

季节和天气因素对麋鹿非损伤性遗传取样的影响

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[摘要] 非损伤性遗传取样在濒危动物遗传多样性的研究中具有重要作用. 在运用非损伤性遗传取样对麋鹿 (*Elaphurus davidianus*) 进行研究时发现, 冬季采集粪样提取的核酸质量优于夏季. 利用 QIAamp DNA Stool Mini Kit 试剂盒, 冬季样品获得的 DNA OD 260/280 在 1.60 ~ 1.90 之间的比例为 61.57%, 而夏季的仅为 43.51%. 基于 PCR 扩增的性别鉴定和微卫星分型表明, 冬季样品的成功率分别为 84.98% 和 96.25%, 夏季的分别为 93.13% 和 95.25%. 天气因素会对样品质量造成极显著的影响, 非雨雪天气采集样品的 OD 260/280 在 1.60 ~ 1.90 之间的比例为 61.57%, 大雪后的为 23.68%, 大雨后的为 23.88%. 雨雪可能会冲刷掉部分粪样表面的肠壁组织、粘膜或者血液, 并且降解其中的 DNA. 对麋鹿的非损伤性遗传取样建议选择晴朗的冬天进行.

[关键词] 非损伤性遗传取样, 麋鹿, OD 260/280, DNA 质量

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The Season and Weather Impacts on Noninvasive Genetic Sampling of the David's Deer (*Elaphurus davidianus*)

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Abstract: In recent years, noninvasive genetic sampling plays an important role in genetic researches of many endangered animals. When we used this method to analyze the genetic diversity of David's Deer, *Elaphurus davidianus*, one of the world endangered species, we found the DNA quality from fecal samples which collected in winter was better than that in summer. The proportion of OD 260/280 ratio which between 1.60 and 1.90 was about 61.57% for the winter samples, and about 43.51% for the summer samples, according to the standard procedure of the QIAamp DNA Stool Mini Kit. The success rate of PCR amplification for gender identification and microsatellite genotyping was 84.98% and 96.25% for winter samples, and 93.13% and 95.25% for summer samples respectively. There was no significant difference between the different seasons. The weather such as rain and snow influenced the DNA quality significantly. The proportion of OD 260/280 ratio which between 1.60 and 1.90 was about 61.57% for the sunny winter samples, about 23.68% and 23.88% for the samples after raining and snowing respectively. The rain would wash out the intestinal tissue, mucosa or blood on the surface of fecal samples, and made the degradation of the DNA. The results suggest that the noninvasive genetic sampling for David's Deer should be done in sunny winter, as it can improve the DNA quality effectively.

Key words: noninvasive genetic sampling, *Elaphurus davidianus*, OD 260/280, DNA quality

非损伤性遗传取样即在不打扰动物的情况下, 从动物的毛发^[1]、粪便^[2]、尿液^[3]等中提取 DNA, 进行遗传学或者保护生态学的相关研究. 该方法因不伤害动物即可获取分析所需的 DNA 样本, 简便易行, 因而

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在动物保护遗传学研究中已广泛应用于动物的个体识别^[4]、种群大小测定^[5]、基因流检测^[6,7]、性别比分析^[8]、种群丰富度趋势分析^[9]、物种鉴定^[10]以及种群历史动态分析^[11]等研究。因此,如何通过非损伤性遗传取样获得高质量的DNA,特别是通过动物粪便来获取研究用的DNA越来越受到研究者的关注^[12]。近年来,围绕样品保存^[13-16]、样品的类型^[17]、DNA提取^[16,18,19]与扩增方法^[19,20]、样品的新鲜度^[21-23]以及动物的食物类型对样品的影响^[24,25]等均开展了深入的探讨,许多研究表明影响实验成功率的最主要因素是样品的新鲜程度,新鲜样品的扩增成功率明显高于久置的样品。

麋鹿(*Elaphurus davidianus*),又名“四不像”,属于国家一级保护动物,世界濒危物种。麋鹿在我国为重要引入物种,经过瓶颈效应和奠基者效应,麋鹿的遗传多样性极低,目前仍处于极度濒危状态^[26]。在江苏大丰麋鹿国家级自然保护区,经过多次的野放,目前麋鹿野生种群数量已达215头^[27],由于难以获得野生麋鹿个体的组织、血液及毛发样本等,因此对现生野生种群的遗传多样性状况知之甚少。本研究拟通过对不同季节粪便样品获得DNA的纯度与浓度比较,以及对性别鉴定和微卫星扩增成功率的分析,探讨季节(冬季与夏季)和天气(晴天与雨、雪天气)对非损伤性遗传取样的影响,以期对麋鹿及其他有蹄类动物的遗传多样性研究提供参考。

