Sex identification in the European mole (*Talpa europaea*) using genetic tools.

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Abstract:
The European mole (*Talpa europaea*) is difficult to census and sex due to its fossorial lifestyle and low sexual dimorphism. Traditional morphological sex determination requires trapping, highlighting the need for non-invasive molecular methods. Previous genetic approaches relying on the male-specific SRY gene are problematic, as the absence of amplification in females cannot be distinguished from a standard PCR failure without using additional control reactions. While amplifying an X-linked marker as a positive control for females is feasible, it requires an additional, costly, and time-consuming PCR reaction, whereas our approach offers a superior solution by performing sex identification and control for both X and Y chromosomes within a single PCR reaction.The aim of this study was to evaluate the efficacy of a single-reaction molecular marker based on the length polymorphism of intron 7 of the ZFX gene (ZF(i7)X/Y) as a reliable alternative for unambiguous sex determination in the European mole, specifically testing its viability on low-concentration DNA from non-invasive samples. Three molecular markers (SRY, ZFX/ZFY, and ZF(i7)X/Y) were tested using muscle tissue and hair samples. The sex of the reference individuals was confirmed by morphological measurements and post-mortem dissection. Our results showed that the use of either the SRY marker alone or in combination with existing X-chromosome markers did not yield reliable and reproducible results for sex identification, whereas the newly proposed marker (ZF(i7)X/Y) provided reproducible, easily interpretable, and reliable results in this regard.By eliminating the need for separate internal controls and proving effective on low-quality, non-invasively collected hair samples, this approach offers a practical tool for future ecological and population studies of the species without the need for trapping.

Keywords: non-invasive sampling, genetic sex, ZFX gen, European mole, Talpa europaea.

Received: 2026-07-01
Revised: 2026-11-10
Accepted: 2026-11-23
Final review: 2026-09-09

Short title
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Sex identification in the European mole (*Talpa europaea*) using genetic tools.

2. Abstract

The European mole (*Talpa europaea*) has a fossorial lifestyle and limited external sexual dimorphism, which complicate field studies and sex identification. Traditional sex identification in living individuals requires trapping and examination, highlighting the need for molecular alternatives. However, assays relying on the male-specific SRY gene require an amplification control to distinguish female samples from unsuccessful PCR reactions. The aim of this study was to evaluate the ZF(i7)$_{X/Y}$ marker, based on intron-length polymorphism, as a practical tool for sex identification in the European mole and to compare its performance with that of the SRY-HMG and short ZF$_X$/ZF$_Y$ markers. All three markers were tested using DNA extracted from muscle tissue of 15 individuals whose sex had been independently confirmed by morphological examination and post-mortem dissection. PCR conditions and the effect of template dilution were evaluated, and amplification products were characterised by Oxford Nanopore sequencing. The SRY-HMG assay generated multiple amplification products in both sexes, including non-specific fragments of the expected diagnostic size. A low-level SRY signal was also detected in the reference female, although its origin remained unresolved. The short ZF$_X$/ZF$_Y$ marker amplified in both sexes but did not produce distinguishable banding patterns. In contrast, ZF(i7)$_{X/Y}$ consistently yielded a single band in females and two distinct bands in males, with sex assignments matching anatomical observations in all examined individuals. Sequencing supported the identification of the shorter product as ZFX and the longer product as a candidate ZFY homologue. Diagnostic amplification was also maintained in diluted muscle-derived DNA extracts. These findings support ZF(i7)$_{X/Y}$ as a straightforward method for sex identification in the European mole using a single PCR and standard agarose gel electrophoresis, without routine sequencing. Further validation in larger, independent samples and confirmation of the chromosomal origin of the longer product are needed.

Key words: genetic sex, ZFX gene, European mole, *Talpa europaea*

3. Introduction

The European mole (*Talpa europaea*, Linnaeus, 1758) is found in most parts of Europe except Norway, Ireland, most of Sweden, regions close to the Mediterranean Sea and most small islands (Mitchell-Jones *et al.* 1999). In Poland, moles are common, widespread, native mammals that inhabit underground systems comprising hundreds of metres of permanent and

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semi-permanent tunnels (Pucek and Raczyński 1983). Their presence in the field is indicated by small mounds of soil produced during the excavation or maintenance of burrows and tunnels. Moles are small, secretive animals that are rarely seen above ground, where signs of their presence (molehills) are readily visible and recognisable (Skoczeń 1958). Their fossorial lifestyle makes direct observation and population surveys difficult, while their limited external sexual dimorphism complicates sex identification. Little is still known about the breeding season of this species; in the European mole, reproductive activity is seasonal, with breeding occurring from March to May. Moreover, the timing of the breeding season varies with latitude (Racey 1978). One litter per year has been reported, with litter sizes ranging from two to seven young. Young moles searching for new habitats generally travel no more than two kilometres from their parental nest, and the species is not known for long-distance migration (Pucek 1984).

Early studies suggested that sex determination of the European mole based on external morphological features was difficult outside the breeding season (Abelentsev *et al.* 1956, Deparma 1951, Ognev 1928), particularly in juveniles (Godet 1951). However, Skoczeń (1958) demonstrated that sex identification is feasible even outside the reproductive period. He established that the distance between the anus and the urethra in males (10–13 mm), compared with the distance between the anus and the clitoris in females (2–3 mm), provides a diagnostic criterion. This method is also applicable to juveniles. In young females, the distance from the clitoris to the base of the anal protuberance ranges from 1 to 2 mm, whereas the corresponding distance in young males is 5–7 mm. However, this method of sex determination assumes the need for trapping animals, which, due to their fossorial and secretive lifestyle, could be very problematic.

Female *T. europaea* possess bilateral ovotestes, each comprising an ovarian component and a testicular region known as the interstitial gland. The ovarian component develops during the spring breeding season, whereas the interstitial gland enlarges in autumn as the ovarian component decreases in size (Whitworth *et al.* 1999). The European mole is classified as Least Concern on the IUCN Red List (Amori *et al.* 2017). In Poland, it is partially protected, except for individuals occurring in gardens, horticultural crops, forest nurseries, grassy airfields, earth hydraulic structures and sports facilities (Minister of the Environment 2016).

Molecular sex determination is useful when tissue samples are available but the animal cannot be examined, or when sex-specific characteristics are absent or difficult to observe (Griffiths *et al.* 1998, Griffiths and Tiwari 1993).

In mammals, molecular sex identification is most commonly based on the amplification of a conserved fragment of the SRY gene (Bryja and Konečný 2003, Pomp *et al.* 1995). SRY is a male-specific gene present in most placental and marsupial mammals (Foster *et al.* 1992, Sinclair *et al.* 1990), typically occurring as a single-copy *locus* located in the non-recombining region of the Y chromosome. This marker should be amplified only in male mammals. Therefore, no PCR product is obtained in females. This approach has been applied to selected insectivorous mammals, including three mole species: Roman mole (*Talpa romana*), Iberian mole (*Talpa occidentalis*), and European mole (*Talpa europaea*) (Sánchez *et al.* 1996). In that study, the PCR assay produced a single band corresponding to the HMG box fragment of the SRY gene in males, whereas no amplification product was detected in females. However, failure to amplify a male-specific marker cannot distinguish a female sample from an unsuccessful PCR (Ortega *et al.* 2004, Shaw *et al.* 2003). An internal amplification control is therefore needed to interpret negative results. Such controls may target homologous ZFX/ZFY sequences on the X and Y chromosomes (Joshi *et al.* 2019, Bryja and Konečný 2003, Aasen and Medrano 1990). When a second nuclear *locus* is amplified as a control, it should ideally occur in the same copy number as the target *locus* (Wilson and White 1998, Bérubé and Palsbøll 1996). Mitochondrial DNA (mtDNA) sequences have also been used as internal controls in multiplex reactions (Joshi *et al.* 2019, Palsbøll *et al.* 1992), but their use introduces the problem of the substantially higher copy number of mtDNA target sequences compared with the nuclear target sequence used for sex determination. The requirement to use multiple primer pairs is not an optimal solution, as additional primer sets in a reaction may lead to sequence competition and consequently preferential amplification of one target sequence over another (Shaw *et al.* 2003). Amplifying different markers in separate reactions increases the workload and the time required to obtain the results. A marker that produces distinguishable products from homologous loci on both sex chromosomes could address these limitations. Markers of this type have been successfully applied in various groups of birds (Kroczak *et al.* 2022, Kroczak *et al.* 2021, Griffiths *et al.* 1998). In mammals, Shaw *et al.* (2003) proposed an assay based on the length polymorphism of intron 7 of ZFX/ZFY, here referred to as ZF(i7)$_{X/Y}$. This assay produces amplification products in both sexes, but with different banding patterns. Consequently, the results are unambiguous and easy to interpret: males are expected to yield two products of different sizes, whereas females yield products of the same size that appear as a single band on the gel. Although this marker has been used in mammalian species from different taxonomic groups (Xu *et al.* 2010, Shaw *et al.* 2003), its applicability to the European mole has not yet been evaluated. Therefore, in the present study, we aimed to evaluate ZF(i7)$_{X/Y}$

as a candidate marker for molecular sex identification in the European mole and compare its performance with that of the SRY-based assay proposed by Sánchez *et al.* (1996). We also examined whether the distinct amplification patterns could allow sex identification without a separate control reaction in female samples.

4. Materials and Methods

4.1. Sex determination by external features and necropsy in moles

To develop an effective PCR-based test for sex identification, dead mole specimens found in the field were used. All individuals died of natural causes and were not killed for the purposes of this study. The dead specimens were collected under an appropriate permit issued by the General Directorate for Environmental Protection and Regional Directorate for Environmental Protection in Wrocław (permit no. GDOŚ/DOPozgiz-4200/IV-13/505/10/bm and WPN.6402.1.16.2011.MK). The measurements proposed by Skoczeń (1958) allowed for preliminary sex identification of dead individuals (Figure S1). However, because, in young individuals, morphological assessment alone may be insufficient to confirm their sex, all individuals were subjected to post-mortem dissection. In a male, clearly visible testes and a penis were present, while in a female, a clearly visible uterus was observed (Figure S1).

4.2. Molecular markers

To confirm the usefulness of sex determination markers, markers previously used for mammalian species, including insectivores, were applied (Bryja and Konečný 2003, Sánchez *et al.* 1996, Pomp *et al.* 1995). These included the SRY-HMG marker, which allows identification of the presence of the Y chromosome carrying the SRY gene (Sánchez *et al.* 1996), and the ZF_X/ZF_Y marker used as a control for female samples in which no amplification product is expected for the SRY-HMG marker (Bryja and Konečný 2003). Since methods based on markers that do not produce a product for one of the sexes are often misleading and require additional control reactions to confirm the correctness of PCR master mix preparation and template quality, the effectiveness of the marker $ZF(i7)_{X/Y}$, based on length polymorphism of intron 7 of the ZFX gene was also tested (Shaw *et al.* 2003). Amplicon sizes for each marker were estimated *in silico* based on available reference nuclear genomes for the European mole (*Talpa europaea*) (GenBank accession no. GCA_964194135.1) and the Iberian mole (*Talpa occidentalis*) (GenBank accession no. GCA_014898055.3), and are presented together with the corresponding primer sequences and relevant literature references in Table 1.

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Table 1. Marker name, primer sequences used for amplification, and the literature source from which the sequences were obtained. Amplicon sizes for each marker were estimated *in silico* based on the available reference nuclear genomes of the European mole (*Talpa europaea*) (GenBank accession no. GCA_964194135.1) and the Iberian mole (*Talpa occidentalis*) (GenBank accession no. GCA_014898055.3). "–" indicates that the sequence is absent from the X chromosome.

