JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

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Case-Study Investigation of Equine Maternity via PCR-RFLP: A Biochemistry Laboratory Experiment

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Supporting Information

ABSTRACT: A simple and robust biochemistry laboratory experiment is described that uses restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) products to verify the identity of a potentially valuable horse. During the first laboratory period, students purify DNA from equine samples and amplify two loci of mitochondrial DNA. During the second laboratory period, students digest PCR products with restriction enzymes and analyze the fragment sizes through agarose gel electrophoresis. An optional step of validating DNA extracts through real-time PCR can expand the experiment to three weeks. This experiment, which has an engaging and versatile scenario, provides students with exposure to key principles and techniques of molecular biology, bioinformatics, and evolution in a forensic context.

KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Collaborative/Cooperative Learning, Hands-On Learning/Manipulatives, Bioanalytical Chemistry, Forensic Chemistry, Nucleic Acids/DNA/RNA*

The case-study approach to biochemistry has been growing in popularity as a way to improve student engagement with course material.[1,2] Recently, our entire biochemistry laboratory curriculum was changed so that students use biochemical techniques to solve real-world problems. This paper describes one such experiment, in which mitochondrial DNA (mtDNA) is used to investigate the maternity of a potentially valuable colt before purchase. Lineage determination has widespread applications, not only among breeders and purebred aficionados, but also in such other diverse fields as forensics,[3] the food industry,[4] and the preservation of endangered species.[5]

Mitochondrial DNA analysis has several advantages for DNA profiling of animals.[4] Its high copy number makes amplification relatively easy, even in the hands of novices, and analysis can be performed noninvasively on samples such as hair shafts, urine, and feces. Finally, because of maternal inheritance and a high mutation rate, mother−child pairs normally have identical mtDNA sequences that are distinct from those of unrelated individuals. Potential forensic applications of equine mtDNA analysis include investigations of doping in the racing industry and theft of valuable horses,[6] as well as verifying stud-book records for maternal lineages.[7]

In this exercise, analysis of mtDNA is used to authenticate a colt said to have an impressive pedigree. Substitution of horses can be a lucrative, although illegal, practice. Indeed, the term "ringer" has its origins in horse racing, referring to the substitution of a fast horse for a slower one of similar appearance in order to benefit from longer odds. An example is the Fine Cotton Affair of 1984, when a superior horse disguised as a competitor at 33−1 odds won an Australian racing event.[8] This substitution would have been worth over a million dollars had it succeeded, but the white paint dripping from the horse's feet alerted officials to the scam.

Typically, analysis of mtDNA necessitates expensive and time-consuming sequencing protocols to distinguish between samples. This exercise was designed to take advantage of a simple, indirect method to detect single-nucleotide sequence differences: the polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP). PCR-RFLP combines the original method of DNA profiling, RFLP analysis,[9] with amplification via PCR.[10] Its advantages include increased sensitivity, elimination of the need for radioactive labeling, and the ability to resolve products that do not vary in size, only sequence. PCR-RFLP can be used to distinguish between populations within a species,[11] to classify individuals of similar species,[12−14] and to perform pedigree analysis.[15,16] Previously published undergraduate experiments have been better suited to biologists than chemists, using PCR-RFLP to monitor genetic variation among mushroom species[17] and to identify common flies.[18]

In this experiment, equine DNA is purified from hair or hoof, amplified via PCR at two loci within the mitochondrial genome, and then cleaved with select restriction enzymes. Because of point mutations at key restriction sites, fragments from unrelated individuals can differ in length, as monitored by agarose gel electrophoresis. A comparison of the cleavage patterns of the mother and colt in question can disprove relatedness if the band sizes differ and provide reasonable assurance of maternity if they are the same. With mtDNA

Published: October 18, 2013

analysis techniques becoming increasingly useful in the real world (often in a forensic setting[3,19]), this experiment provides an engaging context for introducing basic molecular biology techniques to undergraduate biochemistry students.

MATERIALS AND METHODS

This experiment was designed for an upper-division undergraduate biochemistry course and requires two laboratory periods to complete. An optional step of using real-time PCR to validate DNA extracts can expand the experiment to three weeks (Table 1) as described in the Supporting Information.

Table 1. Timeline for the Individual Steps of this Experiment

Two-Week Experiment		Three-Week Experiment	
Procedure	Time Required/h	Procedure	Time Required/h
DNA extraction, week 1	2	DNA extraction, week 1	2
PCR amplification setup, week 1	0.5[a]	Real-time PCR setup, week 1	0.5[c]
Restriction digest, week 2	1	Real-time PCR data analysis, week 2	1
Agarose gel preparation, week 2	1[b]	PCR amplification setup, week 2	0.5
Running agarose gel, week 2	1	Restriction digest, week 3	1
Imaging agarose gel, week 2	0.5	Agarose gel preparation, week 3	1[b]
		Running agarose gel, week 3	1
		Imaging agarose gel, week 3	0.5

[a]Thermocycling takes about 3 h, but the thermocycler can be programmed to run overnight and hold the samples at 4 °C. [b]Agarose gel is poured during the restriction digest incubation so these times actually overlap. [c]Including real-time PCR expands the experiment from two weeks to three. Thermocycling takes about 3 h, but students do not have to be present.

