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BIOTEHNIŠKA FAKULTETA

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VARSTVENA GENETIKA RJAVEGA MEDVEDA (*Ursus arctos* L.) V SLOVENIJI

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CONSERVATION GENETICS OF BROWN BEAR (*Ursus arctos L.*) IN SLOVENIA

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AI Varovanje rjavega medveda in upravljanje z njim potrebuje trdne znanstvene podatke, če želimo, da bi upravljavske odločitve dejansko delovale v realnem svetu. Orodja, ki jih je prinesel hiter razvoj molekularne genetike v zadnjih dveh desetletjih, nam pri tem dajejo popolnoma nov, prej nepredstavljiv vpogled v dogajanja v naravnih populacijah. Ta varstveno genetska orodja smo uporabili za raziskavo parametrov, pomembnih za spremljanje varstvenega statusa populacije medveda v Sloveniji. S pomočjo neinvazivnega genetskega vzorčenja in modeliranja označevanja in ponovnega ulova smo ocenili število medvedov v Sloveniji ob koncu letne smrtnosti leta 2007, pred reprodukcijo 2008 (424 osebkov, 95 % CI 383-458) in spolno strukturo populacije (40,5 % samcev, 59,5 % samic). Razvili smo novo metodo za primerjavo genetske pestrosti med prej neprimerljivimi populacijami in jo uporabili v meta-analizi za pregled globalne porazdelitve tega parametra pri rjavem medvedu. Kot prvi smo pokazali, da je mogoče spremljati efektivno velikost populacije v prosti naravi in to spremljanje tudi vključiti v rutinske programe monitoringa. Pokazali smo tudi, da je efektivna velikost populacije medvedov v severnih Dinaridih (276, 183-350 95 % CI) sicer velika, da pa ne dosega številčnosti potrebne za dolgoročno ohranitev evolucijskega potenciala. Z genetsko potrditvijo identitete v Sloveniji označenega medveda, ubitega v krivolovu v Avstriji, smo prispevali k razumevanju krivolova kot ključnega problema za naravno rekolonizacijo medveda v Alpe. Rezultati raziskav predstavljajo trden temelj upravljanju s to veliko zverjo pri nas in kaže, da bodo nekatere izmed teh raziskav prerastle v trajen genetski monitoring populacije.

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AI Conservation and management of the brown bear requires solid scientific data if we want the management decisions to work in the real world. Tools provided by rapid development of molecular genetics over the last two decades provide us with a completely new, previously unimaginable insight into processes in natural populations. We used these conservation genetics tools to research parameters required for monitoring of the population status of brown bear in Slovenia. We used noninvasive genetic sampling and mark-recapture modeling to estimate the number of bears in Slovenia at the end of annual mortality in 2007 and before the reproduction of 2008 (424 individuals, 95 % CI 383-458) and sex structure of the population (40.5 % males, 59.5 % females). We developed a new method for comparing genetic diversity between previously incomparable populations and used it for a meta-analysis of global distribution of this parameter in the brown bear. We were the first to show that the effective population size can be tracked through time in a natural population and included in routine monitoring programs. We also showed that the effective population size of bears in northern Dinaric Mts. (276, 183-350 95 % CI), although large, still does not meet the threshold required to retain the population's evolutionary potential. Through genetic confirmation of identity of a GPS-collared bear poached in Austria we contributed to understanding of poaching as a key obstacle for natural recolonization of bears into the Alps. Results of our research represent a solid foundation for brown bear management in Slovenia and it seems that at least a part of it may evolve into a long-term genetic monitoring of the population.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

KAZALO PREGLEDNIC

KAZALO SLIK

2.1 Objavljena znanstvena dela

2.1.1 Monitoring efektivne velikosti populacije rjavega medveda (Ursus arctos) z uporabo novih pristopov, ki potrebujejo en sam vzorec genotipov

