Škola doktorských studií v biologických vědách
Jihočeská Univerzita v Českých Budějovicích
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VÝVOJ MIKROSATELITŮ U NEMODELOVÝCH DRUHŮ ŽIVOČICHŮ

Disertační práce

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Annotation:

This thesis is a compilation of six publications describing development of microsatellite markers for four mammal species and five insect species. Most of these species are endangered and the aim of developing microsatellites was their use in conservation-genetic studies. The thesis thus represents a contribution to methodological tools for investigation of endangered animals. Different approaches were used for the development of the loci, including inovative pyrosequencing, which represents a trend in DNA analyses with variety of practical aplications. Besides other things, this thesis documents increasing availability of the most modern molecular biology methods and their utilization in classical biological disciplines such as zoology, botany and ecology.

Prohlášení

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 3.8.2016 …….

Mgr. Jan Zima

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Poděkování

Děkuji školiteli, spoluautorům, zaměstnavatelům, kolegům, rodičům, sourozencům, manželce, dětem a kamarádům.

Seznam publikací, na nichž je disertace založena a podíl autora na jejich vzniku

1. Lučan R.K., Benda P., Reiter A., **Zima Jr. J.** 2011: Reliability of field determination in three cryptic whiskered bats (*Myotis alcathoe, M.mystacinus, M.brandtti*) and basic biometric characters: evidence from the Czech Republic. Vespertilio 15: 55-62 **(bez IF)**

JZ provedl veškeré molekulárně-genetické práce, včetně stanovení genotypů a následných populačně-genetických analýz; sepsal příslušné části metodiky a výsledků

2. Zemanová B., Hájková P., Bryja J., **Zima Jr. J.,** Hájková A., Zima J. 2011: Development of multiplex microsatellite sets for nonivasive population genetic study of the endangered Tatra chamois. Folia Zoologica 60: 70-80 **(IF 2011 = 0,56)**

JZ figuroval v projektu jako laboratorní technik (PCR, fragmentační analýza, stanovení genotypů)

3. **Zima Jr. J.**, Lestina D., Konvicka M. 2013: Characterization of ten polymorphic microsatellite markers for an endangered butterfly *Argynnis niobe* and their cross-species utility in the closely related species *A. adippe (Lepidoptera: Nymphalidae)*. European Journal of Entomology 110: 383-387 **(IF 2013 = 1,07)**

JZ provedl veškeré molekulárně-genetické práce, včetně stanovení genotypů a následných populačně-genetických analýz; napsal metodiku, výsledky a diskusi

4. Drag L., **Zima Jr. J.**, Cizek L. 2013: Characterization of nine polymorphic microsatellite loci for a threatened saproxylic beetle Rosalia alpina (Coleoptera: Cerambycidae). Conservation Genetics Resources 5: 907-909 **(IF 2013 = 0,95)**

JZ působil jako konzultant specialista – poskytl know-how vývoje mikrosatelitů de novo a připomínkoval rukopis článku

5. **Zima Jr. J.,** Lestina D., Jansta P., Petru V., Tropek R. 2014: Isolation and characterization of eight microsatellite markers for *Mylothris jacksoni knutsoni (Lepidoptera: Pieridae)*, an endemic butterfly of the Gulf of Guinea Highlands. Conservation Genetics Resources 6: 763-764 **(IF 2014 = 0,95)**

JZ provedl veškeré molekulárně-genetické práce, včetně stanovení genotypů a následných populačně-genetických analýz; napsal většinu textu článku

6. **Zima Jr. J.,** Lebrasseur O., Borovanská M., Janda M. 2016: Identification of microsatellite markers for a worldwide distributed, highly invasive ant species *Tapinoma melanocephalum* (Hymenoptera: Formicidae). European Journal of Entomology 113: 409-414 **(IF 2016 = ?, IF 2015 = 0,98)**

JZ provedl veškeré molekulárně-genetické práce, včetně stanovení genotypů a následných populačně-genetických analýz; napsal část úvodu, metodiku, výsledky a diskusi

OBSAH

Úvod

Mikrosatelity, někdy též označované jako krátké tandemové repetice (angl. short tandem repeats), jsou úseky genomu složené z několika, obvykle 5-50ti, opakujících se repetičních motivů, přičemž tyto repetiční motivy sestávají nejčastěji ze dvou, tří, či čtyř nukleotidů (např. CACACA, GTCGTCGTC, GATAGATAGATA apod.). Tyto lokusy se vyskytují v eukaryotních genomech v hojných počtech, ačkoliv jejich abundance se napříč různými taxony liší (Tóth et al. 2000).

Ačkoliv mikrosatelity nekódují žádné funkční proteiny, výskyt pravidelně se opakujících repetic je z pravděpodobnostního hlediska jednoznačně nenáhodný, zvláště uvážíme-li četnost jejich výskytu v genomech. Proto je na místě otázka, jaké evoluční mechanismy vedly ke vzniku mikrosatelitů a jaká je jejich funkce. Mikrosatelity jsou jedním z nejvariabilnějších typů DNA sekvencí (Weber 1990). Na rozdíl od kódujících sekvencí, kde nejčastějším typem mutace je záměna nukleotidu v sekvenci DNA, polymorfismus mikrosatelitních alel je odvozen především od variability v délce sekvence, která je dána počtem repetičních motivů. Další významnou vlastností mikrosatelitů je vysoká míra heterozygotnosti a přítomnost mnoha různých alel v rámci populace. Tyto vlastnosti, spolu s klasickou Mendelovskou dědičností a selekční neutralitou činí z mikrosatelitů ideální nástroj při hledání odpovědí na celou řadu otázek v oblastech molekulární ekologie a populační genetiky (Beebee & Rowe 2007, Allendorf & Luikart 2007). Za vysokou variabilitou mikrosatelitů stojí vedle selekční neutrality vysoká mutační rychlost, přičemž základní mutační mechanismy jsou dva. Prvním je tzv. replication slippage neboli sklouzávání DNA polymerázy, kdy při replikaci úseku DNA sestávajícího z mnoha krátkých repetičních motivů dojde k přidání či naopak ubrání jednoho repetičního motivu (Tachida & Iizuka 1992). Druhým běžným mutačním mechanismem mikrosatelitů je rekombinace, kdy při crossing-overu může dojít ke změně délky repetitivní sekvence (Harding et al. 1992). Méně často se vyskytují i inzerce nebo delece (souhrnně anglicky indels) jednoho nukleotidu (Dieringer & Schlötterer 2016), což má pak za následek narušení pravidelného sledu repetičních motivů, které je potřeba zohlednit při určování genotypu, resp. skórování alel. Souhrnný přehled mutačních mechanismů mikrosatelitů, včetně neobvyklých typů, zasazený do kontextu evoluční genomiky lze najít v několika přehledných a vyčerpávajících literárních přehledech (Ellegren 2000, Li et al. 2002, Ellegren 2004).

Jak již bylo řečeno, pozorovaná abundance krátkých repetitivních úseků DNA v genomech nemůže být dílem náhody. Mezi doložené funkce mikrosatelitů patří podíl na organizaci chromatinu potažmo chromozomů, především v oblastech centromer a telomer (Areshchenkova & Ganal 1999, Eichler 1999). Mikrosatelity se rovněž podílejí na regulaci metabolických procesů DNA, především rekombinace (Jeffreys et al. 1998, Templeton et al. 2000). Bylo zjištěno, že k rekombinaci dochází preferenčně v oblastech dinukleotidových repetitivních sekvencí, díky jejich vysoké afinitě k rekombinačním enzymům (Biet et al. 1999). Strukturní a podpůrnou úlohu hrají mikrosatelity rovněž při replikaci DNA (Field & Wills 1996), regulaci buněčného cyklu (Codegoni et al. 1999) a korekci replikačních chyb (Kolodner & Marsischky 1999). Další oblastí funkční působnosti mikrosatelitů je regulace genové aktivity. Řada studií dokumentuje vliv přítomnosti repetitivní sekvence v oblasti promotoru na intenzitu a průběh transkripce (Sandaltzopoulos et al. 1995, Chen & Roxby 1997). Častý je rovněž vliv přítomnosti či počtu krátkých tandemových repetic na genovou expresi. Některé geny mohou být exprimovány pouze při specifickém počtu repetic v promotoru (Liu et al. 2000), nebo naopak za určitých okolností je exprese genů při změně počtu repetic vypínána (Miret et al. 1998). Tyto mechanismy mohou mít významný vliv na přežívání organismů v měnících se podmínkách. Kromě toho slouží některé mikrosatelity jako vazebná místa pro regulační proteiny (Csink & Henikoff 1998) a mohou ovlivňovat též intenzitu a průběh translace mRNA (Martin-Farmer & Janssen 1999).

Otázka „K čemu jsou mikrosatelity dobré?“ má samozřejmě dvě zásadně odlišné roviny významu. K čemu jsou dobré organismům v rámci fungování genomu bylo stručně naznačeno v předchozím odstavci. K čemu jsou dobré biologům jakožto nástroj výzkumu bude popsáno v odstavci následujícím. Již zmíněné vlastnosti mikrosatelitů (selekční neutralita, kodominance, mendelovská dědičnost, vysoká variabilita) umožňují při dostatečném počtu analyzovaných lokusů jednoznačně detekovat jednotlivce ze vzorku DNA, což je i nebiologické veřejnosti známo pod termínem „DNA fingerprinting“ neboli genetická daktyloskopie – metoda stále více používaná především v kriminalistice (Roewer 2013). Z možnosti jednoznačně identifikovat konkrétního jednotlivce v kombinaci s mendelovskou dědičností a kodominancí logicky vyplývá další z nejběžnějších praktických aplikací mikrosatelitové analýzy – určení rodičovství a jiných typů příbuznosti (Blouin et al. 1996, Jones & Ardren 2003). Stanovením genotypu většího počtu jedinců v rámci populací lze z frekvence výskytu jednotlivých alel kvantifikovat populačně-genetické fenomény jako genový tok (Bossart & Prowell 1998, Gaggiotti et al. 1999) populační diferenciace (Balloux & Lugon-Moulin 2002), inbreeding (Coltman & Slate 2003), bottleneck (Williamson-Natesan 2005), nebo hybridizace (Randi 2007). Oblíbená je rovněž kombinace genetických a geografických dat užívaná k vysvětlení pozorovaných populačních jevů na pozadí vlivu geografické vzdálenosti a krajinných parametrů. Tento interdisciplinární přístup se vžil pod názvem krajinná genetika (angl. landscape genetics) (Manel et al. 2003, Holderegger & Wagner 2008). Výzkum výše uvedených populačně-genetických parametrů má zvýšený význam v ochranářské biologii (Frankham et al. 2002), případně biologii invazních druhů (Lee 2002), přenašečů chorob (McCoy 2008), hospodářsky významných živočichů a rostlin apod. V neposlední řadě nachází analýza mikrosatelitů uplatnění v humánní medicíně, konkrétně v diagnostice některých nádorových onemocnění (Ribic et al. 2003, Boland & Goel 2010), případně jiných typů onemocnění, obecně způsobených mutacemi, s nimiž jsou konzistentně asociovány určité mikrosatelitové genotypy (Smolik et al. 2010, Korytko & Laczmanska 2016).

Obecná metodika I

K analýze mikrosatelitů potřebujeme vzorky DNA zkoumaného organismu, obvykle dostatečné množství jedinců z více různých subpopulací, abychom byli schopni získat statisticky relevantní dataset alelických frekvencí (Hale et al. 2012). Lokusy amplifikujeme pomocí polymerázové řetězové reakce (PCR), nejlépe v tzv. multiplexu, což znamená že do jedné PCR reakce dáme více párů primerů a získáme tak produkty více lokusů v jediné reakci. Abychom byli schopni alely jednotlivých lokusů v rámci multiplexu od sebe odlišit, jsou forward primery značené různými typy fluorescence, což má primární význam při fragmentační analýze na automatickém sekvenátoru (Thermo Fisher Scientific 2014), což je dnes standardní metoda kvantifikace počtu nukleotidových bází mikrosatelitních lokusů (dříve se využívala rovněž gelová elektroforéza, modifikovaná za účelem extrémní citlivosti s možností rozlišit PCR produkty lišící se ve velikosti v řádu jednotek bází). Získané elektroforetogramy analyzujeme (stanovujeme genotypy) pomocí příslušných softwarů (Pompanon et al. 2005, Guichoux et al. 2011) a konečně genotypické datasety statisticky zpracováváme celou řadou metod a programů (Excoffier & Heckel 2006) v závislosti na otázkách a hypotézách dané studie.

Obligátní komplikací při využití mikrosatelitů vždy bylo a dosud je potřeba specifických primerů, a to v počtu nejlépe několika desítek. Obvyklým postupem při zahájení práce, jejímž cílem je získat genotypy populací studovaného druhu, je hledání v literatuře, zda pro daný druh specifické primery existují. Pokud ano, výzkumník si ušetří mnoho práce a finančních prostředků, na druhou stranu to znamená zvýšenou pravděpodobnost, že daný druh byl již z hlediska populační genetiky zkoumán, což ovšem může být výhoda i nevýhoda – na jednu stranu je možné na existující studii navázat, zaměřit se na jiný aspekt apod., na druhou stranu se vytrácí atraktivita prvenství a neotřelosti.

Druhou variantou je situace, že nalezneme v literatuře primery navržené pro více či méně příbuzný druh a rozhodneme se vyzkoušet zda budou použitelné i pro námi studovaný druh. Tomuto přístupu se anglicky říká cross-species amplification, česky mezidruhová amplifikace. Obecně platí, že čím příbuznější je druh, pro který byly primery navrženy, tím lépe, ačkoliv v tomto ohledu panuje nezanedbatelná míra náhodnosti – někdy jsou použitelné primery navržené pro odlišný řád, jindy zase nebudou fungovat primery navržené pro blízce příbuzný druh (Barbará et al. 2007).

Příkladové studie I

Příkladem využití metody mezidruhové amplifikace jsou dvě publikace, které jsou součástí předkládané dizertační práce. Relativně nedávno byl v rámci rodu netopýrů *Myotis* popsán nový, do té doby tzv. kryptický druh – *Myotis alcathoe* (von Helversen et al. 2001). Na území České Republiky byl předtím zaměňován za jeden ze dvou sesterských druhů – *Myotis mystacinus* a *Myotis brandtti* (Lučan et al. 2009). V naší studii jsme se zabývali spolehlivostí rozlišení a správného určení těchto tří druhů v terénních podmínkách pouze na základě morfologie. Jako kontrolní údaj nám posloužilo srovnání s dodatečně provedeným určením pomocí setu mikrosatelitů. Bylo testováno celkem 33 lokusů, navržených pro jiné druhy netopýrů (*Pipistrellus pipistrellus, Nyctalus noctula, Plecotus auritus, Eptesicus fuscus, Myotis myotis*). Výsledkem bylo nalezení dvanácti lokusů, které byly polymorfní a spolehlivě se amplifikovaly u všech tří zkoumaných druhů. Tyto lokusy byly amplifikovány ve dvou multiplexech. V souladu s výše uvedeným postulátem, bylo devět z těchto dvanácti lokusů původně navrženo pro nejpříbuznější druh – *Myotis myotis*. Bylo zgenotypováno celkem 359 jedinců druhů *M.alcathoe, M.mystacinus* a *M.brandtti*. Data byla analyzována pomocí faktoriální korespondenční analýzy v programu GENETIX (Belkhir et al. 2004). Assignment test, přiřazující každému jedinci hodnotu pravděpodobnosti genetické příslušnosti byl proveden v programu GeneClass (Cornuet et al. 1999). Výsledky obou analýz jednoznačně rozdělily jedince do druhů, mj. díky řadě druhově specifických alel. Tato data pak byla použita pro potvrzení správného určení zvířat v terénu pomocí morfometrie (Lučan et al. 2011). Získaná data později přinesla další zajímavý poznatek v kontextu studie Bogdanovicze et al. (2012), která dokumentovala výskyt mezidruhových hybridů v rámci *M.alcathoe*, *M.mystacinus* a *M.brandtti* na podzimních shromaždištích netopýrů v jižním Polsku. Naše data, analyzovaná totožným způsobem (software STRUCTURE (Pritchard et al. 2000)), výskyt hybridů na území ČR nepotvrdila.

Druhým příkladem využití metody mezidruhové amplifikace je studie zkoumající populační genetiku kamzíka horského na Slovensku. Ve Vysokých Tatrách se v počtu cca sedmi set jedinců vyskytuje endemický autochtonní poddruh *Rupicapra rupicapra tatrica*. Do Nízkých Tater bylo v sedmdesátých letech dvacátého století několik jedinců tohoto poddruhu přemístěno a vznikla zde relativně stabilní populace čítající cca sto jedinců. V sousedících pohořích Vysoké Fatry a Slovenského Ráje žijí populace kamzíků pocházející z Německých a Rakouských Alp (Martínková et al. 2012). Tatranský poddruh je na seznamu kriticky ohrožených druhů. V průběhu minulých sto let docházelo k poměrně dramatickým poklesům početnosti, např. během druhé světové války vlivem lovu. Cílem výzkumu bylo zjistit úroveň genetické variability jednotlivých populací a především detekovat případnou hybridizaci mezi tatranským a alpským poddruhem, kterou bylo možno očekávat především v Nízkých Tatrách. Za tímto účelem bylo testováno celkem 65 mikrosatelitových lokusů, původně navržených pro skot, ovce, kozy a goraly. Bylo nalezeno dvacet lokusů polymorfních a konzistentně se amplifikujících v rámci všech zkoumaných populací, přičemž výrazným trendem byl vysoký podíl (68%) monomorfních lokusů u tatranského podruhu, zatímco u alpských kamzíků byla většina (63%) testovaných lokusů polymorfních. Vybrané lokusy byly amplifikovány pomocí tří multiplexových setů a využity k získání odpovědí na výše uvedené otázky. Ve všech zkoumaných populacích byla zjištěna nízká genetická variabilita a vysoká míra inbreedingu. Nejnižší úroveň variability byla pozorována u tatranského

poddruhu, pravděpodobně jako důsledek opakovaných bottlenecků. Všechny čtyři populace byly od sebe zřetelně geneticky odlišné. Introdukované populace alpského poddruhu vykazovaly vyšší genetickou variabilitu, přestože byly založeny nižším počtem jedinců. V nízkotatranské populaci byla prokázána hybridizace s alpským poddruhem z obou sousedících lokalit (Zemanová et al. 2011, Zemanová et al. 2015).

Obecná metodika II

Pokud chceme studovat populační genetiku druhu, pro který mikrosatelitové primery zatím neexistují a ani metoda cross-species amplifikace nepřinese funkční markery, je potřeba specifické primery identifikovat a charakterizovat. První protokol pro izolaci mikrosatelitů *de novo* byl publikován v roce 1991 (Rassmann et al.). Tento postup zahrnoval vytvoření parciálních genomových knihoven, tj. fragmentů DNA o vhodné velikosti (v řádu stovek bází), získaných pomocí restrikčních enzymů, případně sonikace. Tyto fragmenty byly následně zaklonovány do bakteriálních buněk pomocí plasmidových vektorů. Úspěšně zaklonované knihovny obsahující repetiční motivy byly poté vybrány pomocí Southernovy hybridizace (Southern 1975) za použití sond obsahujících repetiční motivy. Zejména v případě genomů bohatých na mikrosatelity, byl tento postup relativně jednoduchý a efektivní. Na druhou stranu se ukázalo, že v případě nízkých frekvencí výskytu mikrosatelitů v cílovém genomu, je klonování a hybridizace extrémně pracné a málo výnosné. Z těchto důvodů byly vyvinuty různé alternativní strategie za účelem snížení časové náročnosti a zvýšení počtu identifikovaných mikrosatelitů, např. využití RAPD (Wu et al. 1994, Ciffarelli et al. 1995), extenze primerů (Ostrander et al. 1992, Paetkau 1999), nebo selektivní hybridizace (Karagyozov et al. 1993, Armour et al. 1994, Kijas et al. 1994). Postupem času začalo být běžné, že tyto časově náročné laboratorní procedury jsou zajišťovány komerčními poskytovateli. Zejména pro výzkumné týmy, které využívají mikrosatelity pouze příležitostně, případně se zabývají jedním, či několika málo druhy organismů, se jedná o vítanou možnost (Zane et al. 2002).

Nástup next-generation sekvenování (NGS) otevřel v oblasti izolace mikrosatelitů nové možnosti. Množství sekvenčních dat získaných během jediné analýzy se pohybuje v řádu milionů bází. V rámci získaných sekvencí pak stačí najít pravidelné repetiční motivy o vhodné délce (mikrosatelity) a krátké sekvence, které je ohraničují (primery) (Abdelkrim et al. 2009, Allentoft et al. 2009, Castoe et al. 2010). Zvýšení počtu nalezených mikrosatelitů lze dosáhnout využitím 454 GS-FLX (Roche Aplied Science) technologie k sekvenování obohacených DNA knihoven (Santana et al. 2009). Optimalizovaný protokol kombinující klasickou metodu obohacení DNA knihoven pomocí biotinu a streptavidinu (Kijas et al. 1994) s modifikací Titanium 454 GS-FLX technologie (Malausa et al. 2011), byl vytvořen ve společnosti GenoScreen (Francie), která tuto metodu komerčně poskytuje pod názvem Geno Sat. Výhodou této metody je že s využitím dostupných genomových dat vyhledává v cílovém genomu preferenčně repetiční motivy, které jsou v něm nejhojněji zastoupeny. Praktické využití servisu Geno Sat vyžaduje poskytnutí směsného vzorku (cca 12 jedinců) DNA cílového organismu a po obdržení získaných sekvencí primerů, ohraničujících mikrosatelity, je vhodné provést bioinformatickou analýzu za účelem výběru nejvhodnějších primerů a jejich následné testování zda poskytují polymorfní a konzistentně se amplifikující mikrosatelity. Tento postup byl aplikován při vývoji mikrosatelitů čtyř různých druhů hmyzu – ohroženého motýla perleťovce maceškového (*Argynnis niobe*), ohroženého brouka tesaříka alpského (*Rosalia alpina*), endemického motýla Kamerunské vulkanické linie (*Mylothris jacksoni knutsoni*) a vysoce invazního mravence (*Tapinoma melanocephalum*). Výsledky těchto analýz byly publikovány a jsou součástí předkládané dizertační práce.

Příkladové studie II

Většina hojně studovaných ohrožených motýlů tvoří kolonie o vysokých lokálních densitách a nízkém genovém toku. Druhy s opačnou strategií (nízké density, velká mobilita) nebývají ohroženy. Vůbec nejohroženější však bývají druhy spadající mezi tyto dva extrémy, tj. středními densitami i genovým tokem. Takové druhy využívají přirozeně vzácné zdroje, proto vyžadují velké rozlohy biotopů (Thomas 2000). Příkladem jsou dva sesterské druhy perleťovců, p. prostřední (*Argynnis adippe*) a p. maceškový (*Argynnis niobe*). Bývali všeobecně rozšířeni v tradičně obhospodařované krajině střední Evropy, v posledním půlstoletí drasticky ustoupili. *A. niobe*, vyžadující extenzivně pasená stanoviště, v ČR přežívá hlavně v moravských Karpatech, zatímco *A. adippe*, preferující opuštěné louky až křoviny, se v posledním desetiletí vrací (Beneš et al. 2002). Za účelem studia genetické struktury těchto motýlů jsme vyvinuli mikrosatelitní markery pro *A. niobe* a metodou cross-species amplifikace byly tyto lokusy aplikovány i u sesterského druhu *A. adippe*. Společnosti GenoScreen jsme zaslali směsný vzorek DNA čtyř jedinců Perleťovce maceškového. Bylo provedeno pyrosekvenování a následně analýza GenoSat, které poskytla bezmála 70 tisíc sekvencí, z nichž více než osm tisíc obsahovalo repetiční motivy. Bioinformatická analýza vyústila v 388 potenciálně vhodných párů primerů, ohraničujících mikrosatelitové lokusy. Poté, co jsme tyto potenciálně vhodné primery podrobili důkladnější analýze ohledně jejich biochemických vlastností, důležitých pro úspěšnou amplifikaci, s využitím on-line aplikace NetPrimer jsme vybrali třicet nejvhodnějších. Tyto lokusy jsme testovali na polymorfismus u obou druhů simultánně, čímž jsme docílili nebývale vysoké úspěšnosti při aplikaci lokusů u *A. adippe*. Vybrali jsme celkem deset lokusů, z nichž všechny byly polymorfní u *A. niobe* a devět u *A. adippe*. Pomocí těchto markerů jsme zgenotypovali 32 jedinců od každého druhu z údolí Vsetínské Bečvy v CHKO Beskydy. U obou druhů jsme zaznamenali relativně nízkou genetickou variabilitu (průměrný počet alel na lokus 8,2 u *A. niobe* a 4,8 u *A. adippe*), jakož i nižší úroveň pozorované heterozygotnosti než očekávané. Tyto parametry naznačují možný výskyt inbreedingu, bottlenecku nebo genetického driftu – fenomény očekávatelné u ohrožených druhů, které nedávno zaznamenaly výrazný úbytek početnosti.

