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Fast sex identification in wild mammals using PCR amplification of the *Sry* gene

Josef BRYJA[1] and Adam KONEČNÝ[2]

[1] *Department of Population Biology, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, CZ-675 02 Studenec 122, Czech Republic; e-mail: bryja@brno.cas.cz*

[2] *Department of Zoology and Ecology, Faculty of Science, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic; e-mail: konada@post.cz*

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A b s t r a c t . Several primer sets were described for PCR amplification of the *Sry* gene that became very suitable method for sex identification in mammals. We tested applicability of two primer sets for sex identification in one bat and 12 rodent species from Central Europe. Rapid DNA extraction with non-ionic detergents, which is very suitable for large-scale population studies, was used. Primers SRY-A from P o m p et al. (1995) did not amplify *Sry* fragment in males of *Myotis myotis*, four species of *Microtus*, and *Clethrionomys glareolus*, while amplification was successful in four species of *Apodemus*, two species of *Mus*, and *Sciurus vulgaris*. On the other hand, primers from S á n c h e z et al. (1996) gave clear *Sry* band in males of all tested species, only with one exception, i.e. male of *Sciurus vulgaris*.

Key words: sex identification, *Sry* gene, DNA extraction, Y chromosome, Rodentia, population screening

Introduction

Numerous techniques have been developed for genetic sex identification in mammals, mainly in embryos in the earlier stages of gestation where anatomic sexing is not possible. These methods include cytogenetic analysis, detection of H-Y antigen, measurement of X-linked enzymes before Barr body formation, and Y-chromosome specific probes (B o n d i o l i 1992). More recently, PCR amplification of sex-specific DNA fragments has been widely available for sex identification. These techniques use genes *Amg* (S u l l i v a n et al. 1993), *Zfy* (A a s e n & M e d r a n o 1990), and *Sry* (P o m p et al. 1995). The *Sry* gene evolves in a strange manner for sex identification. This single gene is hypothesized to produce a transcription factor of the high mobility group (HMG) type that recognizes specific DNA sequences and bends target DNA (H a r l e y & G o o d f e l l o w 1994). This process starts the differentiation of the embryonic, bipotential gonads as testes (S i n c l a i r et al. 1990, G u b b a y et al. 1990).

Sry is a male-specific gene in most placental and marsupial mammals (S i n c l a i r et al. 1990, F o s t e r et al. 1992) occurring as a single copy normally located on the non-pairing region of the Y chromosome. However, exceptions to this rule have been described in rodents. Some species have several copies of *Sry* on the Y chromosome (*Akodon* spp. – B i a n c h i et al. 1993; several Asian and African murid species – N a g a m i n e 1994, L u n d r i g a n & T u c k e r 1997; *Microtus* spp. and *Arvicola* spp. – B u l l e j o s et al. 1999) or even located on the X chromosome (*Microtus cabrerae* – B u l l e j o s et al. 1997). Conversely, the *Sry* gene is completely missing in two mole-vole species of the genus *Ellobius* (J u s t et al. 1995, V o g e l et al. 1998) and in two subspecies of the spiny rat *Tokudaia osimensis* (S o u l l i e r et al. 1998, S u t o u et al. 2001).

The *Sry* gene contains a DNA-binding motif (HMG box) that is conserved across species of marsupial and placental mammals (Gubbay et al. 1990, Sinclair et al. 1990). Conversely, high rate of evolution was found in the C-terminal (non-HMG box) region, which suggests that the non-box region is either functionally unconstrained or has undergone species-specific adaptive divergence (Tucker & Lundrigan 1993).

In the last years, at least two primer sets have been designed that can be used for sexing various species using the *Sry* gene. First, the SRY-A primers - (Pomp et al. 1995) amplify a 157-bp fragment of the *Sry* in horses, humans, baboons, cats, dogs, rats, mice (Pomp et al. 1995) and coypu (Garcia-Meunier et al. 2001). The primer sequences are rather conservative and are recommended for extensive use (Griffiths 2000). Second, primers designed by Sánchez et al. (1996) amplify a 202-bp fragment of the *Sry* HMG box. They were successfully used in insectivores (Sánchez et al. 1996), voles (Bullejos et al. 1997, 1999) and bats (Bullejos et al. 2000). We tested applicability of these primer sets for sexing various central European mammals. Here we present (1) fast method of DNA extraction suitable mainly for large population genotyping and (2) results of PCR amplification of the *Sry* in various mammal species using both primer sets described above.

Material and Methods

DNA extraction

DNA for PCR reaction was extracted using non-ionic detergents (adapted from Perkin Elmer Cetus: Amplifications: Vol. #2 PEC 1989: 1-3). We put approximately 2 mm^3 of tissue (tail, toe, spleen, half wing punch from bat, embryo) into 200 μl of PCR Buffer with Nonionic Detergents (50 mM KCl, 10mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$. $6H_2O$, 0.1 mg/ml gelatin, 0.45 % v/v Nonidet P40, 0.45 % v/v Tween 20), added 5 μl of proteinase K (10 mg/ml), and incubated at 55°C until tissues were degraded (1-3 hours, better overnight). In the next step the samples were heated to 95°C for 10 minutes in PCR machine to inactivate proteinase K. We used 5 μl of tissue lysate as DNA template for PCR amplification.