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 UVOD

Karizmatične vrste, kot to medved nedvomno je, so pogosto v žarišču javnega interesa. Nekateri jih malodane častijo, drugi bi jih najraje iztrebili, nikogar pa ne pustijo ravnodušnega. Zaradi tega je upravljanje in varovanje takšnih vrst težek in velik zalogaj za vsakega upravljavca, saj se bo ne glede na odločitev zmeraj našel kdo, ki mu ta odločitev ne bo všeč. Ključno vlogo pri sprejemanju upravljalskih in varstvenih odločitev mora imeti znanost, saj lahko samo znanstveno preverjeni, verodostojni podatki omogočijo suverene, argumentirane odločitve, ki zagotavljajo ustreznost upravljanja in upravljavcu nudijo varnost pred neizogibnimi kritikami, vrsti pa ustrezno varstvo in dolgoročni obstoj. Prav pri zagotavljanju takšnih podatkov postaja vedno pomembnejša vloga varstvene genetike. Mnoga vprašanja o številčnosti, populacijski dinamiki, spolni strukturi, sposobnosti dolgoročnega preživetja in drugih pomembnih parametrih za varstvo in upravljanje so bila nedolgo tega praktično nerešljiva in predmet bolj ali manj grobih ocen. Genetika nam lahko na ta vprašanja ponudi odgovore, dovoljuje pa nam tudi nova vprašanja, ki si jih prej nismo upali niti postaviti.

Molekularna genetika je naredila dolgo pot od zamudne, drage metode neuporabne za ekološke študije naravnih populacij do hitrega, poceni in široko uporabnega orodja, ki ga poznamo danes. Že v času, ki sem ga porabil za izdelavo doktorskega dela, sem bil priča občutnim spremembam in napredku. Analize, ki so nam leta 2005 vzele skoraj pol leta, lahko zdaj ponovimo v nekaj dneh, študije, o katerih si takrat nismo upali niti razmišljati, postajajo danes realnost. In videti ni nobenih znakov, da bi se ta trend kakorkoli upočasnjeval. Prav nasprotno, vse kaže, da genetika svojo pravo mesto in veljavo v ekoloških študijah šele dobiva.

Dobro zasnovan genetski monitoring ima velik potencial, da razumevanje populacije medveda v Sloveniji postavimo na popolnoma novo raven in znatno izboljšamo varovanje in upravljanje te karizmatične vrste pri nas. Tako je bil glavni cilj mojega dela vzpostavitev temeljev varstvene genetike medveda pri nas: raziskava trenutnega stanja genetske pestrosti in efektivne velikosti populacije medvedov v Sloveniji ter postavitev temeljev za trajen genetski monitoring in forenzične raziskave te vrste pri nas, tako s pomočjo vzorcev ulovljenih, ustreljenih ali drugače umrlih medvedov, kot s pomočjo neinvazivne genetike. Paleta problemov, ki sem jih pri tem obravnaval, je široka. O znanstveni zanimivosti dela pričajo objave v vodilnih znanstvenih revijah s področja molekularne ekologije in varstvene genetike, ki so sestavni del tega doktorskega dela. Zlasti pa upam, da bo to delo imelo tudi širši vpliv na varovanje in upravljanje naravnih populacij redkih in ogroženih živalskih vrst, saj bo le tako naloga, ki sem si jo ob začetku zadal, dokončno izpolnjena.

1.2 RJAVI MEDVED (*URSUS ARCTOS*)

Zgodovina medveda v Evropi je dolga zgodba izumiranja in kratka zgodba vrnitve. Stoletja preganjanja so to vrsto izbrisale iz večjega dela Evrope, tako da so v začetku XX. stoletja ostali samo izolirani ostanki populacij v Apeninih, Italijanskih Alpah, Kantabrijskem gorovju in Pirenejih (Zedrosser in sod., 2001). Nekoliko bolje je šlo medvedom v Srednji, Vzhodni in Severni Evropi, kjer so ostale avtohtone populacije v Dinaridih, Karpatih in Severni Evropi, ki pa so bile prav tako mnogo manjše, kot so danes.

