

TECHNICAL NOTES

Representational difference analysis to distinguish cryptic species

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In the study of biodiversity it is important to have a reliable system for identification of various genetically distinct units (species, subspecies, etc.). One of the most efficient tools available today is the polymerase chain reaction (PCR) with diagnostic primers that yield a detectable product for one taxon but not for other taxa. Critical to this method is the identification of diagnostic DNA fragments from which primers can be designed. Representational difference analysis (RDA) can reliably isolate DNA fragments that specify a particular taxon. In this report we demonstrate the utility of the technique by the development of binary markers that distinguish between two cryptic species of voles (genus *Microtus*).

Although it is possible to distinguish cryptic species using common methods such as sequencing or karyotyping, a fast yes/no-type of assay is highly desirable. Such an assay is especially valuable when large-scale studies are conducted in which significant numbers of individuals need to be unambiguously identified. The real challenge, however, is isolating markers that can be used in this assay. RDA provides a solution to this problem, allowing complex mammalian genomes to be compared and differences between them to be isolated. We employed RDA for the development of genetic markers capable of distinguishing two closely related species of voles (*Microtus arvalis* and *M. rossiaemeridionalis*). These two taxa are

indistinguishable under field conditions and are truly sympatric over expansive portions of their ranges (Zagorodnyuk 1991), but can be reliably identified based on karyotypes (*M. arvalis* $2n = 46$, FN = 58–90; *M. rossiaemeridionalis* $2n = 54$, FN = 54). Knowing the karyotypes of animals makes it easy to test the efficacy of developed markers. As a result of our experiments, we obtained two primer pairs: one specific for *M. arvalis*, another for *M. rossiaemeridionalis* (Table 1 and Fig. 1).

Specimen collection, identification and isolation of genomic DNA. All specimens, including ones used in the original RDA experiments and in marker testing, were collected in northern Ukraine from several localities around Chernobyl and karyotyped as described in Baker *et al.* (1996). High-molecular-weight genomic DNA was isolated from frozen liver samples.

RDA and analysis of difference products. RDA was carried out as described by Lisitsyn & Wigler (1995), with slight modifications concerning difference products purification between rounds of enrichment. (For a complete and well-written step-by-step explanation of the RDA procedure see Lisitsyn (1995). We used *Bgl*III restriction enzyme and oligonucleotide adapters RBgl12 and RBgl24 (Lisitsyn *et al.* 1993) to prepare amplicons from *M. arvalis* and *M. rossiaemeridionalis* genomic DNAs. Two reciprocal experiments were then performed using (A) *M. arvalis* amplicons as the tester (*M. rossiaemeridionalis* as the driver), and (B) *M. rossiaemeridionalis* as the tester (*M. arvalis* as the driver). Difference products obtained after three rounds of RDA were analysed on agarose gel and the most prominent bands were excised and cloned into pGEM-T vector (Promega). Fifteen clones per cloned band were amplified using vector-situated primers. Amplification products were then digested with the restriction endonuclease *Sau*3AI (Promega) and run on an agarose gel. Products displaying identical restriction fragment patterns were considered to be a 'family' of sequences representing the same RDA product. To identify diagnostic RDA products, a member of each 'family' was then labelled with [α^{32} P]-dCTP (NEN laboratories) using a Random Primed DNA labelling kit (Boehringer Mannheim). Radioactively labelled RDA products were hybridized to blots prepared using genomic DNAs of *M. arvalis* and *M. rossiaemeridionalis* digested with

Primer	Sequence	Product length (bp)	Optimal Mg amount*	Optimal PCR profile
Mar14F	CCCCCTAAAGATGTGTTACAGAAACCATC		25 nM	94 °C 60 s
Mar14R	AAGCCCAACTTGTAAACCAGATAAAGCAC	533		60 °C 30 s
Mro16F	GCTCGCTGGTAGAGACAGTCACC		10 nM	73 °C 45 s
Mro16R	GAAGGCAGATTGACCAAGATTTC	447		35 cycles

Table 1 Diagnostic primers designed from RDA products

*Other component are 5 μ M of each primers, 0.625 nM (each) dNTPs, 1.25 units of *Taq* polymerase, 1–5 ng of genomic DNA (typically 1 μ L of 1/100 dilution of the original stock).