PCR amplification

Part of the *Sry* gene was amplified by duplex PCR reaction using two sets of primers. In both cases a 447/445-bp region of the *Zfy-Zfx* genes was co-amplified as a positive control of successful PCR reaction (primers were taken from Aasen & Medrano (1990) where additional details can be found). First set of primers that amplify a 157-bp region of the *Sry* gene was modified from Pomp et al. (1995) – the 5' upstream primer SRYA-5 (5'-TGA ACG CAT TCA TGG TGT GGT-3') and the 3' downstream primer SRYA-3 (5'-AAT CTC TGT GCC TCC TGG AA-3'). The amplifications were performed in the following conditions: 1.5 mM $MgCl_2$, 0.4 μM SRY primers, 0.2 μM ZFY/ZFX primers, 200 μM dNTPs, 0.8 U recombinant Taq polymerase (Invitrogen), and 5 μL of tissue lysate in a 25 μL reaction volume. The cycling conditions were 30 cycles of denaturation at 94 °C (45 sec), annealing at 54°C (1 min) and extension at 72°C (1 min).

The second set of primers that amplify a 202-bp fragment of the SRY-HMG box was taken from Sánchez et al. (1996). Primer sequences were (5'-3') GTC AAG CGC CCC ATG AAT GCA T and AGT TTG GGT ATT TCT CTC TGT G that corresponded to the

HMG box of the mouse *Sry* gene (G u b b a y et al. 1990). The conditions for PCR amplification were the same as in the first set with the only exception – we used 0.2 μM of each HMG-SRY and ZFY/ZFX primers. The thermal profile started with initial denaturation at 93°C (5 min) followed by 30 cycles of denaturation at 93°C (1 min), annealing at 50°C (1 min) and extension at 72°C (1 min) and final extension at 73°C for 5 min. The PCR amplifications were performed in the PCR Robocycler (Stratagene). PCR products were visualised by horizontal electrophoresis in 1.5 % ethidiumbromid-stained agarose gels.

Results and Discussion

The method of DNA extraction using non-ionic detergents yielded sufficient amount of DNA for successful PCR reaction. When no PCR products were observed, the failure could be ascribed either to high concentration of template DNA or other tissue residues. In these cases we decreased volume of tissue lysate for PCR reaction. After this modification we obtained clear PCR products. Nevertheless, the amount of amplified DNA varied significantly. Thus, this method of DNA extraction is usable for large-scale qualitative PCR analyses. The tissue lysate were stored at –20°C for several weeks. We have not tested the effect of long-time storage of samples but successful PCR reactions were performed up to 2 months after extraction. The results are comparable with similar rapid DNA-extraction techniques such as tissue boiling (V a l s e c c h i 1998) or Chelex extraction (G a r c i a - M e u n i e r et al. 2001).

We tested applicability of two primer sets for sex identification in one bat and 12 rodent species from Central Europe (Table 1). Widely recommended SRY-A primers from P o m p et al. (1995) did not amplify the *Sry* fragment in males of *Myotis myotis*, four species of *Microtus*, and *Clethrionomys glareolus*, while amplification was successful in four species

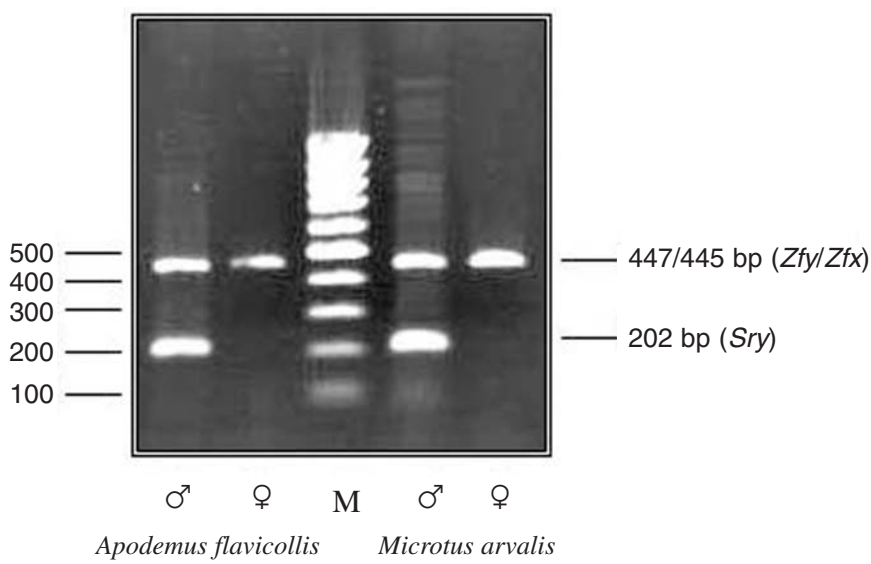

Fig. 1. Example of the PCR-based sex identification in two rodent species. Amplified fragments of duplex PCR reaction using HMG-SRY-primers from S á n c h e z et al. (1996) were visualised by electrophoresis in 1.5 % ethidiumbromid-stained agarose gel. Amplification of the 202-bp *Sry* fragment was successful only in males. M = 100 bp DNA ladder (Fermentas). Fragment sizes (in base pairs) of the ladder are written on the left margin.