Zgodba se je spremenila v drugi polovici prejšnjega stoletja. Naravne populacije so si opomogle in se začele širiti (Zedrosser in sod., 2001), zadnje desetletje dvajsetega stoletja pa je prineslo tudi prve poskuse ponovnih naselitev te vrste na območja, kjer je izumrla (Clark in sod., 2002).

Medvedi v Sloveniji predstavljajo severozahodni rob dinarske populacije, ene od preostalih avtohtonih populacij medveda v Evropi. Celotna populacija se razprostira preko 11 držav z ocenjeno številčnostjo 2800 osebkov in se na večjem delu območja smatra kot stabilna (Zedrosser in sod., 2001). Morda je populacija razdrobljena v več manjših subpopulacij, saj je habitat na nekaterih območjih prekinjen (Zedrosser in sod., 2001). Zgodovinski zapisi kažejo, da so prišli medvedi v Sloveniji v prvi polovici dvajsetega stoletja na rob izumrtja (Švigelj, 1961), populacija pa si je opomogla in se začela širiti šele v drugi polovici tega stoletja (Jerina in Adamič, 2008b). Prav dinarska populacija je genetsko najprimernejša za ponovne naselitve te vrste v Zahodno Evropo (Taberlet in Bouvet, 1994), medvedi iz Slovenije pa so bili že ponovno naseljeni v Italijo, Francijo in Avstrijo (Clark in sod., 2002).

1.3 VARSTVENA GENETIKA

Pri varovanju in upravljanju in upravljanju z redkimi in ogroženimi vrstami, tudi z medvedom, dobiva vse večji pomen razmeroma mlada znanstvena disciplina, varstvena genetika. Frankham (2002) opredeljuje varstveno genetiko kot »uporabo genetike za ohranjanje vrst kot dinamičnih subjektov, ki so se sposobni prilagajati spremembam v okolju«. Je del varstvene biologije, krizne znanstvene vede, ki se ukvarja z biotsko pestrostjo, naravnimi procesi, ki jo ustvarjajo in načini za njeno ohranjanje pri spremembah okolja, ki jih povzroča človek. Tako kot so grožnje biotski pestrosti raznolike, so raznolika tudi orodja, ki jih v varstveni genetiki uporabljamo, njihov skupen cilj pa je boljše razumevanje razlogov ogrožanja in iskanje rešitev, ki bodo omogočale dolgotrajno preživetje populacij, v skrajnem primeru pa tudi rešitev vrste pred izumrtjem.

1.3.1 Genetski monitoring

Pomemben del varstvene genetike je genetski monitoring. Čeprav postaja zaradi človekovega poseganja v naravo monitoring pomembnih vrst in ekosistemskih procesov vedno bolj ključen del varovanja biotske pestrosti, velika večina programov monitoringa še vedno ne izkorišča vseh potencialov, ki jih nudi sodobna molekularna genetika (Schwartz in sod., 2007). Genetski pristopi omogočajo pridobivanje podatkov, ki so relevantni tako v ekoloških kot evolucijskih časovnih okvirjih, nam nudijo odgovore na vprašanja, na katera z drugimi metodami ni mogoče odgovoriti, ob tem pa so pogosto znatno cenejši od klasičnih ekoloških pristopov k monitoringu (Schwartz in sod., 2007). Čeprav je v Sloveniji v monitoring populacije medveda že desetletja vloženega precej truda in sredstev, so rezultati, ki jih dajejo klasične ekološke metode, v marsičem omejeni. Genetske metode pa nam omogočajo vpogled v temeljne procese, ki določajo sposobnost populacije, da preživi preko daljših časovnih obdobij v spreminjajočem se okolju.