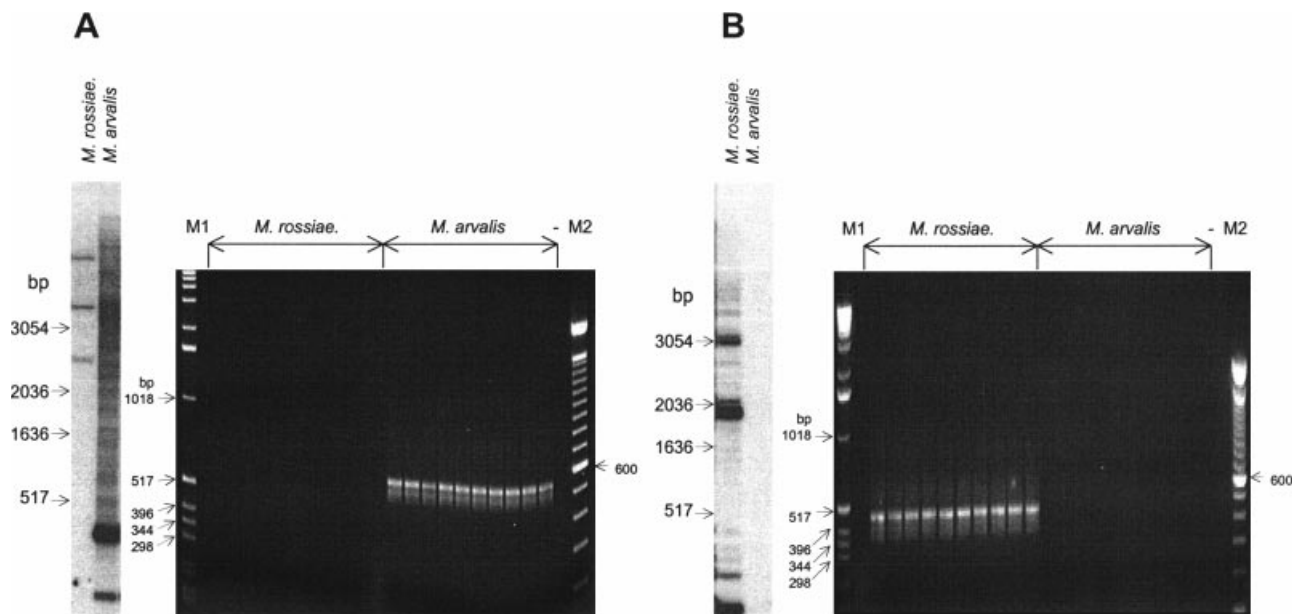


Fig. 1 Results of two reciprocal RDAs performed to obtain primers specific to (A) *Microtus arvalis* and (B) *M. rossiaemeridionalis*. Autoradiograms represent results of Southern blot analyses where radioactively labelled DNA from a cloned RDA product (A, *Mar14*; B, *Mro16*) was hybridized to filters carrying *Bgl*III-digested genomic DNA of *M. arvalis* and *M. rossiaemeridionalis*. Photographs of electrophoretic gels show the results of PCR amplification with primers designed to difference products *Mar14* (A) and *Mro16* (B). Genomic DNA from 10 individuals of *M. rossiaemeridionalis* and 10 individuals of *M. arvalis* was used as the template. *M. rossiae.* = *M. rossiaemeridionalis*; M1 = 1 kb ladder, M2 = 100 bp ladder (with 600 bp band indicated); - = negative (no template) control of amplification.

*Bgl*III endonuclease (Promega). To ensure complete digestion of the genomic DNA we used an excess of the restriction enzyme and long incubation times. Additionally, sets of filters with digested genomic DNA of different individuals were prepared and hybridized, producing patterns identical to those in Fig. 1. Products identified as diagnostic based on hybridization (either presence of hybridization in the case of tester DNA and absence in the case of driver or different patterns of hybridization between tester and driver; Fig. 1) were sequenced using the dRhodamine Terminator Ready kit (Perkin-Elmer) and an ABI 310 autosequencer. Primers to individual difference products were designed using the Oligo 4.05 program and tested on the genomic DNA of previously karyotyped animals.

Results. We performed two reciprocal RDA experiments using DNA from *M. arvalis* as the tester and DNA from *M. rossiaemeridionalis* as the driver in experiment A (Fig. 1A) and reciprocal experiment B (Fig. 1B).

In experiment A RDA using DNA from *M. arvalis* as the tester yielded a single, most abundant product. It was cloned (clone *Mar14*) and used as a probe in a Southern hybridization experiment with digested genomic DNA from *M. arvalis* and *M. rossiaemeridionalis*. The hybridization pattern (Fig. 1A) was different for tester and driver DNA, suggesting that we isolated a genomic difference. Based on the hybridization pattern it is possible to conclude that *Mar14* is a so-called polymorphic amplifiable restriction fragment (PARF, see Lisitsyn *et al.* 1993), that is, a sequence that is present

in both tester and driver genomes but differently flanked by restriction sites. However, as shown below, *Mar14*-specific primers generate amplification products only when *M. arvalis* DNA is used as the template, suggesting that there is only a partial homology between *Mar14* sequence and the hybridizing region of the *M. rossiaemeridionalis* genome. The nucleotide sequence of *Mar14* was determined (Accession no. AF093582) and compared to the ENTREZ database ([HTTP://www.ncbi.nlm.nih.gov/Entrez](http://www.ncbi.nlm.nih.gov/Entrez)). No matching entries were found. Primers complementary to the sequence of *Mar14* were then designed and tested on genomic DNA isolated from individuals of *M. arvalis* ($N = 10$) and *M. rossiaemeridionalis* ($N = 10$) that were collected in different locations and identified by karyotyping. As shown in Fig. 1A, these primers (*Mar14F* and *Mar14R*; Table 1) produced amplification products only with genomic DNA from *M. arvalis*; therefore, within this experimental design they are diagnostic for this species.