1 材料和方法

1.1 样品采集

研究用粪便样采集于野放麋鹿种群的栖息地,位于江苏大丰麋鹿国家级自然保护区第三核心区。其中,2012年1月份采集141个粪样(晴天),2月份采集38个粪样(小雪),6月份采集131个粪样(晴天),12月份采集134个粪样(阴雨)。采集的球状新鲜粪样置于灭菌过的50 mL离心管中,-20℃保存,并在采集后1周内完成DNA的提取工作。

1.2 DNA提取

每个样品先用干净灭菌的牙签剥取等量的粪样表层,利用QIAGEN公司提取粪样DNA试剂盒(QIAamp DNA Stool Mini Kit)进行DNA的提取。获得的DNA母液(100 μL)用Nano Drop 1000分光光度计(ND-1000 V 3.5.2)测定其OD_{260/280}比值以及DNA浓度,DNA样置-20℃保存备用。

1.3 性别鉴定和微卫星扩增

从鹿科动物已知的19对性别鉴定引物^[28-34]中,筛选出引物对CerZFXyf/CerZFXYr(Accession No. EF077165)进行性别鉴定。电泳检测PCR扩增产物,其中具有300 bp和500 bp两条带的为雄性个体,仅出现300 bp一条带的为雌性个体。每个样品重复2遍,并做阴性对照。

在麋鹿及其他鹿科动物利用过的54个微卫星位点^[35-39]中,成功筛选出20个位点,进一步检测有9个位点具有多态性,分别是:URBO21B(60℃,140 bp),T172(58℃,250 bp),Elda-16(55℃,144 bp),BM1225(58℃,230 bp),Rt5(56℃,100 bp),F65(54℃,199 bp),P2G6(58℃,180 bp),BM4107(57℃,150 bp),FCB48(60℃,150 bp)。引物经荧光标记后运用多管法进行扩增^[40,41]。

1.4 数据分析

利用OD_{260/280}来测定样品DNA纯度,统计样品中OD_{260/280}在1.60~1.90之间的比例。样品浓度数据先用Kolmogorov-Smirnov和Shapiro-Wilk(Statistica统计软件包)检验其正态性和方差同质性。经检验,数据经各种转化后都不符合参数统计条件,故采用非参数统计Kruskal-Wallis检验(H-检验)进行不同季节及气候条件下浓度差异分析。

通过性别鉴定以及微卫星扩增的成功率来分析季节及天气因素对DNA质量的影响。有研究表明,非损伤性遗传取样进行微卫星多态分析时,应尽量选取产物小于200 bp的引物,以提高扩增成功率^[42]。因此,研究中将小于200 bp和大于200 bp的分开进行统计分析。

2 结果

2.1 不同季节样品DNA质量的差异

2012年1月和6月份的粪便样均为晴天采集,其中冬季(1月份)样品中OD_{260/280}在1.60~1.90之间的比例为61.57%,夏季的为43.51%。H-检验表明,冬季样品与夏季样品DNA浓度差异显著($P=0.016$),冬

季样品浓度高于夏季样品.可见,从获得 DNA 的纯度和浓度来看,冬季采集的样品质量明显比夏季采集的样品好.从 PCR 扩增成功率来看,在性别鉴定上夏季的成功率高于冬季;在微卫星扩增上,小片段比大片段扩增具有更高的成功率,但 9 个位点扩增的成功率在冬季和夏季间差异不显著 ($P=0.65$) (表 1).

2.2 气候对样品 DNA 质量的影响

在本研究中,冬季采集的样品有晴天、阴雨及小雪 3 种天气状况.结果表明 OD 260/280 在 1.60 ~ 1.90 之间的样品所占比例:晴天采集的为 61.57%,雪天的为 23.68%,阴雨天的为 23.88%.从获取 DNA 的浓度来看,H-检验表明晴天和小雪天气获得的样品间无显著差异,但阴雨天采集的样品和其他之间均有极显著的差异(表 2).

从 PCR 扩增的成功率来看,性别鉴定和微卫星扩增上都显示晴天采集的样品的质量显著好于雨天和雪天后采集的样品(表 3).

表 2 冬季不同天气采样对样本 DNA 浓度的影响

Table 2 Effects of sampling in different weather on concentration of the extracted DNA from samples

天气状况	晴天	小雪	阴雨
中位数 R	180.63 ^a	168.95 ^a	128.75 ^b

Multiple Comparisons p values(2-tailed), Kruskal-Wallis test:
 $H(2, N=313)=23.330\ 47, P<0.001$.

