populations (habitat improvements, population management

actions e.g. rearing of young turtles) have to be continued after the LIFE-project to maintain the genetic diversity of the populations. For the population management only animals with known origin and the right haplotype Ib are allowed to release in the west-polish areas. In the future, status and possible inbreeding in the turtle populations should be periodically investigated in West-Poland when adequate genetic analyses are available e.g. the microsatellite analysis. The most suitable methods for the conservation of the last west-polish populations of *Emys orbicularis* have to be continuously improved by experts.
The actual status of the east-polish population is still unclear and has to be investigated further including genetic investigations. At least no allochthonous individuals could be found up to now. With the help of more studies protection activities and adequate population management methods for these populations have to be created.

3.3 Germany

Results

Three animals from the project area Märkische Schweiz DA 02 were identified as autochthonous by reference to their mitochondrial haplotype IIb (tab. 3).
Because they were the last single individuals of the extinct population in this area the animals were integrated into the breeding group of animals of the pond turtle project.
Additionally animals of the project area of Poratz DA 03 and animals from the area of Kölpinsee DA 04 were examined. For all animals the analysis revealed the expected haplotype IIb. Thus, the results confirmed that until now both populations form genetically pure, autochthonous relicts.

Table 3: Results of genetic analysis of the *Emys orbicularis* mucous membrane (Germany)

No.	Subspecies	Population	Individual no.	Age	Sex	Haplotype	Sample: date, person
2	*Emys o. orbicularis*	DA 02	17.7.2005	ad	f	IIb	18.7.2005, M. Wolf
3	*Emys o. orbicularis*	DA 02	6/05a	ad	f	IIb	14.6.2005, N. Schneeweiß
4	*Emys o. orbicularis*	DA 02	6/05b	ad	f	IIb	14.6.2005, N. Schneeweiß
5	*Emys o. orbicularis*	DA 03	1+3/2	sad		IIb	11.9.2008, N. Schneeweiß
6	*Emys o. orbicularis*	DA 03	1+3/3	sad		IIb	11.9.2008, N. Schneeweiß
7	*Emys o. orbicularis*	DA 03	1+3/1+2	sad		IIb	11.9.2008, N. Schneeweiß
8	*Emys o. orbicularis*	DA 03	1+3/7	sad		IIb	11.9.2008, N. Schneeweiß
9	*Emys o. orbicularis*	DA 03	1+3/1+5	sad		IIb	11.9.2008, N. Schneeweiß
10	*Emys o. orbicularis*	DA 03	Hh 97 „Kn“	sad		IIb	11.9.2008, N. Schneeweiß
11	*Emys o. orbicularis*	DA 01	Station	sad		IIb	11.9.2008, N. Schneeweiß
12	*Emys o. orbicularis*	DA 04	4/1+8	sad		IIb	11.9.2008, N. Schneeweiß
13	*Emys o. orbicularis*	DA 04	4/1+9	sad		IIb	11.9.2008, N. Schneeweiß

14	*Emys o. orbicularis*	DA 04	4/1+12	sad		IIb	11.9.2008, N. Schneeweiß
15	*Emys o. orbicularis*	DA 04	4/8	sad		IIb	11.9.2008, N. Schneeweiß
16	*Emys o. orbicularis*	DA 04	4/1+9	sad		IIb	27.8.2009, N. Schneeweiß
17	*Emys o. orbicularis*	DA 04	4/1+12	sad		IIb	27.8.2009, N. Schneeweiß
18	*Emys o. orbicularis*	DA 04	4/1+10	sad		IIb	27.8.2009, N. Schneeweiß
19	*Emys o. orbicularis*	DA 04	4/1+11	sad		IIb	27.8.2009, N. Schneeweiß
20	*Emys o. orbicularis*	DA 04	4/2+3	sad		IIb	27.8.2009, N. Schneeweiß
21	*Emys o. orbicularis*	DA 04	4/1+8	sad		IIb	27.8.2009, N. Schneeweiß
22	*Emys o. orbicularis*	DA 03	2+3/1+7	sad		IIb	27.8.2009, N. Schneeweiß
23	*Emys o. orbicularis*	DA 03	3/6(2x)	sad		IIb	27.8.2009, N. Schneeweiß

Conclusions

The currently low number of individuals of the last 5 autochthonous relict populations of Germany as well as their complete isolation inevitably lead to a high degree of in-breeding. In relation to the distance to each other (9 - 60 km) it is to be expected that there was no exchange of genes among the populations over the last decades or centuries. As long as the single occurrences had a relatively high number of individuals and there were nearby sub-populations there should have hardly been any negative genetic effects on the populations. Having a severe decrease in the number of individuals during the last 40 years inbreeding depression is an increasing issue. Even though there have been no signs of inbreeding according to fitness parameters like fertility, reproduction rate etc. so far, 2006 prophylactic exchange of young animals between the populations commenced as a protective measure.

In other words 50 to 60 % of young animals that are added to a specific population originate from one or more other relict populations of Brandenburg. This applies to young animals which originate from open land nesting sites. Within the scope of conservational breeding, animals from different populations of Brandenburg are purposely combined to breeding pairs. In this way an exchange of genes among populations is ensured.

At present it is unclear to which extent this strategy has an effect on stabilizing the populations.

An intense accompanying monitoring which includes the gathering of data related to fertility, reproduction rate, survival rate of the juveniles etc. promises new insights concerning the issue at hand.

4. Literature

LENK, P., U. FRITZ, U. JOGER & M. WINKS (1999): Mitochondrial phylogeography of the European pond turtle, Emys orbicularis (LINNAEUS 1758). – Molecular Ecology (1999) **8**: 1911-1922.