Three difference products were obtained in experiment B. All three displayed different patterns of hybridization to the tester and driver DNAs. One of these products, designated *Mro16*, was analysed in detail (Southern hybridization of labelled *Mro16* to the tester and driver DNA is shown on Fig. 1B). Based on the hybridization pattern (presence of multiple bands) it is possible to conclude that *Mro16* represents a repetitive element unique to *M. rossiaemeridionalis*. *Mro16* was sequenced and its sequence (Accession no. AF093583) was compared to the ENTREZ database. This analysis revealed a highly similar sequence representing a *M. rossiaemeridionalis* B1-like element containing repeat (Accession

no. U36930; Mayorov *et al.* 1996). Primers complementary to the sequence of *Mro16* (*Mro16F* and *Mro16R*; Table 1) were designed and tested as described above. Subsequent amplification generated products only from the genomic DNA of *M. rossiaemeridionalis* and, within our sample, primers *Mro16F* and *Mro16R* were diagnostic for *M. rossiaemeridionalis*.

Our goal was to employ RDA for the isolation of DNA fragments unique to each of two cryptic species of voles (*M. arvalis* and *M. rossiaemeridionalis*). It allowed us to design PCR primers that generate a diagnostic amplification product unique to each species.

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DNA extraction from urine and sex identification of birds

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Sex of sexually monomorphic birds can only be determined during the breeding season by the confirmation of the position in copulation or by checking of sexual hormonal

levels. However, these sexing methods are not applicable to nestlings. A simple and rapid method of sexing birds was established by Griffiths *et al.* (1996). This method is based on the difference in the nucleotide sequence between CHD (chromodomain-helicase-DNA-binding protein) genes on W-linked and non-W-linked chromosomes. Griffiths *et al.* (1996) used blood as a source of DNA but such blood collections require the capture of birds. In some mammals, DNA extraction from urine or faeces for PCR has been successfully performed (Constable *et al.* 1995). Our report describes DNA extraction for sex determination from bird excrements, especially white urine parts, without capture.

Samples of excreta were obtained from 13 male and 12 female captive White Leghorn chickens. Excrement from most birds, except water birds, consists of white part and brown part, which are solid and separated from each other. The white parts (urine) may come from the urinary tract. Brown parts (faeces) come from the digestive tract. Urine was fixed with 70% ethanol. Faeces was wiped out with a cotton swab soaked in 0.9% NaCl, flushed into the solution several times and fixed in 70% ethanol. DNA was extracted from blood by a conventional method using sodium dodecylsulphate (SDS) and proteinase K. Hydrolysates were extracted with phenol and chloroform–isoamylalcohol (CIAA) and dialysed against TE (10 mM Tris buffer and 1 mM EDTA, pH 8.0). Fixed cells from excrement were collected by centrifugation at 9000 g for 5 min, and washed twice with 1 mL of STE (0.1 M NaCl, 10 mM Tris and 1 mM EDTA, pH 8.0). Sediments were added with 250 µL of STE, 25 µL of 10% SDS and digested with proteinase K (20 µL of 5 mg/mL) at 55 °C for 2 h. The hydrolysates were added with 30 µL of 5 M NaCl and extracted with 200 µL each of CIAA and phenol for 2 h. DNA was precipitated using a DNA precipitation kit (Nippon Gene, Ethachin Mate) and the precipitates were dissolved with 50 µL of TE.

A part of the CHD gene was amplified using the primer set of Griffiths *et al.* (1996). A reaction mixture of 25 µL contained 0.625 units of *Taq* DNA polymerase Gold (Applied Biosystems), 15 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl₂ and 400 µM dNTP and 10% dimethylsulphoxide. The PCR cycles were as follows: 6 min (preheating) at 94 °C, 32 cycles of 1 min at 94 °C, 2 min at 53 °C, 2 min at 72 °C. Two µL of PCR product was cut with 1 unit of *Hae*III for 4 h and analysed by 5% polyacrylamide gel electrophoresis using a TBE (Tris–Borate–EDTA) buffer of pH 8.2. The band patterns were visualized by silver staining (Tegelström 1986).

DNA from blood samples of male and female chickens gave a clear PCR product of 110 bp. *Hae*III cutting of the product from female chickens gave three bands: 45 bp, 65 bp and the original 110 bp as reported by Griffiths *et al.* (1996) (lanes 4–6 in Fig. 1). In the case of male chickens, however, there were two genetic types: one with all the 110 bp band cut into two fragments, 45 bp and 65 bp (seven males); and in the other six males, the original 110 bp remained in addition to 45 bp and 65 bp as in female chickens (six males) (Fig. 2). All 10 jungle fowls, however, including five males and five females, gave the same results reported by Griffiths *et al.* (1996). As White Leghorns have been under strong artificial selection pressure, a mutated CHD gene without the *Hae*III site may be maintained in these domestic chickens.