Research Article

ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., Suppl. 10, 2017: 15-22

Evaluation of 11 Microsatellite Loci for Reconstructing of Kinship Groups in the European Pond Turtle, *Emys orbicularis* (Linnaeus, 1758)

Marta Dux[1], Daniel Doktór[2], Adam Hryniewicz[3]& Beata Prusak[1*]

[1]Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Postępu 36A, Jastrzębiec, Poland;
E-mails: martadux@wp.pl, beprusak@gmail.com
[2]Foundation for Oświn Lake, Wyskok 16, 11-420 Srokowo, Poland; E-mail: daniel_oswin@wp.pl
[3]City Zoological Garden in Warsaw, Ratuszowa 1/3, 03-461 Warsaw, Poland; E-mail: ahryniewicz7@o2.pl

Abstract: Multiple paternity has been investigated in many reptile taxa, including *Emys orbicularis*. In the present study, we evaluated a set of eleven microsatellite loci for the detection of multiple paternity and the number of potential fathers in the clutches of the European Pond Turtle. We presented paternity analysis of hatchlings from six nests (64 samples in total) collected at three locations situated in the Zwolenka River Valley, central Poland. We estimated allele frequencies, observed and expected heterozygosities, the Power of Exclusion (PE), the Probability of Siblings Identity (PI) and Polymorphism Information Content (PIC). In all but one of analyzed clutches, we have identified sibling groups with the number of potential fathers from two to five. Detected multiple paternity may increase the effective population size and the genetic diversity as well as may decrease the levels of inbreeding. We concluded that the panel of eleven polymorphic microsatellite loci used could be applied as an effective and reliable tool for the paternity testing in *E. orbicularis*.

Key words: *Emys orbicularis*, multiple paternity, microsatellites, polymorphism information content

Introduction

The European Pond Turtle, *Emys orbicularis* (Linnaeus, 1758) belongs to the family Emydidae and nowadays lives in southern and central Europe, West Asia and North Africa (Fritz 2003). The turtle is a highly polytypic species with nine mitochondrial lineages recognized (Fritz et al. 2007). In Poland, the existence of two lineages of the subspecies *E. orbicularis orbicularis* were reported (Fritz et al. 2005, 2007, 2009, Lenk et al. 1999), albeit the distributional data were published only in recent years (Prusak et al. 2011). The species inhabits almost the entire lowland area of Poland but in many sites occurs only locally and is considered as infrequent (Najbar 2001). *Emys orbicularis* is listed as NT "Lower Risk/Near Threatened" category in the IUCN Red List (Tortoise & Freshwater Turtle Specialist Group 1996), and in the Polish Red Data Book of Animals as an endangered species (EN) (Jabłonski 1992).

Multiple paternity has been reported in many taxa, including both invertebrates (Xue et al. 2014) and vertebrates (Labrecque et al. 2014). In reptiles, the occurrence of the phenomenon has been detected in the European Pond Turtle (Roques et al. 2006) among several other turtle species (Fitzsimmons et al. 1998, Crim et al. 2002, Moore & Ball 2002, Johnston et al. 2006), and also in the garter snake *Thamnopis sirtalis* (Mccraken et al. 1999).

*Corresponding author: beprusak@gmail.com

Coleman & Jones (2011) demonstrated that reptiles have significantly higher rates of multiple paternity than other vertebrate classes (i.e. birds), though the rates in reptiles are not significantly different from mammals or parental care class of fishes. Fishes, for example, exhibit various mating systems like polygynandry (when both males and females mate with more than one partner, see Parker & Kornfield 1996), polygyny (when males mate with several females but females mate with a single male, see Ziadi-Künzli & Tachihara 2016) and polyandry (when females mate with multiple males but males mate with a single female, see Avise et al. 2002). In reptiles, especially in turtles and tortoises, polyandry is particularly common (Lee & Hays 2004), although single mating is often enough for the fertilization and does not limit the reproductive success (Trivers 1972), promiscuity is quite common in nature and it is considered to be biologically favourable (Lee & Hays 2004). Moreover, parentage analysis confirmed also the sperm storage in short as well as in long periods (Pearse & Avise 2001). In females of the European Pond Turtle, sperm storage has been also detected, though the level of multiple paternity was assessed as low (Roques et al. 2006).

In the studies of paternity microsatellite markers are the most utilized tools (Moore & Ball 2002, Ireland et al. 2003, Roques et al. 2004). Due to the significant polymorphism, microsatellites are widely used in genetic studies in the studies of migration (Zeng et al. 2012), kinship and population genetics (Velo-Antón et al. 2008, Prusak et al. 2013, Stuckas et al. 2014, Vamberger et al. 2015).