1.3.2 Genetska pestrost populacije

Poznavanje in spremljanje genetske pestrosti in efektivne velikosti populacije nam omogoča hitro zaznavanje pomembnih sprememb, ki bi lahko populacijo ogrozile. Genetska pestrost je temelj fitnesa in evolucijskega potenciala populacije, posledično pa tudi njene sposobnosti za prilagajanje na spremembe v okolju (Allendorf in Luikart, 2007; Frankham in sod., 2002; Reed in Frankham, 2003). Populacija, ki izgubi velik del genetske pestrosti, bo v večini primerov obsojena na propad. Kljub temu pa smo do nedavnega vedeli o genetski pestrosti naših medvedov zelo malo. Problematika ima še večji pomen, ker bodo slovenski medvedi kot genetsko najustreznejši (Taberlet in Bouvet, 1994) skoraj gotovo še naprej uporabljani pri ponovnih naselitvah, kjer je visoka genetska pestrost izvorne populacije ključnega pomena (Frankham, 2009). Po drugi strani pa visoka genetska pestrost naših medvedov ni sama po sebi umevna, če vemo, da je populacija nedavno prišla na sam rob izumrtja (Huber in sod., 2009; Huber in Frković, 1993; Švigelj, 1961). Izguba genetske pestrosti v populacijskem "ozkem grlu" je odvisna od obsega redukcije populacije in trajanja »ozkega grla« (Allendorf in Luikart, 2007; Frankham in sod., 2002), o njenem obsegu pa je iz zgodovinskih zapisov težko sklepati.

1.3.3 Efektivna velikost populacije

Genetski parameter, ki je podobno kot genetska pestrost neločljivo povezan z varstvom populacije in verjetnostjo njenega dolgotrajnega preživetja, je efektivna velikost populacije (N_e). Efektivna velikost populacije je verjetno en izmed najbolj elegantno preprostih konceptov v vsej biologiji (Waples, 2010) in je ključen parameter tako v varstveni (Frankham, 2005) kot v evolucijski biologiji (Charlesworth, 2009). Opredeljena je kot velikost Wright-Fisherjeve populacije (Fisher, 1930; Wright, 1931), ki bi izgubljala genetsko pestrost tako hitro kot naravna populacija, ki jo preučujemo (Crow in Kimura,

1970). Opisuje hitrost naključnih genetskih procesov in jo lahko razumemo kot neposreden kazalnik evolucijskega potenciala populacije in njene ranljivosti na naključne genetske procese. Lahko jo neposredno uporabimo kot temelj za predvidevanje usode majhnih populacij (Leberg, 2005; Palstra in Ruzzante, 2008), preko nje pa lahko zgodaj zaznamo fragmentacijo (England in sod., 2010) in zmanjšanje populacije (Antao in sod., 2010). N_e se uporablja tudi kot kriterij sposobnosti populacije za dolgoročno preživetje (viabilnosti), kot je v svojem klasičnem delu predstavil Franklin (1980). Pri tem velja N_e = 50 kot minimum za izogibanje parjenja v sorodstvu, N_e = 500 pa kot meja za ohranitev evolucijskih procesov in dolgoročno viabilnost. Razmerje med efektivno in dejansko oziroma ocenjeno velikostjo populacije (N_c) je v povprečju okrog 1:10, se pa od vrste do vrste (in populacije) razlikuje (Frankham in sod., 2002). Pri medvedu so s pomočjo simulacij prišli do ocen razmerja N_e/N_c od 0.2 do 0.38 (Harris in Allendorf, 1989), do podobnih ocen pa so prišli tudi z empiričnimi študijami (Miller in Waits, 2003), čeprav lahko te vrednosti padejo tudi na 0.1 glede na velikost populacije in sistem upravljanja (Luikart in sod., 2010).