表 1 冬季和夏季晴天采样性别鉴定和微卫星成功率

Table 1 The success rate of gender identification and microsatellite genotyping between winter and summer samples

	样本量	性别鉴定成功率	微卫星成功率		
			<200 bp	>200 bp	9 个位点
冬季	141	84.98%	98.38%	88.80%	96.25%
夏季	131	93.13%	96.84%	89.69%	95.25%

表 3 冬季不同天气下采集样品的性别鉴定和微卫星成功率比较

Table 3 The success rate of gender identification and microsatellite under different weather in winter

	样本量	性别鉴定成功率	微卫星成功率	
			<200 bp	>200 bp
晴天	141	84.98%	98.38%	88.80%
小雪	38	65.79%	79.70%	56.58%
阴雨	134	58.21%	83.69%	57.46%

3 讨论

3.1 样品保存与 DNA 提取方法

非损伤性遗传取样采集的样品可以用冷冻法、酒精浸泡法或硅胶干燥法进行保存^[13-16],新鲜的样品获得的 DNA 其 PCR 扩增成功率高并且基因分型错误率低^[21-23]. Nsubuga 等研究影响猿类粪样 DNA 质量的因素时,认为使用酒精-硅胶两步法可以显著提高扩增成功率^[43].

目前普遍认为利用 QIAamp DNA Stool Mini Kit 是提取粪样 DNA 的最佳方法^[42].该试剂盒应用硅胶膜技术从新鲜或冷冻的动物粪便、或其他含有高浓度 PCR 抑制剂的样品中纯化得到基因组 DNA,独特的吸附树脂配合经优化的缓冲液可去除样品中的 PCR 抑制剂.本研究中,使用该试剂盒提取 DNA 时,尽可能剥取麋鹿粪球的表层,减少粪球内部食物残渣的混入,以免目标 DNA 中带入太多植物或者肠道微生物的 DNA;同时,通过延长消化时间,增加过柱次数,有效地提高了 DNA 的质量和浓度.结果表明,获得的 DNA 能够满足后续的遗传多样性分析.

3.2 季节和天气对麋鹿粪样 DNA 质量的影响

许多研究表明,影响样本 DNA 质量最关键的因素是样本的新鲜程度^[21-23]. Lucchini 等通过采集狼的粪样进行阿尔卑斯山脉狼群种群大小监测时发现,夏天采集样品的 PCR 扩增成功率低于冬天的,新鲜粪样的成功率高于放置时间较长的^[23].研究中对线粒体基因、核基因和性别鉴定成功率进行了比较,其中线粒体和核基因成功率是冬季采集样品显著高于夏季,但是性别鉴定则没有明显差异.研究认为冬季雪地里采集的粪样,能够提取更高质量的 DNA^[23]. Piggott 通过对澳大利亚东南部袋鼠粪样的研究,发现夏季采集样品的 PCR 扩增成功率高于冬季采集的样品,夏季样品的基因分型错误率也低于冬季的,分析认为澳大利亚东南部冬天雨水较多,对样品的保存有很大的影响^[21].

本研究采集的均为新鲜样品,获得后立即置-20℃冷冻保存并且尽快完成 DNA 提取工作.结果表明,冬季样品中 OD 260/280 在 1.60 ~ 1.90 之间的比例为 61.57%,夏季的为 43.51%;DNA 浓度数据也显示冬季采集的样品优于夏季样品,且差异显著(H-检验, $P=0.016$).可见,麋鹿野生种群的非损伤性取样在冬季采集样品的质量是优于夏季的.从 PCR 扩增成功率来看,微卫星扩增成功率差异不大,但性别鉴定成

功率夏季样品高于冬季样品,这还有待我们进一步研究。

本研究样品均取自江苏大丰麋鹿国家级自然保护区。保护区位于北亚热带向暖温带过渡的黄海滩涂湿地,冬季温暖雨水较少,1月份平均气温为 -0.8°C ,最低温度为 -12°C ;7月份平均气温为 26.8°C ,最高温度为 31°C ;6~8月降雨量为675mm,占全年降雨量的68%^[44]。从冬季获得的样品来看,晴天采集的样品的性别鉴定成功率显著高于小雪天和阴雨天采集的样品;微卫星扩增成功率也一样,且大于200 bp片段受到的影响更大。由于该地区冬天雪后极易融化,融化以后的雪水和雨水会冲刷掉部分粪样表面的肠壁组织、粘膜或者血液,并降解其中的DNA。可见,天气状况对粪样DNA的质量影响较大。