The aim of the present study was to determine whether multiple paternity occurs in the examined clutches of the European Pond Turtle from Poland and to evaluate the usefulness of the used panel of microsatellite loci for reconstructing kinship groups.

Materials and Methods

The biological material (tissue samples) was derived from turtles collected from six nests (families), at three locations situated in the Zwolenka River Valley, central Poland (Borowiec Reserve, Siekierka and Zastocze). In total, samples from 64 hatchlings of the European Pond Turtle were taken. Family A consisted of 19 individuals (Siekierka), Family B of 10, D of 11, E of 7, F of 5 (Borowiec Reserve) and Family C of 12 individuals (Zastocze). The individuals collected from a certain breeding chamber were identified as belonging to the particular family. We analyzed only those hatchlings that were found dead in a breeding chamber after wintering.

Total genomic DNA was extracted according to the standard organic procedure (Wilson et al. 1995) from tissues of the European Pond Turtle hatchlings. We used 11 previously published polymorphic microsatellite loci: msEo2, msEo21, msEo29, msEo4 (Pedall et al. 2009), GmuD107, GmuD16, GmuD88, GmuD93 (King & Julian 2004), Emys2, Emys6 and Emys11 (Ciofi et al. 2009). Two multiplex PCRs amplifying seven and four loci were performed as described earlier (Prusak et al. 2013), except loci Emys4 and Emys5 discarded from the present analysis due to detection of null alleles in previous studies of Polish populations of the European Pond Turtles (Prusak et al. 2013). Amplified PCR products were resolved on ABI 3130 Genetic Analyzer (Life Technologies, USA) using an internal size standard GeneScan™ 500 LIZ® Size Standard (Life Technologies, USA). Fragment sizes were determined with the GeneMapper v.4.0 software (Life Technologies, USA).

GenAlex 6 program (Peakall & Smouse 2006) was used to estimate separately for each family, allele frequencies, effective number of alleles, observed and expected heterozygosities, the probability of identity (PI), the power of exclusion (PE), and the power of the increasing number of loci for PI and PE. The polymorphism information content (PIC) for each marker was calculated separately for the six groups of offspring with PICcalc (Nagy et al. 2012) from the allele frequencies. The PIC value is a measure of polymorphism (Shete et al. 2000), thus allowing characterizing genetic variation in a population (or in the family, as in the present case). The parameter is also defined as the probability that the marker genotype of a given offspring will allow to determine his parents (Nagy et al. 2012). Paternity analysis was performed with Kinalyzer software (Berger-Wolf et al. 2007, Ashley et al. 2009). The procedure enabled division of examined individuals into sibling sets with common progenitor, thus reconstructing kinship groups (Berger-Wolf et al. 2007, Ashley et al. 2009).

Results and Discussion

We observed genetic variability at all analyzed loci (from 5 to 11 alleles per locus). The number of effective alleles ranged between 2.818 and 4.455 (mean 3.515). The observed heterozygosity (H_o) ranged from 0.792 to 0.818, while the expected heterozygosity (H_e) ranged from 0.547 to 0.658 (Table 1). Maximal probabilities of exclusion, calculated for each locus and family, had different values depending on the analyzed index (P1E, P2E or P3E).

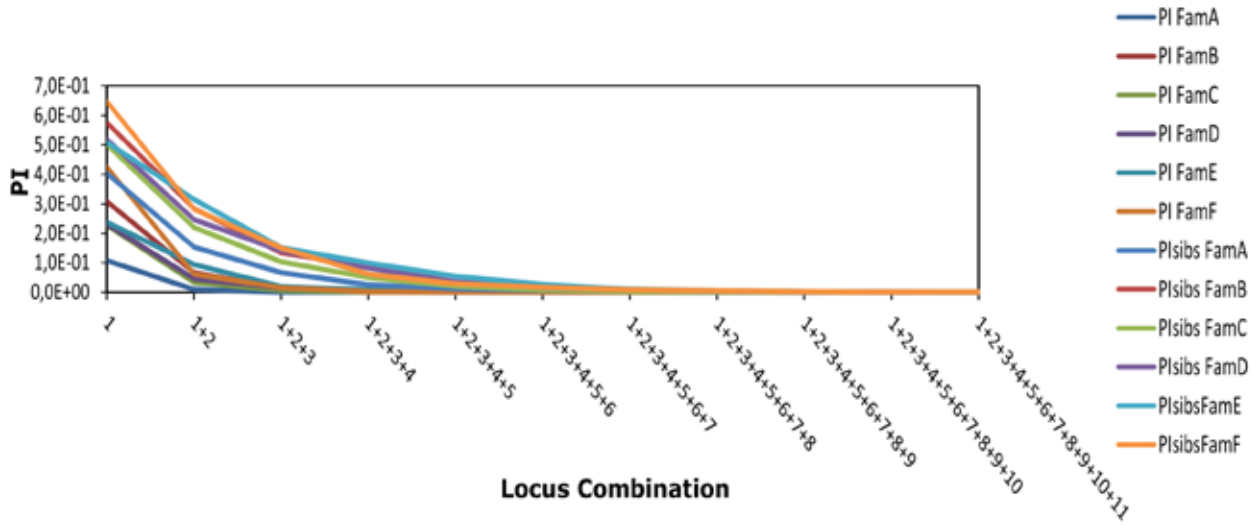

Fig. 1. Probability of Identity (PI) for each locus and for increasing combinations of the 11 loci: 1 – for one locus, 1+2 – for two loci, 1+2+3 – for three loci, etc.