V zadnjih štiridesetih letih je bilo razvitih več metod, ki omogočajo oceno efektivne velikosti populacije iz genetskih podatkov. Daleč največ uporabljana je časovna metoda (Leberg, 2005; Luikart in sod., 2010). Metodo sta prva opisala (Krimbas in Tsakas, 1971), temelji pa na vzorčenju genetske pestrosti v več časovnih obdobjih, spremembe v frekvencah alelov zaradi genetskega drifta pa so signal za oceno harmonične sredine N_e v obdobju med vzorčenji. V zadnjem času so bile razvite metode, ki omogočajo oceno N_e iz enega genetskega vzorčenja populacije v določenem trenutku. Metoda vezavnega neravnovesja (linkage disequilibrium), ki uporablja kot signal za oceno N_e vezavno neravnovesje med nevtralnimi lokusi, je bila razvita že pred dvajsetimi leti (Hill, 1981), pristop, ki pa omogoča njeno dejansko uporabo, pa je bil razvit šele nedavno (Waples, 2006). Še ena nedavno razvita metoda je simulacijska metoda z Bayesovim pristopom (Tallmon in sod., 2008). V zadnjih letih sta bili razviti tudi dve zanimivi metodi, ki sta pravzaprav hibrid med genetskimi in demografskimi metodami (Wang, 2009; Wang in sod., 2010). Obe uporabljata genetske podatke za oceno sorodnosti ali starševstva, nato pa preko tega ocenita N_e. Zlasti zanimiva je metoda EPA (Estimator by Parentage Assignment, (Wang in sod., 2010), ki je edina do zdaj razvita metoda, ki omogoča neposredno in korektno oceno N_e pri vrstah, ki imajo prekrivajoče generacije, ne da bi morali zelo podrobno poznati demografske značilnosti vrste.

Čeprav ima potencial kot idealen parameter za genetski monitoring, je spremljanje N_e skozi čas zelo zahtevno (Schwartz in sod., 2007). Najpogosteje uporabljano časovno metodo je zelo težko uporabiti kot osnovo za monitoring (Schwartz in sod., 2007), saj potrebuje najmanj dva vzorca populacije, med katerima mora preteči najmanj ena generacija. V praksi mora biti ta interval znatno daljši, zlasti če se generacije prekrivajo. Omenjene

sodobne metode, ki omogočajo oceno N_e z enim vzorcem genotipov zajetih iz populacije v določenem trenutku, so zaenkrat še premalo uporabljane, imajo pa ogromen potencial (Waples in Do, 2010). Pred našo raziskavo še ni bila nobena uporabljena v kontekstu monitoringa efektivne velikosti populacije pri sesalcih.

1.3.4 Neinvazivno genetsko vzorčenje, ocena številčnosti s pomočjo genetskega označevanja in ponovnega ulova in forenzična genetika

Razmeroma novo in zelo uporabno orodje za genetski monitoring »klasičnih« ekoloških parametrov je neinvazivna genetika (Waits in Paetkau, 2005), kjer za genetske analize uporabljamo material, ki ga žival pusti v okolju (iztrebki, urin, dlaka, slina…). Na ta način lahko zelo učinkovito spremljamo klasične demografske kazalnike populacije kot so njena velikost in spolna struktura (Luikart in sod., 2010). Metode analize takih vzorcev so zahtevne, pri učinkoviti vpeljavi v rutinski monitoring pa lahko nudijo vpogled v dogajanja v populaciji, ki ga ne moremo dobiti na noben drug način.