Tesařík alpský (*Rosalia alpina*) je ikonickým druhem ochrany Evropské přírody. Atraktivní zvíře vázané svými potřebami na staré vzrostlé listnaté stromy, především buky, ale též např. javory, jilmy a jasany. Dříve rozšířený na většině území Evropy, během posledních desetiletí vyhynul v severní části svého areálu a v České Republice nyní nalézáme okraj jeho rozšíření. Nejvíce ohrožené jsou malé izolované populace, jelikož dospělý brouk, který během několika málo dní nenalezne sexuálního partnera se již nerozmnoží (Drag et al. 2011). Navzdory velkému významu v ochranářské biologii, nebyla genetická struktura populací tesaříka alpského donedávna vůbec zkoumána. Analýza GenoSat poskytla 59 párů primerů, z nichž bylo vybráno 40 nejvhodnějších, z nichž 28 poskytovalo konzistentní PCR produkty v rozmezí 100 – 300 bází. Z těchto lokusů bylo 15 monomorfních, 4 tvořily nespecifické produkty těžko odlišitelné od skutečných alel a 9 lokusů bylo polymorfních se spolehlivě určitelnými alelami, které jsme amplifikovali pomocí dvou multiplexových setů. Pro potřeby krátkého článku, popisujícího vývoj a základní parametry těchto mikrosatelitů, bylo zgenotypováno 45 jedinců ze západního Slovenska. V rámci této jedné subpopulace jsme detekovali nízkou alelickou variabilitu (2-4 alely na lokus) a úroveň heterozygotnosti dosáhla v průměru hodnoty 0,34. Lokusy byly v Hardy-Weinbergově, jakož i ve vazebné rovnováze. Nově vyvinuté mikrosatelity byly následně použity k analýze populačně genetické struktury tesaříka alpského ve střední a jihovýchodní Evropě. Byly stanoveny genotypy celkem sedmi set jedinců ze třiceti dvou různých lokalit. Byla zjištěna negativní korelace genetické diverzity a zeměpisné šířky. Nejvyšší alelická diverzita byla detekována v Řecku, což naznačuje funkci

tamních pohoří jako glaciálních refugií. Klastrová analýza poukázala na existenci dvou geneticky diferencovaných linií – jihovýchodní a severozápadní, oddělených hranicí mezi východními a západními Karpatami (Drag et al. 2015).

Mylothris jacksoni knutsoni je endemický motýl Kamerunských hor, známých též jako Kamerunská vulkanická linie. Toto pohoří je jediným rozsáhlým územním celkem afromontánního habitatu na západ od Konžské pánve a je významným centrem endemismu a biodiverzity. Současně je zde vysoká (lidská) populační hustota s příslušnými tlaky na životní prostředí, což z této oblasti činí jedno z nejohroženějších míst tropické Afriky (Tropek & Konvička 2010). Přesto byl vliv lidské činnosti na genetickou strukturu živočišných populací v této oblasti dosud studován minimálně. *Mylothris jacksoni knutsoni* je závislý na afromontánních habitatech v nadmořské výšce nad 1500 m n.m., kde obývá okraje horských lesů a křovinaté submontánní porosty, přičemž se vyhýbá zdegradovaným pastvinám i lesním pasekám po těžbě dřeva. Populačně genetická struktura tohoto druhu, jakož ani žádného jiného motýla z této oblasti dosud nebyla studována. Analýza GenoSat společnosti GenoScreen nám poskytla 263 párů primerů lokusů, obsahujících repetitivní motiv. Bioinformatickou analýzou pomocí on-line aplikace NetPrimer bylo vybráno 24 nejvhodnějších párů primerů, které byly testovány na osmi jedincích. Ve výsledku bylo popsáno osm polymorfních lokusů, které jsme sdružili do dvou multiplexových panelů, pomocí nichž jsme stanovili genotypy třiceti jedinců z jedné z lokalit (Nkogam Masiv, Západní Kamerun). Podrobnosti ohledně parametrů jednotlivých lokusů lze dohledat v příslušné publikaci (Zima Jr. et al. 2014), zde uvedu pouze že všechny lokusy byly v Hardy-Weinbergově rovnováze a nebyly mezi sebou ve vazebné nerovnováze. Pomocí těchto lokusů jsme následně analyzovali 350 jedinců z patnácti lokalit pokrývajících celkový areál výskytu zkoumaného druhu. Výsledky těchto analýz jsou aktuálně připravovány k publikaci, proto zde pouze naznačím, že jsme zaznamenali pozitivní korelaci mezi genetickou a geografickou vzdáleností a genetickou diferenciaci a sníženou variabilitu v okrajových a izolovaných populacích (Tropek et al. in prep.).

Tapinoma melanocephalum je celosvětově rozšířený, vysoce invazní, synantropní druh mravence, jehož šíření a distribuce jsou do značné míry zprostředkovány a ovlivněny lidskou činností. Původní areál tohoto druhu není znám, nicméně v úvahu připadá oblast Indo-Pacifiku, kde se vyskytuje největší počet různých druhů rodu *Tapinoma* (Wetterer 2009). Z hlediska významu může tento druh představovat ohrožení některých složek původní bioty, škodit v zemědělství a také bylo zjištěno, že je potenciálním přenašečem patogenů (Moreira et al. 2005). Vzhledem k vysoké míře synantropie může detailní znalost fylogeografie a populační struktury těchto mravenců mimo jiné poskytnout zajímavé srovnání s migrační historií druhu *Homo sapiens* (Lebrasseur 2014). Za tímto účelem jsme vyvinuli dvanáct mikrosatelitových lokusů, stejnou metodou jako v předchozích studiích – tj. pyrosekvenování genomových knihoven obohacených o repetitivní motivy s následnou bioinformatickou analýzou, výběr nejvhodnějších primerů a testování polymorfismu získaných PCR produktů. Kromě nově popsaných lokusů, jsme testovali nedávno popsané primery, které jsou univerzální pro všechny mravence (Butler et al. 2014). Z třiadvaceti testovaných jsme zaznamenali dvanáct polymorfních. Celkem dvacet čtyři mikrosatelitů tak bylo použito ke stanovení populačně genetických parametrů zkoumaného druhu. Primárně byly určeny genotypy dvaceti jedinců z jedné populace na Papui-Nové Guinei (PNG). V rámci této jedné populace byla detekována extrémně nízká genetická variabilita, jak ve smyslu počtu alel na lokus (průměr = 1,66), tak ve smyslu proporce heterozygotů (průměr = 0,008). Abychom se podívali na alelickou diverzitu v rámci (mnohem) širšího území a většího počtu různých populací, genotypovali jsme dalších třicet jedinců z deseti lokalit (PNG, Mikronésie). Zjištěná alelická variabilita u nově vyvinutých lokusů dosáhla průměrné hodnoty 6,6 alel na lokus, nicméně pozorovaná proporce heterozygotů (0,144) byla výrazně nižší než očekávaná (0,645). Obdobné hodnoty jsme zaznamenali i při užití univerzálních mikrosatelitů (Na=6,4; Ho=0,142; He=0,545). Abychom ukázali, že tento trend výrazného nadbytku homozygotů je charakteristický pro *T.*

melanocephalum, analyzovali jsme s využitím totožných univerzálních mikrosatelitů vzorky populací šesti jiných druhů resp. rodů Indo-Pacifických mravenců, u nichž jsme detekovali obvyklou úroveň alelické diverzity a víceméně vyrovnané hodnoty pozorované a očekávané heterozygotnosti. Kromě srovnání s jinými druhy jsme metodicky vyloučili možné artefakty, které by mohly úroveň pozorované heterozygotnosti zkreslit. Výrazný nadbytek homozygotů u *T.melanocephalum* tak interpretujeme jako průvodní jev efektu zakladatele (angl.. founder effect), efektu hrdla láhve (angl.. bottleneck) a inbrídingu. Všechny tyto jevy jsou u tohoto druhu očekávatelné, vzhledem k jeho tendenci šířit se na velké vzdálenosti, zakládat kolonie z minimálního počtu jedinců a dalším reprodukčně-ekologickým vlastnostem, které vyúsťují ve vysokou příbuznost jedinců v rámci kolonie.

Závěr

Navzdory rozvoji a vzrůstající dostupnosti tzv. next-generation sekvenování (NGS) (Ekblom & Galindo 2011), klasické genetické markery jako mitochondriální DNA a mikrosatelity zůstávají nenahraditelným nástrojem pro většinu molekulárních ekologů, zejména vzhledem ke snadné a dlouhodobě zavedené metodologii zpracování a vyhodnocování výsledků, cenové dostupnosti a v neposlední řadě kontinuálně vzrůstajícímu počtu organismů, které s pomocí těchto klasických markerů byly analyzovány a je tak možné čím dál více organismů v různých typech analýz vzájemně porovnávat.

Mikrosatelity mají oproti NGS několik důležitých výhod. S využitím genomových dat byly například popsány primery mikrosatelitů, konzervativních (čili použitelných pro amplifikaci příslušných lokusů) v rámci veškerých mravenců, přičemž většina těchto lokusů je zároveň polymorfní v rámci jednotlivých druhů (Butler et al. 2014). Tato studie ukazuje možný trend v budoucím využití mikrosatelitů a představuje inspirativní výzvu pro badatele zabývající se ostatními taxonomickými skupinami. Mesak et al. (2014) provedli srovnávací studii, charakterizující příbuzenské vztahy v populacích ryb *Kryptolebias marmoratus*. Za použití mikrosatelitů a RAD-seq SNP technologie zpracovali stejné vzorky a dospěli k závěru, že NGS RAD-seq technologie může mít při detekci skutečných populačně-genetických parametrů významné nedostatky ve srovnání s klasickými mikrosatelity.

Předkládaná dizertační práce je kompilací šesti publikací, popisujících mikrosatelitové markery čtyř druhů savců a pěti druhů hmyzu. Většinou se jedná o druhy ohrožené a cílem vývoje mikrosatelitů pro tyto druhy bylo jejich následné využití v ochranářsko-genetických studiích. Práce tak představuje obohacení dostupných metodických nástrojů k výzkumu ohrožených živočichů. K vývoji lokusů bylo využito různých postupů, včetně moderního pyrosekvenování, které představuje trend v analýzách DNA s celou řadou praktických aplikací. Tento text mimo jiné dokumentuje vzrůstající dostupnost nejmodernějších metod molekulární biologie a jejich využití v klasických biologických oborech jako je zoologie, botanika a ekologie.

Reference

Abdelkrim J., Robertson B.C., Stanton J.A.L., Gemmell N.J. 2009: Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. Bio Techniques 46: 185-191

Allendorf F.W. & Luikart G. 2007: Conservation and the genetics of populations. Blackwell Publishing

Allentoft M.E., Schuster S.C., Holdaway R.N. et al. 2009: Identification of microsatellites from an extinct moa species using high-throughput (454) sequence data. Bio Techniques 46: 195-200

Areshchenkova T. & Ganal M.W. 1999: Long tomato microsatellites are predominantly associated with centromeric regions. Genome 42: 536-544

Armour J.A., Neumann R., Gobert S., Jeffreys A.J. 1994: Isolation of human simple repeat loci by hybridization selection. Human Molecular Genetics 3: 599-565

Balloux F. & Lugon-Moulin N. 2002: The estimation of population differentiation with microsatellite markers. Molecular Ecology 11: 155-165

Barbará T., Palma-Silva C., Paggi G.M., Bered F., Fay M.F., Lexer C. 2007: Cross-species transfer of nuclear microsatellite markers: potential and limitations. Molecular Ecology 16: 3759-3767

Beebee T. & Rowe G. 2007: An introduction to molecular ecology. Oxford University Press

Belkhir K., Borsa P., Chikhi L., Raufaste N., Bonhomme F. 2004: GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier (France)

Beneš J., Konvička M., Dvořák J. et al. 2002: Butterflies of the Czech Republic: Distribution and Conservation I. SOM, Prague, 478 pp.

Biet E., Sun J., Duetreix M. 1999: Conserved sequence preference in DNA binding among recombination proteins: an effect of ssDNA secondary structure. Nucleic Acid Research 27: 596-600

Blouin M.S., Parsons M., Lacaille V., Lotz S. 1996: Use of microsatellite loci to classify individuals by relatedness. Molecular Ecology 5: 393-401

Bogdanowicz W., Piksa K., Tereba A. 2012: Hybridization hotspots at bat swarming sites. PLoS ONE 7: e53334

Boland C.R. & Goel A. 2010: Microsatellite instability in colorectal cancer. Gastroenterology 138: 2073-2087

Bossart J.L. & Prowell D.P. 1998: Genetic estimates of population structure and gene flow: Limitations, lessons and new directions. Trends in Ecology & Evolution 13(5): 202-206

Butler I.A., Siletti K., Oxley P.R., Kronauer D.J.C. 2014: Conserved microsatellites in ants enable population genetic and colony pedigree studies across a wide range of species. Plos One 9

Castoe T.A., Poole A.W., Gu W. et al. 2010: Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence. Molecular Ecology Resources 10: 341-347

Chen Y., Roxby R. 1997: Identification of a functional CT-element in the *Phytophthora infestans* piypt1 gene promoter. Gene 198: 159-164

Cifarelli R.A., Gallitelli M., Cellini F. 1995: Random amplified hybridization microsatellites (RAHM): isolation of a new class of microsatellite-containing DNA clones. Nucleic Acid Research 23: 3802-3803

Codegoni A.M., Bertoni F., Collela G. et al. 1999: Microsatellite instability and frameshift mutations in genes involved in cell cycle progression or apoptosis in ovarian cancer. Oncology Research 11: 297-301

Coltman D.W. & Slate J. 2003: Microsatellite measures of inbreeding: a meta-analysis. Evolution 57: 971-83

Cornuet J.M., Piry S., Luikart G., Estoup A., Solignac M. 1999: New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153: 1989-2000

Csink A.K. & Henikoff S. 1998: Something from nothing: the evolution and utility of satellite repeats. Trends in Genetics 14: 200-204

Dieringer D. & Schlötterer C. 2016: Two distinct modes of microsatellite mutation processes: evidence from the complete genomic sequences of nine species. Genome Research 13: 2242-2251

Drag L., Hauck D., Pokluda P., Zimmermann K., Čížek L. 2011: Demography and dispersal ability of a threatened Saproxylic Beetle: a mark-recapture study of the Rosalia Longicorn (*Rosalia alpina*). PLoS ONE 6: e21345

Drag L., Hauck D., Bérces S., Michalcewicz J., Jelaska L., Aurenhammer S., Cizek L. 2015: Genetic differentiation of populations of the threatened saproxylic beetle Rosalia longicorn, Rosalia alpina (Coleoptera: Cerambycidae) in Central and South-east Europe. Biological Journal of the Linnean Society 116: 911-925

Eichler E.E. 1999: Repetitive conundrums of centromere structure and function. Human Molecular Genetics 8: 843-854

Ekblom R. & Galindo J. 2011: Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity 107: 1-15

Ellegren H. 2000: Microsatellite mutations in the germline: implications for evolutionary inference. Trends in Genetics 16: 551-558

Ellegren H. 2004: Microsatellites: Simple sequences with complex evolution. Nature Reviews Genetics 5: 435-445

Excoffier L. & Heckel G. 2006: Computer programs for population genetics data analysis: a survival guide. Nature Review Genetics 7: 745-758

Field D. & Wills C. 1996: Long, polymorphic microsatellite in simple organisms. Proceedings of Royal Society of London B Biological Sciences 263: 209-215

Frankham R., Ballou J.D., Briscoe D.A. 2002: Introduction to Conservation Genetics. Cambridge University Press

Gaggiotti O.E., Lange O., Rassmann K., Gliddon C. 1999: A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. Molecular Ecology 8: 1513-1520

Guichoux E., Lagache L., Wagner S. et al. 2011: Current trends in microsatellite genotyping. Molecular Ecology Resources 11: 591-611

Hale M.L., Burg T.M., Steeves T.E. 2012: Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. Plos One 7(9): e45170

Harding R.M., Boyce A.J., Clegg J.B. 1992: The evolution of tandemly repetitive DNA: recombination rules. Genetics 132: 847-859

Helversen O. von, Heller K.G., Mayer F., Nemeth A., Volleth M., Gombkötö P. 2001: Cryptic mammalian species: a new species of whiskered bat (Myotis alcathoe n. sp.) in Europe. Naturwissenschaften 88: 217-23

Holderegger R. & Wagner H.H. 2008: Landscape genetics. BioScience 58: 199-207

Jeffreys A.J., Murray J., Neumann R. 1998: High-resolution mapping of crossovers in human sperm defines a minisatellite-associated recombination hotspot. Molecular Cell 2: 267-273

Jones A.G. & Ardren W.R. 2003: Methods of parentage analysis in natural populations. Molecular Ecology 12: 2511-2523

Karagyozov L., Kalcheva I.D., Chapman V.M. 1993: Construction of random small-insert genomic libraries highly enriched for simple sequence repeats. Nucleic Acid Research 21: 3911-3912

Kijas J.M., Fowler J.C., Garbett C.A., Thomas M.R. 1994: Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotechniques 16: 656-662

Kolodner R.D. & Marsischky G.T. 1999: Eukaryotic DNA mismatch repair. Current Opinion in Genetics & Development 9: 89-96

Korytko M. & Laczmanska I. 2016: Microsatellites and their application in diagnostics. Kosmos 65: 11-16

Lebrasseur O.M.M. 2014: Dogs, Chickens and Ants: Investigating the reliability of modern maternal genetic data in retracing early dispersals. PhD Thesis, Durham University.

Lee C.E. 2002: Evolutionary genetics of invasive species. Trends in Ecology & Evolution 17: 386-391

Li Y., Korol A.B., Fahima T., Beiles A., Nevo E. 2002: Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Molecular Ecology 11: 2453-2465

Liu L., Dybvig K., Panangala V.S., Van Santen V.L., French C.T. 2000: GAA trinucleotide repeat region regulates M9/pMGA gene expression in *Mycoplasma gallisepticum*. Infection and Immunity 68: 871-876

Lučan R.K., Andreas M., Benda P. et al. 2009: Alcathoe bat (*Myotis alcathoe*) in the Czech Republic: distributional status, roosting and feeding ecology. Acta Chiropterologica 11: 61-69

Lučan R.K., Benda P., Reiter A., Zima Jr. J. 2011: Reliability of field determination in three cryptic whiskered bats (*Myotis alcathoe, M.mystacinus, M.brandtti*) and basic biometric characters: evidence from the Czech Republic. Vespertilio 15: 55-62

Malausa T., Gilles A., Meglécz E. et al. 2011: High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. Molecular Ecology Resources 11: 638-644

Manel S., Schwartz M.K., Luikart G., Taberlet P. 2003: Landscape genetics: landscape ecology and population genetics. Trends in Ecology and Evolution 18: 189-197

Martin-Farmer J. & Janssen G.R. 1999: A downstream CA repeat sequence increases translation from leadered and unleadered mRNA in *Escherichia coli*. Molecular Microbiology 31: 1025-1038

Martínková N., Zemanová B., Kranz A., Giménez M.D., Hájková P. 2012: Chamois introductions to Central Europe and New Zealand. Folia Zoologica 61: 239-245

McCoy K.D. 2008: The population genetic structure of vectors and our understanding of disease epidemiology. Parasite 15: 444-448

Mesak F., Tatarenkov A., Early R.L., Avise J.C. 2014: Hundreds of SNPs vs. dozens of SSRs: which dataset better characterizes natural clonal lineages in a self-fertilizing fish? Frontiers in Ecology and Evolution 2: 74

Miret J.J., Pessoa-Brandao L., Lahue R.S. 1998: Orientation-dependent and sequence-specific expansions of CTG/CAC trinucleotide repeats in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences USA 95: 12438-12444

Moreira D.D.O., Morais V., Vieira-da-Motta O., Campos-Farinha A.E.C., Tonhasca Jr. A. 2005: Ants as carriers of antibiotic-resistant bacteria in hospitals. Neotropical Entomology 34: 999-1006

Ostrander E.A., Jong P.M., Rine J., Duyk G. 1992: Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. Proceedings of the National Academy of Sciences of the USA 89: 3419-3423

Paetkau D. 1999: Microsatellites obtained using strand extension: An enrichment protocol. Biotechniques 26: 690-697

Pompanon F., Bonin A., Bellemain E., Taberlet P. 2005: Genotyping errors: causes, consequences and solutions. Nature Reviews Genetics 6: 847-859

Pritchard J.K., Stephens M., Donnelly P. 2000: Inference of population structure using multilocus genotype data. Genetics 155: 945-959

Randi E. 2007: Detecting hybridization between wild species and their domesticated relatives. Molecular Ecology 17: 285-293

Rassmann K., Schlotterer C., Tautz D. 1991: Isolation of simple sequence loci for use in polymerase chain reaction-based DNA fingerprinting. Electrophoresis 12: 113-118

Ribic C.M., Sargent D.J., Moore M.J. et al. 2003: Tumor microsatellite-instability as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. The New England Journal of Medicine 349: 247-257

Roewer L. 2013: DNA fingerprinting in forensics: past, present, future. Investigative Genetics 4: 22

Sandaltzopoulos R., Mitchelmore C., Bonte E., Wall G., Becker P.B. 1995: Dual regulation of the *Drosophila hsp26* promoter *in vitro*. Nucleic Acid Research 23: 2479-2487

Santana Q.C., Coetzee M.P.A., Steenkamp E.T. et al. 2009: Microsatellite discovery by deep sequencing of enriched genomic libraries. Bio Techniques 46: 217-223

Smolik S., Domal-Kwiatkowska D., Kapral M., Weglarz L. 2010: Microsatellite marker analysis in the treatment and diagnosis of familial hypertrophic cardiomyopathy. Acta Poloniae Pharmaceutica – Drug Research 67: 669-672

Southern E.M. 1975: Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98: 503-517

Tachida H. & Iizuka M. 1992: Persistence of repeated sequences that evolve by replication slippage. Genetics 131: 471-478

Tautz D. & Renz M. 1984: Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Research 12(10): 4127-4138

Templeton A.R., Clark A.G., Weiss K.M., Nickerson D.A., Boerwinkle E., Sing C.F. 2000: Recombinational and mutational hot spots within the human lipoprotein lipase gene. Americal Journal of Human Genetics 66: 69-83

Thermo Fisher Scientific Inc. 2014: DNA fragment analysis by capillary elecrophoresis. Publication number 4474504

Thomas C.D. 2000: Dispersal and extinction in fragmented landscapes. Proceedings of Royal Society London (B) 267: 139-145

Tóth G., Gáspári Z., Jurka J. 2000: Microsatellites in different eukaryotic genomes: survey and analysis. Genome Research 10: 967-981

Tropek R., Konvička M. 2010: Forest Eternal? Endemic butterflies of the Bamenda highlands, Cameroon, avoid close-canopy forest. African Journal of Ecology 48: 428-437

Weber J.L. 1990: Informativeness of human $(dC\text{-}dA)_n$ $(dG\text{-}dT)_n$ polymorphisms. Genomics 7: 524-530

Wetterer J.K. 2009: Worldwide spread of the ghost ant, *Tapinoma melanocephalum* (Hymenoptera: Formicidae). Myrmecological News 12: 23-33

Williamson-Natesan E.G. 2005: Comparison of methods for detecting bottlenecks from microsatellite loci. Conservation Genetics 6: 551-562

Wu K., Jones R., Danneberger L., Scolnik P.A. 1994: Detection of microsatellite polymorphisms without cloning. Nucleic Acid Research 22: 3257-3258

Zane L., Bargelloni L., Patarnello T. 2002: Strategies for microsatellite isolation: a review. Molecular Ecology 11: 1-16

Zemanová B., Hájková P., Bryja J., Zima Jr. J., Hájková A., Zima J. 2011: Development of multiplex microsatellite sets for nonivasive population genetic study of the endangered Tatra chamois. Folia Zoologica 60: 70-80

Zemanová B., Hájková P., Hájek B., Martínková N., Mikulíček P., Zima J., Bryja J. 2015: Extremely low genetic variation in endangered Tatra chamois and evidence for hybridization with an introduced Alpine population. Conservation Genetics 16: 729-741

Zima Jr.J., Lestina D., Jansta P., Petru V., Tropek R. 2014: Isolation and characterization of microsatellite markers for *Mylothris jacksoni knutsoni* (Lepidoptera: Pieridae), an endemic butterfly of the Gulf of Guinea Highlands. Conservation Genetics Resources 6: 763-764

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Reliability of field determination in three cryptic whiskered bats (*Myotis alcathoe*, *M. mystacinus*, *M. brandtii*) and basic biometric characters: evidence from the Czech Republic

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Abstract. The Alcathoe bat (*Myotis alcathoe*), the whiskered bat (*Myotis mystacinus*) and the Brandt's bat (*Myotis brandtii*) represent three cryptic species living in sympatry across much of Europe. Although their determination based solely on external characters has been widely used in field research, there is no study addressing the reliability of such determination. Based on material of bats identified with the help of genetic methods, we aimed (1) to analyse the reliability of field determination, (2) to compare basic quantitative measurements (forearm length, length of tibia, body mass) and (3) to provide information on the reliability of using these measurements for field identification of these three species based on material from the Czech Republic. Fourteen of 359 individuals (3.9%) were originally erroneously determined based on external characters. Eight per cent of bats originally determined as *M. alcathoe* were in fact different species. In all *M. mystacinus* bats, the original species determination was confirmed using the molecular analysis (i.e. 0% determination error). Four per cent of bats (5 inds.) originally determined as *M. brandtii* were assigned to *M. mystacinus* using molecular methods. The three species significantly differed in forearm length, the length of tibia and body mass. Although a considerable overlap of marginal values always existed, the lowest one was recorded between *M. alcathoe* and *M. brandtii*. The best model for discrimination among the three species included sex, forearm length and body mass. However, ca. 69–94% correctness of assignment based solely on these three variables stresses the importance of using a combination of both metric and qualitative characters (i.e. colouration, ear and dental morphology) to further improve the reliability of determination.