Marker Name	Primer F		Primer R		Amplicon length [bp]		Fragment	Reference
	Name	Sequence	Name	Sequence	X chromosome	Y chromosome		
SRY	SRY-HMG-F	GTCAAGCGCCCCATGAATGCAT	SRY-HMG-R	AGTTTGGGTATTTCTCTCTGTG	-	200	fragment of the SRY HMG box	Sánchez *et al.* 1996
ZF_X/ZF_Y	P1-5EZ	ATAATCACATGGAGAGCCACAAGCT	P2-3EZ	GCACTTCTTTGGTATCTGAGAAAGT	447	447	8 exon fragment of the ZFX gene	Aasen and Medrano 1990
$ZF(i7)_{X/Y}$	LGL331	CAAATCATGCAAGGATAGAC	LGL335	AGACCTGATTCCAGACAGTACCA	912	1100	7 intron of the ZFX gene	Shaw *et al.* 2003

4.3. DNA Extraction and Biological Samples

Total DNA was extracted from muscle tissue samples collected from 15 mole individuals (Table 2) using the Sherlock AX Kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's protocol. For each individual, 20 mg of muscle tissue was used for DNA isolation.

Table 2. Individuals tested in this study, along with the location where each individual was found, the type of biological material collected, and the molecular markers used for sex identification. "Yes" indicates that the marker was amplified for the respective individual. "*" indicates that the amplicon was sequenced.

No. of the individual	Sex	Biological sample	Source	Molecular marker		
				SRY-HMG	ZFX/ZFY	ZF(i7)X/Y
1	Female	tissue	51°03'03"N 17°24'26"E	yes*	yes*	yes*
2	Male	tissue	50°17′15″N 16°48′54″E	yes*	yes*	yes*
3	Male	tissue	51°03′03″N 17°24′25″E	yes	yes	yes*
4	Female	tissue	51°03′33″N 17°11′55″E	yes	yes	yes*
5	Male	tissue	50°16′13″N 16°53′07″E	yes	yes	yes*
6	Female	tissue	51°03′02″N 17°24′25″E	yes	yes	yes*
7	Female	tissue	51°02′59″N 17°24′25″E	yes	yes	yes*
8	Female	tissue	51°03′03″N 17°24′29″E	yes	yes	yes*
9	Male	tissue	51°03′03″N 17°24′13″E	yes	yes	yes*
10	Female	tissue	51°03′14″N 17°24′17″E	yes	yes	yes*
11	Male	tissue	51°03′14″N 17°23′40″E	yes	yes	yes*
12	Male	tissue	51°03′21″N 17°23′21″E	yes	yes	yes*
13	Male	tissue	50°58′33″N 16°32′21″E	yes	yes	yes*
14	Female	tissue	51°02′17″N 17°23′28″E	yes	yes	yes*
15	Male	tissue	51°02′52″N 17°24′34″E	yes	yes	yes*

4.4. DNA concentration in muscle-derived extracts

DNA concentrations in muscle tissue extracts obtained from all individuals included in the study are presented in Table 3. DNA quantification was performed using a fluorometric method with a Qubit fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol for the Qubit 1X dsDNA High Sensitivity (HS) Assay.

Table 3. DNA concentrations in the analyzed samples were determined fluorometrically using a Qubit fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol for the Qubit dsDNA High Sensitivity (HS) Assay.

Sample ID	DNA concentration in muscle tissue extracts (ng/µL)
1	33.8
2	38.4
3	40
4	120
5	47.8
6	99.6
7	120
8	44.8
9	120
10	88.4
11	28.2
12	31.6
13	44.8
14	44.8
15	17.8

4.5. Testing conditions of the PCR reaction

4.5.1. Temperature gradient

For all three markers (SRY-HMG, ZF_X/ZF_Y, and $ZF(i7)_{X/Y}$), a temperature gradient PCR was performed to determine the optimal annealing temperature. The optimization of reaction conditions was carried out using two selected individuals: no. 1 and no. 2 (Table 2), whose sex had been confirmed post-mortem by dissection. PCR amplifications were performed in a total reaction volume of 25 µL, containing 1 µL of template DNA (concentration reported in Table 3), 1 U of DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL of 10× buffer, 0.6 µL of 10 mM dNTP mix, and 0.6 µL of each primer (10 µM). A temperature gradient was applied for all three markers to identify the most suitable annealing temperature. The tested annealing temperatures (X) were 50°C, 52°C, 54°C, 56°C, 58°C, and 60°C. The PCR cycling conditions were as follows: SRY-HMG program: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, annealing at X°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min. ZF_X/ZF_Y program: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, annealing at X°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min. $ZF(i7)_{X/Y}$ program: initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s,

annealing at X°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min.

4.5.2. Testing of template concentrations

Next, under the optimized conditions established for the most effective molecular marker, $ZF(i7)_{X/Y}$, the effect of template DNA concentration on PCR amplification results was evaluated using DNA extracted from muscle tissue. PCR reactions were performed in a total volume of 25 µL, each containing 1 µL of either undiluted template DNA or DNA diluted 10-, 20-, 40-, 80-, 160-, 320-, 640-, 1280-, or 2560-fold. Nuclease-free water was used as the diluent. The initial DNA concentration and the concentrations corresponding to each dilution are presented in Table 4. The remaining components of the reaction mixture remained unchanged: 1 U DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL of 10× buffer, 0.6 µL of 10 mM dNTPs, and 0.6 µL of each primer (10 µM).

Table 4. DNA concentrations in diluted template samples extracted from muscle tissue of selected individuals (no. 1 and 2, Table 2) were determined fluorometrically using a Qubit fluorometer (Thermo Fisher Scientific), according to the manufacturer's protocol for the Qubit dsDNA High Sensitivity (HS) Assay. The initial DNA concentration in each extract and the concentrations measured after 10-fold, 20-fold, 40-fold, 80-fold, 160-fold, 320-fold, 640-fold, 1,280-fold, and 2,560-fold dilution are presented. "Too Low" indicates that the DNA concentration was below the quantification range of the Qubit dsDNA HS Assay, resulting in a fluorescence signal insufficient for reliable quantification.

Dilution	DNA concentration (ng/µL)	
	1	2
Undiluted isolate	33.8	38.4
10-fold	2.86	8.02
20-fold	1.34	5.5
40-fold	0.53	2.32
80-fold	0.166	1.3
160-fold	0.124	0.454
320-fold	0.106	0.304
640-fold	Too Low	0.112
1280-fold	Too Low	Too Low
2560-fold	Too Low	Too Low

4.6. DNA amplification

4.6.1. DNA Amplification of marker SRY-HMG

The SRY marker was amplified in all 15 examined European mole (*Talpa europaea*) individuals whose sex had been confirmed by dissection. The PCR amplifications were

performed in a total volume of 25 µL of reaction mixture containing 1 µL of template DNA (concentration reported in Table 3), 1 U DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL of 10× buffer, 0.6 µL of 10 mM dNTPs, and 0.6 µL of each primer (10 µM). A no-template control (NTC) was included for the reaction mix, and filter tips were used during pipetting to minimize the risk of potential sample contamination. The reaction conditions were as follows: 95°C for 10 min; (95°C for 30 s, 50°C for 30 s, 72°C for 60 s) repeated 35 times; and 72°C for 5 min.

4.6.2. DNA Amplification of marker ZF_X/ZF_Y

The ZF_X/ZF_Y marker was amplified in all 15 examined European mole (*Talpa europaea*) individuals whose sex had been confirmed by dissection. The PCR amplifications were performed in a total volume of 25 µL of reaction mixture containing 1 µL of template DNA (concentration reported in Table 3), 1 U DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL of 10× buffer, 0.6 µL of 10 mM dNTPs, and 0.6 µL of each primer (10 µM). A no-template control (NTC) was included for the reaction mix, and filter tips were used during pipetting to minimize the risk of potential sample contamination. The reaction conditions were as follows: 95°C for 10 min; (95°C for 30 s, 50°C for 30 s, 72°C for 60 s) repeated 35 times; and 72°C for 5 min.

4.6.3. DNA Amplification of marker $ZF(i7)_{X/Y}$

The $ZF(i7)_{X/Y}$ marker was amplified in all 15 examined European mole (*Talpa europaea*) individuals whose sex had been confirmed by dissection. The PCR amplifications were performed in a total volume of 25 µL of reaction mixture containing 1 µL of template DNA (concentration reported in Table 3), 1 U DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL of 10× buffer, 0.6 µL of 10 mM dNTPs, and 0.6 µL of each primer (10 µM). A no-template control (NTC) was included for the reaction mix, and filter tips were used during pipetting to minimize the risk of potential sample contamination. The reaction conditions were as follows: 95°C for 10 min; (95°C for 30 s, 50°C for 30 s, 72°C for 60 s) repeated 35 times; and 72°C for 5 min.

4.7. Visualization of PCR reaction results on an agarose gel

PCR products were separated by electrophoresis in a 1.5% agarose gel. The gel contained the fluorescent dye Syngen GreenDNA Gel Stain (Syngen Biotech Sp. z o.o.), added at a volume of 0.8 µL per 120 mL of dissolved agarose. Electrophoresis was carried out in a 1× TAE buffer at a voltage of 2–5 V/cm. To assess DNA fragment size, the GeneRuler 100 bp

Plus DNA Ladder (Thermo Scientific) was used, which is recommended for determining the size of double-stranded DNA in the range of 100 bp to 3000 bp in agarose gels. The results of the separation were visualized under ultraviolet light using a UPV GelDoc-It2 Imaging System (Analytik Jena).

4.8. Nanopore library preparation and data analysis

Selected SRY-HMG and ZF_X/ZF_Y PCR products and all $ZF(i7)_{X/Y}$ amplicons were sequenced on an Oxford Nanopore MinION using the Native Barcoding Kit 96 V14 and R10.4.1 flow cells. POD5 files were basecalled with GPU-enabled Dorado v2.1.2 using the super high-accuracy model.

BAM integrity was checked with SAMtools 1.22.1, and read counts, yield, length and quality distributions were calculated per barcode. Mean read quality was derived from mean base-error probability. An empirical catalogue of 27 product families was constructed using VSEARCH 2.31.0 clustering at 88% identity and ≥85% query and target coverage, followed by preliminary consensus generation and dereplication (Rognes *et al.* 2016). Read ends were trimmed in 5-nt steps until terminal 20-nt windows reached Q10. Reads ≥80 nt with mean quality ≥Q15 were aligned to the catalogue using minimap2 2.31 (Li 2018). Product assignment required a primary alignment with ≥95% identity, ≥95% reference coverage, ≥90% query coverage and pre-clipping read length within 90–112% of the empirical product length.

Sample-specific consensuses required ≥100 retained reads per product; additional products also required ≥1% of input reads, with exceptions for expected assay products. Reads within 3 nt or 2% of the family median length, whichever was greater, were selected preferentially at Q20, using Q15 reads when necessary. Consensuses were generated from deterministic subsets of up to 1,000 reads using pyabpoa 1.5.6 and Medaka 2.2.2 with model r1041_e82_400bps_sup_v5.2.0 (Gao *et al.* 2021). Subset comparisons and remapping of all retained product reads assessed consensus reliability. Positions with <20 observations or <70% nucleotide support were masked as N, counting bases ≥Q10 and deletions. The mixed 447-nt product from male 78 was resolved using 14 linked variable sites; subgroup assignment required ≥7 informative sites at Q≥15 and ≥85% concordance. Remaining reads were classified as unresolved.