Necessary equipment includes a thermal cycler, agarose gel boxes, power supplies, and a transilluminator. Detailed procedures are given in the Supporting Information.

The first laboratory period requires about 2.5 h to isolate template DNA and set up the PCR reactions. Working in pairs, students purify DNA from the colt and the alleged mother using a commercially available kit (DNA IQ System from Promega) that is reliable and robust in the hands of novices. (The QIAamp DNA Stool Kit from Qiagen can be used for feces, if desired.) Following purification, samples are concentrated with Amicon centrifugal filtration units (Millipore), which improves the success of PCR. Without this step, DNA from some horses did not amplify well, either because it was too dilute or because of copurified inhibitors.

Following extraction of DNA from the alleged mother−son pair, students set up PCR reactions with these samples and an unrelated control (commercially available horse DNA) for comparison. PCR is performed to amplify two loci within the hypervariable D-loop of the mitochondrial control region (Table 2).[16,20]

The second laboratory period, which takes about 3 h, involves digesting the PCR products with various restriction enzymes and analyzing the fragments using agarose gel electrophoresis. Following visualization of products, a standard curve is generated from a molecular weight ladder to determine the sizes of all restriction fragments.

HAZARDS

Horses are unpredictable and can be dangerous. Only experienced equestrians should obtain samples for this experiment. Gloves, lab coats, and goggles should be worn at all times, both in the laboratory and when handling equine samples. Detailed information about hazardous reagents is provided in the Supporting Information. Avoid contact with and inhalation of all reagents. It is recommended that pregnant women do not use the DNA IQ kit. Other potentials hazards include UV light and the possibility of electrical shock during electrophoresis if the apparatus is not used correctly. Eye protection opaque to UV light should be used with transilluminators that do not have built-in safety shields.

RESULTS

PCR at two different loci produced a 397-bp[20] and a 232-bp[16] amplicon, with the former showing more variability between unrelated individuals (Figure 1). This PCR product was

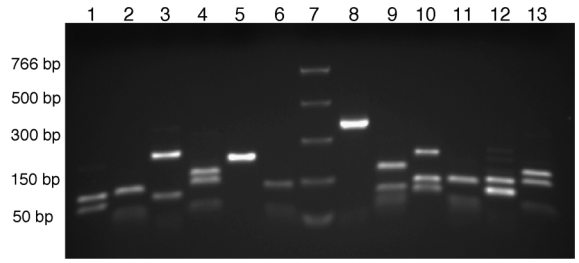

Figure 1. Representative student-generated cleavage patterns of equine PCR products **A** (232 bp) and **B** (397 bp). Lanes 1−4 are an Arabian horse; lanes 5, 6, 8, 9 are a miniature horse; lanes 10−13 are a commercial standard horse. Lane 1, **A**, AciI digest; lane 2, **A**, MseI digest; lane 3, **B**, AciI digest; lane 4, **B**, MluCI digest; lane 5, **A**, AciI digest; lane 6, **A**, MseI digest; lane 7, size markers: lane 8, **B**, AciI digest; lane 9, **B**, MluCI digest; lane 10, **A**, AciI digest; lane 11, **A**, MseI; lane 12, **B**, AciI; lane 13, **B**, MluCI.

designed for lineage determination through sequencing rather than via RFLP analysis, but two restriction enzymes were found that readily provided discriminating power between our subjects. Students were easily able to observe molecular differences between the samples that allowed them to conclude that the horse in question was an imposter.

ASSESSMENT

Student feedback for this experiment was very favorable, noting its many positive aspects, such as the real-world relevance that

Table 2. Sequences of PCR Primers Used in this Experiment

PCR Product	Forward Primer	Reverse Primer
232-bp D-loop fragment[16]	5′-AGGACTATCAAGGAAGAAGCTCTA	5′-GTACATGCTTATTATTCATGGGGCA
397-bp D-loop fragment[20]	5′-AACGTTTCCTCCCAAGGACT	5′-GTAGTTGGGAGGGTTGCTGA

dx.doi.org/10.1021/ed300740r | J. Chem. Educ. 2013, 90, 1518−1521

gave a purpose to the work, the opportunity to purify DNA themselves from crude samples, and the forensic flavor of the scenario. Pre- and postassessment revealed self-reported learning in several key areas (Table 3), with students also agreeing that they learned more from laboratory experiments that have real-life applications (4.4 out of a possible 5). Negative comments included the waiting time, which is a common problem in biochemistry, and that some students did not like working with feces.