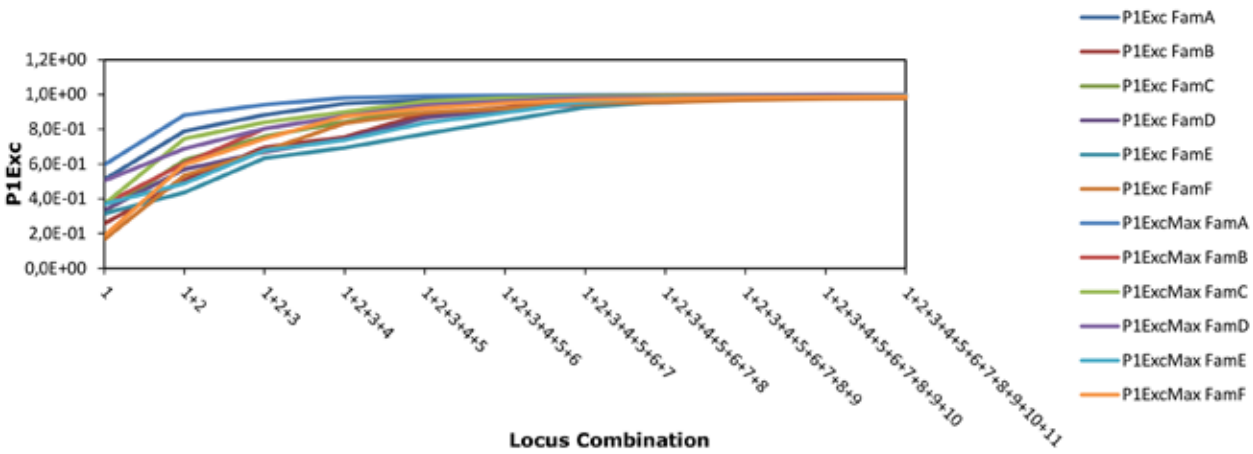

Fig. 2. Probability of Exclusion (P1Exc) for each locus and for increasing combinations of the 11 loci – when the other parent is known: 1 – for one locus, 1+2 – for two loci, 1+2+3 – for three loci, etc.

The maximal probability of exclusion for a single locus and with the known both parental genotypes ($P1Ex_{max}$) ranged between 18.75% and 59.52%. The maximal probability of exclusion for a single locus and with one known parental genotype ($P2Ex_{max}$) had values between 12.50% and 41.60%. The maximal probability of exclusion of both putative parents for a single locus ($P3Ex_{max}$) ranged between 28.13% and 77.18%. Probability of sibling identity and probability of exclusion were also calculated for the combinations of increasing number of loci (Figs. 1–4). The use of an increasing number of loci led to increasing values of probability of exclusion (exceeded 99.00% for the cumulation from 3 to 10 loci depending on the analyzed family) and decreasing values of PIC_{sibs} (lower than 0.10% for the cumulation from 9 and to 10 loci depending on the analyzed family). The maximal probabilities of exclusion and sibling identity for the cumulation of eleven analyzed microsatellite loci are shown in Table 2. Our analyses showed that the number of microsatellite loci used and their level of polymorphism are critical for the reliable reconstruction of sibling relationships, in particular when parental genotypes cannot be assigned (are unknown). Obtained results of cumulative values for probability of exclusion proved that the error probability in paternity testing for the used panel of microsatellites in all analyzed hatchlings reached low values. Polymorphic information content (PIC), within all investigated individuals, had values between 0.269 and 0.759 (Table 3), mean 0.551.