***Myotis alcathoe*, *Myotis mystacinus*, *Myotis brandtii*, determination, external morphology**

Introduction

The Alcathoe bat (*Myotis alcathoe*), the whiskered bat (*Myotis mystacinus*) and the Brandt's bat (*Myotis brandtii*) represent three distinct species from the *Myotis mystacinus* morpho-group living in sympatry across most of Europe (Dietz et al. 2007). Although they are not closely related species (see Ruedi & Mayer 2001), their morphology is so similar that until the 1960s they were treated as a single species. Gauckler & Kraus (1970) and Hanák (1970) showed *M. brandtii* (Eversmann, 1845) to represent a species separate from *M. mystacinus* (Kuhl, 1817), based on

body size and dental and penial morphology. Since that time, *M. mystacinus* and *M. brandtii* were easily distinguished in the field based on the presence of prominent cingular cusp on the upper large premolar (P^4) and the widened distal part of penis in the latter species (Gauckler & Kraus 1970, Hanák 1970, 1971). However, the influx of molecular methods into bat taxonomy at the beginning of the 21st century revealed another species within the morpho-group, whose existence had been predicted based on the results of karyologic studies by Volleth (1987) and field observations by von Helversen (1989). This new species was described as *Myotis alcathoe* von Helversen et Heller, 2001 (von Helversen et al. 2001) and the growing literature has confirmed its sympatric (and even syntopic) occurrence over much of the European continent (Niermann et al. 2007, Spitzenberger et al. 2008, Lučan et al. 2009, Jan et al. 2010, Bashta et al. 2011).

External characters used for field identification of these three bats (summarised by Dietz et al. 2007) are as follows:

Myotis alcathoe – smallest European *Myotis*; at first sight it resembles *Myotis daubentonii* but is clearly smaller; dorsal pelage is uniformly brown or reddish brown; face and ears are pale-coloured; feet are smaller than in *M. mystacinus* and *M. brandtii*; tragus is short, it does not reach the notch on the posterior edge of the ear or only scarcely; the large upper premolar (P^4) bears a distinct cingular cusp that is, however, not so prominent as in *M. brandtii*; penis is evenly narrow or slightly thickened at the end; forearm length <32.8 mm, fifth finger length <44 mm, third finger length <56 mm, thumb length <4.7 mm, tibia length <14.8 mm, foot length <5.8 mm.

Myotis mystacinus – slightly larger than *M. alcathoe*; dorsal pelage is very dark, frequently with yellowish tips giving bi-coloured ("frosty") appearance; face and ears are dark brown to black; tragus extends beyond the notch on the posterior edge of the ear; penis is evenly narrow for its whole length; tragus, tibia, foot and thumb lengths larger than in *M. alcathoe* (see above); cingular cusp on the large upper premolar as well as prominent protoconuli on upper molars are mostly missing or are very minute; the second small premolars in both jaws (P^3 and P_3) are markedly smaller than the first ones (P^2 and P_2).

Myotis brandtii – similar or same in size to *M. mystacinus*; dorsal pelage has light-golden hair tips; skinny parts on the face and the base and inner part of ears are pale (pinkish); tragus extends behind notch on the posterior edge of the ear; high cingular cusp on the large upper premolar (P^4) which is equal in height or even higher than the second small premolar (P^3); the two small upper premolars (P^2 and P^3) are almost equal in size; penis is club-shaped at its end.

Although basic external qualitative and quantitative characters discriminating *M. alcathoe, M. mystacinus* and *M. brandtii* were well defined (see above) and have been frequently used (see e.g. Niermann et al. 2007, Lučan et al. 2009, Danko et al. 2010), there is little information on the reliability of discrimination based on these characters particularly in *M. alcathoe*. This bat is a rare species and field workers do not often have an opportunity to train their identification skills on a large number of individuals. Moreover, the size characters given in the literature were taken from material originating from different parts of Europe, thereby they can include possible geographic variation, while there may be lower variation within a smaller geographic region (e.g. within Central Europe) which could be useful-to-know for local researchers to improve the reliability of their identification.

The aim of this study was (1) to analyse the reliability of field identification of *M. alcathoe, M. mystacinus* and *M. brandtii*, (2) to compare basic field measurements (forearm length, tibia length, body mass) in genetically identified individuals, and (3) to provide information on the reliability of discrimination based on these measurements for field identification of the respective three species based on relatively extensive data from the Czech Republic.

Material and methods

During various fieldworks focused on bats, *Myotis alcathoe*, *M. mystacinus* and *M. brandtii* were captured using mist-netting in suitable habitats (foraging sites, swarming sites) or by hand nets at roosting sites. Upon their identification based on external qualitative and quantitative characters summarized above, wing membrane samples were taken from each individual using the sterile biopsy punch (Worthington Wilmer & Barratt 1996) and stored in 96% ethanol. Sex, age, forearm length and body mass were recorded in most of the sampled bats (n=310), while the length of tibia was only measured in a subsample of each species (n=77) only. Forearm length was taken including wrist. Only measurements of full-grown bats were used for analyses while juveniles measured before 15 July were excluded because of the possibility they did not reach adult size at that time.

Genomic DNA was extracted from the wing punch, with the DNeasy Blood & Tissue kit (QIAGEN). All individuals were genotyped for 12 microsatellite loci, which were selected for their ability to distinguish among the three species (Zima et al. 2011). Methodology of the PCR amplification, fragment analysis, genotyping and details of the microsatellites will be described elsewhere (Zima et al., in prep.). All individual genotypes were analysed as a single dataset, with three distinct "populations", which were represented by individuals of one of the three species. The most probable "population" of origin for each individual was determined using the Bayesian assignment test implemented in the GeneClass software (Cornuet et al. 1999). Original field determination was then compared with true species identity based on the molecular genetic analysis.

Given the normal distribution of the data we used factorial analysis of variance (ANOVA) to test the effect of species and sex on the forearm and tibia lengths and the body mass. We used Tukey test for post-hoc comparisons. The discriminant function analysis (DFA) was used to find best discriminating variables. In the first run we used the dataset including species, sex, forearm length, tibia length and body mass. Given the missing data on tibia length, this dataset was ca. four-times smaller (n=77) than the dataset used for final model that included sex, forearm length and body mass only (n=310). All analyses were performed using the Statistica 8.0 (Statsoft Inc.) software. If not specified, the values are presented as mean±S.D.

Results

Reliability of Field Determination

Altogether 359 individuals of *M. alcathoe, M. mystacinus* and *M. brandtii* were sampled at 18 localities (6 of them hosting *M. alcathoe*, 13 *M. mystacinus*, 9 *M. brandtii*) and identified to species using molecular genetic methods. Of these, 14 individuals (3.9%) were erroneously identified in the field based on external characters. Of 113 bats originally identified as *M. alcathoe* six were genetically identified as *M. mystacinus* (4 males, 2 females) and three as *M. brandtii* (2 males, 1 female), which means that 8% of the bats originally determined as *M. alcathoe* were in fact different species. In all 121 individuals of *M. mystacinus*, the original species determination was confirmed using the molecular genetic analysis (i.e. 0% determination error). Of 125 bats originally determined as *M. brandtii*, five (5 females) were assigned to *M. mystacinus* using molecular methods (4% determination error). While 9 of 14 erroneously determined bats were captured at a swarming site, where high numbers of bats (usually >100 in a netting event) of up to 16 species are usually captured, the remaining five misidentified bats were sampled at much less "busy" sites. Thirteen of these bats were adults and one was a juvenile.

External Characters

The average forearm length [in millimetres] was 31.9±0.82 (n=90) in *M. alcathoe*, 34.7±1.2 (n=118) in *M. mystacinus*, and 35.8±1.1 (n=102) in *M. brandtii*. It significantly differed between the three species ($F_{2,304}$=297.5; p<0.0001) but also varied with sex within each species sample ($F_{1,304}$=30.3; p<0.0001). While males and females did not differ in forearm length in *M. alcathoe* (p=0.56), males were significantly smaller than females in the two remaining species, *M. mystacinus* (p<0.001) and *M. brandtii* (p<0.05). Both sexes of *M. alcathoe* had a smaller forearm length than all *M. mystacinus* and *M. brandtii* (p<0.001 in all cases). While males of *M. mystacinus* had a smaller forearm length than both sexes of *M. brandtii* (p<0.001 in both cases), females of

Table 1. Biometric data given separately for each species and sex of *Myotis alcathoe*, *M. mystacinus* and *M. brandtii*. CI – confidence intervals, min – minimum, max – maximum
Tab. 1. Biometrické údaje v závislosti na druhu a pohlaví u *Myotis alcathoe*, *M. mystacinus a M. brandtii*. CI – konfidenční interval, min – minimum, max – maximum

	sex / pohlaví	n	mean / průměr	S.D.	–95 CI	+95 CI	min	max
forearm length / délka předloktí [mm]								
Myotis alcathoe	♂♂	47	31.8	0.7	31.6	32.0	30.3	33.5
	♀♀	43	32.1	0.9	31.8	32.4	30.0	33.6
Myotis mystacinus	♂♂	26	33.9	0.9	33.5	34.2	31.7	35.6
	♀♀	92	35.0	1.2	34.7	35.2	31.8	38.2
Myotis brandtii	♂♂	31	35.3	1.1	34.9	35.7	32.6	37.4
	♀♀	71	36.0	1.1	35.7	36.2	32.9	38.2
tibia length / délka tibie [mm]								
Myotis alcathoe	♂♂+♀♀	46	14.7	0.7	14.5	14.9	12.8	16.0
Myotis mystacinus	♂♂+♀♀	23	15.9	0.6	15.6	16.1	14.7	16.9
Myotis brandtii	♂♂+♀♀	8	16.2	1.1	15.3	17.1	14.5	17.4
body mass / hmotnost [g]								
Myotis alcathoe	♂♂	46	4.4	0.5	4.3	4.6	3.6	5.5
	♀♀	42	4.8	0.7	4.6	5.1	3.5	6.8
Myotis mystacinus	♂♂	26	4.9	0.5	4.7	5.1	4.0	5.8
	♀♀	91	5.7	0.6	5.6	5.9	4.5	7.8
Myotis brandtii	♂♂	31	5.9	0.9	5.6	6.2	4.8	9.0
	♀♀	68	6.4	0.7	6.2	6.5	4.7	8.5

M. mystacinus had a smaller forearm length than females of *M. brandtii* ($p<0.001$) but did not differ from males of *M. brandtii* ($p=0.59$). Detailed data on forearm length for each species and sex are given in Table 1. Despite statistically significant differences in forearm length among the

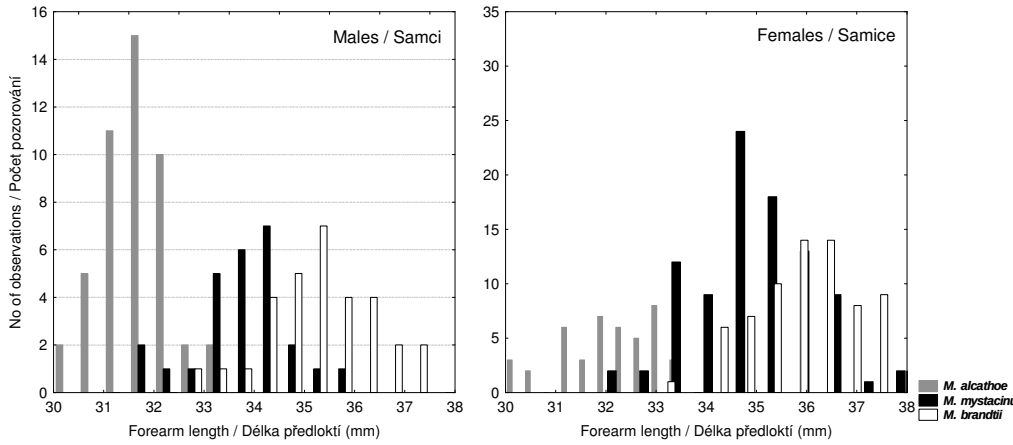

Fig. 1. Distribution of forearm length in males (left) and females (right) of *Myotis alcathoe*, *M. mystacinus* and *M. brandtii*.
Obr. 1. Rozložení délek předloktí u samců (vlevo) a samic (vpravo) *Myotis alcathoe*, *M. mystacinus* a *M. brandtii*.

species, there was a considerable overlap (Fig. 1). In males, the overlap of forearm lengths was 43.8% between *M. alcathoe* and *M. mystacinus*, 73.7% between *M. mystacinus* and *M. brandtii* and 7.7% between *M. alcathoe* and *M. brandtii*. In females, the overlap of forearm lengths was 29.6% between *M. alcathoe* and *M. mystacinus*, 96.9% between *M. mystacinus* and *M. brandtii* and 9.6% between *M. alcathoe* and *M. brandtii*.

The average length of tibia [in millimetres] was 14.7±0.7 (n=46) in *M. alcathoe*, 15.9±0.6 (n=23) in *M. mystacinus*, and 16.2±1.1 (n=8) in *M. brandtii*. While species identity had a significant effect on the length of tibia ($F_{2,71}$=12.6; p<0.0001), sex did not ($F_{1,71}$=0.98; p=0.16). *M. alcathoe* had a smaller tibia length than either of the remaining two species (p<0.001 in both cases) but *M. mystacinus* did not significantly differ from *M. brandtii* (p=0.44). Data on tibia length are given in Table 1. The overlap in tibia lengths was 55.1% between *M. alcathoe* and *M. mystacinus*, 80.6% between *M. mystacinus* and *M. brandtii* and 51.9% between *M. alcathoe* and *M. brandtii*.

The average body mass [in grams] was 4.6±0.6 in *M. alcathoe* (n=88), 5.6±0.7 in *M. mystacinus* (n=117) and 6.2±0.8 in *M. brandtii* (n=99). Species identity ($F_{2, 298}$=108.4; p<0.0001) and sex ($F_{1, 298}$=44.8; p<0.0001) had a significant effect on body mass. Males of *M. alcathoe* had a slightly (p=0.06) lower body mass than females and both sexes had a lower body mass than all *M. mystacinus* and *M. brandtii*, except for males of *M. mystacinus* which did not differ from females of *M. alcathoe* (p=0.9). Males of *M. mystacinus* had a lower (p<0.001) body mass than females and also had a lower body mass than both sexes of *M. brandtii* (p<0.001 in both cases). Females of *M. mystacinus* (p<0.001) had a lower body mass than females of *M. brandtii*, but did not differ (p=0.9) from males of *M. brandtii*. Detailed data on body mass are given in Table 1. In males, the overlap in body mass was 72.2% between *M. alcathoe* and *M. mystacinus*, 57.9% between *M. mystacinus* and *M. brandtii*, and 24.7% between *M. alcathoe* and *M. brandtii*. In females, it was 84.2% between *M. alcathoe* and *M. mystacinus*, 98.1% between *M. mystacinus* and *M. brandtii*, and 63.6% between *M. alcathoe* and *M. brandtii*.

External Quantitative Characters and Their Discrimination Power

The best model in DFA in the restricted dataset included sex ($F_{2, 69}$=4.93; p<0.01), forearm length ($F_{2, 69}$=14.55; p<0.0001), and body mass ($F_{2, 69}$=9.10; p<0.001), while the effect of tibia length was not significant ($F_{2, 69}$=2.20; p=0.12). Therefore, we built a new model including sex, forearm length and body mass only, which enabled us to considerably enlarge the analysed dataset (see Methods). All variables in a new model were also highly significant (sex: $F_{2, 299}$=11.10; p<0.0001; forearm length: $F_{2, 299}$=130.58; p<0.0001; body mass: $F_{2, 299}$=14.30; p<0.0001). Based on these three variables, the model correctly classified 94.3% of *M. alcathoe*, 70.9% of *M. mystacinus* and 68.7% of *M. brandtii*.

Forearm Length in Erroneously Determined Individuals

Forearm length of all *M. mystacinus* (n=6) and *M. brandtii* (n=3) misidentified as *M. alcathoe* was well bellow the mean values and confidence limits for the respective species. It ranged between 31.7–33.1 mm and 32.6–33.2 mm in misidentified *M. mystacinus* and *M. brandtii*, respectively. Forearm length of all *M. mystacinus* (n=5) misidentified as *M. brandtii* ranged between 34.8–36.1 mm, which is well within or slightly above the mean values and confidence limits of *M. mystacinus*.

Discussion

Our results showed that despite high precision in field identification, there were some misidentifications in our material and these were unevenly distributed among the three species of bats under

study. While no *M. alcathoe* was confused with the other two species, 11/132 individuals (8.3%) of *M. mystacinus* were confused with either *M. alcathoe* (4.5%) or *M. brandtii* (3.8%), and 3/128 individuals (2.3%) of *M. brandtii* were confused with *M. alcathoe*. This fact generally reflects the enormous phenotypic variation in the morpho-group, as well as the higher degree of overlap in external morphological characters between *M. mystacinus* and *M. brandtii* than between one of the latter species and *M. alcathoe*.

It is worth mentioning, however, that most of the erroneously identified bats (9/14) were captured and determined under busy circumstances, i.e. bat researchers were forced to quickly process a high number of individuals and, consequently, the error in determination could be higher than under usual conditions when a researcher can carefully inspect a captured bat. Furthermore, all individuals of *M. mystacinus* or *M. brandtii* that were confused with *M. alcathoe* were unusually small individuals and their size fell within the range of the latter species, which most probably affected the evaluation of the individual more than the other discrimination characters (e.g. ear colouration or dentition traits).

Spitzenberger et al. (2008) pointed out that as the fur and membrane colour of subadult individuals of *M. alcathoe* and *M. mystacinus* are similar, reliable records based on field identification should be restricted to adult individuals. However, despite our material consisted of a mixture of both adults and juveniles (the latter made up ca. 25% of all inds.), most of the misidentified bats in our analysis were adults. Hence we assume that the determination bias was mostly due to abnormal size (quantitative character) rather than to colouration (qualitative character).

Our analysis revealed that the three species significantly differ in forearm length, tibia length as well as body mass and that there is no overlap of forearm length values lying within 95% confidence limits when sex is taken into account. Application of these values as determination criteria may further improve the reliability of field determination for populations from Central Europe. Although the combination of sex, forearm length and body mass alone is not sufficient for a reliable discrimination among the three species (cf. ca. 69–94% correctness in determination by the results of DFA), inclusion of further discrimination characters (particularly the qualitative ones, see Introduction) may obviously largely improve precision as demonstrated by ca. 92–100% correct field determination in our study.

It is virtually useless to compare our measurements with those published for *M. mystacinus* prior to the end of 20th century (e.g. Hanák 1965, 1970, 1971, Benda & Tsytsulina 2000) as they most probably contain mixed data for *M. mystacinus* and *M. alcathoe* (see also Benda et al. 2003). However, it is possible to carry out such comparison for the other two species. The forearm and tibia lengths of *M. alcathoe* in our material are in accordance with the values reported from other European countries, e.g. Slovakia (Benda et al. 2003, Danko et al. 2010), Spain (Agirre-Mendi et al. 2004), Poland (Niermann et al. 2007, Bashta et al. 2011), Czech Republic (Řehák et al. 2008), Austria (Spitzenberger et al. 2008), Germany (Schorcht et al. 2009) and Ukraine (Bashta et al. 2011). The overall variation in forearm length was somewhat larger than reported in the original species diagnosis by von Helversen et al. (2001) but smaller than reported by Dietz et al. (2007). The upper limits of body mass in our material (6.8 g for females) exceeded the values given by von Helversen et al. (2001) as well as those by Dietz et al. (2007). However, the values at the upper limit of the range were obtained from pregnant females.

Also in *M. brandtii* our data on forearm and tibia lengths and body mass well correspond with the published information (e.g. Hanák 1965, 1970, 1971, Benda & Tsytsulina 2000, Dietz et al. 2007). Only the lower limit in forearm length in our material was somewhat smaller than in majority of the above cited studies.

Souhrn

Spolehlivost určení tří kryptických druhů netopýrů (*Myotis alcathoe, M. mystacinus, M. brandtii*) podle vnějších znaků a jejich základní biometrické údaje: zkušenosti z České republiky. Netopýr alkathoe, netopýr vousatý a netopýr Brandtův jsou nepříbuzné, ale morfologicky velmi podobné druhy žijící sympatricky na většině evropského území. Přestože v rámci terénní praxe jsou tyto druhy dnes víceméně rutinně odlišovány na základě vnější morfologie, doposud nebyla provedena žádná analýza, která by spolehlivost takovéhoto určování ověřila. Cílem naší studie bylo (1) pomocí molekulárně genetických metod ověřit správnost terénního určení druhu, (2) porovnat proměnlivost základních a v terénu standardně zaznamenávaných vnějších rozměrů (délka předloktí a holeně, tělesná hmotnost) a (3) ověřit spolehlivost jejich použití pro správné druhové určení na základě materiálu z území České republiky.

Čtrnáct z celkem 359 jedinců (3,9 %) těchto tří druhů bylo v terénu určeno nesprávně. Osm procent z počtu 113 jedinců původně určených jako *M. alcathoe* náleželo ve skutečnosti k jednomu ze dvou ostatních druhů (6 *M. mystacinus,* 3 *M. brandtii*). U všech jedinců (celkem 121) určených jako *M. mystacinus* byla správnost tohoto určení potvrzena. U pěti ze 125 jedinců (4 %) původně určených jako *M. brandtii* byla pomocí molekulárně genetických metod přiřazena druhová příslušnost k *M. mystacinus*. Většina chybně určených zvířat byla tvořena dospělci, avšak v případě záměn s *M. alcathoe* velikostně výrazně podprůměrnými jedinci. Dalším možným faktorem nesprávného určení (kromě samotné matoucí velikosti) mohla být skutečnost, že většina nesprávně identifikovaných jedinců byla určována během odchytů, při nichž bylo zpracováváno velké množství netopýrů, čímž mohla být míra pozornosti výzkumníků ovlivněna.

Všechny tři studované druhy se vzájemně významně lišily v délkách předloktí a holeně i v tělesné hmotnosti, přičemž vzájemný překryv byl vždy nejmenší mezi *M. alcathoe* a *M. brandtii*. Nejlepší model pro mezidruhovou diskriminaci zahrnoval pohlaví, délku předloktí a váhu jedince. Na základě diskriminační analysy byla s využitím těchto tří proměnných správnost druhové determinace 69–94 %. Tato skutečnost zdůrazňuje nutnost zohlednění nemetrických znaků (zbarvení srsti, čenichu, ucha, tvar a velikost tragu (kozlíku), penisu, a jednohrotých zubů), pro dosažení co možná nejvyšší míry spolehlivosti druhového určení těchto tří druhů.

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References

Agirre-Mendi P. T., García-Mudarra J. L., Juste J. & Ibáñez C., 2004: Presence of *Myotis alcathoe* Helversen & Heller, 2001 (Chiroptera: Vespertilionidae) in the Iberian Peninsula. *Acta Chiropterologica*, **6**: 49–57.

Bashta A. T., Piskorski M., Mysłajek R.W., Tereba A., Kurek K. & Sachanowicz K., 2011: *Myotis alcathoe* in Poland and Ukraine: new data on its status and habitat in Central Europe. *Folia Zoologica*, **60**: 1–4.

Benda P. & Tsytsulina K. A., 2000: Taxonomic revision of *Myotis mystacinus* group (Mammalia: Chiroptera) in the western Palearctic. *Acta Societatis Zoologicae Bohemiceae*, **64**: 331 – 398.

Benda P., Ruedi M. & Uhrin M., 2003: First record of *Myotis alcathoe* (Chiroptera: Vespertilionidae) in Slovakia. *Folia Zoologica*, **52**: 359–365.

Cornuet J. M., Piry S., Luikart G., Estoup A. & Solignac M., 1999: New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*, **153**:1989–2000

Danko Š., Krištín A. & Krištofík J., 2010: *Myotis alcathoe* in eastern Slovakia: occurrence, diet, ectoparasites and notes on its identification in the field. *Vespertilio*, **13–14**: 77–91.

Dietz C., von Helversen O. & Nill D., 2007: *Handbuch der Fledermäuse Europas und Nordwestafrikas*. Franckh-Kosmos Verlags GmBH & Co. KG, Stuttgart, 399 pp.

Gauckler A. & Kraus M., 1970: Kennzeichen und Verbreitung von *Myotis brandtii* (Eversman, 1845). *Zeitschrift fur Säugetierkunde*, **35**: 113–124.

Hanák V., 1965: Zur Systematik der Bartfledermaus *Myotis mystacinus* Kuhl, 1819 und über das Vorkommen von *Myotis ikonnikovi* Ognev, 1912 in Europa. *Věstník Československé Společnosti Zoologické*, **29**: 353 – 367.

Hanák V., 1970: Notes on the distribution and systematics of *Myotis mystacinus* Kuhl, 1819. *Bijdragen tot de Dierkunde*, **40**: 40 – 44.

Hanák V., 1971: *Myotis brandtii* (Eversmann, 1845) (Vespertilionidae, Chiroptera) in der Tschechoslowakei. *Věstník Československé Společnosti Zoologické*, **35**: 175–185.

von Helversen O., 1989: Bestimmungsschlüssel für die europäischen Fledermäuse nach äusseren Merkmalen. *Myotis*, **27**: 41–60.

von Helversen, O., K. G. Heller, F. Mayer, A. Nemeth, M. Volleth & Gombkőtő, P., 2001: Cryptic mammalian species: a new species of whiskered bat (*Myotis alcathoe* n. sp.) in Europe. *Naturwissenschaften*, **88**: 217–223.

Jan C. M. I., Frith K., Glover A. M., Butlin R. K., Scott C. D., Greenaway F., Ruedi M., Frantz A. C., Dawson D. A. & Altringham J. D., 2010: *Myotis alcathoe* confirmed in the UK from mitochondrial and microsatellite DNA. *Acta Chiropterologica*, **12**: 471–483.

Lučan R. K., Andreas M., Benda P., Bartonička T., Březinová T., Hoffmannová Š., Hulva P., Neckářová J., Reiter A., Svačina T., Šálek M. & Horáček I., 2009: Alcathoe bat (*Myotis alcathoe*) in the Czech Republic: distributional status, roosting and feeding ecology. *Acta Chiropterologica*, **11**: 61–69.