Product identity was assessed using NCBI core_nt megablast searches of 28 representatives (26 October 2026; word size 28, $E \leq 10^{-5}$) and local BLAST+ 2.16.0 searches of 55 nonredundant sequences (word size 11, $E \leq 10^{-8}$; Camacho *et al.* 2009). References

comprised the annotated *T. europaea* assembly GCF_964194135.1, *T. occidentalis* contig RCFO01000018.1 and *T. europaea* SRY sequence PP841022.1. Assignments considered alignment coverage and annotation. Because the *T. europaea* assembly lacks a Y chromosome, putative Y homologues were designated candidate ZFY.

Percentages used all delivered pass reads per barcode as the denominator unless stated otherwise; retained reads included assigned off-target products. The candidate-Y fraction was calculated as Y/(X+Y). The ≥100-read and ≥1% thresholds described substantial product representation, not validated diagnostic cutoffs. All lower counts were reported. Read proportions were not interpreted as genomic copy number, initial DNA concentration or contamination fraction; assays were analysed separately, and reads were not treated as biological replicates.

5. Results

5.1. Optimization of reaction conditions

For all three markers, a temperature gradient PCR was performed to determine the optimal reaction conditions. The primers enable amplification of the SRY gene fragment encoding the HMG box region of the SRY gene (Bryja and Konečný 2003). According to the findings of Sánchez *et al.* (1996), a product of approximately 200 bp was expected in the male mole, whereas no amplicon should be observed in the female mole. A diagnostic fragment of 200 bp was clearly visible in the male. However, numerous additional products were also detected. These were most intense at an annealing temperature of 50°C, at which the correct amplicon was also amplified most efficiently (Figure 1 – lane 1). At higher annealing temperatures, nonspecific products were less prominent, but the amplification efficiency of the correct SRY gene fragment also decreased (Figure 1 – lane 11). An unexpected result was the presence of amplification products in the female (Figure 1 – lanes 2, 4, 6, 8, 10, and 12). These amplicons were comparable in size to those obtained from the male sample. However, they consistently exhibited greater signal intensity, particularly at lower annealing temperatures. Due to the possibility that the female DNA isolate had been contaminated with male DNA, the DNA extraction for the female was repeated using newly collected muscle tissue samples (taken from different locations than previously and not directly beneath the skin). However, the same result was obtained, even when different isolates and the same gradient conditions were used. The presence of numerous additional amplicons, consistently observed across different annealing temperatures, may indicate the existence of multiple copies of the SRY gene in the male

genome of *Talpa europaea*, as suggested by Bogdanov *et al.* (2024). In the case of the female, this result is unexpected. However, since it was reproducible across independent DNA isolates, it cannot be excluded that sequences present on the X chromosome may serve as binding sites for primers presumed to be specific only to conserved regions of the SRY gene, allowing for potential hybridization. For this reason, the products were sequenced to confirm the origin of the observed amplicons.

Amplification of the ZF_X/ZF_Y marker yielded the expected product without any nonspecific bands at all tested annealing temperatures, for both male and female individuals (Figure 1 - ZF_X/ZF_Y).

Amplification of the $ZF(i7)_{X/Y}$ marker, which is intron 7 of the ZFX gene, was carried out using the LGL331 and LGL335 primers (Shaw *et al.* 2003) for 15 individuals of known sex: 8 males and 7 females (Table 2). Intron 7 of the ZFX gene is characterized by length polymorphism and has been successfully used in other mammal species (Xu *et al.* 2010, Shaw *et al.* 2003). According to the available nuclear genome references for moles, the expected amplicon sizes were approximately 900 bp for the X chromosome and a slightly longer fragment of approximately 1100 bp for the Y chromosome. The expected amplicons were successfully obtained for both tested individuals. In the male sample, two distinct products differing in size were observed, whereas the female sample exhibited only a single band of approximately 900 bp.

The annealing temperature of 50°C was selected for subsequent amplification because it yielded the best results for the candidate marker $ZF(i7)_{X/Y}$ proposed in this study. The ZF_X/ZF_Y marker amplified successfully across the entire temperature gradient, including at 50°C. Although additional amplification products were observed for the SRY marker at this temperature, this was considered advantageous, as their origin was one of the aspects investigated in this study.

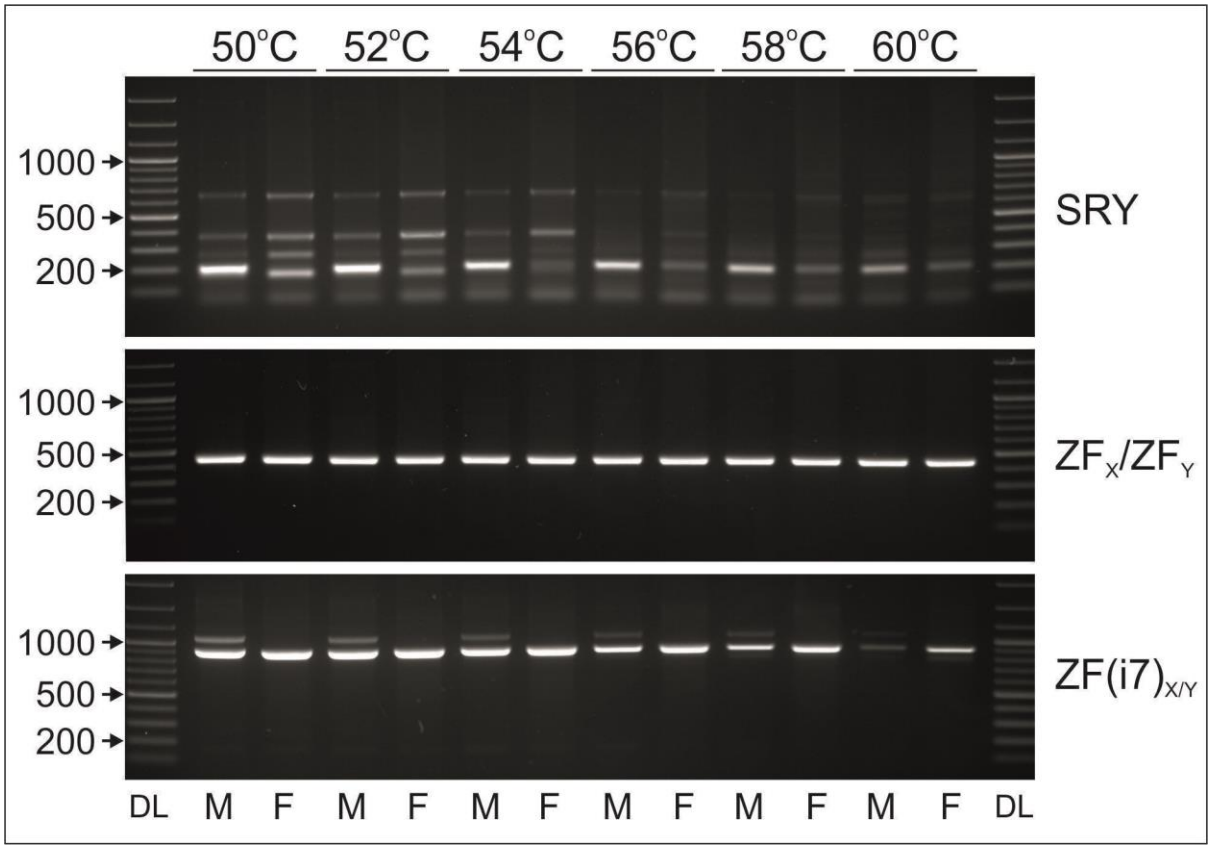

Figure 1. Results of amplification across an annealing temperature gradient of 50°C, 52°C, 54°C, 56°C, 58°C, and 60°C for markers SRY, ZF_X/ZF_Y and $ZF(i7)_{X/Y}$. M - male; F - female. A total of 5 µL of the reaction mixture was loaded onto a 1.5% agarose gel. DL – 5 µL of DNA Ladder 100 bp Plus (Thermo Scientific).

5.2. Amplification Results for the SRY-HMG Marker

Amplification of the SRY-HMG marker using the SRY-HMG-F and SRY-HMG-R primers (Sánchez *et al.* 1996; Table 1) was performed on DNA samples from 15 individuals of known sex: eight males and seven females (Table 2). The amplification products obtained from all individuals were consistent with those observed in the initial temperature-gradient PCR (Figure 2: SRY). No amplification was detected in the no-template control (NTC), and filter tips were used throughout the procedure, further reducing the likelihood of contamination. The resulting amplification products were subsequently sequenced to determine their origin.

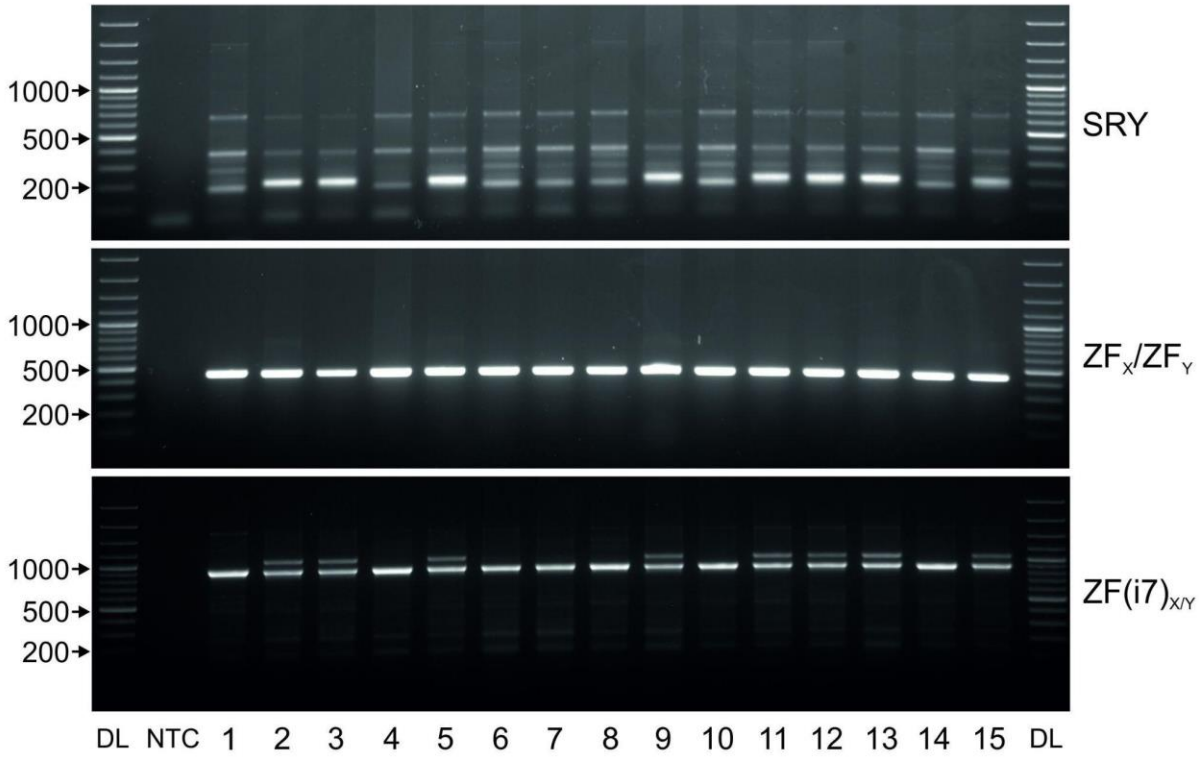

Figure 2. Results of amplification for markers SRY, ZF_X/ZF_Y and $ZF(i7)_{X/Y}$. Lanes 1–15: European moles. The sex of each individual, determined by dissection, is provided in Table 2. A total of 5 µL of the reaction mixture was loaded onto a 1.5% agarose gel. NTC - no template control, DL – 5 µL of DNA Ladder 100 bp Plus (Thermo Scientific).