Kinship analysis showed that offspring of five out of six examined nests had more than a single father (Table 4). These families were divided into 2–5 groups of siblings, with different genotypes at

Table 1. Descriptive statistics of 11 microsatellite markers screened on six sibling groups (N – No of individuals in particular families, N_a – No of alleles, N_e – No of effective alleles, H_o – observed heterozygosity, H_e – expected heterozygosity, I – Shannon's information index)

Family code	Diversity indices					
	N	N_a	N_e	H_o	H_e	I
FamA	19	4.45	3.181	0.804	0.658	1.226
FamB	10	3.818	2.843	0.797	0.614	1.119
FamC	12	3.633	2.972	0.818	0.614	1.119
FamD	11	3.364	2.726	0.719	0.606	1.041
FamE	7	3.000	2.508	0.792	0.578	0.961
FamF	5	2.818	2.391	0.800	0.547	0.902

Table 2. The maximal probabilities of exclusion and sibling identity for the cumulation of eleven analyzed microsatellite loci: $P1Exc_{max}$ – the maximal probability of exclusion with the known both parental genotypes; $P2Exc_{max}$ – the maximal probability of exclusion with one known parental genotype; $P3Exc_{max}$ – the maximal probability of exclusion of both putative parents; PIC_{sibs} – probability of siblings identity

Family code	Probability indices			
	$P1Exc_{max}$	$P2Exc_{max}$	$P3Exc_{max}$	PIC_{sibs}
FamA	1.000	0.993	1.000	0.000
FamB	0.998	0.983	1.000	0.000
FamC	0.999	0.978	1.000	0.000
FamD	0.997	0.967	1.000	0.000
FamE	0.993	0.942	1.000	0.000
FamF	0.989	0.925	0.999	0.000

Table 3. Polymorphism information content (PIC) for each marker in each sibling group

Family code	PIC/microsatellite locus										
	msEo2	msEo41	GmuD107	GmuD88	GmuD16	msEo21	GmuD93	Emys11	Emys2	msEo29	Emys6
FamA	0.704	0.741	0.744	0.647	0.586	0.354	0.495	0.587	0.690	0.567	0.528
FamB	0.442	0.347	0.548	0.587	0.729	0.528	0.759	0.675	0.499	0.512	0.530
FamC	0.536	0.561	0.641	0.583	0.699	0.663	0.683	0.346	0.533	0.547	0.624
FamD	0.526	0.407	0.573	0.422	0.677	0.567	0.555	0.373	0.726	0.672	0.356
FamE	0.523	0.325	0.354	0.567	0.454	0.551	0.703	0.626	0.551	0.354	0.502
FamF	0.703	0.740	0.645	0.499	0.586	0.410	0.548	0.365	0.492	0.365	0.269

analyzed microsatellite loci. The highest number of potential fathers (five) we found in Family A. Family F with a single father identified, consisted of a small number of tested turtles. In Family B, D and E, a single hatchling was assigned to two different sibling groups. Although Kinalyzer performs well when few loci are sampled or the allelic diversity is low, paternity analysis of candidate fathers caught in the population or analysis of all hatchlings from the nest may further resolve the observed ambiguity.

Considering the available literature on the paternity analysis in the European Pond Turtle, such a comprehensive panel of microsatellite loci as in the present study have been used in *E. orbicularis* for the first time. In the previous study (Prusak et al. 2013), we have also shown that the microsatellites analyzed here were successfully used to assess the genetic diversity of Polish populations of *E. orbicularis*. In order to exclude paternity on the basis of inconsistency with Mendelian law, it is necessary to use sufficient number of polymorphic loci to minimize errors (Christie 2010). These conditions were fulfilled in this study, suggesting that a similar set of microsatellite markers can also be used in other studies on the

multiple paternity analyses in wild species. It seems that the high efficiency of the methodology used in the study is confirmed by the high values of PIC. The testing of multiple paternity phenomenon may be carried out using various tools. We applied the analysis on the basis of the offspring genotypes using Kinalyzer software (Berger-Wolf et al. 2007, Ashley et al. 2009). The alternative to determine the most likely number of sires consistent with the progeny genotypes may be the use of COLONY (Wang 2004) or GERUD (Jones 2001) software. However, the program's algorithm requires knowing parental genotype, i.e. known mother that is in many cases of wild species impossible to determine.

For the majority of similar analyses biological material was blood samples from the newly hatched individuals and the females-mothers (Fitzsimmons 1998, Crim et al. 2002). Some sources report that analyzed DNA was purified from tissue biopsy, too (Moore & Ball 2002, Lee & Hays 2004). For the comparison, in forensic medicine analyses the DNA is isolated from blood, buccal mucosa or sperm samples (Weber-Lehmann et al. 2014). For the present analysis, we extracted DNA only from the tissue samples of hatchlings, which were found dead in a breeding chamber. Analysis of all hatchlings from the nest may certainly increase the resolution and accuracy of our analysis. In humans, in most cases of alleged paternity, a set of 15–20 microsatellite loci is enough for exclusion or acknowledgment of paternity (O'Connor et al. 2010). However, in some cases, the set of loci used should be extended over the additional genetic markers to increase the power of discrimination (O'Connor et al. 2010). For example, in humans in the case of monozygotic twins, ultra-deep next generation sequencing (NGS) is used to identify extremely rare mutations (Weber-Lehmann et al. 2014). In case of wild species, the microsatellite markers are often not easy available and the analyses have to be performed with the use