Niermann, N., Biedermann M., Bogdanowicz W., Brinkmann R., Le Bris Y., Ciechanowski M., Dietz C., Dietz I., Estok P., von Helversen O., Le Houedec A., Paksuz S., Petrov B. P., Özkan B., Piksa K., Rachwald A., Roue S. Y., K. Sachanowicz, Schorcht W., Tereba A. & Mayer, F., 2007: Biogeography of the recently described *Myotis alcathoe* von Helversen and Heller, 2001. *Acta Chiropterologica*, **9**: 361–378.

Řehák Z., Bartonička T., Bryja J. & Gaisler J., 2008: New records of the Alcathoe bat, *Myotis alcathoe* in Moravia (Czech Republic). *Folia Zoologica*, **57**: 465–19.

Ruedi M. & Mayer F., 2001: Molecular systematics of bats of the genus *Myotis* (Vespertilionidae) suggests deterministic ecomorphological convergences. *Molecular Phylogenetics and Evolution*, **21**: 436–448.

Schorcht W., Karst I. & Biedermann M., 2009: Die Nymphenfledermaus (*Myotis alcathoe* von Helversen & Heller, 2001) im Kyffhäusergebirge/Thüringen (Mammalia: Chiroptera) – aktuelle Kenntnisse zu Vorkommen und Habitatnutzung. *Vernate*, **28**: 115–129.

Spitzenberger F., Pavlinić I. & Podnar M., 2008: On the occurrence of the *Myotis alcathoe* von Helversen and Heller, 2001 in Austria. *Hystrix* (*n. s.*), **19**: 3–12.

Volleth M., 1987: Differences in the location of nucleolus organizer regions in European vespertilionid bats. *Cytogenetics and Cell Genetics*, **44**: 186–197.

Worthington Wilmer J. & Barratt E., 1996: A non-lethal method of tissue sampling for genetic studies of chiropterans. *Bat Research News*, **37**: 1–3.

Zima J. jr., Jandová M., Bryja J. & Lučan R. K., 2011: Využití mikrosatelitů ke stanovení populačně-genetických parametrů a determinaci druhů *alcathoe*, *mystacinus* a *brandtii* rodu *Myotis*, Vespertilionidae. P.: 256. In: Bryja J., Řehák Z. & Zukal J. (eds.): *Zoologické dny. Brno 2011. Sborník abstraktů z konference 17.–18. února 2011*. Ústav biologie obratlovců AV ČR, Brno, 282 pp.

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Development of multiplex microsatellite sets for noninvasive population genetic study of the endangered Tatra chamois

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Abstract. The only autochthonous population of Tatra chamois (*Rupicapra rupicapra tatrica*) occurs in the Tatra Mountains (northern Slovakia and southern Poland). Another population has been introduced to the Low Tatra Mts., while Alpine chamois (*R. r. rupicapra*) has been introduced to the neighbouring mountain ranges, Veľká Fatra and Slovenský raj. All these populations have undergone intensive bottlenecks. Any resulting low genetic variability would mean that only few genetic markers could be used for population genetic studies due to prevailing monomorphism. We tested 65 markers previously used in chamois or other Caprinae species, from which 20 most suitable loci for noninvasive genetic study of the Tatra chamois were selected. These polymorphic loci were used for optimisation of three multiplex sets and revealed a mean number of alleles of 2.1 and mean expected heterozygosity of 0.331 for the Tatra population. Low genetic diversity was also observed in the Low Tatra population while slightly higher values were obtained for Alpine chamois population in Slovenský raj. We subsequently assessed the amplification success rate for noninvasively obtained samples (faeces), which ranged from 85.1% to 92.7% for particular loci. The developed polymorphic microsatellite sets provide a unique tool for population genetic study of the endangered Tatra chamois, even when using noninvasive sampling, and is also suitable for Alpine chamois.

Key words: noninvasive genetic sampling, amplification success rate, ungulates

Introduction

Chamois (*Rupicapra* spp.) are mountain ungulates distributed over Southern and Central Europe, the Balkans, Turkey and the Caucasus (Shackleton 1997). As they inhabit only higher altitudes, their distribution is markedly fragmented and thus gene flow between populations is restricted. Under such conditions, populations within particular mountain regions may differentiate through mutation, selection and genetic drift (Slatkin 1987, Frankham et al. 2002). Chamois occurring in the Tatra Mountains (northern Slovakia and southern Poland) have been recognized as a separate subspecies, *Rupicapra rupicapra tatrica*, based on their morphological characteristics (Blahout 1972). Only one autochthonous population of Tatra chamois now exists, and this has been evolving

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separately from other chamois populations for at least 10000 years (Pérez et al. 2002, Jamrozy 2006). The population, therefore, can be regarded as an evolutionary significant unit (Crandall et al. 2000).

During the first half of the 20th century, this population underwent two significant bottlenecks, each time declining from around 1000-1500 to 200-300 individuals. Relatively low numbers were found over most of the second half of the century, with a further drop to ca. 200 individuals at the end of the century (Jurdíková 2000, Janiga & Zámečníková 2002, Jamrozy 2006). Consequently, in 2001, a conservation action plan was begun with the aim of saving the Tatra chamois from extinction (Koreň et al. 2001). Strong protection has led to an increase in the Tatra population, with recent estimate of ca. 700 individuals. Tatra chamois nevertheless remain classified as critically endangered in the IUCN Red List of Threatened Species (Aulagnier et al. 2008), even if the species as a whole is not considered endangered in Europe (Temple & Terry 2009).

As a consequence of the dramatic decrease in Tatra chamois abundance in the second half of 1960s, a 'reserve' population of this endangered subspecies was established in the Low Tatra Mts. of Slovakia in the 1970s (Radúch & Karč 1983). At present, the Low Tatra population has ca. 100 individuals (P. Bačkor, S. Ondruš pers. comm.). Nevertheless, in the early 1960s, i.e. prior to recognition of the Tatra chamois as a separate subspecies, Alpine chamois (*R. r. rupicapra*) were introduced into the Veľká Fatra and Slovenský raj Mts. of Slovakia. As these mountain ranges are geographically close to the Low Tatra Mts., hybridisation between the two subspecies may have occurred.

The disturbance of vulnerable Tatra chamois populations is highly undesirable, making it almost impossible to obtain a sufficient number of tissue samples. In such cases, noninvasive genetic sampling (e.g. Kohn & Wayne 1997, Taberlet et al. 1999) remains the only feasible method for studying genetic composition of populations. However, as PCR amplification of DNA extracted from noninvasively obtained material is still rather problematic (e.g. Pompanon et al. 2005, Waits & Paetkau 2005, Hájková et al. 2006, Beja-Pereira et al. 2009), the selection of markers with appropriate parameters is one of the most important factors affecting the success of analysis. Although a higher number of analysed loci reveals a more accurate description of genetic variability and structure, with noninvasive samples, a higher number of genotyped loci has the disadvantage of an increased risk of genotyping errors and higher costs (Waits & Leberg 2000). It is essential, therefore, that the most informative markers are identified. Furthermore, the length of amplified loci may also be important as shorter loci tend to be amplified with a higher success rate (Sefc et al. 2003, Buchan et al. 2005, Broquet et al. 2007). Lastly, DNA quantity is often very limited in noninvasive samples. If multiplex sets are developed, the study requires less DNA and time- and financial costs decrease (Skrbinšek et al. 2010).

In this study, we attempt to ascertain appropriate microsatellite markers in order to produce multiplex sets for the population genetic study of the Tatra chamois using noninvasive samples. Due to the risk of hybridisation with introduced Alpine chamois, the markers were also tested on this subspecies.

Material and Methods

Sixty-five microsatellite loci (Table 1 and 2) that had previously been used in other studies of chamois or other Caprinae species (e.g. Pérez et al. 2002, Maudet et al. 2004a, An et al. 2005, Cassar et al. 2007, Glowatzki-Mullis et al. 2008) were tested for amplification. Tissue samples were used in the tests, Tatra chamois samples being obtained from natural deaths and those of Alpine chamois from legal hunting. Singleplex PCRs were performed on a Mastercycler ep gradient S (Eppendorf) using a mixture of 1 μL of PCR buffer with 25 mM Mg^{2+}, 200 μM of each dNTP, 3.2 μg of BSA, 0.2 U of HotMaster Taq DNA Polymerase (Eppendorf), 0.25 μM of each primer (forward ones fluorescently labelled), 1 μL of extracted DNA and ddH_2O to a volume of 10 μL in each reaction. Cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 65°C for 60 s, and a final extension at 65°C for 10 min. The gradient of annealing temperatures was tested when necessary. PCR products were checked on 1.5% agarose gel by electrophoresis and, when a clear band was present, the products were electrophoresed on an ABI 3130 Genetic Analyzer with GeneScan 500 LIZ Size Standard and analysed using GENEMAPPER 3.7 (Applied Biosystems).

First, the microsatellite markers were tested using three Tatra and four Alpine chamois tissue samples. Loci that proved polymorphic in initial testing were subsequently genotyped using a more extensive set of 11, 5 and 22 samples from the Tatra, Low Tatra and Slovenský raj populations, respectively. Number of alleles (A), allelic richness (AR) and expected (H_E) and observed (H_O) heterozygosity were then calculated in order to characterise the polymorphism of the markers. The allelic richness for particular loci and populations was

computed using the rarefaction procedure implemented in FSTAT 2.9.3 (Goudet 2001), expected (unbiased estimate according to Nei 1978) and observed heterozygosity was calculated using GENETIX 4.05.2 (Belkhir et al. 1996–2004). Hardy-Weinberg equilibrium for particular loci in populations was tested using GENEPOP 3.4 (Raymond & Rousset 1995). We corrected for multiple testing with QVALUE software (Storey 2002) using the false discovery rate (FDR) approach (Benjamini & Hochberg 1995). Frequency of null alleles was computed using FREENA software (Chapuis & Estoup 2007).

Based on (i) the polymorphism rate of the locus, especially in the Tatra chamois populations, (ii) length of the amplified fragment, (iii) frequency of null alleles, and (iv) previous use of the marker in other studies of European chamois populations, the most suitable loci for noninvasive genotyping were selected. For all the populations studied, genotypic linkage disequilibrium between the selected microsatellites was tested using exact tests based on the Markov chain method implemented in GENEPOP 3.4. The FDR correction for multiple testing was done using QVALUE software. Unbiased probability of identity ($PI_{unbiased}$, corrected for small sample size) and probability of identity for siblings (PI_{sibs}) were calculated for selected loci using software GIMLET 1.3.3 (Valière 2002).

Three multiplex sets were designed from the selected loci and in addition, an ungulate species based SRY marker was included into the second multiplex set to enable sex identification (Wilson & White 1998); the microsatellite loci serving as a positive control of successful amplification. For all three sets, PCR was performed using the Qiagen Multiplex PCR Kit (Qiagen). Each reaction contained 5 μL of Multiplex PCR Master Mix, 1 μL of Q-Solution, primers at various concentrations (Table 2), 1 μL of extracted DNA and ddH_2O to a volume of 10 μL. Cycling conditions for the first set were as follows: an initial activation step at 95°C for 15 min, followed by 10 cycles of touch down PCR at 94°C for 30 s, 59°C−0.2°C per cycle for 90 s and 72°C for 60 s, followed by 28 cycles at 94°C for 30 s, 57°C for 90 s and 72°C for 60 s, with a final extension at 60°C for 30 min. The cycling conditions were the same for each set except annealing temperatures, i.e. 59°C−0.4°C per cycle for 90 s (10 ×) and 55°C for 90 s (28 ×) for the second set and 63°C−0.8°C per cycle for 90 s (10 ×) and 55°C for 90 s (28 ×) for the third set. The PCR products were electrophoresed on the ABI 3130 Genetic Analyzer and analysed as described above. Polymorphism of the multiplex sets was assessed based on 17, 5 and 22 tissue samples from the Tatra, Low Tatra and Slovenský raj Mts., respectively.

Subsequently, 347 samples of faeces obtained from specimens from all the Slovak chamois populations were analysed in order to assess the amplification success rate using the three multiplex sets. Very fresh faeces were collected in cold weather, usually from snow. These were either stored in 96% ethanol and put into a cool-box in the field and then into a freezer as soon as possible, or placed straight into silica gel and stored at room temperature. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) with washing of the pellet surface in the ASL buffer (Qiagen) for ca. 50 min as the first step. The PCR protocols, cycling conditions and fragment analysis were the same as those described for multiplex PCR of tissue samples, with 2 μL of extracted DNA used in the reaction instead of 1 μL. Two PCRs per sample and per multiplex set were performed, representing 670–694 PCR reactions for each multiplexed microsatellite locus. Subsequently the amplification success rate was assessed as a proportion of positive PCRs from all the PCRs performed. In this study, we did not aim at construction of consensus genotypes (based on multiple tubes approach, Taberlet et al. 1996), thus we were not able to assess the frequency of genotyping errors (i.e. allelic dropout and false alleles). Instead, we implemented comparison of two PCRs for every particular sample and locus as another indicator of reliability of the faecal DNA amplification using our protocols. Only the samples with both PCRs positive were taken into account, i.e. 276–321 PCR (552–642 allele) pairs were compared for particular loci and proportion of matching alleles was subsequently assessed. Furthermore, 'homozygotes' (i.e. samples that were genotyped as a homozygote for the identical allele in both PCR repetitions) were excluded and the proportion of matching alleles was computed from only the remaining ('heterozygote') PCRs, which represented 90–410 allele pairs for particular loci.

When using faecal DNA, we were not able to clearly amplify two of the loci, NRAMP1 and SRCRSP06, with any of the sets. For this reason, we excluded SRCRSP06 from faecal DNA analysis and NRAMP1 (the more informative locus) was amplified separately using the same PCR protocol and cycling conditions as for the initial testing of microsatellites (Eppendorf HotMaster Taq DNA Polymerase, annealing temperature 52°C), with only the amount of DNA extract used differing, i.e. 2 or 3 μL of faecal DNA in a reaction. Nevertheless, due to considerably lower success rate achieved using this protocol when compared to the

Table 1. *List of 45 microsatellite markers tested in this study but not selected for multiplex sets. n = number of tissue samples; A = number of alleles; AR = allelic richness (based on n = 5); H_E = expected heterozygosity – unbiased estimate according to Nei (1978); H_O = observed heterozygosity; Ref = reference. After the reference, the species/domesticated form for which the locus was developed is noted. All polymorphic loci were in Hardy-Weinberg equilibrium in all populations when p-values were corrected for multiple testing (FDR correction in QVALUE software).*

	Tatra chamois						Alpine chamois				
	Tatra Mts. (n = 11)			Low Tatra Mts. (n = 5)			Slovenský raj Mts. (n = 22)				
Locus	A/AR	H_E	H_O	A/AR	H_E	H_O	A/AR	H_E	H_O	Size (bp)	Ref
BM203	Monomorphic			Monomorphic			2/2.00	0.474	0.546	215–230	1)
BM848	2/2.00	0.524	0.636	3/3.00	0.511	0.600	Monomorphic			232–238	1)
BM1329	Monomorphic			Monomorphic			2/1.95	0.359	0.273	165–174	1)
BM1818	Monomorphic			Monomorphic			2/2.00	0.507	0.546	258–269	1)
BM4505	2/2.00	0.507	0.455	2/2.00	0.467	0.200	2/2.00	0.507	0.546	245–264	1)
HSC	Monomorphic			2/2.00	0.200	0.200	2/2.00	0.485	0.409	268–272	2)
ILSTS005	Monomorphic			Monomorphic			2/1.74	0.206	0.136	166–172	3)
ILSTS019	2/1.86	0.247	0.273	Monomorphic			2/2.00	0.474	0.364	168–191	4)
INFG = OarKP6	Monomorphic			Monomorphic			2/1.98	0.426	0.500	199–205	5)
INRA003	Monomorphic			Monomorphic			2/2.00	0.511	0.500	185–187	6)
INRA005	Monomorphic			Monomorphic			2/1.23	0.046	0.046	156–160	7)
INRA023*	2/2.00	0.525	0.375	2/2.00	0.467	0.600	Monomorphic			198–202	8)
INRA049	Monomorphic			Monomorphic			2/1.99	0.444	0.455	164–166	7)
McM218	2/2.00	0.520	0.546	3/3.00	0.600	0.600	3/2.92	0.639	0.773	278–298	9)
OarFCB11	Monomorphic			3/3.00	0.378	0.400	Monomorphic			156–160	10)
SRCRSP01	Monomorphic			Monomorphic			2/2.00	0.485	0.409	126–128	11)
SRCRSP08	2/1.99	0.416	0.364	2/2.00	0.533	0.400	3/2.94	0.661	0.546	225–243	12)
SY3A	2/1.99	0.416	0.364	2/2.00	0.533	0.400	Monomorphic			296–298	13)
SY12A	Monomorphic			2/2.00	0.200	0.200	2/1.55	0.130	0.136	189–193	13)
SY84B	Monomorphic			Monomorphic			2/1.93	0.333	0.318	215–223	13)
SY93	2/1.86	0.247	0.273	Monomorphic			2/1.98	0.426	0.409	86–90	13)
BM757	Monomorphic										1)
BMC1009	No PCR product										1)
HEL1	Non-specific PCR products										14)
ILSTS011	Monomorphic										15)
INRA036	No PCR product										7)
INRA040	Monomorphic										7)
INRA063**	Monomorphic on gel										7)
MAF65	No PCR product										16)
MAF70	Non-specific PCR products										17)
MB026	Monomorphic										18)
McM527	Monomorphic										19)
OarAE119	Monomorphic										20)
OarCP20	No PCR product										21)
OarCP34	Monomorphic										22)
SRCRSP12	No PCR product										23)
SRCRSP14	No PCR product										23)
SRCRSP15	No PCR product										23)
SRCRSP23	Non-specific PCR products										24)
SY3B	Non-specific PCR products										13)
SY12B	Monomorphic										13)
SY17	Monomorphic										13)
SY48	Non-specific PCR products										13)
SY50	Monomorphic										13)
SY242	Monomorphic										13)

** INRA023: corrected sequence of reverse primer as referred on http://projects.roslin.ac.uk/cdiv/markers.html (CaDBase, Roslin Institute, Edinburgh). ** INRA063: analysed for polymorphism only on 4% agarose gel.*

1) Bishop et al. 1994, cattle; 2) Blattman & Beh 1992, domestic sheep; 3) Brezinsky et al. 1993a, cattle; 4) Kemp et al. 1993, cattle; 5) Paterson & Crawford 2000, domestic sheep; 6) Vaiman et al. 1992, cattle; 7) Vaiman et al. 1994a, cattle; 8) Vaiman et al. 1994b, cattle; 9) Hulme et al. 1996, domestic sheep; 10) Buchanan & Crawford 1993, domestic sheep; 11) Arevalo et al. 1994, domestic goat; 12) Bhebhe et al. 1994, domestic goat; 13) An et al. 2005, Korean goral; 14) Kaukinen & Varvio 1993, cattle; 15) Brezinsky et al. 1993b, cattle; 16) Buchanan et al. 1992, domestic sheep; 17) Buchanan & Crawford 1992a, domestic sheep; 18) Cassar et al. 2007, cattle; 19) Hulme et al. 1994, domestic sheep; 20) Penty et al. 1993, domestic sheep; 21) Ede et al. 1995a, domestic sheep; 22) Ede et al. 1995b, domestic sheep; 23) Kogi et al. 1995, domestic goat; 24) Yeh et al. 1997, domestic goat.

multiplexed loci, the Qiagen Multiplex PCR Kit was later used also for the singleplex PCR of NRAMP1. The PCR protocol was the same as that described for multiplex sets, using 0.25 μM of each primer and 2 μL of faecal DNA extract. Cycling conditions differed from those described for the first multiplex set only during the annealing steps, i.e. touch down at 59°C−0.5°C per cycle for 90 s (10 ×) and 54°C for 90 s (28 ×). In total, 346 and 342 PCRs were analysed using the first ('Eppendorf HotMaster Taq DNA Polymerase') and the second ('Qiagen Multiplex PCR Kit') protocol, respectively. The amplification success rate was calculated for each protocol.

Results

From the 65 tested microsatellites, 41 loci proved polymorphic in at least one of studied populations (Table 1 and 2). Twenty-one loci (32.3%) were polymorphic in both Tatra chamois populations and 16 (24.6%) in all three populations. None of the loci deviated from Hardy-Weinberg equilibrium in any of the populations after FDR correction for multiple testing. Following the above described criteria, 20 loci deemed most suitable for noninvasive genetic analyses of population structure of Slovak chamois were selected (Table 2). No evidence of linkage disequilibrium was observed between any of the loci analysed, i.e. no pair of loci remained significant after FDR correction. Multi-loci $PI_{unbiased}$ for all 20 loci was 3.101×10^{-7}, 1.696×10^{-10} and 4.335×10^{-10} in Tatra, Low Tatra and Slovenský raj population, respectively, while multi-loci PI_{sibs} was 1.088×10^{-3}, 1.669×10^{-3} and 5.645×10^{-5} in the particular populations. Based on these calculations, nine, ten and seven most informative loci should be sufficient to find less than 1% of individuals with identical genotype in the Tatra, Low Tatra and Slovenský raj population, respectively, even if the individuals are closely related (siblings).

Analysis of tissue samples using three multiplex sets of selected loci revealed low genetic diversity in all the Slovak chamois populations studied (Table 2). Mean number of alleles and mean allelic richness calculated for the lowest sample size (n = 5) were 2.1 and 1.88 in the Tatra population, 2.0 and 1.95 in the Low Tatra population, and 2.4 and 2.25 in the Slovenský raj population, respectively. Expected heterozygosity was also lower in endemic Tatra chamois (0.331 in the Tatra Mts. and 0.341 in the Low Tatra Mts.) as compared to Alpine chamois in the Slovenský raj Mts. (0.460). Genetic identification of sex (using SRY marker) was consistent with morphological assessments, which were undertaken after legal hunting or, if possible, after finding of carcasses.

Success rate of faecal DNA amplification was 90.7%, ranging from 85.1% to 92.7% for particular loci, and was 91.5%, 91.5% and 88.4% for the first, second, and third set, respectively. The first and second PCR of individual faecal samples matched in 98.8% of the total number of alleles and the proportion of matching alleles ranged from 97.1% to 99.8% for particular loci. When 'homozygotes' were excluded, the proportion of matching alleles remained very high. The first and second PCR of 'heterozygous loci' matched in 97.1% of alleles and the values for the particular loci ranged from 94.5% to 99.7%, with the exception of locus SY58 which reached only 88.8%. Amplification success rate of 36.7% was detected in singleplex PCR of NRAMP1 locus using the 'Eppendorf HotMaster Taq DNA Polymerase' protocol. Application of the Qiagen Multiplex PCR Kit increased the proportion of positive PCRs to 91.5%.

Discussion

Low genetic variability was found in all the studied Slovak chamois populations, with lower values in both autochthonous and introduced population of Tatra chamois when compared to the Slovenský raj population of Alpine chamois. This result was obtained despite the fact that polymorphism in the Tatra chamois was one of the criteria for microsatellite selection, and that the Slovenský raj population was established through the introduction of a very low number of individuals. Slightly higher values for Tatra chamois, especially for the Low Tatra population, were found in the study of Crestanello et al. (2009), ascertaining $H_E = 0.33$ and 0.41 and $A = 2.18$ and 2.82 in the Tatra and Low Tatra population, respectively, when six and nine samples from the populations were analysed. This probably resulted from the different microsatellite markers used as well as different, and rather small, number of individuals analysed in those two studies.