Table 1. Applicability of two primer sets (adapted from P o m p et al. (1995) and S á n c h e z et al. (1996)) for sex identification of various mammals based on male-specific amplification of a *Sry* fragment.

Species	P o m p et al. (1995)	S á n c h e z et al. (1996)	References
Insectivora			
Talpa occidentalis		yes	S á n c h e z et al. (1996)
Talpa romana		yes	S á n c h e z et al. (1996)
Talpa europaea		yes	S á n c h e z et al. (1996)
Crocidura suaveolens		yes	S á n c h e z et al. (1996)
Neomys anomalus		yes	S á n c h e z et al. (1996)
Erinaceus algirus		yes	S á n c h e z et al. (1996)
Chiroptera			
Rousettus aegyptiacus		yes	B u l l e j o s et al. (2000)
Pteropus poliocephalus		yes	B u l l e j o s et al. (2000)
Pteropus alecto		yes	B u l l e j o s et al. (2000)
Pteropus scapulatus		yes	B u l l e j o s et al. (2000)
Myotis myotis	no	yes	B u l l e j o s et al. (2000), this study
Miniopterus schreibersi		yes	B u l l e j o s et al. (2000)
Eptesicus fuscus		yes	B u l l e j o s et al. (2000)
Eptesicus serotinus		yes	B u l l e j o s et al. (2000)
Rodentia			
Myocastor coypus	yes		G a r c i a - M e u n i e r et al. (2001)
Sciurus vulgaris	yes	no	this study
Microtus agrestis	no	yes	B u l l e j o s et al. (1999), this study
Microtus arvalis	no	yes	B u l l e j o s et al. (1999), this study
Microtus cabrerae		no	B u l l e j o s et al. (1997)
Microtus nivalis		yes	B u l l e j o s et al. (1999)
Microtus duodecimcostatus		yes	B u l l e j o s et al. (1999)
Microtus lusitanicus		yes	B u l l e j o s et al. (1999)
Microtus saviii		yes	B u l l e j o s et al. (1999)
Microtus tatricus	no	yes	this study
Microtus subterraneus	no	yes	this study
Arvicola sapidus		yes	B u l l e j o s et al. (1999)
Arvicola terrestris		yes	B u l l e j o s et al. (1999)
Clethrionomys glareolus	no	yes	B u l l e j o s et al. (1999), this study
Apodemus agrarius	yes	yes	this study
Apodemus flavicollis	yes	yes	this study
Apodemus sylvaticus	yes	yes	this study
Apodemus microps	yes	yes	this study
Mus musculus	yes	yes	this study
Mus domesticus	yes	yes	P o m p et al. (1995), this study
Rattus sp.	yes		P o m p et al. (1995)
Artiodactyla			
cattle	no		P o m p et al. (1995)
sheep	no		P o m p et al. (1995)
goat	no		P o m p et al. (1995)
llama	no		P o m p et al. (1995)
pig	yes		P o m p et al. (1995)
Perrisodactyla			
horses	yes		P o m p et al. (1995)
Primates			
humans	yes		P o m p et al. (1995)
baboons	yes		P o m p et al. (1995)
Carnivores			
cats	yes		P o m p et al. (1995)
dogs	yes		P o m p et al. (1995)

of *Apodemus*, two species of *Mus*, and *Sciurus vulgaris*. On the other hand, primers from Sánchez et al. (1996) gave a clear 202-bp *Sry* band in males of all tested species, with the only exception, i.e. male of *Sciurus vulgaris* (Table 1). Based on results of PCR amplifications in females, we conclude that no *Sry*-related gene (f.e. *Sox3* or *Sox9*) was amplified (Fig. 1). Co-amplification of the *Zfy*/*Zfx* genes resulted in 447/445-bp fragments in both sexes of all species (Fig. 1). Variable success of SRY amplification with particular primers can be caused by rapid evolution of this gene in rodents (Tucker & Lundrigan 1993, Lundrigan & Tucker 1997, Bullejos et al. 1999) and also in bats (Bullejos et al. 2000). Consistent results of amplification in 5 species of voles indicate mutation at primer sites in a common ancestor of the family. Similar event could occur in bats but it is necessary to test this hypothesis by *Sry* amplification in additional bat species. Based on our results, it is necessary to test applicability of the method of molecular sexing in specimens with known sex before sexing unknown samples.

Combination of primer sets that we used is able for sexing central European rodents and bats. Nevertheless, they were not tested in other mammal groups. In such cases, it is possible to try to amplify *Sry* fragment also with primers that were designed for ungulates (Pomp et al. 1995), cervids (Wilson & White 1998), whales (Richard et al. 1994) and Old World murids (Tucker & Lundrigan 1993, Nagamine 1994, Lundrigan & Tucker 1997).

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