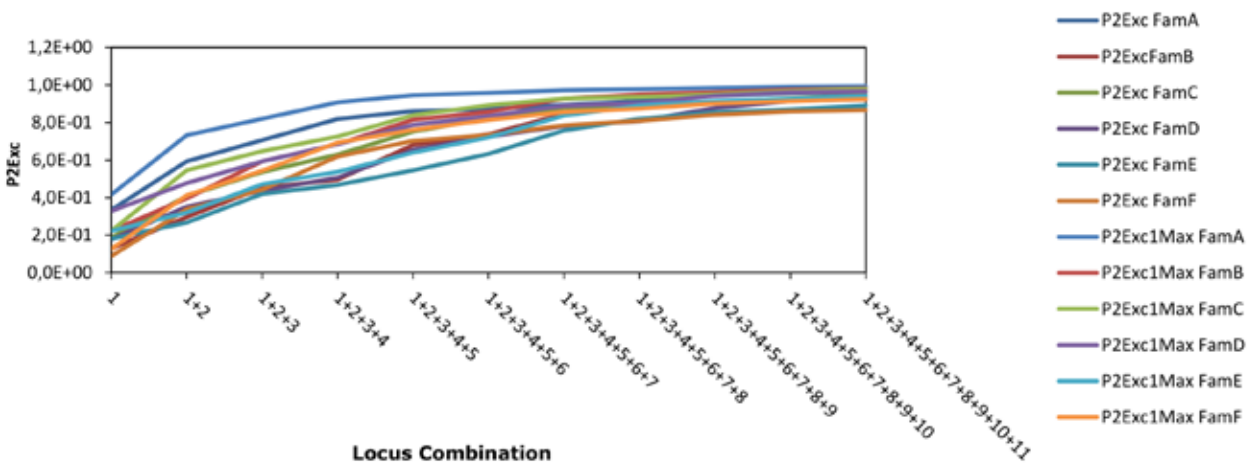

Fig. 3. Probability of Exclusion (P2Exc) for each locus and for increasing combinations of the 11 loci – when genotype of one parent is missing: 1 – for one locus, 1+2 – for two loci, 1+2+3 – for three loci, etc.

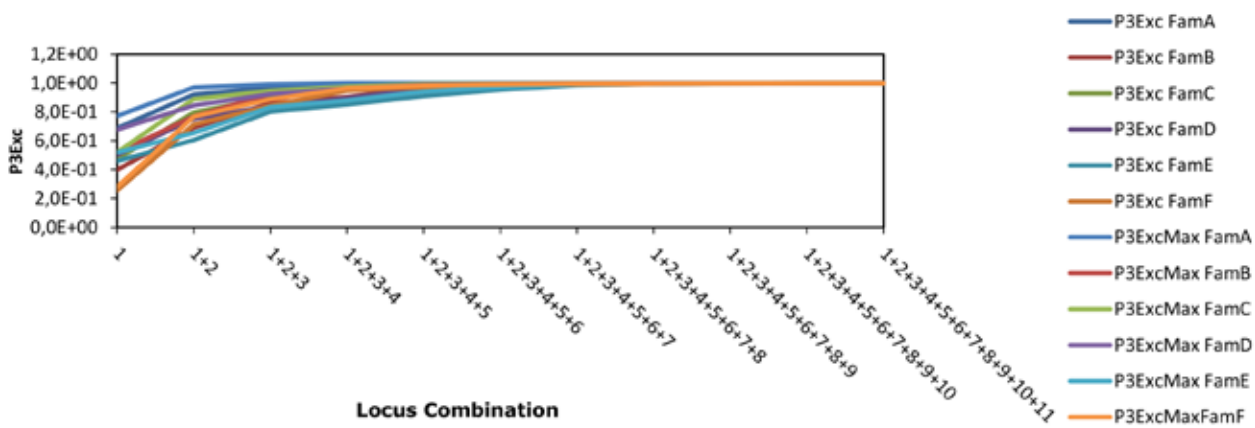

Fig. 4. Probability of Exclusion (P3Exc) for each locus and for increasing combinations of the 11 loci – excluding a putative parent pair: 1 – for one locus, 1+2 – for two loci, 1+2+3 – for three loci, etc.