Table 3. Students' Self-Reported Knowledge in Several Key Areas before and after this Experiment

Area	Before[a]	After[a]
Understanding of the RFLP technique	2.1	4.1
Familiarity with the types of sample that yield forensic DNA	2.6	4.4
Understanding of DNA purification	3.1	4.3
Understanding of the differences between mitochondrial and nuclear DNA	3.5	4.6
Familiarity with mutations used to characterize DNA samples (e.g., STRs and SNPs)	2.3	3.9

[a]Responses were on a 5-point scale, where 5 is the highest.

DISCUSSION

In this exercise, students used the relatively simple method of PCR-RFLP for analysis of equine samples to solve an engaging problem, writing a two-page journal-style report in which they were expected to put their experiment into a general forensic context. They were instructed to obtain reference horse sequence information from PubMed (GenBank X79547) to examine putative restriction sites, which is helpful with assignment of restriction fragment sizes. (Mutations in the form of single-nucleotide polymorphisms relative to the reference sequence can either add or remove restriction sites.) An interesting complication is that horse mtDNA can be heteroplasmic,[21] which can be difficult to distinguish from incomplete digestion. However, even without examining genomic information, students can readily compare banding patterns to determine whether the two subjects match at all loci. Although this is not proof of relatedness, if they do not match, a mother−child relationship can be excluded. Comparison to the unrelated control allows assessment of the uniqueness of a particular cleavage pattern, which provides support for relatedness if the RFLP patterns do match.

The DNA source is flexible and includes hair and hoof, both of which are collected less invasively than liver[16] or blood samples,[15] and also fit well with this scenario. Because successful DNA extraction is essential for effective PCR amplification, a simple, but effective, kit is recommended in lieu of traditional methods that require exposure to potentially hazardous reagents (such as phenol−chloroform) or do not fit well into the time frame of an undergraduate laboratory. Kits are now widespread in the field, and the only failures our students have had with extraction from hair and hoof samples were the clear result of operator error (such as a tube breaking during an unbalanced centrifugation). For those who wish to avoid the expense of kits, alternative methods for extraction from hair[22,23] are given in the Supporting Information.

Experience with genetic analysis techniques, including DNA purification and PCR, is expected of modern biochemistry graduates. This straightforward procedure for DNA profiling of samples from horses (*Equus caballus*) illustrates several essential tools for genetic analysis, including DNA purification, PCR, restriction enzyme digestion, and electrophoresis. Protocols were optimized for reliability and minimal use of hazardous materials. The context can be very versatile, with scenarios ranging from verification of pedigree[20,16] to crimes involving horses.[6,24] Equine fraud can be lucrative, with its perpetrators sometimes going to great lengths to deceive, such as substituting their own urine during equine drug testing.[25] Because some scenarios could benefit from sex determination of samples, protocols were also explored for amplifying regions of the X and Y chromosomes. Good success was obtained with a 429-bp amplicon of the *SRY* gene (see Supporting Information), which would confirm that a sample came from a male.[26] Although our scenario involved comparison of only two subjects, plus a purchased unrelated control, instructors could include more horses, if desired. Having replicates in the same laboratory section is advisable to maximize chances of success.

Learning goals specific to this experiment included gaining familiarity with the differences between mitochondrial and nuclear DNA, manipulating biological samples in their original state, predicting relatedness through interpretation of agarose gel electrophoresis data, and the types of mutations that lead to RFLP. More general learning goals, such as following standard protocols for DNA purification, PCR, DNA digestion, and gel electrophoresis, were also met. Unique features of this experiment, in comparison to those previously published involving PCR-RFLP,[17,18] include its appealing forensic slant, its use of a novel and versatile case study, and its ability to be performed on a variety of samples that might otherwise be viewed as trash. There are also some recent exercises[19,27] that rely on size differences of target DNA via the PCR-STR method,[28] but these are less relevant for lineage determination problems, such as this one, and require higher-resolution electrophoresis to perform. Additionally, the use of horse samples rather than human ones minimizes amplification of contaminating DNA, reduces the risk of human pathogens, and avoids ethical concerns that arise when students test their own DNA.[29]

ASSOCIATED CONTENT

Supporting Information

Instructor notes and a student handout including background information, potential hazards, and instructions. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the BI/CH 368 students at Colby College for testing this experiment (especially Maxwell Hogue for the gel image), Brenda Fekete and Adam Spierer for expert technical assistance, Ethan Kohn and Paul Greenwood for helpful contributions, and the Hart to Hart Farm for biological samples. We are grateful for financial support from the William D. Adams Presidential Scholars Program at Colby College. This project was supported by grants from the National Center for Research Resources

(5P20RR016463-12) and the National Institute of General Medical Sciences (8 P20 GM103423-12) from the National Institutes of Health.

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