研究表明,利用粪便进行麋鹿非损伤性遗传取样时可选择在晴朗的冬季进行。在微卫星变异分析时,选择目的片段比较小的(<200 bp)位点具有较高的扩增成功率。

[参考文献]

- [1] Walker F M, Sunnucks P, Taylor A C. Evidence for habitat fragmentation altering within-population processes in wombats[J]. *Molecular Ecology*, 2008, 17(7): 1 674–1 684.
- [2] Regnaut S, Lucas F S, Fumagalli L. DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations[J]. *Conservation Genetics*, 2006, 7(3): 449–453.
- [3] Sastre N, Francino O, Lampreave G, et al. Sex identification of wolf (*Canis lupus*) using non-invasive samples[J]. *Conservation Genetics*, 2009, 10(3): 555–558.
- [4] Sloane M A, Sunnucks P, Alpers D, et al. Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censusing method[J]. *Molecular Ecology*, 2000, 9(9): 1 233–1 240.
- [5] Solberg K H, Bellemain E, Drageset O M, et al. An evaluation of field and non-invasive genetic methods to estimate brown bear (*Ursus arctos*) population size[J]. *Biological Conservation*, 2006, 128(2): 158–168.
- [6] Zhu L F, Zhan X J, Meng T, et al. Landscape features influence gene flow as measured by cost-distance and genetic analyses: a case study for giant pandas in the Daxiangling and Xiaoxiangling Mountains[J]. *BMC Genetics*, 2010, 11(1): 72–83.
- [7] Zhu L, Zhang S, Gu X, et al. Significant genetic boundaries and spatial dynamics of giant pandas occupying fragmented habitat across southwest China[J]. *Molecular Ecology*, 2011, 20(6): 1 122–1 132.
- [8] Baumgardt J A, Goldberg C S, Reese K P, et al. A method for estimating population sex ratio for sage-grouse using noninvasive genetic samples[J]. *Molecular Ecology Resources*, 2013, 13(3): 393–402.
- [9] Sawaya M A, Stetz J B, Clevenger A P, et al. Estimating grizzly and black bear population abundance and trend in Banff National Park using noninvasive genetic sampling[J]. *Plos One*, 2012, 7(5): e34777.
- [10] Barbosa S, Pauperio J, Searle J B, et al. Genetic identification of Iberian rodent species using both mitochondrial and nuclear loci: application to noninvasive sampling[J]. *Molecular Ecology Resources*, 2013, 13(1): 43–56.
- [11] Dutta T, Sharma S, Maldonado J E, et al. Gene flow and demographic history of leopards (*Panthera pardus*) in the central Indian highlands[J]. *Evolutionary Applications*, 2013, 6(6): 949–959.
- [12] Beja-Pereira A, Oliveira R, Alves P C, et al. Advancing ecological understandings through technological transformations in non-invasive genetics[J]. *Molecular Ecology Resources*, 2009, 9(5): 1 279–1 301.
- [13] Wasser S K, Houston C S, Koehler G M, et al. Techniques for application of faecal DNA methods to field studies of Ursids[J]. *Molecular Ecology*, 1997, 6(11): 1 091–1 097.
- [14] Murphy M A, Waits L P, Kendall K C. Quantitative evaluation of fecal drying methods for brown bear DNA analysis[J]. *Wildlife Society Bulletin*, 2000: 951–957.
- [15] Murphy M A, Waits L P, Kendall K C, et al. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples[J]. *Conservation Genetics*, 2002, 3(4): 435–440.
- [16] Piggott M P, Taylor A C. Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species[J]. *Australian Journal of Zoology*, 2003, 51(4): 341–355.
- [17] Broquet T, Ménard N, Petit E. Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates[J]. *Conservation Genetics*, 2007, 8(1): 249–260.
- [18] Flagstad Ø, Røed K, Stacy J E, et al. Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol[J]. *Molecular Ecology*, 1999, 8(5): 879–883.
- [19] Goossens B, Chikhi L, Utami S S, et al. A multi-samples, multi-extracts approach for microsatellite analysis of faecal samples