5.3. Amplification Results for the ZF_X/ZF_Y Marker

Amplification of the ZF_X/ZF_Y marker using the P1-5EZ and P2-3EZ primers was performed for 15 individuals of known sex: 8 males and 7 females (Table 2). The ZFX gene has its *locus* on both sex chromosomes; therefore, in both males and females, two amplicons are expected for females and males. This marker was originally proposed by Aasen and Medrano (1990) and later employed by other authors as a suitable control for the SRY marker (Bryja and Konečný 2003, Pomp *et al.* 1995), which should theoretically yield no amplification product in females because it is absent from their genome. The amplification product originating from the X chromosome was approximately 447 bp in both sexes of moles (Figure 2: ZF_X/ZF_Y). Nevertheless, because it amplifies reliably under various PCR conditions for both sexes, it constitutes a suitable control fragment, as reported by other authors for different mammalian species (Bryja and Konečný 2003, Pomp *et al.* 1995).

5.4. Amplification Results for the $ZF(i7)_{X/Y}$ Marker

Intron 7 of the ZFX gene is characterized by length polymorphism and has been successfully used for sex determination in other mammalian species (Xu *et al.* 2010, Shaw *et*

al. 2003). Based on the available nuclear genome reference sequences for moles, the expected amplicon sizes were approximately 900 bp for the X chromosome and 1,100 bp for the Y chromosome. To evaluate the suitability of the proposed ZF(i7)$_{X/Y}$ marker for reliable sex determination, we assessed the effect of template DNA quantity on amplification efficiency, considering that samples available for analysis may vary in quality and DNA concentration. The analysis was performed using DNA extracts from one female and one male (individuals numbers 1 and 2, respectively; Table 2), selected because their initial DNA concentrations were comparable. For each individual, PCR amplification was performed using 1 µL of either undiluted DNA or DNA diluted 10-, 20-, 40-, 80-, 160-, 320-, 640-, 1,280-, or 2,560-fold (Table 4). The undiluted DNA samples contained approximately 33.8 ng of DNA for the female and 38.4 ng for the male. The expected amplification products were successfully obtained for both individuals. As anticipated, the male sample showed two distinct bands corresponding to the X- and Y-linked amplicons, whereas the female sample exhibited a single band of approximately 900 bp (Figure 3A, B). In the male sample, both expected products were detected at all tested DNA concentrations (Figure 3A). Interestingly, as the amount of template DNA decreased, the relative intensities of the X- and Y-linked bands became more similar. At the highest dilution (2,560-fold), the Y-linked band, corresponding to the longer amplicon, was unexpectedly more intense than the X-linked band (Figure 3A, lane 2560). In the female sample, the expected X-linked product was detected up to the 320-fold dilution, with amplification efficiency decreasing as the amount of template DNA was reduced (Figure 3B). DNA concentrations in the diluted samples were also assessed using the Qubit dsDNA HS Assay (Table 4). However, at the highest dilutions, DNA concentrations fell below the assay's quantification range. This occurred at the 1,280- and 2,560-fold dilutions for the male sample and from the 640-fold dilution onwards for the female sample. These results are consistent with the lower initial DNA concentration in the female extract compared with the male extract, as indicated by the measurements of the undiluted samples (33.8 ng and 38.4 ng, respectively). Despite this difference, the marker showed higher amplification tolerance to template dilution in the male sample, in which both diagnostic products remained detectable even when the DNA concentration was below the Qubit assay's quantification range. In contrast, in the female sample, the expected product was no longer detected at dilutions of 640-fold and higher. This suggests that the lower initial DNA concentration in the female sample contributed to the earlier loss of detectable amplification, although differences in amplification efficiency between the two samples cannot be excluded. The results obtained for the male sample indicate that the marker can successfully amplify both target fragments even when the amount of

template DNA is very low. Additional weak, non-specific products larger than 1,200 bp were observed in some reactions involving the female sample (Figure 3B, lanes 40–320 and 2560). However, none of these products corresponded in size to the diagnostic Y-linked amplicon observed in males. Notably, at the highest dilution (2,560-fold), only a non-specific product was detected, while the expected X-linked product was absent. Similar non-specific amplification products have previously been reported in other species tested with this marker, but they did not interfere with sex determination (Shaw *et al.* 2003).

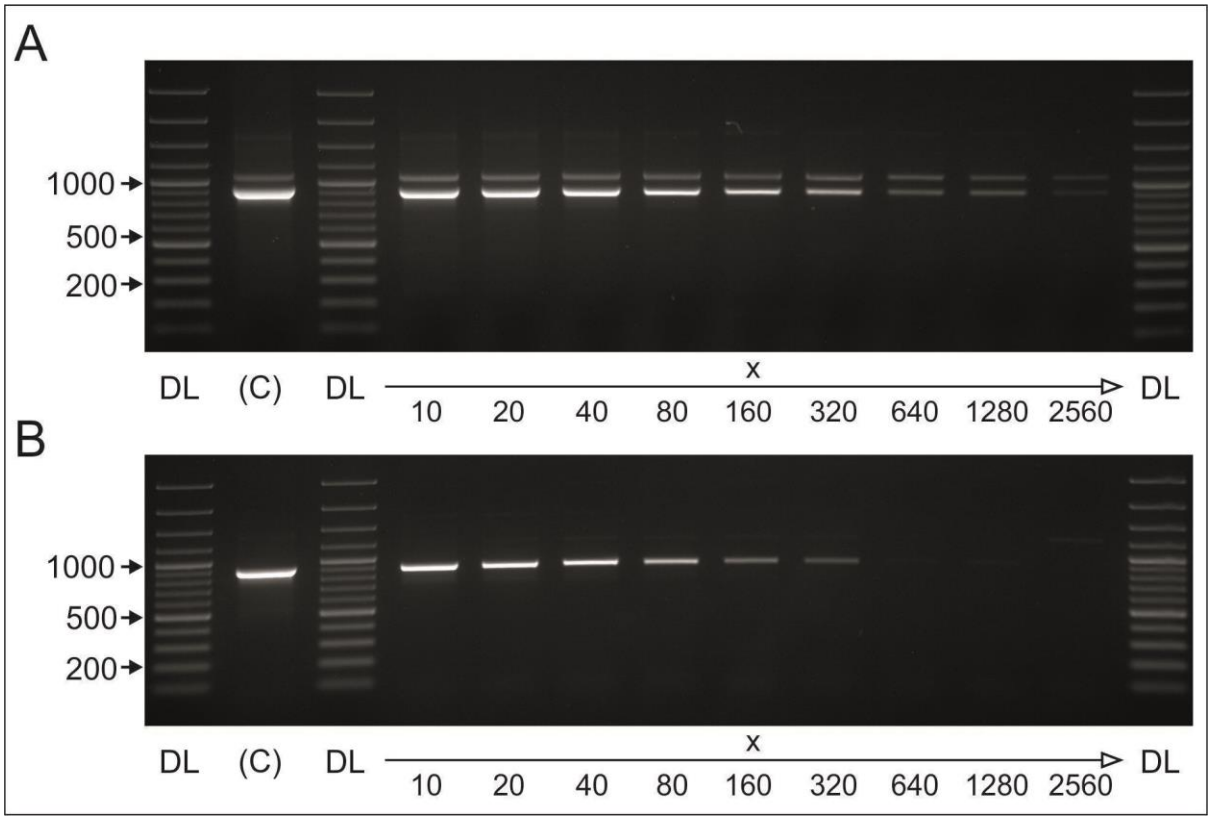

Figure 3. Amplification results of the ZF(i7)$_{X/Y}$ marker in a muscle tissue template gradient: concentrated DNA and template diluted 10-fold, 20-fold, 40-fold, 80-fold, 160-fold, 320-fold, 640-fold, 1280-fold, and 2560-fold for the male (A) and female (B). A total of 5 µL of the reaction mixture was loaded onto a 1.5% agarose gel. DL – 5 µL of DNA ladder 100 bp plus (Thermo Scientific).

Amplification of the ZF(i7)$_{X/Y}$ marker, corresponding to intron 7 of the ZFX gene, was performed using the LGL331 and LGL335 primers (Shaw *et al.* 2003) on DNA samples from 15 individuals of known sex: eight males and seven females (Table 2; Figure 2: ZF(i7)$_{X/Y}$). The marker successfully amplified the expected products in all tested individuals, despite differences in DNA concentrations among the extracts (Table 3), which were not normalized prior to PCR. The amplification patterns observed on the gels were consistent within each sex, with all males showing the expected two-band pattern and all females exhibiting a single band. Importantly, the molecular sex assignments were fully consistent with those determined by

anatomical and morphological examination. Although some reactions produced additional non-specific amplification products, these were weak and did not correspond in size to either of the expected amplicons. Consequently, they did not interfere with the interpretation of the results or the unambiguous determination of sex. All amplification products were subsequently sequenced for confirmation that the amplified fragments corresponded to the targeted genomic regions. Overall, the ZF(i7)$_{X/Y}$ marker proved effective for sex determination in all tested individuals, despite variation in DNA concentration among the muscle-derived extracts. These findings indicate that the marker can provide reliable sex assignments without the need for prior normalization of DNA concentration. Nevertheless, further validation using a larger number of individuals from diverse populations is recommended to confirm the applicability and reliability of this method, which is based on length polymorphism in a selected intron of the ZFX gene.

5.5. Sequencing results

Sequencing analysis comprised 19 tissue-derived PCR samples: ZF(i7)$_{X/Y}$ from all 15 individuals, and SRY-HMG and short ZF$_X$/ZF$_Y$ from reference female no. 1 and male no. 2 (Table 2). All data were deposited in NCBI and are available under accession number PRJNA1557058.

5.5.1. Marker SRY-HMG

The SRY-HMG reactions yielded 34,321 input reads for female 1 and 29,754 for male 2, with median read qualities of Q22.84 and Q21.70, respectively (Table 5). After quality and product-assignment filtering, 11,821 and 14,856 reads were retained. SRY accounted for 233 female reads (0.68% of input; 1.97% of retained reads) and 12,393 male reads (41.65% of input; 83.42% of retained reads). Both SRY consensuses were 202 nt long, contained no masked bases and matched the *T. europaea* SRY reference PP841022.1 at 98.48% identity over 98% of the query. Median consensus depths were 230 and 12,203.5, respectively.

Most retained reads from the female reaction represented off-target products. The dominant product mapped to an unannotated region of chromosome 3 (4,964 reads), followed by products overlapping CREB5 and PTPRT introns and other unannotated regions. SOX30 was represented by 410 female and 396 male reads. Its consensus was 202 nt long in both samples, matching the SRY consensus length. Thus, the approximately 200-bp size class was not specific to SRY. The low-level, sequence-confirmed SRY signal in the female was distinct from these off-target products; its origin could not be determined from the sequencing data.

Table 5. Sequencing yield and identified products of the SRY-HMG assay in muscle-derived DNA from female 1 and male 2.