Table 4. Sibling groups in analyzed families of the European Pond Turtle hatchlings determined with Kinalyzer (Berger-Wolf et al. 2007, Ashley et al. 2009)

Family code	Sibling groups				
	SibsSet 1	SibsSet 2	SibsSet 3	SibsSet 4	SibsSet 5
FamA	A9,A18	A1,A5,A7, A8,A12,A16	A2,A17,A19	A3,A4,A13,A14	A6,A10,A11,A15
FamB	B5,B8,B10	B1,B3,B4, B6,B7	B2,B5,B9		
FamC	C1,C11,C12	C3,C8,C10	C2,C4,C5, C6,C9,C10	C1,C7,C12	
FamD	D4,D11	D2,D3,D5, D6,D7	D1,D8,D9, D10		
FamE	E1,E3,E4, E5,E7	E2,E3,E6			
FamF	F1,F2,F3, F4,F5				

of a limited number of loci or supplementary set have to be adopted from other related species, as in the case of *E. orbicularis* (Pedall et al. 2009). It should also be pointed out that the major disadvantage of using microsatellite markers is the risk of wrong alleles typing (Ingvarsson et al. 2000). In the present study, amplification of all eleven microsatellite markers gave the clear readable PCR products and we did not observe any typical problems with the identification of the individual alleles.

The significance of polyandry results from a number of phenomena like fertilization assurance, the sperm selection, and also shows the direct adaptation benefits arising from an increasing genetic variability of the offspring (Birkhead 2000). According to Lee & Hays (2004), a female mating with multiple partners "makes the best of a bad job". Previous studies showed that multiple paternity occurred in the Italian populations of *E. orbicularis* (Roques et al. 2006), while results of the present study proved the occurrence of this phenomenon in the European Pond Turtle in central Poland. Earlier, multipaternity has been also shown in other turtle species (Pearse & Avise 2001, Ireland et al. 2003, Lee & Hays 2004). The results also confirmed observations of Rovero et al. (1999) on the reproductive behaviour in the Italian populations of *E. orbicularis*, i.e. that females may mate with more than one male during the same season. Our results have shown that the number of used microsatellite loci and their level of polymorphism are critical for the reliable reconstruction of sibling relationships. In our case, the use of an increasing number of loci led to increasing and high values of PE, and decreasing and low values of PI showing that the maximal values obtained for eleven microsatellite markers gave reliable assessment of sibling groups.

Acknowledgements: This study was funded by the Polish Ministry of Science and Higher Education (N304016133). The Polish Ministry of Environment and National Ethics Commission for Animal Experiments approved this research (permits: DOPogiz-4200/II-02/749/05/aj, DOPogiz-4200/II-13/730/10/ed and 433/2005, 3/2009).

References

Ashley M. V., Caballero I. C., Chaovalitwongse W., Dasgupta B., Govindan P., Sheikh S. & Berger-Wolf T. Y. 2009. KINALYZER, a computer program for reconstructing sibling groups. Molecular Ecology Resources 9 (4): 1127–1131.

Avise J. C., Jones A. G., Walker D., DeWoody J. A., Dakin B., Fiumera A., Fletcher D., Mackiewicz M., Pearse D., Porter B. & Wilkins S. D. 2002. Genetic mating systems and natural histories of fishes: lessons for ecology and evolution. Annual Review of Genetics 36: 19–45.

Berger-Wolf T. Y., Sheikh S. I., Dasgupta B., Ashley M. V., Caballero I. C., Chaovalitwongse W. & Putrwevu S. L. 2007. Reconstructing sibling relationships in wild populations. Bioinformatics 23 (13): 149–156.

Birkhead T. 2000. Promiscuity: an evolutionary history of sperm competition and sexual conflict. London: Faber. 272 p.

Christie M. R. 2010. Parentage in natural populations: novel methods to detect parent-offspring pairs in large data sets. Molecular Ecology Resources 10: 115–128.

Ciofi C., Tzika A. C., Natali C., Chelazzi G., Naziridis T. & Milinkovitch M. C. 2009. Characterization of microsatellite loci in the European Pond Turtle *Emys orbicularis*. Molecular Ecology Resources 9: 189–191.

Coleman S. W. & Jones A. G. 2011. Patterns of multiple paternity and maternity in fishes. Biological Journal of the Linnean Society 103: 735–760.

Crim J. L., Spotila L. D., Spotila J. R., O'Connor M., Reina R., Williams C. J. & Paladino F. V. 2002. The Leatherback

Turtle, *Dermochelys coriacea*, exhibits both polyandry and polygyny. Molecular Ecology 11: 2097–2106.

Fitzsimmons N. N. 1998. Single paternity of clutches and sperm storage in the promiscuous Green Turtle (*Chelonia mydas*). Molecular Ecology 7: 575–584.

Fritz U. 2003. Die Europäische Sumpfschildkröte (*Emys orbicularis*). Bielefeld: Laurenti-Verlag. 224 p.

Fritz U., Fattizzo T., Guicking D., Tripepi S., Pennisi M. G., Lenk P., Joger U. & Wink M. 2005. A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys*. Zoologica Scripta 34: 351–371.