- in an arboreal ape[J]. *Conservation Genetics*,2000,1(2):157–162.
- [20] Bellemain E, Taberlet P. Improved noninvasive genotyping method: application to brown bear (*Ursus arctos*) faeces [J]. *Molecular Ecology Notes*,2004,4(3):519–522.
- [21] Piggott M P. Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA [J]. *Wildlife Research*,2005,31(5):485–493.
- [22] Fernando P, Pfrender M E, Encalada S E, et al. Mitochondrial DNA variation, phylogeography and population structure of the Asian elephant [J]. *Heredity*,2000,84(3):362–372.
- [23] Lucchini V, Fabbri E, Marucco F, et al. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps [J]. *Molecular Ecology*,2002,11(5):857–868.
- [24] Murphy M A, Waits L P, Kendall K C. The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*) [J]. *Molecular Ecology*,2003,12(8):2 261–2 265.
- [25] Maudet C, Luikart G, Dubray D, et al. Low genotyping error rates in wild ungulate faeces sampled in winter [J]. *Molecular Ecology Notes*,2004,4(4):772–775.
- [26] Zeng Y, Jiang Z, Li C. Genetic variability in relocated Père David's deer (*Elaphurus davidianus*) populations—Implications to reintroduction program [J]. *Conservation Genetics*,2007,8(5):1 051–1 059.
- [27] 丁玉华. 麋鹿生长三支角罕见案例的剖析 [J]. *野生动物*,2013,34(6):320–322.
- [28] 张亮, 邹方东, 陈三, 等. 林麝及马麝 SRY 基因片段克隆及其在系统进化分析中的应用 [J]. *动物学研究*,2004,25(4):334–340.
- [29] Pfeiffer I, Brenig B. X- and Y-chromosome specific variants of the amelogenin gene allow sex determination in sheep (*Ovis aries*) and European red deer (*Cervus elaphus*) [J]. *BMC Genetics*,2005,6(1):16.
- [30] Kim B J, Lee H, Lee S. Erratum to: Species- and sex-specific multiple PCR amplifications of partial cytochrome b gene and Zfx/Zfy introns from invasive and non-invasive samples of Korean ungulates [J]. *Genes and Genomics*,2010,32(1):103–104.
- [31] Kobolák J, Majzinger I, Szabari M, et al. Sexing of Roe Deer (*Capreolus capreolus* L.) by PCR amplification reaction [C]// 55th EAAP annual Meeting, Session G4. 50. Bled:EAAP,2004.
- [32] Lindsay A R, Belant J L. A simple and improved PCR-based technique for white-tailed deer (*Odocoileus virginianus*) sex identification [J]. *Conservation Genetics*,2008,9(2):443–447.
- [33] Brinkman T J, Hundertmark K J. Sex identification of northern ungulates using low quality and quantity DNA [J]. *Conservation Genetics*,2009,10(4):1 189–1 193.
- [34] Wilson P J. Sex identification of elk (*Cervus elaphus canadensis*), moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*) using the polymerase chain reaction [J]. *Journal of Forensic Sciences*,1998,43:477–482.
- [35] Bishop M D, Kappes S M, Keele J W, et al. A genetic linkage map for cattle [J]. *Genetics*,1994,136(2):619–639.
- [36] Jiao Y, Ge Y F, Fang S G. Eight novel microsatellite markers from the Père David's deer (*Elaphurus davidianus*) [J]. *Conservation Genetics*,2008,9(3):771–773.
- [37] Wu H L, Ni X W, Zhang L Y, et al. Eighteen novel polymorphic microsatellite loci developed from the Pere David's deer (*Elaphurus davidianus*) [J]. *Conservation Genetics*,2008,9(6):1 679–1 682.
- [38] 李文斌, 闻亮, 郑维平, 等. 微卫星标记分析 5 种鹿类动物群体遗传多样性 [J]. *野生动物*,2010,31(5):227–231.
- [39] 张林源, 吴海龙, 钟震宇, 等. 北京麋鹿苑麋鹿种群的微卫星多态性及遗传结构分析 [J]. *四川动物*,2010,29(5):505–508.
- [40] Taberlet P, Waits L P, Luikart G. Noninvasive genetic sampling: look before you leap [J]. *Trends in Ecology and Evolution*,1999,14(8):323–327.
- [41] Taberlet P, Griffin S, Goossens B, et al. Reliable genotyping of samples with very low DNA quantities using PCR [J]. *Nucleic Acids Research*,1996,24(16):3 189–3 194.
- [42] Renan S, Speyer E, Shahar N, et al. A factorial design experiment as a pilot study for noninvasive genetic sampling [J]. *Molecular Ecology Resources*,2012,12(6):1 040–1 047.
- [43] Nsubuga A M, Robbins M M, Roeder A D, et al. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method [J]. *Molecular Ecology*,2004,13(7):2 089–2 094.
- [44] 张国斌, 薛建辉, 吴永波. 半圈养状态下麋鹿对生境的影响 [J]. *中国农学通报*,2007,23(7):180–184.