Low genetic diversity is obviously not unusual in populations of highly endangered mountain ungulates that have suffered dramatic declines in their demographic history. Lorenzini (2005) observed a similarly low level of polymorphism (only 10 polymorphic microsatellites from 60 tested, all but one with only two alleles, although with slightly higher heterozygosity values) in Apennine chamois (*R. pyrenaica ornata*), which became nearly extinct in the late 1940s (less than 100 animals). Very low genetic variability was also recorded for alpine populations of ibex (*Capra ibex*) by Maudet et al. (2002), who rank their results (mean H_E = 0.29–0.45, but only 0.13

Table 2. *Characteristics of 20 multiplexed microsatellite markers found suitable for noninvasive genetic sampling study of Slovak chamois populations (+ ungulate species based SRY marker). n = number of tissue samples; A = number of alleles; AR = allelic richness (based on n = 5); H_E = expected heterozygosity – unbiased estimate according to Nei (1978); H_O = observed heterozygosity; Set = multiplex set in which the locus was included; FC = final concentration of primers in multiplex PCR; Dye = fluorescent dye of forward primer. After the reference, the species/domesticated form for which the locus was developed is noted – sheep = domestic sheep, goat = domestic goat, goral = Korean goral. NRAMP1 and SRCRSP06 loci were only amplified in the third multiplex set when tissue samples were analysed; for analyses of faeces, NRAMP1 was amplified in singleplex PCR and SRCRSP06 was not used. All loci were in Hardy-Weinberg equilibrium in all populations when p-values were corrected for multiple testing (FDR correction in QVALUE software).*

	Tatra chamois						Alpine chamois							
	Tatra Mts. (n = 17)			Low Tatra Mts. (n = 5)			Slovenský raj (n = 22)							
Locus	A/AR	H_E	H_O	A/AR	H_E	H_O	A/AR	H_E	H_O	Set	FC (μM)	Dye	Size (bp)	Reference
BM1258	2/1.77	0.214	0.118	2/2.00	0.200	0.200	3/2.74	0.517	0.455	3	0.08	VIC	111–124	Bishop et al. 1994, cattle
BOBT24	2/2.00	0.499	0.471	2/2.00	0.467	0.200	3/2.89	0.630	0.455	2	0.25	VIC	161–174	Buitkamp et al. 1996, cattle
CSSM66	3/2.94	0.656	0.765	3/3.00	0.600	0.600	2/2.00	0.495	0.455	1	0.20	FAM	193–227	Barendse et al. 1994, cattle
ETH10	2/2.00	0.487	0.529	2/2.00	0.200	0.200	2/1.90	0.304	0.273	1	0.20	NED	206–208	Toldo et al. 1993, cattle
ETH225	2/2.00	0.513	0.471	2/2.00	0.533	0.400	3/2.86	0.635	0.682	3	0.10	FAM	142–150	Steffen et al. 1993, cattle
ILSTS030	3/2.62	0.437	0.471	2/2.00	0.533	0.800	3/2.73	0.563	0.546	3	0.50	PET	160–169	Kemp et al. 1995, cattle
INRA121	2/2.00	0.508	0.412	2/2.00	0.467	0.600	1/1.00	0	0	2	0.10	FAM	109–111	Vaiman et al. 1994a, cattle
MAF214	2/2.00	0.499	0.706	2/2.00	0.467	0.200	3/2.88	0.621	0.455	3	0.80	PET	214–252	Buchanan & Crawford 1992b, sheep
NRAMP1	1/1.00	0	0	2/2.00	0.356	0.400	3/2.39	0.487	0.409	3	0.30	VIC	195–213	Matthews & Crawford 1998, sheep
OarFCB20	2/1.51	0.114	0.118	2/2.00	0.200	0.200	3/2.69	0.513	0.636	1	0.80	PET	81–101	Buchanan et al. 1994, sheep
OarFCB304	4/2.25	0.271	0.294	1/1.00	0	0	3/2.74	0.595	0.455	1	0.10	FAM	127–148	Buchanan & Crawford 1993, sheep
SRCRSP05	2/1.66	0.166	0.177	1/1.00	0	0	2/2.00	0.502	0.500	1	0.15	VIC	163–169	Arevalo et al. 1994, goat
SRCRSP06	2/1.77	0.214	0.235	2/2.00	0.200	0.200	1/1.00	0	0	3	0.60	PET	140–142	Bhebhe et al. 1994, goat
SRCRSP09	2/1.98	0.401	0.529	2/2.00	0.556	0.600	2/1.55	0.130	0.136	3	0.30	NED	124–132	Bhebhe et al. 1994, goat
SRCRSP11	2/2.00	0.471	0.471	2/2.00	0.356	0.400	2/2.00	0.474	0.364	2	0.90	PET	114–120	Kogi et al. 1995, goat
SY58	1/1.00	0	0	2/2.00	0.356	0.400	3/2.81	0.622	0.500	1	0.15	PET	199–203	An et al. 2005, goral
SY84	1/1.00	0	0	2/2.00	0.200	0.200	2/2.00	0.495	0.546	1	0.70	NED	172–176	An et al. 2005, goral
SY259	2/1.29	0.059	0.059	2/2.00	0.200	0.200	2/1.99	0.460	0.500	2	0.10	FAM	157–163	An et al. 2005, goral
SY434	3/2.92	0.628	0.647	2/2.00	0.467	0.600	3/2.89	0.647	0.682	2	0.30	NED	84–100	An et al. 2005, goral
TGLA53	2/2.00	0.487	0.177	2/2.00	0.467	0.600	2/2.00	0.502	0.682	2	0.40	VIC	139–143	Barendse et al. 1994, cattle
SRY	-	-	-	-	-	-	-	-	-	2	0.15	PET	180	
mean*	2/1.88	0.331	0.332	2/1.95	0.341	0.350	2/2.25	0.460	0.436					

** Mean values were calculated including monomorphic loci.*

when monomorphic loci were included) amongst the lowest reported from microsatellites in mammal species. These results all highlight the significance of demographic history (especially population bottlenecks and prolonged periods at low numbers) in affecting genetic variability within a population.

We reached a very high amplification success rate (90.7%) when faecal DNA was analysed, which, in general, is not typical for noninvasive genetic sampling. However, also other noninvasive studies of ungulates report very high success rate. Flagstad et al. (1999) analysed reindeer (*Rangifer tarandus*) and domestic sheep (*Ovis aries*) faecal samples with 95% and 96.5% PCR success rate, respectively. Further, 99% and 95% of Corsican mouflon (*Ovis musimon*) and Alpine ibex (*Capra ibex*) genotyping repetitions, respectively, provided correct genotype when winter samples were examined (Maudet et al. 2004b). Even in Central Africa (Gabon), where climate enhances quick DNA degradation, forest ungulate faeces of ≥ 50 pg/μL DNA concentration were analysed with 83% amplification success or greater (Soto-Calderón et al. 2009). Although in some other mammals, e.g. in Asian elephant (*Elephas maximus*), very high PCR success was achieved (99.6%, Fernando et al. 2003), usually in the non-ungulate species the success is lower (for overview of amplification success rate in extensive set of mammal species see Broquet et al. (2007) and Hájková et al. (2009).

Many factors influence quality and quantity of target DNA in a sample as well as our ability to utilise this DNA during the analyses (e.g. Waits & Paetkau 2005, Broquet et al. 2007). In ungulates, that produce pellet-like faeces, this can be an important factor for high success of the analyses, as it is possible to wash surface of the pellet and thus utilise only outer part of the sample, which seems to be very efficient (Flagstad et al. 1999, Wehausen et al. 2004, Stenglein et al. 2010). Also in the study of Fernando et al. (2003) the outer parts of elephants' dungs were collected by scraping of the surface and used for analysis. Together with the other factors that have strong impact on PCR success, we found that also chemicals, not only those used for sample storage or DNA extraction (e.g. Piggott & Taylor 2003), but also PCR chemicals may affect the success rate significantly. In our study, the use of Qiagen Multiplex PCR Kit increased PCR success in NRAMP1 locus two and a half times.

High proportion of matching alleles between two PCR repetitions in faecal samples – 97.1% (when 'homozygotes' were excluded) – may indicate future low allelic dropout in our data. This is in concordance with high amplification success rate achieved. Locus SY58 showed lower proportion (88.8%) of matching alleles than other loci, thus higher frequency of allelic dropout is expected in this case. This might be an effect of higher length of the locus (Buchan et al. 2005); another possible reason is mutation in primer sequence of one/some of the alleles and thus lower frequency of its/their amplification in faecal DNA analysis (Okello et al. 2005).

Through the testing of an extended set of microsatellite markers that had previously been used in studies of chamois or other Caprinae, we were able to identify those that showed the highest level of polymorphism in Slovak chamois populations. These proved suitable for studies of population structure and demographic history of the highly endangered Tatra chamois. As we considered amplification fragment length through a marker selection process, we were able to develop a unique tool enabling the study of our target species using noninvasive genetic sampling, the only feasible method for large scale analyses of population structure, e.g. using a landscape genetic approach. The usability of our sets in faecal samples was confirmed also through achievement of high amplification success rate.

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Literature

An J., Min M.-S., Sommer J., Louis E., Brenneman R., Kwon S.-W., Shin N.S. & Lee H. 2005: Isolation and characterization of 15 microsatellite loci in the Korean goral (*Nemorhaedus caudatus*). *Mol. Ecol. Notes 5: 421–423.*

Arevalo E., Holder D.A., Derr J.N., Bhebhe E., Linn R.A., Ruvuna F., Davis S.K. & Taylor J.F. 1994: Caprine microsatellite dinucleotide repeat polymorphisms at the *SR-CRSP-1*, *SR-CRSP-2*, *SR-CRSP-3*, *SR-CRSP-4*, *SR-CRSP-5* loci. *Anim. Genet. 25: 202.*

Aulagnier S., Giannatos G. & Herrero J. 2008: *Rupicapra rupicapra*. In: IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1. *Downloaded on 07 August 2009. www.iucnredlist.org*

Barendse W., Armitage S.M., Kossarek L.M., Shalom A., Kirkpatrick B.W., Ryan A.M., Clayton D., Li L., Neibergs H.L., Zhang N., Grosse W.M., Weiss J., Creighton P., McCarthy F., Ron M., Teale A.J., Fries R., McGraw R.A., Moore S.S., Georges M., Soller M., Womack J.E. & Hetzel D.J.S. 1994: A genetic linkage map of the bovine genome. *Nature Genet. 6: 227–235.*

Beja-Pereira A., Oliveira R., Alves P.C., Schwartz M.K. & Luikart G. 2009: Advancing ecological understandings through technological transformations in noninvasive genetics. *Mol. Ecol. Res. 9: 1279–1301.*

Belkhir K., Borsa P., Chikhi L., Raufaste N. & Bonhomme F. 1996–2004: GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. *Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier.*

Benjamini Y. & Hochberg Y. 1995: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. B, Statistical Methodology* 57*: 289–300.*

Bhebhe E., Kogi J., Holder D.A., Arevalo E., Derr J.N., Linn R.A., Ruvuna F., Davis S.K. & Taylor J.F. 1994: Caprine microsatellite dinucleotide repeat polymorphisms at the *SR-CRSP-6*, *SR-CRSP-7*, *SR-CRSP-8*, *SRCRSP-9* and *SR-CRSP-10* loci. *Anim. Genet. 25: 203.*

Bishop M.D., Kappes S.M., Keele J.W., Stone R.T., Sunden S.L.F., Hawkins G.A., Toldo S.S., Fries R., Grosz M.D., Yoo J. & Beattie C.W. 1994: A genetic linkage map for cattle. *Genetics 136: 619–639.*

Blahout M. 1972: Zur Taxonomie der Population von *Rupicapra rupicapra* (Linné, 1785) in der Hohen Tatra. *Zool. listy 21: 115–132.*

Blattman A.N. & Beh K.J. 1992: Dinucleotide repeat polymorphism within the ovine major histocompatibility complex. *Anim. Genet. 23: 392.*

Brezinsky L., Kemp S.J. & Teale A.J. 1993a: *ILSTS005*: a polymorphic bovine microsatellite. *Anim. Genet. 24: 73.*

Brezinsky L., Kemp S.J. & Teale A.J. 1993b: Five polymorphic bovine microsatellites (*ILSTS010-014*). *Anim. Genet. 24: 75–76.*

Broquet T., Ménard N. & Petit E. 2007: Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conserv. Genet. 8: 249–260.*

Buchan J.C., Archie E.A., van Horn R.C., Moss C.J. & Alberts S.C. 2005: Locus effects and sources of error in noninvasive genotyping. *Mol. Ecol. Notes 5: 680–683.*

Buchanan F.C. & Crawford A.M. 1992a: Ovine dinucleotide repeat polymorphism at the *MAF70* locus. *Anim. Genet. 23: 185.*

Buchanan F.C. & Crawford A.M. 1992b: Ovine dinucleotide repeat polymorphism at the *MAF214* locus. *Anim. Genet. 23: 394.*

Buchanan F.C. & Crawford A.M. 1993: Ovine microsatellites at the *OarFCB11*, *OarFCB128*, *OarFCB193*, *OarFCB266* and *OarFCB304* loci. *Anim. Genet. 24: 145.*

Buchanan F.C., Galloway S.M. & Crawford A.M. 1994: Ovine microsatellites at the *OarFCB5*, *OarFCB19*, *OarFCB20*, *OarFCB48*, *OarFCB129* and *OarFCB226* loci. *Anim. Genet. 25: 60.*

Buchanan F.C., Swarbrick P.A. & Crawford A.M. 1992: Ovine dinucleotide repeat polymorphism at the *MAF65* locus. *Anim. Genet. 23: 85.*

Buitkamp J., Obexer-Ruff G., Kessler M. & Epplen J.T. 1996: A microsatellite (*BOBT24*) located between the bovine *IL4* and *IL13* loci is polymorphic in cattle and goat. *Anim. Genet. 27: 212–213.*

Cassar S., Galan M. & Loison A. 2007: A set of 21 polymorphic microsatellites in Alpine chamois (*Rupicapra rupicapra*). *Mol. Ecol. Notes 7: 243–247.*

Chapuis M.P. & Estoup A. 2007: Microsatellite null alleles and estimation of population differentiation. *Mol. Biol. Evol. 24: 621–631.*

Crandall K.A., Bininda-Emonds O.R.P., Mace G.M. & Wayne R.K. 2000: Considering evolutionary processes in conservation biology. *Trends Ecol. Evol. 15: 290–295.*

Crestanello B., Pecchioli E., Vernesi C., Mona S., Martínková N., Janiga M., Hauffe H.C. & Bertorelle G. 2009: The genetic impact of translocations and habitat fragmentation in chamois (*Rupicapra*) spp. *J. Hered. 100: 691–708.*

Ede A.J., Pierson C.A. & Crawford A.M. 1995a: Ovine microsatellites at the *OarCP9*, *OarCP16*, *OarCP20*, *OarCP21*, *OarCP23* and *OarCP26* loci. *Anim. Genet. 26: 129–130.*

Ede A.J., Pierson C.A. & Crawford A.M. 1995b: Ovine microsatellites at the *OarCP34*, *OarCP38*, *OarCP43*, *OarCP49*, *OarCP73*, *OarCP79* and *OarCP99* loci. *Anim. Genet. 26: 130–131.*

Fernando P., Vidya T.N.C., Rajapakse C., Dangolla A. & Melnick D.J. 2003: Reliable noninvasive genotyping: Fantasy or reality? *J. Hered. 94: 115–123.*

Flagstad Ø., Røed K., Stacy J.E. & Jakobsen K.S. 1999: Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. *Mol. Ecol. 8: 879–883.*

Frankham R., Ballou J.D. & Briscoe D.A. 2002: Introduction to conservation genetics. *Cambridge University Press, Cambridge, UK.*

Glowatzki-Mullis M.-L., Muntwyler J., Bäumle E. & Gaillard C. 2008: Genetic diversity measures of Swiss goat breeds as decision-making support for conservation policy. *Small Ruminant Res. 74: 202–211.*

Goudet J. 2001: FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). *http://www.unil.ch/izea/softwares/fstat.html*

Hájková P., Zemanová B., Bryja J., Hájek B., Roche K., Tkadlec E. & Zima J. 2006: Factors affecting success of PCR amplification of microsatellite loci from otter faeces. *Mol. Ecol. Notes 6: 559–562.*

Hájková P., Zemanová B., Roche K. & Hájek B. 2009: An evaluation of field and noninvasive genetic methods for estimating Eurasian otter population size. *Conserv. Genet. 10: 1667–1681.*

Hulme D.J., Davies K.P., Beh K.J. & Maddox J.F. 1996: Ovine dinucleotide repeat polymorphism at the *McM218*, *McM150* and *McM138* loci. *Anim. Genet. 27: 57–64.*

Hulme D.J., Silk J.P., Redwin J.M., Barendse W. & Beh K.J. 1994: Ten polymorphic ovine microsatellites. *Anim. Genet. 25: 434–435.*

Jamrozy G. 2006: The Tatra chamois *Rupicapra rupicapra tatrica* Blahout, 1971 – environmental and population changes, from the Pleistocene until the present time. *Nat. Conserv. 62: 53–62.*

Janiga M. & Zámečníková H. 2002: Zoologická charakteristika historických údajov o kamzíkoch (*Rupicapra rupicapra tatrica* Blahout, 1971) v Tatrách ako podklad pre vyhodnotenie ich súčasnej početnosti [Zoological characteristics of the historical data on chamois (*Rupicapra rupicapra tatrica* Blahout, 1971) as a base for the evaluation of their current abundance in the Tatra Mountains]. In: Janiga M. & Švajda J. (eds.), Ochrana kamzíka [Chamois protection]. *TANAP, NAPANT, IHAB: 99–182. (in Slovak with English summary)*

Jurdíková N. 2000: The decline of the Tatra chamois. *Caprinae Newsletter of the IUCN/SSC Caprinae Specialist Group: 4–6.*

Kaukinen J. & Varvio S.-L. 1993: Eight polymorphic bovine microsatellites. *Anim. Genet. 24: 148.*

Kemp S.J., Brezinsky L. & Teale A.J. 1993: A panel of bovine, ovine and caprine polymorphic microsatellites. *Anim. Genet. 24: 363–365.*

Kemp S.J., Hishida O., Wambugu J., Rink A., Longeri M.L., Ma R.Z., Da Y., Lewin H.A., Barendse W. & Teale A.J. 1995: A panel of polymorphic bovine, ovine and caprine microsatellite markers. *Anim. Genet. 26: 299–306.*

Kogi J., Yeh C.C., Bhebhe E., Burns B.M., Ruvuna F., Davis S.K. & Taylor J.F. 1995: Caprine microsatellite dinucleotide repeat polymorphisms at the *SR-CRSP-11*, *SR-CRSP-12*, *SR-CRSP-13*, *SR-CRSP-14* and *SR-CRSP-15* loci. *Anim. Genet. 26: 449.*

Kohn M.H. & Wayne R.K. 1997: Facts from feces revisited. *Trends Ecol. Evol. 12: 223–227.*

Koreň M., Radúch J., Chovancová B., Šturcel M., Kováč J., Gašinec I., Ksiažek J., Vančura V., Hummel M. & Ondruš S. 2001: Program záchrany kamzíka vrchovského tatranského na roky 2001–2005 [Conservation action plan for Tatra chamois for 2001–2005]. *Tatranská Lomnica. (in Slovak)*

Lorenzini R. 2005: A panel of polymorphic microsatellites in the threatened Apennine chamois (*Rupicapra pyrenaica ornata*). *Mol. Ecol. Notes 5: 372–374.*

Matthews G.D. & Crawford A.M. 1998: Cloning, sequencing and linkage mapping of the NRAMP1 gene of sheep and deer. *Anim. Genet. 29: 1–6.*

Maudet C., Beja-Pereira A., Zeyl E., Nagash H., Kence A., Özüt D., Biju-Duval M.-P., Boolormaa S., Coltman

D.W., Taberlet P. & Luikart G. 2004a: A standard set of polymorphic microsatellites for threatened mountain ungulates (Caprini, Artiodactyla). *Mol. Ecol. Notes 4: 49–55.*

Maudet C., Luikart G., Dubray D., Von Hardenberg A. & Taberlet P. 2004b: Low genotyping error rates in wild ungulate faeces sampled in winter. *Mol. Ecol. Notes 4: 772–775.*

Maudet C., Miller C., Bassano B., Breitenmoser-Würsten C., Gauthier D., Obexer-Ruff G., Michallet J., Taberlet P. & Luikart G. 2002: Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in Alpine ibex [*Capra ibex (ibex)*]. *Mol. Ecol. 11: 421–436.*

Nei M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics 89: 583–590.*

Okello J.B.A., Wittemeyer G., Rasmussen H.B., Douglas-Hamilton I., Nyakaana S., Arctander P. & Siegismund H.R. 2005: Noninvasive genotyping and Mendelian analysis of microsatellites in African savannah elephants. *J. Hered. 96: 679–687.*

Paterson K.A. & Crawford A.M. 2000: Ovine microsatellite *OarKP6* isolated from a BAC containing the ovine interferon gamma gene. *Anim. Genet. 31: 343.*

Penty J.M., Henry H.M., Ede A.J. & Crawford A.M. 1993: Ovine microsatellites at the *OarAE16*, *OarAE54*, *OarAE57*, *OarAE119* and *OarAE129* loci. *Anim. Genet. 24: 219.*

Pérez T., Albornoz J. & Domínguez A. 2002: Phylogeography of chamois (*Rupicapra* spp.) inferred from microsatellites. *Mol. Phylogenet. Evol. 25: 524–534.*

Piggott M.P. & Taylor A.C. 2003: Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. *Aust. J. Zool. 51: 341–355.*

Pompanon F., Bonin A., Bellemain E. & Taberlet P. 2005: Genotyping errors: causes, consequences and solutions. *Nature Rev. Genet. 6: 847–859.*

Radúch J. & Karč P. 1983: Súčasný stav a perspektívy kamzíčej populácie v Národnom parku Nízke Tatry [Present state and perspectives of the chamois population in the National Park of the Low Tatras]. *Zborník prác o Tatranskom národnom parku 24: 61–82. (in Slovak with English summary)*

Raymond M. & Rousset F. 1995: GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered. 86: 248–249.*

Sefc K.M., Payne R.B. & Sorenson M.D. 2003: Microsatellite amplification from museum feather samples: effects of fragment size and template concentrations on genotyping errors. *Auk 120: 982–989.*

Shackleton D.M. (ed.) IUCN/SSC Caprinae Specialist Group 1997: Wild sheep and goats and their relatives. Status survey and conservation Action Plan for Caprinae. *IUCN, Gland, Switzerland and Cambridge, UK.*

Skrbinšek T., Jelenčič M., Waits L., Kos I. & Trontelj P. 2010: Highly efficient multiplex PCR of noninvasive DNA does not require pre-amplification. *Mol. Ecol. Res. 10: 495–501.*

Slatkin M. 1987: Gene flow and the geographic structure of natural populations. *Science 236: 787–792.*

Soto-Calderón I.D., Ntie S., Mickala P., Maiseles F., Wickings E.J. & Anthony N.M. 2009: Effects of storage type and time on DNA amplification success in tropical ungulate faeces. *Mol. Ecol. Res. 9: 471–479.*

Steffen P., Eggen A., Dietz A.B., Womack J.E., Stranzinger G. & Fries R. 1993: Isolation and mapping of polymorphic microsatellites in cattle. *Anim. Genet. 24: 121–124.*

Stenglein J.L., De Barba M., Ausband D.E. & Waits L.P. 2010: Impacts of sampling location within a faeces on DNA quality in two carnivore species. *Mol. Ecol. Res. 10: 109–114.*

Storey J.D. 2002: A direct approach to false discovery rates. *J. Roy. Stat. Soc. B, Statistical Methodology 64: 479–498.*

Taberlet P., Griffin S., Goossens B., Questiau S., Manceau V., Escaravage N., Waits L.P. & Bouvet J. 1996: Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res. 24: 3189–3194.*

Taberlet P., Waits L.P. & Luikart G. 1999: Noninvasive genetic sampling: look before you leap. *Trends Ecol. Evol. 14: 323–327.*

Temple H.J. & Terry A. 2009: European mammals: Red List status, trends, and conservation priorities. *Folia Zool. 58: 248–269.*

Toldo S.S., Fries R., Steffen P., Neibergs H.L., Barendse W., Womack J.E., Hetzel D.J.S. & Stranzinger G. 1993: Physically mapped, cosmid-derived microsatellite markers as anchor loci on bovine chromosomes. *Mamm. Genome 4: 720–727.*

Vaiman D., Imam-Ghali M., Moazami-Goudarzi K., Guérin G., Grohs C., Levéziel H. & Saïdi-Mehtar N. 1994b:

Conservation of syntenic group of microsatellite loci between cattle and sheep. *Mamm. Genome 5: 310–314.*

Vaiman D., Mercier D., Moazami-Goudarzi K., Eggen A., Ciampolini R., Lépingle A., Velmala R., Kaukinen J., Varvio S.L., Martin P., Levéziel H. & Guérin G. 1994a: A set of 99 cattle microsatellites: characterization, synteny mapping, and polymorphism. *Mamm. Genome 5: 288–297.*

Vaiman D., Osta R., Mercier D., Grohs C. & Levéziel H. 1992: Characterization of five new bovine dinucleotide repeats. *Anim. Genet. 23: 537–541.*

Valière N. 2002: GIMLET: a computer program for analysing genetic individual identification data. *Mol. Ecol. Notes 2: 377–379.*

Waits J.L. & Leberg P.L. 2000: Biases associated with population estimation using molecular tagging. *Anim. Conserv. 3: 191–199.*

Waits L.P. & Paetkau D. 2005: Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *J. Wildl. Manage. 69: 1419–1433.*

Wehausen J.D., Ramey R.R. & Epps C.W. 2004: Experiments in DNA extraction and PCR amplification from bighorn sheep feces: the importance of DNA extraction method. *J. Hered. 95: 503–509.*

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 Sc. 43: 477–482.*

Yeh C.C., Kogi J.K., Holder M.T., Guerra T.M., Davis S.K. & Taylor J.F. 1997: Caprine microsatellite dinucleotide repeat polymorphisms at the *SR-CRSP21*, *SR-CRSP22*, *SR-CRSP23*, *SR-CRSP24*, *SR-CRSP25*, *SR-CRSP26* and *SR-CRSP27* loci. *Anim. Genet. 28: 380–381.*

NOTE

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Characterization of ten polymorphic microsatellite markers for an endangered butterfly *Argynnis niobe* and their cross-species utility in the closely related species *A. adippe* (Lepidoptera: Nymphalidae)

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Abstract. The Niobe Fritillary, *Argynnis niobe*, is a habitat specialist and as a consequence is highly endangered in contemporary Europe. To investigate its genetic diversity and population structure, 10 polymorphic microsatellite loci were developed and characterized, using a recently developed pyrosequencing method. The number of alleles per locus ranged from 2 to 21, and the observed and expected heterozygosities varied from 0.17 to 0.53 and from 0.24 to 0.92, respectively. These loci were also successfully used to study the genetic diversity of a closely related species, the High Brown Fritillary, *Argynnis adippe*, and will be used in future population structure studies of both these species.

INTRODUCTION

Molecular markers are increasingly used in insect conservation biology as they are a cheaper and more reliable way of estimating critical population parameters important for population management, such as intra-population genetic diversity, gene flow, spatial population differentiation and effective population size (Amos & Balmford, 2001; Palsboll et al., 2007). By using these techniques, researchers can consider scales that are much larger than the local scales of pre-molecular population ecology (Sigaard et al., 2008; Brattstrom et al., 2010) and make it possible to compare the genetic makeup over large regions (Finger et al., 2009; Habel et al., 2010), even up to continent-wide comparisons in order to unravel the colonization-extinction history within the current ranges of species (Todisco et al., 2010; Zachos & Hartl, 2011; Runquist et al., 2012). Microsatellite markers are particularly widely used because they are co-dominant, hyper variable, mostly neutral and reproducible (Jarne & Lagoda, 1996). Despite these advantages microsatellite markers have not been widely used in studies on some well studied model groups, notably butterflies and other Lepidoptera, because the sequences flanking microsatellites in this group are very similar for different loci. These similarities are generated by recombination mediated events, such as unequal crossing-over or gene conversion and through transposition of mobile elements (Van't Hof et al., 2007). These problems have now been resolved thanks to next-generation sequencing methodology, which allow the rapid identification of large numbers of bioinformatically variable loci, without the necessity of laborious and costly cloning (Vandewoestijne et al., 2012).