Fritz U., Guicking D., Kami H., Arakelyan M., Auer M., Ayaz D., Fernández C. A., Bakiev A., Celani A., Džukić G., Fahd S., Havaš P., Joger U., Khabibullin V., Mazanaeva L., Široký P., Tripepi S., Vélez A. V., Antón G. V. & Wink M. 2007. Mitochondrial phylogeography of European Pond Turtles (*Emys orbicularis*, *Emys trinacris*) – an update. Amphibia-Reptilia 28: 418–426.

Fritz U., Ayaz D., Hundsdörfer A. K., Kotenko T., Guicking D., Wink M., Tok C. V., Çiçek K. & Buschbom J. 2009. Mitochondrial diversity of European Pond Turtles (*Emys orbicularis*) in Anatolia and the Ponto-Caspian Region: multiple old refuges, hotspot of extant diversification and critically endangered endemics. Organisms Diversity & Evolution 9: 100–114.

Ingvarsson S., Finnsdottir V., Sigurdsson A. & Geirsson G. 2000. Population studies and validation of paternity determinations by six microsatellite loci. Journal of Forensic Sciences 45 (3): 692–695.

Ireland J. S., Broderick A. C., Glen F., Godley B. J., Hays G. C., Lee P. L. M. & Skibinski D. O. F. 2003. Multiple paternity assessed using microsatellite markers, in Green Turtles *Chelonia mydas* (Linnaeus, 1758) of Ascension Island, South Atlantic. Journal of Experimental Marine Biology and Ecology 291: 149–160.

Jabłonski A. 1992. European Pond Turtle. In: Głowacinski Z. (Ed.): Polish red book of animals. Warsaw: State Publishing House for Agriculture and Forestry, pp. 231–232 (In Polish, English Summary).

Johnston E. E., Rand M. S. & Zweifel S. G. 2006. Detection of multiple paternity and sperm storage in a captive colony of the central Asian tortoise, *Testudo horsfieldii*. Canadian Journal of Zoology 84: 520–526.

Jones A. G. 2001. GERUD 1.0: a computer program for the reconstruction of parental genotypes from progeny arrays using multilocus DNA data. Molecular Ecology Notes 1: 215–218.

King T. L. & Julian S. E. 2004. Conservation of microsatellite DNA flanking sequence across 13 Emydid genera assayed with novel Bog Turtle (*Glyptemys muhlenbergii*) loci. Conservation Genetics 5 (5): 719–725.

Labrecque J. R., Alva-Campbell Y. R., Archambeault S. & Crow K. D. 2014. Multiple paternity is a shared reproductive strategy in the live-bearing surfperches (Embiotocidae) that may be associated with female fitness. Ecology and Evolution 4 (12): 2316–2329.

Lee P. L. M. & Hays G. C. 2004. Polyandry in a marine turtle: females make the best of a bad job. Proceedings of the National Academy of Sciences of the USA 101 (17): 6530–6535.

Lenk P., Fritz U., Joger U. & Wink M. 1999. Mitochondrial phylogeography of the European Pond Turtle, *Emys orbicularis* (Linnaeus 1758). Molecular Ecology 8: 1911–1922.

McCraken G. F., Burghard G. M. & Houts S. E. 1999. Microsatellite markers and multiple paternity in the garter snake *Thamnopis sirtalis*. Molecular Ecology 8: 1475–1479.

Moore M. K. & Ball R. M. 2002. Multiple paternity in Loggerhead Turtle (*Caretta caretta*) nests on Melbourne Beach, Florida: a microsatellite analysis. Molecular Ecology 22: 281–288.

Nagy S., Poczai P., Cernák I., Gorji A. M., Hegedűs G. & Taller J. 2012. PICcalc: an online program to calculate polymorphic information content for molecular genetic studies. Biochemical Genetics 50 (9–10): 670–672.

Najbar B. 2001. European Pond Turtle. The natural monographs No 7. The Publishing House of Lubuski Naturalist Club. 135 p. (In Polish, English Summary).

O'Connor K. L., Butts E., Hill C. R., Butler J. M. & Vallone P. M. 2010. Evaluating the effect of additional forensic loci on likelihood ratio values for complex kinship analysis. Proceedings of the 21st International Symposium on Human Identification (October 12, 2010), 1–27 pp.

Parker A. & Kornfield I. 1996. Polygynandry in *Pseudotropheus zebra*, a cichlid fish from Lake Malawi. Environmental Biology of Fishes 47 (4): 345–352.

Peakall R. & Smouse P. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295.

Pearse D. E. & Avise J. C. 2001. Turtle mating systems: behaviour, sperm storage and genetic paternity. The American Genetics Association 92: 206–211.