Metric or product	1 — female	2 — male
Input reads	34321	29754
Median input read Q	22.84	21.7
After quality trimming and filtering	27,934 (81.39%)	20,642 (69.38%)
Retained and assigned to products	11,821 (34.44%)	14,856 (49.93%)
SRY, 202 nt	233 (0.68%)	12,393 (41.65%)
SOX30, 202 nt	410 (1.19%)	396 (1.33%)
Chr 3 unannotated region, ~181 nt	4,964 (14.46%)	1,041 (3.50%)
CREB5 intron, ~271 nt	1,752 (5.10%)	157 (0.53%)
PTPRT intron, ~376 nt	1,589 (4.63%)	674 (2.27%)
Chr X unannotated region, ~179 nt	1,449 (4.22%)	95 (0.32%)
Chr 16 unannotated region, ~222 nt	1,423 (4.15%)	96 (0.32%)
SRY / retained reads (%)	1.97	83.42
SRY consensus median depth	230	12203.5

5.5.2. Marker ZF_X/ZF_Y

The short ZF_X/ZF_Y reactions yielded 93,018 input reads for female 1 and 98,946 for male 2, with median read qualities of Q22.32 and Q22.21. The 447-nt product family accounted for 65,047 reads (69.93% of input) in the female and 68,355 reads (69.08%) in the male. The female consensus matched ZFX on chromosome X. In the male, 14 linked variable sites resolved two 447-nt sequence groups: 46,790 reads were assigned to ZFX and 12,900 to candidate ZFY, while 8,665 reads remained unresolved. These groups represented 68.45%, 18.87% and 12.68% of the male parent family, respectively. The two resolved male consensuses and the female consensus contained no masked bases; their median depths were 46,022, 12,758 and 63,999, respectively.

The ZFX consensuses matched NC_140081.1 at 99.33% identity over 99% of the query, whereas the candidate ZFY consensus matched the homologous *T. occidentalis* sequence RCFO01000018.1 at 99.09% identity over 98% of the query. The two male products were therefore distinguishable by sequence, but not by length. The Y-associated assignment remains comparative because the *T. europaea* reference assembly used here does not contain a Y chromosome.

5.5.3. Marker ZF(i7)$_{X/Y}$

Across the 15 ZF(i7)$_{X/Y}$ reactions, 1,132,624 input reads were available, of which 660,532 (58.32%) were retained after quality and product-assignment filtering (Table 6). Median input read quality ranged from Q23.32 to Q23.61. ZFX and candidate ZFY contributed 460,966 and 78,132 reads, respectively: together, these represented 47.60% of input reads and 81.62% of retained reads. The remaining 121,434 retained reads belonged to other product families.

ZFX consensuses were recovered from all 15 individuals and ranged from 899 to 907 nt. They aligned across the full query length to *T. europaea* chromosome X (NC_140081.1:12,826,355–12,827,257), with identities of 98.35–99.23%. The longer product yielded a 1069-nt consensus in each of the eight males and matched the homologous region on *T. occidentalis* contig RCFO01000018.1 at 97.01% identity over the full query. The corresponding reference introns were 820 nt and 987 nt long, supporting an intron-length difference as the basis of the approximately 900/1100-bp gel profiles. The conventional ZF(i7)$_{X/Y}$ assay name is retained because intron numbering depends on the transcript used.

All eight males had 7,038–12,372 candidate-Y reads, representing 24.74–31.94% of X+Y reads. The seven females had 0–22 candidate-Y reads (0–0.060% of X+Y): female 1 had 22, females 6, 7 and 14 had one, two and one, respectively, and the remaining females had zero. None of these female signals met the minimum read count for a candidate-Y consensus. Dominant sequence profiles were therefore concordant with independently assigned sex in all 15 individuals, while retaining the trace Y-like signals in the quantitative results.

The 15 ZFX and eight candidate-ZFY consensuses had minimum per-position depths of 13,182–31,351 and 5,717–9,907, respectively. Eighteen of the 23 consensuses contained no masked bases. A single base was masked in ZFX from female 8 and in candidate ZFY from males 5, 9, 11 and 12. Residual homopolymer and indel uncertainty precludes treating small consensus-length differences as validated allelic variation, but did not affect separation of the two major product-length classes.

Table 6. Quantitative sequencing results for the ZF(i7)$_{X/Y}$ assay in muscle-derived DNA from 15 independently sexed European moles. ID - individual identifier; F - female; M - male. Sex was determined independently by morphology and dissection.

ID	Sex	Input reads	Retained reads	ZFX reads	Candidate ZFY reads	Y/(X+Y) (%)
1	F	74,382	42,282	36,782	22	0.060
2	M	63,863	36,390	20,811	8,068	27.937
3	M	56,282	30,857	16,757	7,038	29.578
4	F	73,938	45,973	39,504	0	0.000
5	M	83,794	51,319	32,219	11,165	25.735
6	F	84,268	45,898	33,697	1	0.003
7	F	77,134	44,574	32,955	2	0.006
8	F	80,121	48,314	40,295	0	0.000
9	M	78,810	44,494	24,770	9,548	27.822
10	F	72,932	43,911	37,933	0	0.000
11	M	85,685	49,033	28,467	11,686	29.104
12	M	77,505	44,503	26,772	9,317	25.817
13	M	79,618	46,564	26,358	12,372	31.944
14	F	70,449	42,720	36,531	1	0.003
15	M	73,843	43,700	27,115	8,912	24.737
Total	8 M / 7 F	1,132,624	660,532	460,966	78,132	—

6. Discussion

Although the sex of European moles can be determined based on external morphological characteristics (Skoczeń 1958), this approach is primarily applicable to deceased individuals, as examination of living animals requires their capture and physical handling. Molecular methods for sex determination have been developed for numerous mammalian species, including several insectivores and three mole species (Sánchez *et al.* 1996). Many of these approaches rely on amplification of a conserved fragment of the SRY gene, which is generally located on the Y chromosome and absent from females (Foster *et al.* 1992, Sinclair *et al.* 1990). Under this approach, the presence of an approximately 200-bp amplification product indicates a male, whereas its absence, provided that DNA amplification is confirmed by an additional control marker, indicates a female (Bryja and Konečný 2003, Sánchez *et al.* 1996). However, the reliability of SRY-based sex determination may be affected by several genomic features, including gene duplication, translocation to the X chromosome or, in some species, complete loss of the gene (Sutou *et al.* 2001, Soullier *et al.* 1998, Vogel *et al.* 1998, Bullejos *et al.* 1997,

Just *et al.* 1995). Consequently, the suitability of this marker should be verified for each target species. In the present study, amplification of the SRY-HMG fragment using primers described by Sánchez *et al.* (1996) produced the expected approximately 200-bp product in males, but also generated amplification products in females. This pattern was reproducible across independent DNA extractions and was not accompanied by amplification in the no-template controls. Sequencing confirmed that the male sample contained a substantial proportion of reads corresponding to SRY (12,393 reads), whereas only 233 SRY-assigned reads were recovered from the female sample. Although both consensus sequences were 202 nt long and showed 98.48% identity to the *T. europaea* SRY reference, the female signal was considerably weaker and its origin remains unresolved. Importantly, the approximately 200-bp size class was not specific to SRY, as products assigned to SOX30, which also yielded 202-nt consensuses, were identified in both samples. Most retained female reads represented other off-target products, including sequences mapping to chromosome 3 and regions overlapping CREB5 and PTPRT. These findings demonstrate that gel electrophoresis alone cannot establish the identity of products generated by the SRY-HMG primers.

The repeated occurrence of the female amplification pattern makes simple contamination during an individual DNA extraction less likely. Nevertheless, low-level male DNA admixture, PCR carryover or sequencing-related effects, including barcode misassignment, cannot be excluded. The negative PCR controls reduce the likelihood of contamination of the reaction mixtures but cannot establish the background level of the sequencing procedure, as these controls were not sequenced. Similarly, the low number of female SRY reads does not demonstrate the presence of SRY in the female genome. Therefore, the biological origin of this signal remains uncertain.

The additional amplification products observed in males may partly be considered in the context of the findings of Bogdanov *et al.* (2024), who proposed that *T. europaea* may possess multiple copies of the SRY gene. However, the presence of multiple bands or different sequence products does not, by itself, demonstrate SRY gene duplication. In particular, the sequencing results obtained here showed that several products amplified by the SRY-HMG primers originated from other genomic regions. Thus, neither the number of amplification products nor their relative sequencing abundance can be used to infer SRY copy number or chromosomal location. Regardless of the underlying cause of the female amplification, the SRY-HMG marker cannot be considered sufficiently reliable for unambiguous sex determination in the European mole.

The short ZF_X/ZF_Y marker, based on amplification of exon 8 using primers described by Aasen and Medrano (1990), provided the expected approximately 447-bp product in both sexes and therefore served as a suitable amplification control. However, sequencing revealed that the single band observed in males contained two distinct sequence groups, corresponding to ZFX and a candidate ZFY product. These groups were distinguishable by 14 linked variable sites but were identical in length. The female product corresponded to ZFX, whereas the male sample contained both ZFX and candidate ZFY sequences. The latter assignment was supported by sequence similarity to the homologous region in *T. occidentalis*. Nevertheless, because the available *T. europaea* reference assembly does not contain a Y chromosome, the chromosomal location of this product has not been independently confirmed. These results illustrate that a single electrophoretic band may contain multiple homologous sequences and that the short ZF_X/ZF_Y marker, although useful as an amplification control, cannot independently distinguish the sexes.

To overcome the limitations of the SRY-HMG approach, we evaluated the $ZF(i7)_{X/Y}$ marker, based on length polymorphism within intron 7 of the ZFX gene. This approach was originally proposed by Shaw *et al.* (2003) and subsequently applied to other mammalian species, including the giant panda (Xu *et al.* 2010). Unlike the conventional SRY-based approach, it does not depend on the absence of amplification in females. Instead, it relies on the presence of homologous sequences in both sexes that produce distinguishable amplification patterns due to differences in intron length. In females, the two X-linked copies produce a single band, whereas males exhibit two bands corresponding to the X- and Y-associated products. The direction of the length difference varies among species, with the Y-associated product being either shorter or longer than its X-linked counterpart (Shaw *et al.* 2003). In the European mole, the $ZF(i7)_{X/Y}$ marker consistently produced an approximately 900-bp product in females and two products in males, corresponding to the X- and Y-associated fragments. Sequencing analysis further supported this interpretation. The shorter consensuses (899–907 nt) matched the *T. europaea* X chromosome, whereas the longer 1,069-nt product, recovered from all eight males, showed high sequence similarity to the homologous intronic region of *T. occidentalis*. The corresponding reference introns were 820 and 987 nt long, respectively, supporting intron-length variation as the basis of the observed approximately 900- and 1,100-bp amplification products. Although the longer fragment is strongly supported as a candidate ZFY product, its location on the Y chromosome has not yet been independently confirmed in *T. europaea*, owing to the lack of a Y chromosome in the available reference genome.

Importantly, the dominant ZFX and candidate ZFY sequence profiles were consistent with the independently established sex of all 15 examined individuals. All eight males showed substantial candidate-Y read counts, representing 24.74–31.94% of their combined X- and Y-associated reads. In contrast, the seven females had between zero and 22 candidate-Y reads, accounting for no more than 0.060% of their combined X- and Y-associated reads. None of these low-level female signals was sufficient to generate a candidate-Y consensus. Thus, although trace Y-like reads were detected in some female reactions, they did not affect the interpretation of the dominant sequence profiles or the resulting sex assignments.