Perhaps due to the difficulties stated above and associated high costs, population genetic studies using microsatellites have so far targeted species with only a subset of potentially diverse lepidopteran population structures. The majority of the species so far studied are sedentary, forming high-density populations in discrete patches of habitat, which are interconnected, to varying extents, by gene flow (e.g., Keyghobadi et al., 1999; Harper et al., 2000; Cassel, 2002; Zeisset et al., 2005; Sarhan, 2006; Habel et al., 2008). As a consequence of habitat loss and population isolation, such species frequently suffer declines of within-population genetic diversity caused by inbreeding and/or genetic drift (Habel et al., 2011), making it important in the context of the conservation of these species to consider their population genetic structure. The population structures of these mainly sedentary species, however, were rather well rendered by pre-molecular approaches, such as mark-recapture, and the molecular genetics results often tended to elaborate what was already expected. There are studies on species at the opposite extreme of possible butterfly population structures, i.e. migratory species that occur as panmictic populations spread over huge geographic distances (Vandewoestijne & Van Dyck, 2010), but such species are seldom of concern to conservationists because they easily replace population losses through immigration.

Species with intermediate dispersal ability fall between these two extremes and have been studied much less, although their fates in modern landscapes should be of great concern to conservationists. More than a decade ago, Thomas (2000) observed that in Western Europe butterflies of intermediate mobility were declining more than their sedentary and mobile counterparts and rapid declines were subsequently reported for several intermediately mobile species (e.g., Konvicka et al., 2008; Kadlec et al., 2010). This is due to the inverse relationship between dispersal and population density (Cowley et al., 2001a, b; Konvicka et al., 2012), which predicts that species that are too mobile to be restricted to patches of a few hectares (cf. Ehrlich & Hanski, 2004), but not as mobile as true migrants (cf. Vandewoestijne et al., 1999), will require relatively large areas of habitat in order to sustain viable populations. As many European species depend on habitats maintained for centuries by preindustrial land use patterns (Settele et al., 2009), conserving such species requires maintaining or mimicking traditional land use over scales that are too large for a single-site approach. A better understanding of the genetic makeup of populations of intermediately mobile species' is a necessary first step in delimiting the boundaries of conservation management units from the point of view of the species concerned. Until now, very few of the species that are declining in abundance and have an intermediate gene flow

have been subjected to genetic analyses, a notable exception being the North American fritillary *Speyeria idalia* (Drury, 1773) (Williams et al., 2003).

We developed microsatellite markers for two intermediately mobile, low-density species of high conservation concern in Europe, the Niobe Fritillary *Argynnis niobe* (Linnaeus, 1758) and High Brown Fritillary *Argynnis adippe* (Denis & Schiffermüller, 1775) (Nymphalidae: Heliconinae). These two closely related species belong to the subgenus *Fabriciana* of the genus *Argynnis* (Simonsen, 2006; Simonsen et al., 2006) and both have broad ranges in the Palaearctic, were historically widespread across Central Europe and inhabit a diverse range of landscapes with grassland, pastures, orchards and woodlots (Tolman & Levington, 1997; Kudrna et al., 2011). As a likely result of land use changes, they became less widespread in many countries and retreated to remote regions that still practice small-scale farming (cf. Spitzer et al., 2009), thus indicating their dependency on large areas consisting of a mosaic of habitats as existed before the intensification of agriculture. Examples of such regions include the offshore islands of Germany (Salz & Fartmann, 2009) and Estonia (Sang et al., 2010) or mountainous regions (Spitzer et al., 2009b; Verovnik et al., 2012). The declines in the abundance of *A. adippe* are less severe and of little concern on a continental basis (Van Swaay et al., 2010), but this species is severely threatened in Britain, for example (Fox et al., 2011). The situation appears much worse for *A. niobe*, which has disappeared from most of Germany (Salz & Fartmann, 2009), and is now near-threatened continentally (Van Swaay et al., 2010). In the Czech Republic, where the material for this study was collected, the extent of the distribution of *A. adippe* was at its lowest a decade ago and is now recovering, whereas the current distribution of *A. niobe* is 40 per-cent of what it was in the 1950s and this species is now critically endangered (Benes et al., 2002).

MATERIAL AND METHODS

Samples were collected during summer 2011 in the Vsetínská Bečva valley, a sub-mountainous area extending for approximately 200 km^2 (centroid coordinates: 49°19′N, 18°9′E) close to the Czech-Slovakian border, which is still traditionally farmed and where both species reach their highest densities within the Czech Republic.

Genomic DNA was extracted from a little piece of wing using the DNeasy Blood&Tissue kit (QIAGEN), which is a way of obtaining tissue samples from butterflies without damaging them (Hamm et al., 2010) and especially important in the case of endangered species. Initially, we reviewed the literature for microsatellites already developed for related species in order to try cross-species amplification. The only existing primers potentially suitable for cross-species amplification were those designed by Williams et al. (2002) for a related fritillary, *Speyeria idalia*. Forward primers were fluorescently labelled and PCR products were analyzed using fragment analysis on an automated sequencer. A variety of PCR conditions were tested, but none of the primer pairs provided microsatellite PCR products.

For the next step, primers were developed de novo using the recent and highly efficient pyrosequencing method (Ronaghi, 2001). DNA from four *Argynnis niobe* individuals was pooled and sent to GenoScreen (France) for the GenoSat service, combining DNA enrichment procedures with the use of multiplexed microsatellite probes and the update Titanium of the 454 GS-FLX technology (Malausa et al., 2011) (see Vandewoestijne et al., 2012 for more details). Obtained primers were analyzed for all primer secondary structures including hairpins, self-dimers and cross-dimers in primer pairs, using the on-line application NetPrimer, available at http://www.premierbiosoft.com/netprimer/.

These secondary structures should be avoided if possible, because they could reduce amplification success. Primers were also checked for the presence of G or C bases within the last five bases from the 3' end of primers (GC clamp), which helps to promote specific binding at the 3' end due to the stronger bonding of G and C bases. In general, we followed the PCR primer design guidelines reviewed at http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html.

Initially, we tried PCR amplification with unlabelled primers, followed by 1% agarose gel electrophoresis, on eight individuals (four *A. addipe* and four *A. niobe*), to determine which primer pairs provided suitable PCR products. Subsequently, we ordered fluorescently labelled primers (only forward: 6-FAM, NED, PET, or VIC, Applied Biosystems). We performed monoplex PCR on the same eight individuals for each of the fluorescently labelled primers using Combi PPP-MasterMix (Top-Bio). The total reaction volume was 12 µl, containing 4 µl of MasterMix, 1 µl of each primer (final c = 0.08 nmol), 4 µl of PCR H_2O and 20 ng of DNA. Cycling parameters were: 4 min of initial denaturation at 94°C, followed by 35 cycles of 94°C (30 s), 54°C (60 s), 72°C (60 s), with final elongation at 72°C for 5 min. We also tested different annealing temperatures (50°, 52°, 56°, 58°), but 54°C provided the best results. The PCR products were then analysed by fragment analysis on automated sequencer ABI 3130 and allelic patterns were scored with software GeneMapper (Applied Biosystems) to determine which loci were polymorphic and can be reliably scored.

For the selected loci, we genotyped another 24 individuals of each species and thus had a total of 32 genotyped individuals per species (24 males and 8 females). Basic parameters of loci, such as number of alleles (Na), observed (H_o) and expected (H_e) heterozygosities, were calculated using the software GenAlEx (Peakall & Smouse, 2006). All loci in both species were also tested for Hardy-Weinberg equilibrium (HWE). The occurrence of null alleles was tested using the software FreeNA (Chapuis & Estoup, 2007) and linkage disequilibrium tests were computed in Genepop 4.0 (Rousset, 2008). Because butterflies have heterogametic females and there is always the possibility that genetic markers are sex-linked, we checked for at least one heterozygotic female genotype at every locus.

RESULTS

From the GenoSat service we obtained 67671 sequences, of which 8144 contained microsatellite motifs and the software analysis (provided by GenoScreen) resulted in 388 bioinformatically validated pairs of primers. Based on the NetPrimer analyses, we selected the thirty most suitable primer pairs. Out of these thirty primer pairs, eighteen provided consistent products of between 100 and 300 bp. Of these eighteen loci, analysed with fluorescent labels on the forward primer, two were monomorphic, six exhibited an unclear pattern with unspecific products indistinguishable from real alleles, and ten were polymorphic with reliably scorable alleles.

The basic parameters of the ten loci selected, based on 32 genotyped individuals of each species, are given in Table 1. The mean number of alleles per locus was 8.2 for *A. niobe* and 4.8 for *A. adippe*. The average observed proportion of heterozygotes over all loci (H_o) was 0.33 in *A. niobe* and 0.30 in *A. adippe*, while the level of expected heterozygosity (H_e) reached 0.62 in the former and 0.44 in the latter species. Seven loci in *A. niobe* and four in *A. adippe* were not in HWE. These loci exhibited null alleles with frequencies 0.09–0.34. The exact test for linkage disequilibrium resulted in non-significant P-values, allowing us to consider the analysed loci independent. We found

TABLE 1. Characterization of 10 polymorphic microsatellite loci in *Argynnis niobe* and *A. adippe*. Locus name, forward (F) and reverse (R) primer sequences, repeat motif, size range of alleles (Na), observed (H_o) and expected (H_e) heterozygosities, and statistical significance of the HWE test (ns = not significant, *** P < 0.001) for 32 individuals of each species.

Locus name	Primer sequences (5 → 3′)	Motif	*A. niobe*					*A. adippe*				
			Range	Na	*Ho*	*He*	HWE	Range	Na	*Ho*	*He*	HWE
An_5	F:CGATTGCATATACATCGTGC	CTAT	118–148	11	0.25	0.80	***	118–140	3	0.00	0.23	***
	R:CCTGTTCAAAAGATTCCGTCA											
An_20	F:TAGATCCAGTGGTCGCCTTT	TGTA	186–428	21	0.33	0.92	***	186–206	4	0.50	0.54	ns
	R:ATATGACAGTCGGGAGACGG											
An_21	F:CATCGTGACGAAATCTGCAT	ATAC	213–228	8	0.28	0.68	***	213–229	4	0.10	0.62	***
	R:AGGCTACATTTTGCCCTGTG											
An_22	F:TCCGTTCGCTACCAAACTTC	TA	168–254	14	0.27	0.82	***	176–226	11	0.28	0.64	***
	R:AGTTATCATCGCTTCGCTCG											
An_24	F:GTGCAGGGAAGGAAGAGAAG	AG	74–122	7	0.53	0.66	***	100–106	4	0.19	0.60	***
	R:ATGAATGGAGTTTCGCCAAG											
An_25	F:TTAAAAGAGCTTGCTGCGGT	TG	186–194	4	0.50	0.57	ns	188–200	4	0.40	0.35	ns
	R:TGCATCAAATGTGTTACGTGC											
An_26	F:TGTCGAATCAAGAAATTAGAATGC	ATCT	86–102	5	0.22	0.30	***	90	1	0.00	0.00	–
	R:CAAGAATTGCTCGTTTAAAAGTATT											
An_27	F:ACCAAGTTCCACCCATCTGA	CTT	175–181	2	0.28	0.24	ns	175–190	5	0.44	0.36	ns
	R:CACAGAAGCCACTGCCACTA											
An_28	F:TTACAACATGATTACCATTAGCCA	GT	126–146	6	0.17	0.75	***	134–148	8	0.84	0.83	ns
	R:GGTACGAACCTTCTACCTGGTC											
An_30	F:GCCATTATTGTATCCTCCTTGG	TTG	240–249	4	0.50	0.43	ns	237–246	4	0.25	0.23	ns
	R:CGTCAAAAGAGCAATCAGTGG											

at least one heterozygotic female at each of the loci, indicating that none of the loci were sex-linked (i.e. localised on the W-chromosome).

DISCUSSION AND CONCLUSIONS

The development of microsatellite markers using the pyrosequencing method has greatly facilitated their use in molecular ecology by reducing the cost and the time required to analyze samples (Santana et al., 2009). Now almost anyone can easily access a useful tool for investigating natural populations of non-model organisms. In this report, we describe ten polymorphic microsatellites for two closely related butterfly species of high conservation importance. We see this paper as a pilot study, taking advantage of only a small part of the raw data set, the rest of which can be used to isolate more microsatellite loci in the future. However, the population genetic parameters based on our ten loci are already informative and non-trivial, hinting at the occurrence of interesting evolutionary phenomena within the populations studied.

Primers were designed for *Argynnis niobe*, but the testing of polymorphism was performed for both species simultaneously, which led to relatively high cross-species amplification success, which is uncommon in Lepidoptera. Although some loci exhibited null alleles, the homozygote excess was more likely caused by the strong prevalence of only one type of allele at most loci. We did not detect any locus for which some individuals would fail to amplify at least one allele, which suggests that the homozygote excess was not due to null alleles, but represented genuine homozygosity (Dakin & Avise, 2004). Homozygote excess is usually interpreted as evidence for inbreeding and/or genetic drift (Frankham et al., 2008), but this needs to be confirmed by an analysis of more individuals.

In Table 2, we provide a brief comparison of the microsatellite loci parameters obtained in several other studies on Lepi-

TABLE 2. Comparison of microsatellite loci parameters obtained in other studies on Lepidoptera that used similar sized samples. Mean number of alleles per locus (Na), expected (H_e) and observed (H_o) heterozygosities.

Source study	Species	Sample size	Number of loci	Na	H_e/H_o
Vila et al., 2009	*Erebia palarica*	35	10	14.3	0.82/0.75
Petenian et al., 2005	*Parnassius apollo*	40	6	7.5	0.45/0.33
Petenian et al., 2005	*Euphydryas aurinia*	40	5	14	0.86/0.42
Rousselet et al., 2004	*Thaumetopoea pityocampa*	30	5	6.6	0.70/0.62
This study	*Argynnis adippe*	32	10	4.8	0.44/0.30
This study	*Argynnis niobe*	32	10	8.2	0.62/0.33

doptera that used similar sized samples. Genetic variability, expressed in terms of the number of alleles per locus and observed heterozygosity, was very low in our focal species, which indicates a strong influence of genetic drift and inbreeding on their population genetic structures. Surprisingly, the number of alleles per locus was two times lower in the locally more abundant and less threatened *A. adippe*. The long-term dataset (Benes et al., 2002; and Czech Butterfly Recording) available for this species suggests that this could have been caused by a bottleneck event that happened several decades ago, when there was a more drastic decline in numbers in *A. adippe* than *A. niobe*, but for a more accurate interpretation a more robust dataset is needed.

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REFERENCES

AMOS W. & BALMFORD A. 2011: When does conservation genetics matter? — *Heredity* **87**: 257–265.

BENES J., KONVICKA M., DVORAK J., FRIC Z., HAVELDA Z., PAVLICKO A., VRABEC V. & WEIDENHOFFER Z. 2002: *Butterflies of the Czech Republic: Distribution and Conservation I.* SOM, Prague, 478 pp.

BRATTSTROM O., AKESSON S. & BENSCH S. 2010: AFLP reveals cryptic population structure in migratory European red admirals (*Vanessa atalanta*). — *Ecol. Entomol.* **35**: 248–252.

CASSEL A. 2002: Characterization of microsatellite loci in *Coenonympha hero* (Lepidoptera: Nymphalidae). — *Mol. Ecol. Notes* **2**: 566–568.

CHAPUIS M.P. & ESTOUP A. 2007: Microsatellite null alleles and estimation of population differentiation. — *Mol. Biol. Evol.* **24**: 621–631.

COWLEY M.J.R., THOMAS C.D., ROY D.B., WILSON R.J., LEON-CORTES J.L., GUTIERREZ D., BULMAN C.R., QUINN R.M., MOSS D. & GASTON K.J. 2001a: Density-distribution relationships in British butterflies. I. The effect of mobility and spatial scale. — *J. Anim. Ecol.* **70**: 410–425.

COWLEY M.J.R., THOMAS C.D., WILSON R.J., LEON-CORTES J.L., GUTIERREZ D. & BULMAN C.R. 2001b: Density-distribution relationships in British butterflies. II. An assessment of mechanisms. — *J. Anim. Ecol.* **70**: 426–441.

DAKIN E.E. & AVISE J.C. 2004: Microsatellite null alleles in parentage analysis. — *Heredity* **93**: 504–509.

EHRLICH P.R. & HANSKI I. 2004: *On the Wings of Checkerspots: A Model System for Population Biology*. Oxford University Press, New York, 371 pp.

FINGER A., SCHMITT T., ZACHOS F.E., MEYER M., ASSMANN T. & HABEL J.C. 2009: The genetic status of the violet copper *Lycaena helle* – a relict of the cold past in times of global warming. *Ecography* **32**: 382–390.

FOX R., WARREN M.S., BRERETON T.M., ROY D.B. & ROBINSON A. 2011: A new Red List of British butterflies. *Insect Conserv. Divers*. **4**: 159–172.

FRANKHAM R., BALLOU J. & BRISCOE D. 2008: *Introduction to Conservation Genetics.* Cambridge University Press, Cambridge, 617 pp.

HABEL J.C., FINGER A., MEYER M., SCHMITT T. & ASSMANN T. 2008: Polymorphic microsatellite loci in the endangered butterfly *Lycaena helle* (Lepidoptera: Lycaenidae). *Eur. J. Entomol*. **105**: 361–362.

HABEL J.C., SCHMITT T., MEYER M., FINGER A., RODDER D., ASSMANN T. & ZACHOS F.E. 2010: Biogeography meets conservation: the genetic structure of the endangered lycaenid butterfly *Lycaena helle* (Denis & Schiffermuller, 1775). — *Biol. J. Linn. Soc. Lond*. **101**: 155–168.

HABEL J.C., FINGER A., SCHMITT T. & NEVE G. 2011: Survival of the endangered butterfly *Lycaena helle* in a fragmented environment: Genetic analyses over 15 years. — *J. Zool. Syst. Evol. Res.* **49**: 25–31.

HAMM C.A., AGGARWAL D. & LANDIS D.A. 2010: Evaluating the impact of non-lethal DNA sampling on two butterflies, *Vanessa cardui* and *Satyrodes eurydice*. — *J. Insect Conserv.* **14**: 11–18.

HARPER G.L., PIYAPATTANAKORN S., GOULSON D. & MACLEAN N. 2000: Isolation of microsatellite markers from the Adonis blue butterfly (*Lysandra bellargus*). — *Mol. Ecol*. **9**: 1948–1949.

JARNE P. & LAGODA P.J.L. 1996: Microsatellites, from molecules to populations and back. — *Trends Ecol. Evol*. **11**: 424–429.

KADLEC T., VRBA P., KEPKA P., SCHMITT T. & KONVICKA M. 2009: Tracking the decline of the once-common butterfly: delayed oviposition, demography and population genetics in the hermit *Chazara briseis*. — *Anim. Conserv*. **13**: 172–183.

KEYGHOBADI N., ROLAND J. & STROBECK C. 1999: Influence of landscape on the population genetic structure of the alpine butterfly *Parnassius smintheus* (Papilionidae). — *Mol. Ecol*. **8**: 1481–1495.

KONVICKA M., BENES J., CIZEK O., KOPECEK F., KONVICKA O. & VITAZ L. 2008: How too much care kills species: Grassland reserves, agri-environmental schemes and extinction of *Colias myrmidone* (Lepidoptera: Pieridae) from its former stronghold. — *J. Insect Conserv*. **12**: 519–525.

KONVICKA M., ZIMMERMANN K., KLIMOVA M., HULA V. & FRIC Z. 2012: Inverse link between density and dispersal distance in butterflies: field evidence from six co-occurring species. — *Popul. Ecol.* **54**: 91–101.

KUDRNA O., HARPKE A., LUX K., PENNERSTORFER J., SCHWEIGER O., SETTELE J. & WIEMERS M. 2011: *Distribution Atlas of Butterflies in Europe*. Geselschaft für Schmetterlingsschutz e.V., Halle, 576 pp.

MALAUSA T., GILLES A., MEGLECZ E. ET AL. 2011: High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. — *Mol. Ecol. Resour.* **11**: 638–644.

PALSBOLL P.J., BERUBE M. & ALLENDORF F.W. 2007: Identification of management units using population genetic data. — *Trends Ecol. Evol*. **22**: 11–16.

PEAKALL R. & SMOUSE P. 2006: GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. — *Mol. Ecol. Notes* **6**: 288–295.

PETENIAN F., MEGLÉCZ E., GENSON G., RASPLUS J. & FAURE E. 2005: Isolation and characterization of polymorphic microsatellites in *Parnassius apollo* and *Euphydryas aurinia* (Lepidoptera). — *Mol. Ecol. Notes* **5**: 243–245.

RONAGHI M. 2001: Pyrosequencing sheds light on DNA sequencing. — *Genome Res.* **11**: 3–11.

ROUSSELET J., MAGNOUX E. & KERDELHUÉ C. 2004: Characterization of five microsatellite loci in the Pine Processionary Moth *Thaumetopoea pityocampa* (Lepidoptera: Notodontidae: Thaumetopoeinae). — *Mol. Ecol. Notes* **4**: 213–214.

ROUSSET F. 2008: GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. — *Mol. Ecol. Resour.* **8**: 103–106.

RUNQUIST E.B., FORISTER M.L. & SHAPIRO A.M. 2012: Phylogeography at large spatial scales: incongruent patterns of population structure and demography of Pan-American butterflies associated with weedy habitats. — *J. Biogeogr*. **39**: 382–396.

SALZ A. & FARTMANN T. 2009: Coastal dunes as important strongholds for the survival of the rare Niobe fritillary (*Argynnis niobe*). — *J. Insect Conserv.* **13**: 643–654.

SANG A., TEDER T., HELM A. & PARTEL M. 2010: Indirect evidence for an extinction debt of grassland butterflies half century after habitat loss. — *Biol. Conserv.* **143**: 1405–1413.

SANTANA Q.C., COETZEE M.P.A., STEENKAMP E.T. ET AL. 2009: Microsatellite discovery by deep sequencing of enriched genomic libraries. — *Bio Techniques* **46**: 217–223.

SARHAN A. 2006: Isolation and characterization of five microsatellite loci in the Glanville fritillary butterfly (*Melitaea cinxia*). — *Mol. Ecol. Notes* **6**: 163–164.

SETTELE J., SHREEVE T., KONVICKA M. & VAN DYCK H. 2009: *Ecology of Butterflies in Europe.* Cambridge University Press, Cambridge, 513 pp.

SIGAARD P., PERTOLDI C., MADSEN A.B., SOGAARD B. & LOESCHCKE V. 2008: Patterns of genetic variation in isolated Danish populations of the endangered butterfly *Euphydryas aurinia*. — *Biol. J. Linn. Soc. Lond.* **95**: 677–687.

SIMONSEN T.J. 2006: Fritillary phylogeny, classification, and larval host plants: reconstructed mainly on the basis of male and female genitalic morphology (Lepidoptera: Nymphalidae: Argynnini). — *Biol. J. Linn. Soc. Lond.* **89**: 627–673.

SIMONSEN T.J., WAHLBERG N., BROWER A.V.Z. & DE JONG R. 2006: Morphology, molecules and fritillaries: approaching a stable phylogeny for Argynnini (Lepidoptera: Nymphalidae). — *Insect Syst. Evol*. **37**: 405–418.

SPITZER L., BENES J., DANDOVA J., JASKOVA V. & KONVICKA M. 2009a: The Large Blue butterfly, *Phengaris* [*Maculinea*] *arion*, as a conservation umbrella on a landscape scale: The case of the Czech Carpathians. — *Ecol. Indic.* **9**: 1056–1063.

SPITZER L., BENEŠ J. & KONVIČKA M. 2009b: Oviposition of the Niobe fritillary [*Argynnis niobe* (Linnaeus, 1758)] at submountain conditions in the Czech Carpathians (Lepidoptera, Nymphalidae). — *Nachr. Entomol. Ver. Apollo* **30**: 165–168.

THOMAS C.D. 2000: Dispersal and extinction in fragmented landscapes. — *Proc. R. Soc. Lond. (B)* **267**: 139–145.

TODISCO V., GRATTON P., CESARONI D. & SBORDONI V. 2010: Phylogeography of *Parnassius apollo*: hints on taxonomy and conservation of a vulnerable glacial butterfly invader. — *Biol. J. Linn. Soc. Lond.* **101**: 169–183.

TOLMAN T. & LEVINGTON R. 1997: *Butterflies of Britain and Europe*. Harper Collins, London, 320 pp.

VANDEWOESTIJNE S. & VAN DYCK H. 2010: Population genetic differences along a latitudinal cline between original and recently colonized habitat in a butterfly. — *Plos One* **5**: 11, Article Number: e13810

VANDEWOESTIJNE S., NÈVE G. & BAGUETTE M. 1999: Spatial and temporal population genetic structure of the butterfly *Aglais urticae* L. (Lepidoptera, Nymphalidae). — *Mol. Ecol*. **8**: 1539–1543.

VANDEWOESTIJNE S., TURLURE C. & BAGUETTE M. 2012: Development of novel microsatellite markers for a specialist species of Lepidoptera, *Boloria aquilonaris* (Nymphalidae), based on 454 sequences. — *Eur. J. Entomol*. **109**: 129–134.

VAN SWAAY C., CUTTELOD A., COLLINS S. ET AL. 2010: *European Red List of Butterflies*. Publications Office of the European Union, Luxembourg, 48 pp.