Pedall I., Schäfer H., Fritz U. & Wink M. 2009. Isolation of microsatellite markers in the *Emys orbicularis* complex and development of multiplex PCR amplification. Conservation Genetics 10 (3): 725–727.

Prusak B., Najbar B., Mitrus S., Górecki G., Rogalla U., Grzybowski G., Hryniewicz A., Wróblewski R., Bochen R. & Grzybowski T. 2011. Distribution of mitochondrial haplotypes (cytb) in Polish populations of *Emys orbicularis* (L., 1758). Biologia 66(5): 893–898.

Prusak B., Mitrus S., Najbar B., Pacholewska A., Deas A., Skonieczna K., Górecki G., Grzybowski G., Hryniewicz A., Wróblewski R. & Grzybowski T. 2013. Population differentiation of the European Pond Turtle (*Emys orbicularis*) in Poland inferred by the analysis of mitochondrial and microsatellite DNA: implications for conservation. Amphibia-Reptilia 34 (4): 451–461.

Roques S., Díaz-Paniagua C. & Andreu A. C. 2004. Microsatellite markers reveal multiple paternity and sperm storage in the Mediterranean Spur-thighed Tortoise, *Testudo graeca*. Canadian Journal of Zoology 82 (1): 153–159.

Roques S., Díaz-Paniagua C., Portheault A., Pérez-Santigosa N. & Hidalgo-Vila J. 2006. Sperm storage and low incidence of multiple paternity in the European Pond Turtle, *Emys orbicularis*: A secure but costly strategy? Biological Conservation 129: 236–243.

Rovero F., Lebboroni M. & Chelazzi G. 1999. Aggressive interactions and mating in wild populations of the European Pond Turtle – *Emys orbicularis*. Journal of Herpetology 33: 258–263.

Shete S., Tiwari H. & Elston R. C. 2000. On estimating the heterozygosity and polymorphism information content value. Theoretical Population Biology 57: 265–271.

Stuckas H., Velo-Antón G., Fahd S., Kalboussi M., Rouag

R., Arculeo M., Marrone F., Sacco F., Vamberger M. & Fritz U. 2014. Where are you from, stranger? The enigmatic biogeography of North African pond turtles (*Emys orbicularis*). Organisms Diversity & Evolution 14: 295–306.

Tortoise & Freshwater Turtle Specialist Group 1996. *Emys orbicularis*. The IUCN Red List of Threatened Species 1996: e.T7717A12844431.

Trivers R. L. 1972. Parental investment and sexual selection. In: Campbell B. (Ed.): Sexual selection and the descent of man, 1871–1971. Chicago: Aldine-Atherton, pp. 136–179.

Wang J. 2004. Sibship reconstruction from genetic data with typing errors. Genetics 166: 1963–1979.

Weber-Lehmann J., Schilling E., Gradl G., Richter D. C., Wiehler J. & Rolf B. 2014. Finding the needle in the haystack: differentiating "identical" twins in paternity testing and forensics by ultra-deep next generation sequencing. Forensic Science International: Genetics 9: 42–46.

Wilson M. R., Polanskey D., Butler J., Dizinno J. A., Repogle J. & Budowle B. 1995. Extraction, PCR amplification, and sequencing of mitochondrial DNA from human hair shafts. BioTechniques 18: 662–669.

Vamberger M., Stuckas H., Sacco F., D'Angelo S., Arculeo M., Cheylan M., Corti C., Lo Valvo M., Marrone F., Wink M. & Fritz U. 2015. Differences in gene flow in a twofold secondary contact zone of pond turtles in southern Italy (Testudines: Emydidae: *Emys orbicularis galloitalica*, *E. o. hellenica*, *E. trinacris*). Zoologica Scripta 44: 233–249.

Velo-Antón G., Garcia-Paris M. & Cordero Rivera A. 2008. Patterns of nuclear and mitochondrial DNA variation in Iberian populations of *Emys orbicularis* (Emydidae): conservation implications. Conservation Genetics 9: 1263–1274.

Xue D., Zhang T. & Liu J. X. 2014. Microsatellite evidence for high frequency of multiple paternity in the marine gastropod *Rapana venosa*. PLoS ONE 9 (1): e86508.

Zeng L., Cheng Q. & Chen X. 2012. Microsatellite analysis reveals the population structure and migration patterns of *Scomber japonicus* (Scombridae) with continuous distribution in the East and South China Seas. Biochemical Systematics and Ecology 42: 83–93.

Ziadi-Künzli F. & Tachihara K. 2016. Female defence polygyny and plasticity in the mating system of the demersal triggerfish *Rhinecanthus aculeatus* (Pisces: Balistidae) from Okinawa Island. Marine Biology 163 (27): 1–13.

Accepted: 10.05.2016