The main advantage of the proposed marker is that sex determination does not require sequencing or other advanced molecular analyses for routine sample processing. Instead, it relies on a straightforward interpretation of the electrophoretic pattern: two distinct bands indicate a male, whereas a single band indicates a female. This distinction was consistently observed in all examined individuals and allowed unambiguous sex assignment directly from agarose gel electrophoresis. Although sequencing revealed low numbers of candidate-Y reads in some female samples, these signals did not result in the appearance of a second diagnostic band and therefore did not compromise the interpretation of the gel profiles. In contrast to the SRY-HMG marker, which produced amplification products in both sexes and required sequencing to establish their origin, the $ZF(i7)_{X/Y}$ marker generated distinct sex-specific patterns that could be interpreted directly from the electrophoretic results. This makes the proposed marker particularly useful for routine molecular sex determination, where simple and reliable interpretation of PCR products is essential.

Another important aspect of the proposed marker is its amplification performance under variable DNA template concentrations. The dilution experiment demonstrated that both diagnostic products remained detectable in the male sample at all tested dilutions, including the highest (2,560-fold), whereas the female X-linked product was detected up to the 320-fold dilution. The earlier loss of amplification in the female sample may partly reflect its lower initial DNA concentration, although differences in amplification efficiency between the two extracts cannot be excluded. These results suggest that the marker can tolerate relatively low template input under the tested conditions. Furthermore, successful amplification in all 15 individuals, despite differences in DNA concentration among muscle-derived extracts, suggests that prior normalization of DNA concentration may not be necessary under the conditions tested.

Overall, the ZF(i7)$_{X/Y}$ marker represents a promising tool for molecular sex determination in *T. europaea*. Its principal advantage is the simplicity and clarity of the resulting electrophoretic patterns, which allow males and females to be distinguished directly on the basis of two bands versus one, respectively. The sequencing results provide additional support for the molecular identity of the amplified fragments, while the concordance between electrophoretic profiles and independently determined sex confirms the practical applicability of the method in the examined material. Further validation using a larger, independent sample of individuals from different populations is recommended, together with genomic evidence confirming the Y-chromosomal origin of the longer product. Nevertheless, the results demonstrate that the proposed marker offers a straightforward approach to sex determination that can be reliably interpreted using standard PCR and agarose gel electrophoresis, without the need for routine sequencing.

7. Conclusion

The ZF(i7)$_{X/Y}$ marker provided a straightforward and effective approach to molecular sex identification in the European mole using DNA extracted from muscle tissue. Unlike the tested SRY-HMG assay, which generated non-specific amplification products in both sexes, and the short ZF$_X$/ZF$_Y$ marker, which produced indistinguishable electrophoretic patterns, ZF(i7)$_{X/Y}$ yielded clearly distinguishable results: a single band in females and two bands in males. These patterns were consistent with the sex independently established by morphological examination and post-mortem dissection in all 15 individuals. Sequencing supported intron-length variation between ZFX and a candidate ZFY homologue as the basis of this distinction, although the Y-chromosomal location of the longer product remains to be confirmed.

The principal advantage of this assay is that sex can be identified using a single PCR followed by standard agarose gel electrophoresis, without a separate control reaction for female samples or routine sequencing. Successful amplification across muscle-derived extracts with different DNA concentrations and in the dilution experiments supports its applicability under the conditions tested. Validation using a larger, independent sample of individuals from different populations is needed to establish the broader reliability of the method.

8. Acknowledgements

This work was supported by the Wrocław University of Environmental and Life Sciences (Poland) as the PhD research program "Innowacyjny Naukowiec No. N060/0003/22".

9. References

Aasen E., Medrano J.F., 1990. Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats. Bio/Technology 8(12): 1279–1281. https://doi.org/10.1038/nbt1290-1279

Abelentsev V.I., Pidoplichko I.G., Popov B.M., 1956. Fauna Ukrainy. Vol. 1 Mammals, issue 1 General characteristics of mammals. Insectivores, bats. Academy of Sciences of the Ukrainian SSR, Kyiv. [In Ukrainian].

Amori G., Hutterer R., Mitsainas G., Yigit N., Kryštufek B., Palomo L.J., 2017. *Talpa europaea*. The IUCN Red List of Threatened Species 2017: e.T41481A22320754. https://doi.org/10.2305/IUCN.UK.2017-2.RLTS.T41481A22320754.en [Accessed 7 November 2026]

Bérubé M., Palsbøll P., 1996. Identification of sex in cetaceans by multiplexing with three ZFX and ZFY specific primers. Mol. Ecol. 5(2): 283–287. https://doi.org/10.1111/j.1365-294X.1996.tb00315.x

Bogdanov A., Sokolova M., Bakloushinskaya I., 2024. Specificity of key sex determination genes in a mammal with ovotestes: the European mole *Talpa europaea*. Animals 14(15): 2180. https://doi.org/10.3390/ani14152180

Bryja J., Konečný A., 2003. Fast sex identification in wild mammals using PCR amplification of the Sry gene. Folia Zool. 52(3): 269–274.

Bullejos M., Sánchez A., Burgos M., Hera C., Jiménez R., Díaz de la Guardia R., 1997. Multiple, polymorphic copies of SRY in both males and females of the vole *Microtus cabrerae*. Cytogenet. Cell Genet. 79(3–4): 167–171. https://doi.org/10.1159/000134712

Camacho C., Coulouris G., Avagyan V., Ma N., Papadopoulos J., Bealer K., Madden T.L., 2009. BLAST+: architecture and applications. BMC Bioinformatics 10: 421. https://doi.org/10.1186/1471-2105-10-421

Deparma N.K., 1951. Krot [The mole]. Zagotizdat, Moscow. [In Russian].

Foster J.W., Brennan F.E., Hampikian G.K., Goodfellow P.N., Sinclair A.H., Lovell-Badge R., Selwood L., Renfree M.B., Cooper D.W., Marshall Graves J.A., 1992. Evolution of sex determination and the Y chromosome: SRY-related sequences in marsupials. Nature 359(6395): 531–533. https://doi.org/10.1038/359531a0

Gao Y., Liu Y., Ma Y., Liu B., Wang Y., Xing Y., 2021. abPOA: an SIMD-based C library for fast partial order alignment using adaptive band. Bioinformatics 37(15): 2209–2211. https://doi.org/10.1093/bioinformatics/btaa963

Godet R., 1951. Contribution à l'éthologie de la taupe (*Talpa europaea* L.). Bull. Soc. Zool. Fr. 76: 107–128.

Griffiths R., Tiwari B., 1993. Primers for the differential amplification of the sex-determining region Y gene in a range of mammal species. Mol. Ecol. 2(6): 405–406. https://doi.org/10.1111/j.1365-294X.1993.tb00034.x

Griffiths R., Double M.C., Orr K., Dawson R.J.G., 1998. A DNA test to sex most birds. Mol. Ecol. 7(8): 1071–1075. https://doi.org/10.1046/j.1365-294X.1998.00389.x

Joshi B.D., De R., Goyal S.P., 2019. Utility and applicability of a universal set of primers in identifying the sex of South and Southeast Asian mammals. Zool. Stud. 58: 19. https://doi.org/10.6620/ZS.2019.58-19

Just W., Rau W., Vogel W., Akhverdian M., Fredga K., Graves J.A., Lyapunova E., 1995. Absence of Sry in species of the vole *Ellobius*. Nat. Genet. 11(2): 117–118. https://doi.org/10.1038/ng1095-117

Kroczak A., Wierzbicki H., Urantówka A.D., 2022. *In silico* analysis of seven PCR markers developed from the CHD1, NIPBL and SPIN genes followed by laboratory testing shows how to reliably determine the sex of Musophagiformes species. Genes 13(5): 932. https://doi.org/10.3390/genes13050932

Kroczak A., Wołoszyńska M., Wierzbicki H., Kurkowski M., Grabowski K.A., Piasecki T., Galosi L., Urantówka A.D., 2021. New bird sexing strategy developed in the order Psittaciformes involves multiple markers to avoid sex misidentification: debunked myth of the universal DNA marker. Genes 12(6): 878. https://doi.org/10.3390/genes12060878

Li H., 2018. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics 34(18): 3094–3100. https://doi.org/10.1093/bioinformatics/bty191

Minister of the Environment, 2016. Rozporządzenie Ministra Środowiska z dnia 16 grudnia 2016 r. w sprawie ochrony gatunkowej zwierząt [Regulation of the Minister of the Environment of 16 December 2016 on the protection of animal species]. Dz. U. 2016, poz. 2183. [In Polish]. https://eli.gov.pl/eli/DU/2016/2183/ogl [Accessed 7 November 2026]

Mitchell-Jones A.J., Amori G., Bogdanowicz W., Kryštufek B., Reijnders P.J.H., Spitzenberger F., Stubbe M., Thissen J.B.M., Vohralík V., Zima J., 1999. The atlas of European mammals. T. & A. D. Poyser, London.

Ognev S.I., 1928. Zveri vostochnoi Evropy i Severnoi Azii [Mammals of Eastern Europe and Northern Asia]. Vol. 1 Insectivora and Chiroptera. Gosudarstvennoe Izdatelstvo, Moscow–Leningrad. [In Russian].

Ortega J., Franco M.R., Adams B.A., Ralls K., Maldonado J.E., 2004. A reliable, non-invasive method for sex determination in the endangered San Joaquin kit fox (*Vulpes macrotis mutica*) and other canids. Conserv. Genet. 5(5): 715–718. https://doi.org/10.1007/s10592-004-1862-0

Palsbøll P.J., Vader A., Bakke I., El-Gewely M.R., 1992. Determination of gender in cetaceans by the polymerase chain reaction. Can. J. Zool. 70(11): 2166–2170. https://doi.org/10.1139/z92-292

Pomp D., Good B.A., Geisert R.D., Corbin C.J., Conley A.J., 1995. Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of day-10 or -11 pig embryos. J. Anim. Sci. 73(5): 1408–1415. https://doi.org/10.2527/1995.7351408x

Pucek Z. (Ed.), 1984. Klucz do oznaczania ssaków Polski [Key to the identification of Polish mammals]. 2nd revised edition. Państwowe Wydawnictwo Naukowe, Warszawa. [In Polish].

Pucek Z., Raczyński J. (Eds.), 1983. Atlas rozmieszczenia ssaków w Polsce [Atlas of Polish mammals]. Państwowe Wydawnictwo Naukowe, Warszawa.