VAN'T HOF A.E., BRAKEFIELD P.M., SACCHERI I.J. & ZWAAN B.J. 2007: Evolutionary dynamics of multilocus microsatellite arrangements in the genome of the butterfly *Bicyclus anynana*, with implications for other Lepidoptera. — *Heredity* **98**: 320–328.

VEROVNIK R., REBEUŠEK F. & JEŽ M. 2012: *Atlas of Butterflies (Lepidoptera: Rhopalocera) of Slovenia*. Centre for Cartography of Fauna and Flora, Miklaž na Dravskem Polju, 456 pp

VILA M., LATASA T., PINO J.J. & VERHULST G. 2009: Characterization of ten polymorphic microsatellite markers for the endemic Chapman's ringlet, *Erebia palarica* (Lepidoptera: Nymphalidae). — *Eur. J. Entomol*. **106**: 485–490.

WARREN M.S. 1995: Managing local microclimates for the High Brown fritillary, *Argynnis adippe*. In Pullin A.S. (ed.): *Ecology and Conservation of Butterflies*. Chapman & Hall, London, pp. 198–210.

WILLIAMS B.L., BRAWN J.D. & PAIGE K.N. 2002: Highly polymorphic microsatellite loci for *Speyeria idalia* (Lepidoptera: Nymphalidae). — *Mol. Ecol. Notes* **2**: 87–88.

WILLIAMS B.L., BRAWN J.D. & PAIGE K.N. 2003: Landscape scale genetic effects of habitat fragmentation on a high gene flow species: *Speyeria idalia* (Nymphalidae). — *Mol. Ecol*. **12**: 11–20.

ZACHOS F.E. & HARTL G.B. 2011: Phylogeography, population genetics and conservation of the European red deer *Cervus elaphus*. — *Mamm. Rev*. **41**: 138–150.

ZEISSET I., ALS T.D., SETTELE J. & BOOMSMA J.J. 2005: Microsatellite markers for the large blue butterflies *Maculinea nausithous* and *Maculinea alcon* (Lepidoptera: Lycaenidae) and their amplification in other *Maculinea* species. — *Mol. Ecol. Notes* **5**: 165–168.

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TECHNICAL NOTE

Characterization of nine polymorphic microsatellite loci for a threatened saproxylic beetle *Rosalia alpina* (Coleoptera: Cerambycidae)

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Abstract Nine polymorphic microsatellite loci were developed and characterized for the Rosalia longicorn, an endangered icon of European saproxylic biodiversity. The detected number of alleles per locus ranged from 2 to 4, and the observed and expected heterozygosities varied from 0.044 to 0.622, and from 0.086 to 0.613, respectively. All loci were in Hardy–Weinberg equilibrium as well, as no evidence of linkage disequilibrium was found. Despite the low level of polymorphism, all loci described in this study will provide a useful tool in future genetic studies of the *Rosalia alpina* species.

Keywords SSR markers · Population genetics · Dead wood · Xylophagous

The Rosalia longicorn (*Rosalia alpina*) (Linnaeus, 1758) is among the most attractive and widely known beetles associated with old trees and dead wood. It is classified in the IUCN Red List of Threatened Species as vulnerable (IUCN 2012) and listed in the EU Habitats Directive as a priority species of community interest. Its range covers most of Europe, reaching to the Urals (Sama 2002) and Turkey (Sama et al. 2012). In Europe, this species was distributed from Sicily in the south to Sweden in the north (Sama 2002; Lindhe et al. 2010). During recent centuries, however, it has disappeared from the northern part of its range (e.g. Bense 2002; Starzyk 2004) and now only a single population survives north of the Alps and west of the Carpathians (Drag et al. 2011). Although usually considered as a montane species associated with beech forests (Duelli and Wermelinger 2005; Bosso et al. 2013; Lachat et al. 2013), the Rosalia longicorn also inhabits lowlands and develops in a number of other broadleaved trees, including e.g. maples, elms, and ashes (Ciach et al. 2007; Cizek et al. 2009). The beetle prefers mature, often pollarded or shredded trees growing outside closed canopy conditions (Russo et al. 2011).

Although this beetle is a priority of conservation concern in Europe, nothing is known about its genetic structure and diversity. Here we describe 9 microsatellite loci which will provide a valuable tool for studying the genetic variability of this threatened species.

Genomic DNA was extracted from the tissue of 45 individuals from a population located in western Slovakia using the Genomic DNA Mini Kit Tissue (Geneaid). The DNA of eight individuals was pooled together and sent to Genoscreen (Lille, France) for the Geno Sat service. It includes microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science). For more details see Malausa et al. (2011). The following eight probes were used: $(TC)_{10}$, $(TG)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$ and $(ATCT)_6$. This technique allowed the identification of 31,864 reads (average length = 220 bp), of which 2,249 contained microsatellite motives. It appeared that the microsatellite motives were frequently at the beginning or at the end of the sequences, so the number of validated primers that could be designed was rather low, resulting in 59 pairs of primers.

Using the on-line application NetPrimer (http://www.premierbiosoft.com) we selected the 40 most suitable

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Table 1 Characterization of 9 microsatellite loci for *Rosalia alpina* from 45 individuals

Locus	Primer sequences (5′ → 3′)	Repeat motif	Size range (bp)	N_A	H_O	H_E	GenBank accession no.
RA_08	F: ACGACCACGTGTTGCCTTAT	$(CT)_8$	124–132	2	0.289	0.420	KF114384
	R: CAGAGCGAGTAGAGTAGGGAGA						
RA_15	F: TGTGCATTTGTCTGGGTCTT	$(TG)_{10}$	96–100	3	0.044	0.086	KF114387
	R: GACAAATACAGAGGGGAAAACG						
RA_11	F: CTGGACTACGACGGAACGA	$(AC)_{10}$	85–91	4	0.578	0.613	KF114385
	R: ACGCGTTCATCAGGTATTCA						
RA_13	F: TGCTACTGGATTTTCCGGTACT	$(GA)_8$	215–219	3	0.133	0.126	KF114386
	R: GCAGGCCCACGTATGAATAG						
RA_23	F: AAGCATAAGAATTATTCAAACAGATTT	$(TG)_8$	134–140	4	0.422	0.583	KF114388
	R: TCACAATCAGGCAAAACCCT						
RA_29	F: CCAATGGATTGCGTCAATAA	$(TC)_{10}$	150–156	4	0.356	0.368	KF114390
	R: CATCCTAAAAAGACAGACGCA						
RA_40	F: TTTGTTTCGTCGAATTCCATT	$(CT)_8$	219–227	4	0.622	0.537	KF114392
	R: CGAGGTACTGACCGTGATTG						
RA_37	F: AAAATCCTAAAATCTAGTGCGAATAA	$(TTC)_7$	250–271	3	0.422	0.567	KF114391
	R: AGTACTAAAGAGTTAGGGATGTTCTGA						
RA_28	F: ATGTTACCCTAGAATAATTGAATGGA	$(GT)_6$	139–147	3	0.511	0.531	KF114389
	R: CCATCTCACAGGACTCACGA						

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity

primer pairs. Out of these, 28 provided consistent PCR products of between 100 and 300 bp. We performed monoplex PCR with eight individuals for each of these 28 primers (forward primers were fluorescently labeled with 6-FAM, NED, PET, or VIC dyes, Applied Biosystems). The total reaction volume was 10 µl, and contained 5 µl of Combi PPP-MasterMix (Top-Bio), 0.8 µM of each primer, 3.2 µl of PCR H2O, and 20–50 ng of template DNA. Conditions for the PCR reaction were: 4 min of initial denaturation at 94 °C, followed by 35 cycles of 94 °C (30 s), 54 °C (60 s), 72 °C (60 s), with final elongation at 72 °C for 5 min. Labeled PCR products were analysed on automated sequencer ABI 3730XL (Applied Biosystems) and allelic patterns were scored using the software GeneMapper 3.7 (Applied Biosystems).

Out of the 28 primer pairs, 15 were monomorphic, 4 exhibited an unclear pattern with unspecific products indistinguishable from real alleles, and 9 were polymorphic with reliably scorable alleles. Subsequently we created two multiplex sets, first with loci RA_11, RA_08, RA_13, RA_28, RA_29, RA_37, second with loci RA_15, RA_23, RA_40 and analysed the rest of the individuals under the conditions as described above. Microchecker (Van Oosterhout et al. 2004) was used to investigate the occurrence of null alleles. Genetic diversity measures were estimated using GenAlEx 6 (Peakall and Smouse 2006). Levels of heterozygosity and the linkage disequilibrium were calculated in GenePop 4.1.3 (Rousset 2008).

The level of polymorphism in all loci was rather low, with the number of alleles per locus ranging from 2 to 4 (Table 1). These low values can be attributed to the sampling site, rather than to the low variability of chosen loci, since in other populations a higher level of polymorphism was found (unpublished data). The average observed proportion of heterozygotes over all loci (H_O) and the level of expected heterozygosity (H_E) were 0.34 and 0.43, respectively. After Bonferroni correction, all nine loci were in HWE. Results of Microchecker suggested the presence of null alleles in two loci (RA_23, RA_37); however, all analysed samples amplified (i.e., showed one or two bands), which suggests that the homozygote excess was probably not due to null alleles, but more likely represented genuine homozygosity (Dakin and Avise 2004). Homozygote excess is usually interpreted as evidence for inbreeding and/or genetic drift (Frankham 2008)—expectable in threatened species, but this needs to be confirmed by an analysis of more individuals. The exact test for linkage disequilibrium resulted in non-significant P-values after correction for multiple tests.

These newly described microsatellite loci are potentially valuable in future population and conservation genetic studies and may contribute to more efficient protection of this threatened species.

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References

Bense U (2002) Schutzmaßnahmen für den Alpenbock (*Rosalia alpina*) im Bereich der Schwäbischen Alb. DgaaE Nachrichten 16:57–58

Bosso L, Rebelo H, Garonna AP, Russo D (2013) Modelling geographic distribution and detecting conservation gaps in Italy for the threatened beetle *Rosalia alpina*. J Nat Conserv 21:72–80

Ciach M, Michalcewicz J, Fluda M (2007) The first report on development of *Rosalia alpina* (Linnaeus, 1758) (Coleoptera: Cerambycidae) in wood of Ulmus L. in Poland. Pol J Entomol 76:101–105

Cizek L, Schlaghamerský J, Bořucký J, Hauck D, Helešic J (2009) Range expansion of an endangered beetle: alpine longhorn *Rosalia alpina* (Coleoptera: Cerambycidae) spreads to the lowlands of Central Europe. Entomol Fennica 20:200–206

Dakin EE, Avise JC (2004) Microsatellite null alleles in parentage analysis. Hered 93:504–509

Drag L, Hauck D, Pokluda P, Zimmermann K, Cizek L (2011) Demography and dispersal ability of a threatened Saproxylic Beetle: a mark-recapture study of the Rosalia Longicorn (*Rosalia alpina*). PLoS ONE 6(6):e21345

Duelli P, Wermelinger B (2005) Der Alpenbock (*Rosalia alpina*). Ein seltener Bockkäfer als Flaggschiff–Art. Merkblatt für die Praxis 39:1–8

Frankham R (2008) Genetic adaptation to captivity in species conservation programs. Mol Ecol 17:325–333

IUCN (2012) IUCN red list of threatened species. Version 2012.2. www.iucnredlist.org. Accessed 13 March 2013

Lachat T, Ecker K, Duelli P, Wermelinger B (2013) Population trends of *Rosalia alpina* (L.) in Switzerland: a lasting turnaround? J Insect Conserv. doi:10.1007/s10841-013-9549-9

Lindhe A, Jeppsson T, Ehnström B (2010) Longhorn beetles in Sweden—changes in distribution and abundance over the last two hundred years. (Långhorningar i Sverige—förändringar av utbredning och frekvens under de senaste tvåhundra åren.) Entomologisk Tidskrift, Uppsala, Sweden

Malausa T, Gilles A, Meglécz E et al (2011) High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. Mol Ecol Res 11:638–644

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour 8:103–106

Russo D, Cistrone L, Garonna AP (2011) Habitat selection by the highly endangered long-horned beetle *Rosalia alpina* in Southern Europe: a multiple spatial scale assessment. J Insect Conserv 15:685–693

Sama G (2002) Atlas of Cerambycidae of Europe and the Mediterranean Area. Vol. I. Northern, Western, Central and Eastern Europe, British Isles and Continental Europe from France (excl. Corsica) to Scandinavia and the Urals. Kabourek, Zlín

Sama G, Rapuzzi P, Özdikmen H (2012) Preliminary report of the entomological surveys (2010, 2011) of G. Sama and P. Rapuzzi to Turkey (Coleoptera: Cerambycidae). Munis Entomol Zool 7:22–45

Starzyk JR (2004) *Rosalia alpina* (Linnaeus, 1758), Nadobnica alpejska. In: Głowacinski Z, Nowacki J (ed) Polska czerwona ksiega zwierzat. Bezkregowce. (Polish red data book of animals. Invertebrates.) Kraków: IOP PAN, AR Poznań. pp 148–149

Van Oosterhout C, Hutchison WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

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MICROSATELLITE LETTERS

Isolation and characterisation of microsatellite markers for *Mylothris jacksoni knutsoni* (Lepidoptera: Pieridae), an endemic butterfly of the Gulf of Guinea Highlands

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Abstract Eight polymorphic microsatellite loci were developed and characterized for the *Mylothris jacksoni knutsoni*, an endemic butterfly of endangered montane habitats of the Gulf of Guinea Highlands, West/Central Africa. The loci were tested for polymorphism in 30 individuals from the Nkogam Massif, western Cameroon. The detected numbers of alleles per locus ranged from 2 to 5, and the observed and expected heterozygosities varied from 0.200 to 0.867, and from 0.186 to 0.739, respectively. All loci were in Hardy–Weinberg equilibrium, and no evidence of linkage disequilibrium was found. Despite its uniqueness, the biota of this area is still understudied and this is the first study describing microsatellite loci for any African species of the Pieridae family, as well as of any butterfly of the study area.

Keywords Microsatellites · Lepidoptera · Endemism · Afromontane habitats

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Due to its strong isolation the Gulf of Guinea Highlands (alternatively known as the Cameroon Volcanic Line), the only large Afromontane area west of the Congo Basin, is a highly important centre of biodiversity and endemism. Simultaneously, it is an area with a very high population density and its unique montane habitats thus rank highly among the most threatened parts of tropical Africa (Tropek and Konvicka 2010). On the other hand, its unique biota still remains considerably understudied, especially in terms of effect of the habitat alteration on genetic structure of its species. *Mylothris jacksoni knutsoni* is an endemic butterfly with the clearly disjunctive relatives in mountains of eastern Democratic Republic of Congo and South Sudan (Larsen 2005). This species is dependent on the Afromontane habitats above 1,500 m a.s.l. where it inhabits montane forest edges and shrubby submontane habitats, and avoids strongly degraded pastures and large forest clearings (Larsen 2005; Tropek and Konvicka 2010). So far, population genetic structure of this species has not been studied, as well as of any other butterfly of the area.

Genomic DNA was extracted from tissue of 30 individuals sampled in December 2011 at edges of a degraded montane forest remnant on an eastern ridge of the Nkogam Massif, West Cameroon (N 05.72133°, E 10.70523°, 1,800 m a.s.l.), using the DNeasy Blood&Tissue kit (QIAGEN). The DNA of eight individuals was pooled together and sent to Genoscreen (Lille, France) for the Geno Sat® service. It includes microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science; for more details see Malausa et al. 2011). We obtained 43,470 sequences, of which 3,326 contained microsatellite motifs, resulting in 263 bioinformatively validated pairs of primers. Using the online application NetPrimer (http://www.premierbiosoft.com) we selected the 24 most suitable primer pairs. Initially,

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Table 1 Characterization of 8 polymorphic microsatellite loci for *Mylothris jacksoni knutsoni* based on genotypes of 30 individuals

Locus name	Primer sequences (5 → 3′)	Repeat motif	Size range (bp)	N_A	H_O	H_E
MJK_4	F: TTCATCACTGTTCAACAATCCC	AT	232–270	5	0.800	0.651
	R: TGCTACCAGTTCTCCACTCG					
MJK_5	F: TGTATTTCAGCTCTGGATCGC	GA	151–157	4	0.867	0.739
	R: TTGAAAACTGGACGGGAGAC					
MJK_7	F: CGTGTCCTAATCCATTACATCC	CT	126–134	5	0.300	0.298
	R: ATCGTTGCTTTGGGATAAGG					
MJK_11	F: TTTCACAGACGTGCGAGTTC	AG	90–98	4	0.200	0.186
	R: TCAGGGATTAAACGTAACGGA					
MJK_12	F: CATTGACCACTAGAGTGATGCC	TTG	250–262	2	0.267	0.231
	R: AGAAGACGAGGCTGTATGG					
MJK_14	F: ACCACGATCCTCAAGAGCTG	AG	82–90	5	0.567	0.676
	R: GAACGCCGAATTTGTTTCTG					
MJK_18	F: GGCAAATATGAGGCAACTCTG	AAC	392–454	4	0.467	0.560
	R: TGCTGTCGTATTCGTTCTGC					
MJK_23	F: CTGAGAAATCAGGACGTAGGC	CA	92–94	2	0.333	0.391
	R: GCTCAAGGAATCGAAAGGGT					

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity

we performed monoplex PCR on eight individuals with fluorescently labeled primers, followed by fragment analysis on automated sequencer ABI 3730XL (Applied Biosystems). One PCR reaction consisted of 4 µl of Multiplex PCR MasterMix (QIAGEN), 0.2 µM of each primer, 20–50 ng of template DNA and 3.6 µl of PCR H_2O. Conditions for the PCR reaction were: 15 min of 94 °C, followed by 35 cycles of 94 °C (30 s), 55 °C (90 s), 72 °C (60 s), with final elongation at 60 °C for 30 min. Allelic patterns were scored using the software Genemapper 3.7 (Applied Biosystems). Out of 24 loci, 8 were monomorphic, 8 exhibited unclear allelic pattern and/or unspecific products, and 8 were polymorphic with reliably scorable alleles. We combined these 8 primer pairs into 2 multiplex sets (MJK_4, MJK_5, MJK_7, MJK_14 and MJK_11, MJK_12, MJK_18, MJK_23) and analysed the rest of the samples under the same conditions. The GenAlEx 6.5 software (Peakall and Smouse 2006) was used to calculate observed and expected heterozygosity and to test for Hardy–Weinberg equilibrium. Linkage disequilibrium tests were computed using the software Genepop 4.0 (Rousset 2008). The number of alleles per locus ranged from 2 to 5 and the average expected and observed heterozygosities were 0.467 and 0.475 respectively (Table 1). All loci were in Hardy–Weinberg equilibrium and the tests for linkage disequlibrium resulted in non-significant *P* values.

The newly described microsatellite loci will be used for genetic analyses of distinct populations across the Gulf of Guinea Highlands, and to reveal how the strong human-made degradation of the unique habitats affects the endemic Afromontane butterfly. They are also the first microsatellites developed and tested for any African species of the Pieridae family, as well as of any butterfly of the study area.

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References

Larsen TB (2005) Butterflies of West Africa. Apollo Books, Stenstrup

Malausa T, Gilles A, Meglécz E et al (2011) High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. Mol Ecol Res 11:638–644

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour 8:103–106

Tropek R, Konvicka M (2010) Forest Eternal? Endemic butterflies of the Bamenda highlands, Cameroon, avoid close-canopy forest. Afr J Ecol 48:428–437

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NOTE

Identification of microsatellite markers for a worldwide distributed, highly invasive ant species *Tapinoma melanocephalum* (Hymenoptera: Formicidae)

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Key words. Hymenoptera, Formicidae, *Tapinoma melanocephalum*, microsatellites, invasive species, homozygosity excess

Abstract. *Tapinoma melanocephalum* is a worldwide distributed, highly invasive ant species. It lives in close association with human societies and its distribution is human-mediated in large measure. The geographical origin of this ant species is unknown, but its introduction in areas previously devoided of its presence can represent a threat to the native biota, act as an agricultural pest or as a pathogen vector. To investigate the genetic structure and phylogeography of this species we identified 12 new polymorphic microsatellite markers, and in addition, we tested and selected 12 ant-universal microsatellites polymorphic in *T. melanocephalum*. We genotyped 30 individuals from several islands of Micronesia and Papua-New Guinea. All 24 loci exhibited strong homozygosity excess (45–100%, mean = 86%), while the number of alleles per locus reached usual values (2–18, mean = 6.5), resulting in levels of expected heterozygosity much higher than observed. Based on several robust tests, we were able to exclude artefacts such as null alleles and allelic dropout as a possible cause of the observed pattern. Homozygosity excess might be a consequence of founder effect, bottleneck and/or inbreeding. As our sample population was composed of individuals from several distinct localities, the Wahlund effect might have contributed to the increased homozygosity as well. Despite the provisionally observed deviation from the Hardy-Weinberg equilibrium, the newly developed microsatellites will provide an effective tool for future genetic investigations of population structure as well as for the phylogeographic study of *T. melanocephalum*.

INTRODUCTION

Tapinoma melanocephalum (Fabricius, 1793), also known as the "ghost ant", is one of the most widely distributed ant species on Earth. It is ubiquitous throughout tropical and subtropical areas and is also present in temperate zones, where it is confined to indoor environments. It has been documented at more than 1500 sites across the globe with the highest latitude records from Finland, Scotland, Manitoba, and Quebec in the northern hemisphere and from New Zealand in the southern hemisphere (Wetterer, 2009). This species is one of the most common ants associated with humans and is often restricted to disturbed habitats and human-made environments. Indoor nests are usually found within the structures of buildings such as cracks and wall voids, and outdoor nests in flowerpots or under objects on the ground (Choe et al., 2009).

T. melanocephalum has been so widely distributed by commerce that it is difficult to determine its exact geographical origin (Wen, 2007). Although it has been debated, the current hypothesis is that its natural range is located in tropical Asia or in the Indo-Pacific (Wetterer, 2009) where it is also most abundant. Clearly, a comprehensive phylogeographic study is needed to identify the region where the species originated.

Morphologically, *T. melanocephalum* can be characterized by a length of 1.5–2 mm, a laterally compressed mesonotum broadening anteriorly (Collingwood, 1979) and by a distinctly bicoloured body, with the head and thorax brown to dark brown while the abdomen, legs, and antennae are yellow or whitish. However, variability in the coloration pattern and other characters has been documented and several regional forms are recognized (e.g. *Tapinoma melanocephalum malesianum)*, most of them comprehensively summarized by Wetterer (2009). In Asia and the Indo-Pacific, *T. melanocephalum* can also be confused with several similar species, in particular *Tapinoma minutum* or *Tapinoma indicum*. Furthermore, there are reports of species with intermediate characters between these taxa (Clouse, 2007) and their exact species status remains to be determined by a phylogenetic analysis. Consequently, positive identification of *Tapinoma melanocephalum* in the Indo-Pacific can be difficult.

Surprisingly, the social structure of *T. melanocephalum* has not been studied in close detail. The species was reported to be po-

lygynous with individual nests containing hundreds to thousands of workers (Harada, 1990), but more specific information about the numbers of queens in a colony is absent. The species is also considered unicolonial and polydomous. It reproduces by colony budding and does not exhibit aggressiveness among colonies co-existing in the same area (Smith, 1965; Bustos & Cherix, 1998). However, detailed information about occurrence and population-level variability of these traits is not available. These features of colony organization are typical of many invasive ant species (Tsutsui & Suarez, 2003) and are most likely the underlying reasons behind the unprecedented biological success of this species. The combination of polygyny and polydomy allows for fast and frequent relocations of the colonies and allows the species to occupy temporary habitats (Passera, 1994; Appel et al., 2004).

Despite being virtually omnipresent, *T. melanocephalum* has not received as much attention as other invasive ants, e.g. *Solenopsis invicta* or *Linemiptema humile*. This is likely due to its rather non-aggressive nature, lack of sting and because it does not cause as obvious disturbances to affected environment as some of the other introduced ants.

On the other hand, several studies show that it can be a serious pest. The species is known to dominate some subtropical and tropical agricultural systems where it tends phloem feeding Hemiptera. This leads to plant damage and to an increase of plant pathogens, including viral and fungal infections (Venkataramaiah & Rehman, 1989). In Papua New Guinea, *T. melanocephalum* was one of the only ant species whose number increased greatly in the canopy and understory of primary and secondary forests following a focused eradication of ant assemblages (Klimes et al., 2012).

In indoor habitats, this species has been found abundantly in hospitals in Brazil, where it has the high potential of acting as an agent in the spread of pathogens. Moreira et al. (2005) found multiple types of bacteria associated with *T. melanocephalum* workers, some of them with antibiotic resistance. This suggests that its presence may have, in some cases, serious medical and epidemiological consequences.

Considering how common *T. melanocephalum* is, our lack of knowledge on its social structure and life history is surprising. While most of the literature has focused on practical tasks related to its eradication, studies on its genetic structure and population history are missing. Here, we present the first study investigating and identifying microsatellite markers for this species in order to provide an insight into the genetic structure of *T. melanocephalum* populations. This will establish a baseline which will facilitate future genetic studies on the social structure and phylogeography of *T. melanocephalum*. Indeed, a better knowledge of the genetic relationships between and among the colonies and populations of *T. melanocephalum* will contribute to a more efficient management of this pest. Furthermore, the species-specific markers will allow detailed studies of the species' population genetic and phylogeographic history. This can allow us to determine the region of origin of *T. melanocephalum* and to compare its dispersal routes with the patterns of human movement and/or with trajectories of trade and commerce.