Racey P.A., 1978. Seasonal changes in testosterone levels and androgen-dependent organs in male moles (*Talpa europaea*). J. Reprod. Fertil. 52(2): 195–200. https://doi.org/10.1530/jrf.0.0520195

Rognes T., Flouri T., Nichols B., Quince C., Mahé F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4: e2584. https://doi.org/10.7717/peerj.2584

Sánchez A., Bullejos M., Burgos M., Hera C., Jiménez R., Díaz de la Guardia R., 1996. High sequence identity between the SRY HMG box from humans and insectivores. Mamm. Genome 7(7): 536–538. https://doi.org/10.1007/s003359900158

Shaw C.N., Wilson P.J., White B.N., 2003. A reliable molecular method of gender determination for mammals. J. Mammal. 84(1): 123–128. https://doi.org/10.1644/1545-1542(2003)084<0123:ARMMOG>2.0.CO;2

Sinclair A.H., Berta P., Palmer M.S., Hawkins J.R., Griffiths B.L., Smith M.J., Foster J.W., Frischauf A.M., Lovell-Badge R., Goodfellow P.N., 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346(6281): 240–244. https://doi.org/10.1038/346240a0

Skoczeń S., 1958. Determination of the sex of moles (*Talpa europaea* L.) by means of their external features. Acta Theriol. 2(14): 290–292. https://doi.org/10.4098/AT.arch.58-19

Soullier S., Hänni C., Catzeflis F., Berta P., Laudet V., 1998. Male sex determination in the spiny rat *Tokudaia osimensis* (Rodentia: Muridae) is not Sry dependent. Mamm. Genome 9(7): 590–592. https://doi.org/10.1007/s003359900823

Sutou S., Mitsui Y., Tsuchiya K., 2001. Sex determination without the Y chromosome in two Japanese rodents *Tokudaia osimensis osimensis* and *Tokudaia osimensis* spp. Mamm. Genome 12(1): 17–21. https://doi.org/10.1007/s003350010228

Vogel W., Jainta S., Rau W., Geerkens C., Baumstark A., Correa-Cerro L.S., Ebenhoch C., Just W., 1998. Sex determination in *Ellobius lutescens*: the story of an enigma. Cytogenet. Cell Genet. 80(1–4): 214–221. https://doi.org/10.1159/000014983

Whitworth D.J., Licht P., Racey P.A., Glickman S.E., 1999. Testis-like steroidogenesis in the ovotestis of the European mole, *Talpa europaea*. Biol. Reprod. 60(2): 413–418. https://doi.org/10.1095/biolreprod60.2.413

Wilson P.J., White B.N., 1998. Sex identification of elk (*Cervus elaphus canadensis*), moose (*Alces alces*), and white-tailed deer (*Odocoileus virginianus*) using the polymerase chain reaction. J. Forensic Sci. 43(3): 477–482. https://doi.org/10.1520/JFS16172J

Xu X., Li Y., Wang X., Wei K., Zhang W., Zhang Z., Shen F., Yue B., 2010. Zinc-finger intron 7: a new locus for sex identification of giant panda (*Ailuropoda melanoleuca*). Zoo Biol. 29(4): 526–531. https://doi.org/10.1002/zoo.20274

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Supplementary materials

Figure S1. External genital anatomy and internal reproductive anatomy of European moles (*Talpa europaea*) in spring and autumn: (A) female in spring; (B) male in spring; (C) female in autumn; (D) male in autumn. Each panel shows the same individual before dissection (left) and after dissection (right). External views show the anogenital region used for morphological sex identification.

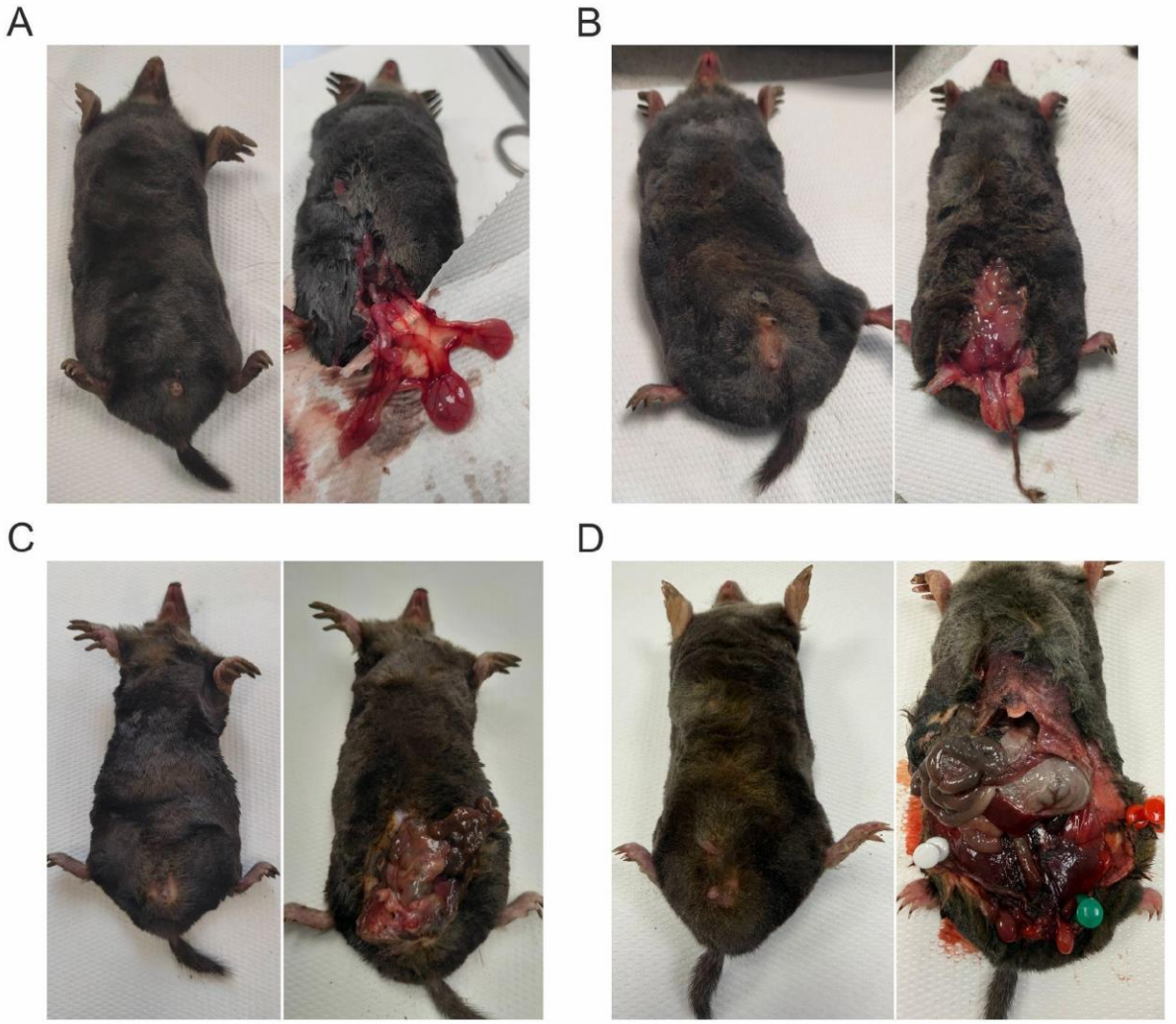

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Table 1. Marker name, primer sequences used for amplification, and the literature source from which the sequences were obtained. Amplicon sizes for each marker were estimated *in silico* based on the available reference nuclear genomes of the European mole (*Talpa europaea*) (GenBank accession no. GCA_964194135.1), and the Iberian mole (*Talpa occidentalis*) (GenBank accession no. GCA_014898055.3). "–" indicates that the sequence is absent from the X chromosome.

Marker Name	Primer F		Primer R		Amplicon length [bp]		Fragment	Reference
	Name	Sequence	Name	Sequence	X chromosome	Y chromosome		
SRY	SRY-HMG-F	GTCAAGCGCCCCATGAATGCAT	SRY-HMG-R	AGTTTGGGTATTTCTCTCTGTG	-	200	fragment of the SRY HMG box	Sánchez *et al.*, 1996
ZF_X/ZF_Y	P1-5EZ	ATAATCACATGGAGAGCCACAAGCT	P2-3EZ	GCACTTCTTTGGTATCTGAGAAAGT	447	447	8 exon fragment of the ZFX gene	Asen and Medrano, 1990
$ZF(i7)_{X/Y}$	LGL331	CAAATCATGCAAGGATAGAC	LGL335	AGACCTGATTCCAGACAGTACCA	912	1100	7 intron of the ZFX gene	Shaw *et al.*, 2003

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Table 2. Individuals tested in this study, along with the location (Lower Silesian Voivodeship) where each individual was found, the type of biological material collected, and the molecular markers used for sex identification. "Yes" indicates that the marker was amplified for the respective individual. "*" indicates that the amplicon was sequenced.

No. of the individual	Sex	Biological sample	Source	Molecular marker		
				SRY-HMG	ZFX/ZFY	ZF(i7)X/Y
1	Female	tissue	51°03'03"N 17°24'26"E	yes*	yes*	yes*
2	Male	tissue	50°17′15″N 16°48′54″E	yes*	yes*	yes*
3	Male	tissue	51°03′03″N 17°24′25″E	yes	yes	yes*
4	Female	tissue	51°03′33″N 17°11′55″E	yes	yes	yes*
5	Male	tissue	50°16′13″N 16°53′07″E	yes	yes	yes*
6	Female	tissue	51°03′02″N 17°24′25″E	yes	yes	yes*
7	Female	tissue	51°02′59″N 17°24′25″E	yes	yes	yes*
8	Female	tissue	51°03′03″N 17°24′29″E	yes	yes	yes*
9	Male	tissue	51°03′03″N 17°24′13″E	yes	yes	yes*
10	Female	tissue	51°03′14″N 17°24′17″E	yes	yes	yes*
11	Male	tissue	51°03′14″N 17°23′40″E	yes	yes	yes*
12	Male	tissue	51°03′21″N 17°23′21″E	yes	yes	yes*
13	Male	tissue	50°58′33″N 16°32′21″E	yes	yes	yes*
14	Female	tissue	51°02′17″N 17°23′28″E	yes	yes	yes*
15	Male	tissue	51°02′52″N 17°24′34″E	yes	yes	yes*

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Table 3. DNA concentrations in the analyzed samples were determined fluorometrically using a Qubit fluorometer (Thermo Fisher Scientific), following the manufacturer's protocol for the Qubit dsDNA High Sensitivity (HS) Assay.

Sample ID	DNA concentration in muscle tissue extracts (ng/µL)
1	33.8
2	38.4
3	40
4	120
5	47.8
6	99.6
7	120
8	44.8
9	120
10	88.4
11	28.2
12	31.6
13	44.8
14	44.8
15	17.8

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Table 4. DNA concentrations in diluted template samples extracted from muscle tissue of selected individuals (no. 1 and 2, Table 2) were determined fluorometrically using a Qubit fluorometer (Thermo Fisher Scientific), according to the manufacturer's protocol for the Qubit dsDNA High Sensitivity (HS) Assay. The initial DNA concentration in each extract and the concentrations measured after 10-fold, 20-fold, 40-fold, 80-fold, 160-fold, 320-fold, 640-fold, 1,280-fold, and 2,560-fold dilution are presented. "Too Low" indicates that the DNA concentration was below the quantification range of the Qubit dsDNA HS Assay, resulting in a fluorescence signal insufficient for reliable quantification.

Dilution	DNA concentration (ng/µL)	
	1 (77)	2 (78)
Undiluted isolate	33.8	38.4
10-fold	2.86	8.02
20-fold	1.34	5.5
40-fold	0.53	2.32
80-fold	0.166	1.3
160-fold	0.124	0.454
320-fold	0.106	0.304
640-fold	Too Low	0.112
1280-fold	Too Low	Too Low
2560-fold	Too Low	Too Low

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Table 5. Sequencing yield and identified products of the SRY-HMG assay in muscle-derived DNA from female 1 and male 2.

Metric or product	1 — female	2 — male
Input reads	34321	29754
Median input read Q	22.84	21.7
After quality trimming and filtering	27,934 (81.39%)	20,642 (69.38%)
Retained and assigned to products	11,821 (34.44%)	14,856 (49.93%)
SRY, 202 nt	233 (0.68%)	12,393 (41.65%)
SOX30, 202 nt	410 (1.19%)	396 (1.33%)
Chr 3 unannotated region, ~181 nt	4,964 (14.46%)	1,041 (3.50%)
CREB5 intron, ~271 nt	1,752 (5.10%)	157 (0.53%)
PTPRT intron, ~376 nt	1,589 (4.63%)	674 (2.27%)
Chr X unannotated region, ~179 nt	1,449 (4.22%)	95 (0.32%)
Chr 16 unannotated region, ~222 nt	1,423 (4.15%)	96 (0.32%)
SRY / retained reads (%)	1.97	83.42
SRY consensus median depth	230	12203.5

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Table 6. Quantitative sequencing results for the ZF(i7)$_{X/Y}$ assay in muscle-derived DNA from 15 independently sexed European moles. ID, individual identifier; F, female; M, male. Sex was determined independently by morphology and dissection.

ID	Sex	Input reads	Retained reads	ZFX reads	Candidate ZFY reads	Y/(X+Y) (%)
1	F	74,382	42,282	36,782	22	0.060
2	M	63,863	36,390	20,811	8,068	27.937
3	M	56,282	30,857	16,757	7,038	29.578
4	F	73,938	45,973	39,504	0	0.000
5	M	83,794	51,319	32,219	11,165	25.735
6	F	84,268	45,898	33,697	1	0.003
7	F	77,134	44,574	32,955	2	0.006
8	F	80,121	48,314	40,295	0	0.000
9	M	78,810	44,494	24,770	9,548	27.822
10	F	72,932	43,911	37,933	0	0.000
11	M	85,685	49,033	28,467	11,686	29.104
12	M	77,505	44,503	26,772	9,317	25.817
13	M	79,618	46,564	26,358	12,372	31.944
14	F	70,449	42,720	36,531	1	0.003
15	M	73,843	43,700	27,115	8,912	24.737
Total	8 M / 7 F	1,132,624	660,532	460,966	78,132	—

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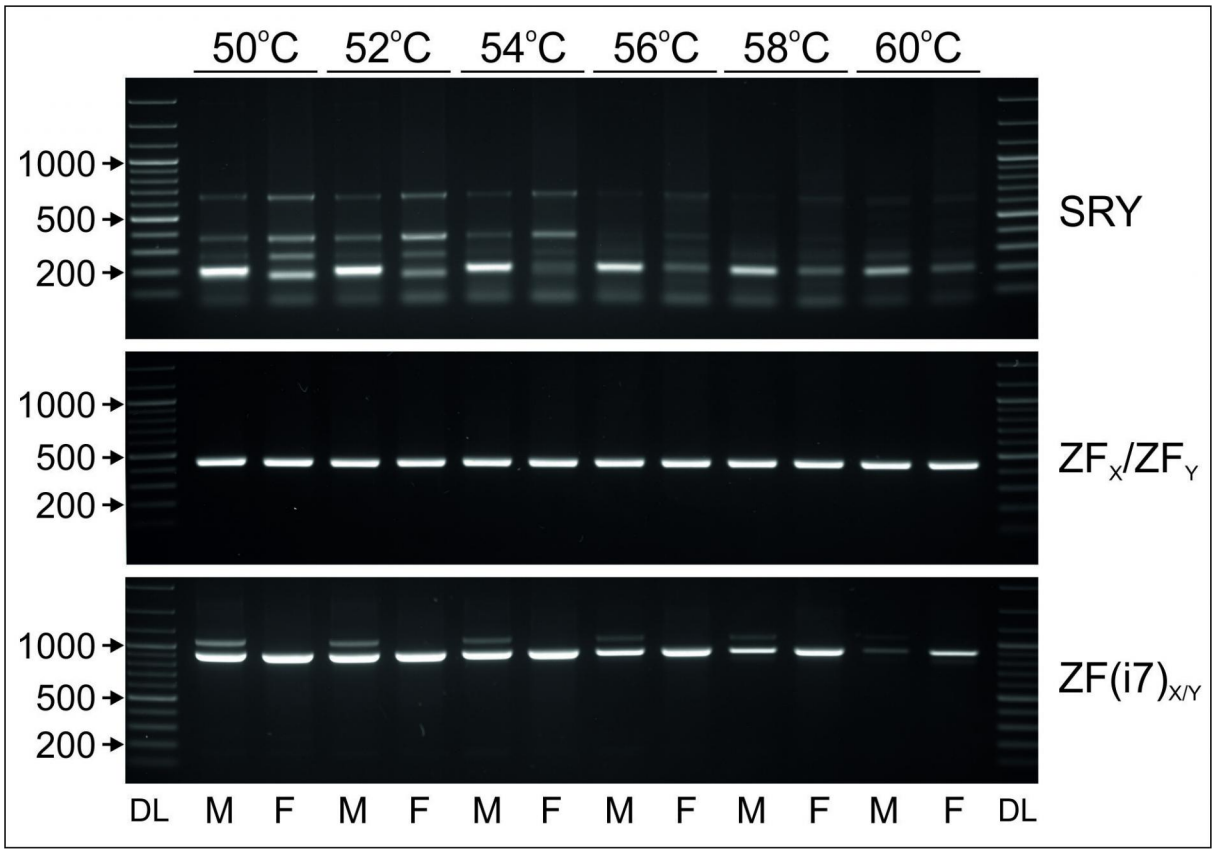

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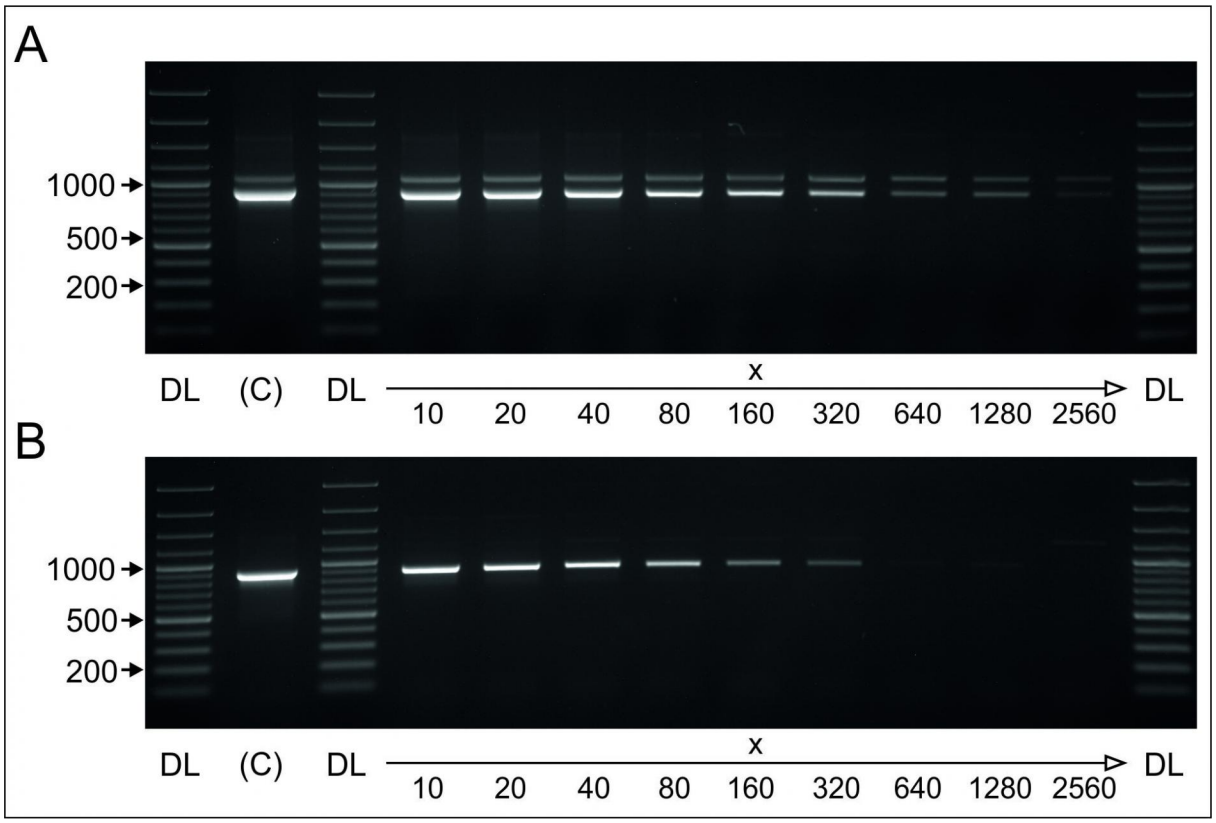
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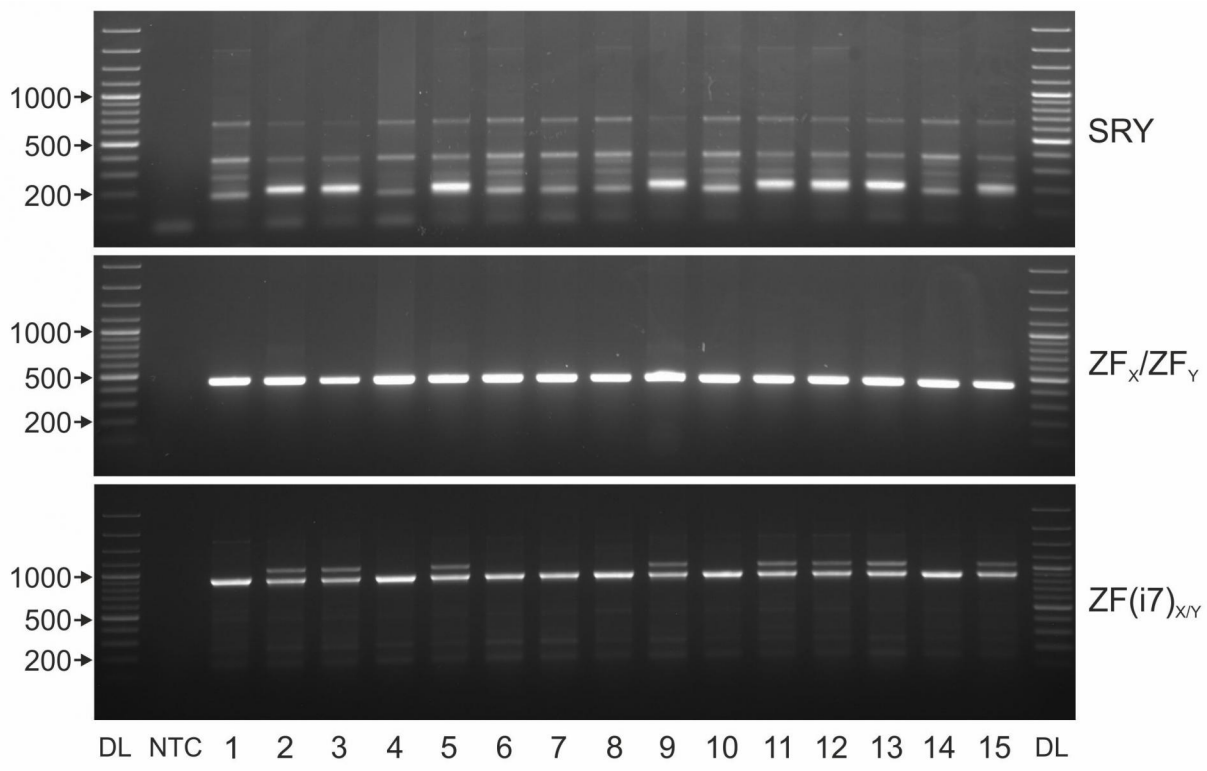

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