Despite the development and increasing availability of next-generation sequencing (NGS) (Ekblom & Galindo, 2011), classical genetic markers such as mitochondrial DNA and microsatellites remain irreplaceable tools for most molecular ecologists. Their sequencing and assessment are user friendly, easy to perform, cheap, and comparable with constantly growing number of analysed organisms. Microsatellites also have several important advantages in comparison to NGS. For example, with the use of genomic data, Butler et al. (2014) developed microsatellite markers that are both conserved and applicable among such large taxonomic group as ants, yet polymorphic within species. Their study highlights possible trend in future utilization of microsatellites for researchers investigating other taxonomic groups. Mesak et al. (2014) compared the performance of microsatellites with RAD-seq SNP methods to characterize clonal patterns in a killifish (*Kryptolebias marmoratus*). They concluded that next-generation RAD-seq technology may have significant constraints in revealing the true genetic pattern compared to classical microsatellites (i.e. phylogenetic noise, issues when lacking a reference genome). Finally, a study by Schlick-Steiner et al. (2014) compared the characteristics of microsatellites to two NGS approaches and found microsatellite-based population genetics to require a smaller amount of DNA, exhibited fewer issues caused by DNA contaminants and were time-efficient (among other advantages). Furthermore, they pointed out that non-model organisms do not benefit as much from NGS as model organisms do due to a lack of background information and financial resources.

MATERIALS AND METHODS

Samples (exclusively female workers) were collected between 2008 and 2014 at several locations in Papua New Guinea (PNG) and the Federate States of Micronesia (Table 1). Specimens were determined preliminarily in the field and their identification later confirmed under stereoscope by comparisons with specimens deposited at Harvard Museum of Comparative Zoology (MCZ) and using online identification resources at www.antkeys.org. To confirm correct species identification and the species limits, a 601 bp fragment of *cytochrome c oxidase 1* (*COI*) subunit was sequenced from each individual and the haplotypes were compared with 41 additional *COI* sequences of four other *Tapinoma* species using Neighbour-Joining and Bayesian phylogenetic reconstruction (Lebrasseur, 2014).

Genomic DNA was extracted from specimens using Genomic DNA Mini Kit Tissue (Geneaid Biotech Ltd., New Taipei City, Taiwan). The concentration of isolated DNA was measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). The DNA of 12 individuals was pooled together and sent to Genoscreen (Lille, France) for the Geno Sat ® service, which includes microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science; for more details see Malausa et al., 2011). The following eight probes were used to enrich the total DNA in these motifs: $(TG)_{10}$, $(TC)_{10}$, $(AAC)_8$, $(AAG)_8$, $(AGG)_8$, $(ACG)_8$, $(ACAT)_6$, $(ACTC)_6$. GsFLX libraries were performed on PCR products. The company processed our samples with three others on an eighteen-sample run, with each sample individualized by the use of a Tag sequence. Concentration of each library was determined by fluorometry in order to get a minimum quantity of 1.46E + 8 mol/µl.

Table 1. Information about localities where the samples were collected.

Area	Locality	GPS	N
Micronesia	Chuuk	7°27′N, 151°53′E	1
Micronesia	Yap	9°31′N, 138°07′E	2
Micronesia	Pohnpei	6°58′N, 158°12′E	9
PNG	Wanang	5°13′S, 145°05′E	3
PNG	Port Moresby	9°14′S, 147°16′E	1
PNG	Weam	8°39′S, 141°08′E	2
PNG	Daru	9°52′S, 143°13′E	2
PNG	Baitabag	5°09′S, 145°47′E	4
PNG	Nagada	5°15′S, 145°79′E	4
PNG	Lepa Island	5°11′S, 145°50′E	1
PNG	Sinu Island	5°08′S, 145°49′E	1

The obtained primers were analysed for all primer secondary structures including hairpins, self-dimers and cross-dimers in primer pairs, using the on-line application NetPrimer (http://www.premierbiosoft.com/netprimer/). These secondary structures should be avoided if possible, because they could reduce amplification success. Primers were also checked for the presence of G or C bases within the last five bases from the 3´end of primer (GC clamp), which helps to promote specific binding at the 3´end due to the stronger bonding of G and C bases. In general, we followed the PCR primer design guidelines reviewed at http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html. Based on the NetPrimer analysis, we selected the 20 most suitable primer pairs for further testing. Initially, we performed monoplex PCR on 16 individuals with fluorescently labelled primers, followed by fragment analysis on the automated sequencer ABI 3730XL (Applied Biosystems, Foster City, California, USA). One PCR reaction consisted of 4 µl of Multiplex PCR Master-Mix (Qiagen, Hilden, Germany), 0.2 µM of each primer, 20 ng of template DNA and 3.6 µl of PCR water. For PCR conditions, we followed the manufacturer's protocol, with the annealing temperature of 54°C. Allelic patterns were scored using the software Genemapper 3.7 (Applied Biosystems).

Out of the tested loci, we selected those which were constantly amplified successfully and which provided clearly scorable and polymorphic PCR products. In the next step we pooled these loci into multiplex panels and used them to genotype all 30 individuals under the same PCR conditions as for the monoplex PCR. Basic parameters of loci, such as number of alleles, observed and expected heterozygosities, and Hardy-Weinberg equilibrium were calculated using the software GenAlEx (Peakall & Smouse, 2006). Exact tests for linkage disequilibrium were performed using the software Genepop 4.0 (Rousset, 2008). In order to obtain detailed information on population-genetic patterns and also for possible comparisons, we analysed the same 30 individuals using recently developed microsatellite markers, which should be universal among ants (Butler et al., 2014). We tested 23 of these loci for polymorphism in *T. melanocephalum* and calculated the same population-genetic parameters using polymorphic loci. To get the comparison of population-genetic patterns, we analysed six other ant species using the set of universal microsatellites which were found polymorphic in *T. melanocephalum*. Finally, to get the information about genetic variability within a single population, we genotyped 20 individuals of *T. melanocephalum* sampled at Wanang village, Papua-New Guinea, using the 12 newly developed loci. In *T. melanocephalum*, PCR, fragment analysis and genotyping were performed twice independently for all 30 individuals and all loci to assess the possible occurrence of allelic dropout (Gagneux et al., 1997).

RESULTS

The concentration of isolated DNA ranged from 0.5 to 15 ng/µl. The library quantification resulted in 5.24E + 09 mol/µl, a sufficient amount for performing emulsion PCR. Emulsion PCR results of the pool which contained our samples showed 88% beads recovery and 10% enrichment. In total, we obtained 31584 sequences (average length = 316 bp), of which 8090 contained microsatellite motifs and the software analysis (provided by GenoScreen) resulted in 450 bioinformatically validated pairs of primers. Out of the 20 loci tested, 12 were amplified consistently, were clearly scorable and polymorphic. We pooled these loci into 3 multiplex panels and the obtained genotypes were used to calculate the basic parameters of the loci (Table 2). The number of alleles per locus ranged from 2 to 18 (mean = 6.6) and the average expected and observed heterozygosities were 0.645 and 0.144, respectively. None of the loci were in Hardy-Weinberg equilibrium (HWE). The tests for linkage disequilibrium resulted in signifi-

Table 2. Characterization of 12 newly developed microsatellite markers in *Tapinoma melanocephalum*. Locus code, GenBank accession number, forward (F) and reverse (R) primer sequences, repeat motif, size range of alleles, number of alleles (Na), observed (*Ho*) and expected (*He*) heterozygosities based on analysis of 30 individuals.

Locus	Primer sequences (5´– 3´)	Repeat motif	Size range (bp)	Na	*Ho*	*He*	Multiplex
TM_2	F: GAAGGATGTCGAAATCGCAG	AC	88–136	18	0,367	0,916	1
KX197932	R: ACAATGTCCGAGAAGCAAGC						
TM_3	F: TGCAATGTAACAGTTTGAGGC	CT	141–150	5	0,033	0,626	1
KX197933	R: GGGAATAATTACCGTGGTTGG						
TM_6	F: GAGCAAGTGGAAGAAAGGAGG	AGG	87–90	2	0,033	0,206	1
KX197934	R: GTAGTGCGAAGACAATGGCA						
TM_9	F: ATACTTCCCATGCACCAAGG	ACA	197–221	5	0,467	0,600	1
KX197935	R: GCGTCGTTGTCTTCTTCCTC						
TM_10	F: TCATCCGTTTGATGAAGTGC	CT	87–97	5	0,067	0,663	1
KX197936	R: CTAGGAGGAAGCAGTGGAGC						
TM_5	F: AGAGCGTCACCCGATGTC	TC	184–216	12	0,300	0,772	2
KX197937	R: TGTCATTTTCTTGCACTTGTGA						
TM_13	F: CTTCTGATGCAGCTAGGGGA	TC	160–166	4	0,133	0,610	2
KX197938	R: ATTTCTTCGCGTCTGATTCG						
TM_16	F: AGCCAAGAGTTTCGTTCTCG	TCG	217–238	7	0,067	0,642	2
KX197939	R: TCTCTTTACGACGGTTCGCT						
TM_17	F: AATCCTTCCTATTCCCACCG	AC	150–154	3	0,033	0,485	3
KX197940	R: TCACCTTACTCAAGTCCAGGG						
TM_18	F: ATTCTCCGTAATCACGCTCG	TC	105–109	3	0,000	0,631	3
KX197941	R: CGTGGTGGAAAGGATATACG						
TM_19	F: AATGTGATCGTGATGAGCCA	CA	217–238	9	0,200	0,806	3
KX197942	R: GGTTCCTAGAACTTTGCGACG						
TM_20	F: TTAGCTGAATTTTCATTGAACG	AT	287–301	6	0,033	0,777	3
KX197943	R: CTCGCTGGCCTGTTCCTC						
Mean				6,6	0,144	0,645	

Table 3. Parameters of universal microsatellite loci polymorphic in *Tapinoma melanocephalum*. Locus code, size range of alleles, number of alleles (Na), observed (*Ho*) and expected (*He*) heterozygosities based on analysis of 30 individuals.

Locus	Size range (bp)	Na	*Ho*	*He*
Ant859	190–203	5	0,037	0,629
Ant7680	274–278	2	0,037	0,036
Ant5035	325–358	9	0,222	0,621
Ant10878	300–399	4	0,032	0,232
Ant7249	339–347	3	0,074	0,201
Ant1343	187–205	3	0,000	0,271
Ant4155	192–219	10	0,556	0,822
Ant2794	256–323	8	0,074	0,527
Ant1368	326–330	5	0,074	0,722
Ant9218	341–379	7	0,370	0,783
Ant3648	332–372	11	0,037	0,864
Ant8424	200–248	10	0,185	0,835
Mean		6,4	0,142	0,545

Table 5. Genetic variability among 20 individuals of *T. melanocephalum* within a single population (Wanang, PNG) and the 12 newly developed microsatellite loci. Number of alleles (Na), observed (*Ho*) and expected (*He*) heterozygosity.

Locus	Na	*Ho*	*He*
TM_2	2	0,000	0,375
TM_3	1	0,000	0,000
TM_5	1	0,000	0,000
TM_6	1	0,000	0,000
TM_9	1	0,000	0,000
TM_10	2	0,000	0,255
TM_13	2	0,050	0,289
TM_16	2	0,000	0,095
TM_17	1	0,000	0,000
TM_18	3	0,050	0,096
TM_19	1	0,000	0,000
TM_20	3	0,000	0,654
Mean	1,66	0,008	0,147

cant values in 166 out of 276 pairs of loci. Out of the 23 universal microsatellites, 12 were polymorphic in *T. melanocephalum* and were analysed under the same conditions as the de novo developed loci. The number of alleles per locus ranged from 2 to 11 (mean = 6.4) and the average expected and observed heterozygosities were 0.545 and 0.142 respectively. None of the loci were in HWE (Table 3). Population genetic parameters of six other ant species obtained with the use of the same 12 universal microsatellites are summarized in Table 4. Values of observed (*Ho*) and expected (*He*) heterozygosities within these six species ranged from 0.23 to 0.63 (mean = 0.46) and from 0.24 to 0.68 (mean = 0.53), respectively, and the mean number of alleles ranged from 2.1 to 4.9 (mean = 3.8). Genetic variability parameters of the single population of *T. melanocephalum* from Wanang village (PNG) are given in Table 5. The number of alleles per locus ranged from 1 to 3 (mean = 1.66) and the average expected and observed heterozygosities were 0.147 and 0.008, respectively. No differences in genotypes were observed comparing the two independent PCRs and fragment analyses.

DISCUSSION

We detected enormously high level of homozygosity in the newly developed microsatellite loci, ranging between 53 and 100% among individual loci and with a mean over all loci of 85.6%. Simultaneously, we detected average levels of genetic diversity, with the number of alleles per locus ranging from 2 to 18 and a mean over all loci of 6.6. Several biological processes might lead to such pattern, in particular founder effect, bottleneck and/or inbreeding (Frankham et al., 2008). These phenomena are expected in *T. melanocephalum* based on what has been described about its life history with the strong ability to disperse over large distances and establishing new colonies with only a few individuals (Wetterer, 2009). The genetic variability detected within the single population from Wanang village (PNG) was extremely low, both in terms of number of alleles per locus and observed heterozygosity. This finding strongly supports the conclusion about the presence of population-genetic phenomena mentioned above in *T. melanocephalum*.

The presence of colony budding, polydomy and lack of aggressiveness among physically separate colonies are suggestive of a decreased genetic variability or high levels of relatedness among individuals. These are often a consequence of within-nest mating, limited dispersal of males and/or females or parthenogenetic reproduction (Pearcy et al., 2006) and have been documented in multiple ant species (Trontti et al., 2005; Thurin & Aron, 2009; Kureck et al., 2012).

To support this interpretation, we performed a comparative analysis of microsatellite genotypic patterns using universal mi-

Table 4. Parameters of 12 universal microsatellite loci, which were polymorphic in *Tapinoma melanocephalum* used for analyses of six other ant species (4 individuals per species). Number of alleles (Na), observed (*Ho*) and expected (*He*) heterozygosities. *Mean values were calculated based on polymorphic loci only.

Locus	*Anonychomyrma*			*Camponotus*			*Odontomachus*			*Pseudolasius*			*Nylanderia*			*Philidris*		
	Na	*Ho*	*He*	Na	*Ho*	*He*	Na	*Ho*	*He*	Na	*Ho*	*He*	Na	*Ho*	*He*	Na	*Ho*	*He*
Ant859	4	0,500	0,688	2	0,000	0,375	2	0,000	0,375	3	1,000	0,625	2	0,000	0,500	1	0,000	0,000
Ant7680	7	1,000	0,844	1	0,000	0,000	3	0,500	0,594	1	0,000	0,000	2	0,000	0,375	7	1,000	0,844
Ant5035	6	0,500	0,813	5	0,750	0,688	5	1,000	0,750	2	0,000	0,500	6	1,000	0,781	1	0,000	0,000
Ant10878	3	0,250	0,406	6	0,750	0,781	5	0,750	0,750	6	0,500	0,813	1	0,000	0,000	3	0,250	0,531
Ant7249	2	0,250	0,219	2	0,000	0,375	3	0,500	0,406	2	0,500	0,375	2	0,250	0,469	2	0,250	0,219
Ant1343	7	1,000	0,844	1	0,000	0,000	1	0,000	0,000	5	1,000	0,750	2	0,250	0,219	7	0,750	0,844
Ant4155	3	0,000	0,625	4	0,500	0,719	1	0,000	0,000	5	1,000	0,781	7	1,000	0,844	7	0,750	0,844
Ant2794	6	1,000	0,813	4	0,500	0,563	1	0,000	0,000	5	1,000	0,781	6	0,500	0,813	8	1,000	0,875
Ant1368	7	1,000	0,844	4	0,750	0,656	1	0,000	0,000	6	0,750	0,813	4	0,500	0,688	6	0,750	0,813
Ant9218	6	0,750	0,813	5	0,750	0,750	1	0,000	0,000	2	0,000	0,500	4	0,250	0,656	5	0,500	0,750
Ant3648	3	0,500	0,531	5	0,750	0,688	1	0,000	0,000	3	0,667	0,667	4	0,250	0,656	7	0,750	0,844
Ant8424	5	0,750	0,688	5	0,250	0,781	1	0,000	0,000	3	0,250	0,594	5	0,500	0,750	4	0,500	0,688
Mean*	4,9	0,625	0,677	4,2	0,500	0,638	4,6	0,550	0,575	3,82	0,606	0,650	4	0,410	0,610	5,6	6,500	0,730

crosatellite loci developed by Butler et al. (2014). We tested 23 universal microsatellites for polymorphism in *T. melanocephalum* and found 12 polymorphic loci (Table 3). The same 30 individuals were genotyped using these universal loci. We detected similar patterns to those of our newly developed loci – high levels of homozygosity (mean over all loci = 86%) and common levels of allelic variability (mean number of alleles per locus = 6.4). To show that such parameters are specific for *T. melanocephalum*, we used the same 12 universal microsatellites to assess genetic diversity in six other Indo-Pacific ant species/genera (*Anonychomyrma scrutator*, *Camponotus maculatus*, *Odontomachus simillimus*, *Pseudolasius australis*, *Nylanderia vaga*, *Philidris cordata*), analysing four individuals per species. In all six species the levels of observed and expected heterozygosities were balanced and no homozygosity excess was observed (Table 4).

The populations of *T. melanocephalum* from PNG and Micronesia exhibited deviations from Hardy-Weinberg equilibrium in all analysed loci. We performed several tests to prove that this observation was not an artefact but represented genuine population-genetic pattern. As we aimed to detect the allelic diversity within the region, we composed the dataset of individuals from three different islands of Micronesia and eight different (and geographically distinct) areas of PNG. Such combination may and obviously did result in the Wahlund effect, e.g. homozygosity excess caused by several genetically distinct units grouped and considered as a single sampling unit (Selkoe & Toonen, 2006). Tests for linkage disequilibrium resulted in significant values in 60% of loci pairs, however this is clearly an artefact caused by the homozygosity excess and Hardy-Weinberg disequilibrium (Sabatti & Risch, 2002; Slatkin, 2008).

Genotypes based on two independent PCRs were identical in all 30 individuals and 24 loci, so the occurrence of allelic dropout can be considered absent or negligible. This conclusion is also supported by the sufficient concentration of isolated DNA and by our success in amplifying 601 bp fragment of mtDNA.

The last possible artefact responsible for these observed patterns would be null alleles, but we can also reject this option as we did not detect any individual that would fail to amplify any allele at just one or several loci, while the rest of the loci would amplify normally (Selkoe & Toonen, 2006) – in this study, we observed homozygote excess in all 24 analysed loci and at least one allele was amplified in all individuals and all loci.

CONCLUSION

In this paper, we described 12 newly developed polymorphic microsatellite markers for *Tapinoma melanocephalum*, a widespread invasive ant species. Recognition of whole-area population structure of this ant species might contribute to reveal the most important colonization pathways of this ubiquitous pest species and means of dispersal. Moreover, reconstructions of its population genetic structure would provide an interesting comparison to the migration patterns and dispersal history of *Homo sapiens*, a worldwide distributed primate species, being reasonably suspected of playing an important role in the ghost ant's dispersal. Besides the newly developed loci, we have tested recently published ant-universal microsatellite markers. In total, we reported 24 microsatellite markers useful for population-genetic investigations of the target species. Within a sample of *T. melanocephalum* populations from Papua-New Guinea and Micronesia, we have detected high levels of homozygosity. This finding was confirmed by a comparison of genetic diversity parameters within six other Indo-Pacific ant species using the same universal loci. Despite the deviation from the Hardy-Weinberg equilibrium in the sampled populations composed of individuals from numerous distinct localities, the newly developed microsatellites provide an effective tool for future investigations of genetic population structure as well as for phylogeographic analyses of *T. melanocephalum*. Moreover, microsatellite analysis can also help clarify the taxonomy and species delimitation within genus *Tapinoma*, which remains until today partially unclear, especially in the Indo-Pacific region where several sister species co-occur sympatrically.

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REFERENCES

Appel A.G.J., Na J.P.S. & Lee C.Y. 2004: Temperature and humidity tolerances of the ghost ant, *Tapinoma melanocephalum* (Hymenoptera: Formicidae). — *Sociobiology* **44**: 89–100.

Bustos X. & Cherix D. 1998: Contribution à la biologie de *Tapinoma melanocephalum* (Fabricius) (Hymenoptera: Formicidae). — *Actes Coll. Insectes Soc.* **11**: 95–101.

Butler I.A., Siletti K., Oxley P.R. & Kronauer D.J.C. 2014: Conserved microsatellites in ants enable population genetic and colony pedigree studies across a wide range of species. — *PloS ONE* **9**(9): e107334, 11 pp.

Choe H.C., Lim J.O. & Lee S. 2009: *Tapinoma melanocephalum* (Fabricius), a new exotic pest in Korea. — *J. Asia-Pac. Entomol.* **12**: 67–69.

Clouse R.M. 2007: The ants of Micronesia (Hymenoptera: Formicidae). — *Micronesica* **39**: 171–275.

Collingwood C.A. 1979: *The Formicidae (Hymenoptera) of Fennoscandia and Denmark*. *Fauna Entomologica Scandinavica. Vol. 8*. Scandinavian Science Press, Klampenborg, 174 pp.

Ekblom R. & Galindo J. 2011: Applications of next generation sequencing in molecular ecology of non-model organisms. — *Heredity* **107**: 1–15.

Frankham R., Ballou J. & Briscoe D. 2008: *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, 617 pp.

Gagneux P., Boesch C. & Woodruff D.S. 1997: Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. — *Mol. Ecol.* **6**: 861–868.

Harada Y. 1990: Ant pests of the Tapinomini tribe. In Vander Meer R.K., Jaffe K. & Cedeno A. (eds): *Applied Myrmecology. A World Perspective*. Westview Press, Boulder, pp. 298–315.

Klimes P., Idigel C., Rimandai M., Fayle T.M., Janda M., Weiblen G.D. & Novotný V. 2012: Why are there more arboreal ant species in primary than in secondary tropical forests? — *J. Anim. Ecol.* **81**: 1103–1112.

Kureck I.M., Jongepier E., Nicolai B. & Foitzik S. 2012: No inbreeding depression but increased sexual investment in highly inbred ant colonies. — *Mol. Ecol.* **21**: 5613–5623.

Lebrasseur O.M.M. 2014: *Dogs, Chickens and Ants: Investigating the Reliability of Modern Maternal Genetic Data in Retracing Early Dispersals*. PhD Thesis, Durham University, 32 pp.

Malausa T., Gilles A., Meglecz E., Blanquart H., Duthoy S., Costedoat C., Dubut V., Pech N., Castagnone-Sereno P.,

Délye C. et al. 2011: High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. — *Mol. Ecol. Resour.* **11**: 638–644.

Mesak F., Tatarenkov A., Early R.L. & Avise J.C. 2014: Hundreds of SNPs vs. dozens of SSRs: which dataset better characterizes natural clonal lineages in a self-fertilizing fish? — *Front. Ecol. Evol.* **2**: 74.

Moreira D.D.O., Morais V., Vieira-da-Motta O., Campos-Farinha A.E.C. & Tonhasca Jr. A. 2005: Ants as carriers of antibiotic-resistant bacteria in hospitals. — *Neotrop. Entomol*. **34**: 999–1006.

Passera L. 1994: Characteristics of tramp species. In Williams D.F. (ed.): *Exotic Ants.* Westview Press, Boulder, CO, pp. 23–43.

Peakall R. & Smouse P.E. 2006: GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. — *Mol. Ecol. Notes* **6**: 288–295.

Pearcy M., Hardy O. & Aron S. 2006: Thelytokous parthenogenesis and its consequences on inbreeding in an ant. — *Heredity* **96**: 377–382.

Rousset F. 2008: Genepop ´007: a complete re-implementation of the genepop software for Windows and Linux. — *Mol. Ecol. Resour.* **8**: 103–106.

Sabatti C. & Risch N. 2002: Homozygosity and linkage disequilibrium. — *Genetics* **160**: 1707–1719.

Schlick-Steiner B.C., Arthofer W., Moder K. & Steiner F.M. 2014: Recent insertion/deletion (reINDEL) mutations: increasing awareness to boost molecular-based research in ecology and evolution. — *Ecol. Evol.* **5**: 24–35.

Selkoe K.A. & Toonen R.J. 2006: Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. — *Ecol. Lett.* **9**: 615–629.

Slatkin M. 2008: Linkage disequilibrium – understanding the evolutionary past and mapping the medical future. — *Nature Rev. Genet.* **9**: 477–485.

Smith M.R. 1965: House-infesting ants of the eastern United States. Their recognition, biology, and economic importance. — *U.S.D.A. Technol. Bull.* **1326**: 1–105.

Thurin N. & Aron S. 2009: Sib-mating in the ant *Plagiolepis pygmaea*: adaptive inbreeding? — *J. Evol. Biol*. **22**: 2481–2487.

Trontti K., Aron S. & Sundstrom L. 2005: Inbreeding and kinship in the ant *Plagiolepis pygmaea*. — *Mol. Ecol.* **14**: 2007–2015.

Tsutsui N.D. & Suarez A.V. 2003: The colony structure and population biology of invasive ants. — *Conserv. Biol.* **17**: 48–58.

Venkataramaiah G.H. & Rehman P.A. 1989: Ants associated with the mealybugs of coffee. — *Indian Coffee* **43**: 13–14.

Wetterer J.K. 2009: Worldwide spread of the ghost ant, *Tapinoma melanocephalum* (Hymenoptera: Formicidae). — *Myrmecol. News* **12**: 23–33.

Wen W.J. 2007: *The Feeding Behaviour of the Ghost Ants,* Tapinoma melanocephalum *(Fabricius) and* Tapinoma indicum *(Forel) (Hymenoptera: Formicidae).* Master Thesis, University Sains Malaysia, 154 pp.

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Vývoj mikrosatelitů u nemodelových druhů živočichů

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