Tip materiala, ki se uporablja kot neinvazivni genetski vzorec, je odvisen od živalske vrste in cilja raziskave. Pri medvedu pa gre v veliki večini primerov za iztrebke ali dlako. Težava prvih tovrstnih raziskav v devetdesetih letih prejšnjega stoletja je bila, da raziskovalci niso razumeli problema, ki ga pri tem predstavljajo napake pri genotipizaciji, in so prihajali do popolnoma napačnih zaključkov (npr. Gagneux in sod., 1997). Pionirsko delo na tem področju je bilo narejeno prav na medvedu (Taberlet in sod., 1997), kjer so problem napak genotipizacije prvič korektno obravnavali. O problemu zanesljivosti genotipov je bilo veliko govora, razumevanje tega problema in metode korekcije pa so danes dovolj razvite, da zanesljivost genotipizacije dosega tisto, ki jo običajno srečamo pri tkivnih vzorcih (Waits in Paetkau, 2005). Neinvazivna genetika doživlja razcvet v 21. stoletju, saj omogoča poceni pridobivanje statistično relevantnega števila vzorcev na ravni populacije, ne da bi živali poškodovali ali motili pri njihovem naravnem obnašanju (Waits in Paetkau, 2005). Pri medvedu so bile metode že večkrat uporabljene, zlasti z namenom ocene velikosti populacije (npr. Bellemain in sod., 2004; Kendall in sod., 2008), kljub temu pa je za njihovo vpeljavo v praktično rabo običajno potrebnega veliko dela, precej prostora pa je še vedno tudi za izboljšave.

Neinvazivno genetsko vzorčenje je idealna metoda za »označevanje« (ponovno prepoznavanje) živali. Ko iz neinvazivnega genetskega vzorca preberemo genotip osebka, lahko to žival kasneje vedno prepoznamo, bodisi v naslednjem neinvazivnem vzorcu, bodisi preko analize tkiva, ko žival pogine. Vzporedno z razvojem neinvazivnega genetskega vzorčenja so se razvijale tudi metode ocene številčnosti po metodi označevanja in ponovnega ulova. Čeprav gre za metode, ki se razvijajo že stoletja, so pravi razcvet doživele v zadnjih dveh desetletjih (Amstrup s sod., 2005). Vedno bolj robustni modeli omogočajo vključevanje številnih dodatnih informacij in vse boljše ocene, ob ocenah

številčnosti pa postaja vedno bolj mogoče slediti tudi parametrom populacijske dinamike (rodnosti, smrtnosti), migracijam med posameznimi območji in podobno. Z razvojem statistike ter informacijsko-teoretičnimi pristopi k izbiri modelov (Burnham in Anderson, 2002) se je postavil tudi formalen, toda zelo fleksibilen analitičen okvir, ki je izjemno dobro implementiran v programskih orodjih (White in Burnham, 1999). V zadnjih letih se pospešeno razvijajo metode, ki upoštevajo značilnosti in posebnosti neinvazivnega genetskega vzorčenja (Lukacs in Burnham, 2005; Miller in sod., 2005). Zaradi tega postaja rutinski, kontinuiran monitoring številnosti ogroženih živalskih vrst s pomočjo neinvazivnega genetskega vzorčenja vedno bolj realna, dostopna in izvedljiva možnost.

1.4 RAZISKAVE GENETIKE RJAVEGA MEDVEDA

Rjavi medved sodi na svetovni ravni med bolje raziskane živalske vrste, kar velja tudi za genetske raziskave (Swenson in sod., 2011). Tudi pri nas je bilo na medvedu opravljenih kar nekaj raziskav, ki so pa bile večinoma usmerjene v raziskave vedenja, prehrane in rabe prostora. O populacijsko-genetskih parametrih naših medvedov je bilo znanega zelo malo.

Pionirske raziskave genetike rjavih medvedov so se v severni Ameriki začele konec prejšnjega stoletja (Paetkau in sod., 1998) in so se ukvarjale zlasti z genetsko pestrostjo in genetskim pretokom med različnimi bolj ali manj povezanimi populacijami. Približno sočasno so raziskave potekale tudi v Evropi (Taberlet in Bouvet, 1994), glavna tematika pa je bila opredelitev najprimernejših populacijskih enot, ki bi lahko služile kot vir živali za ponovne naselitve. Večina evropskih raziskav v tem času je temeljila na raziskavah mitohondrijske DNA. Iz tega obdobja je posebej pomembno delo Taberleta s sodelavci (1997), ki je eden temeljnih člankov na področju genetike neinvazivnih vzorcev. Problematika strukture in zgodovine populacij medveda v Evropi je še vedno tema številnih raziskav (pregled v Swenson in sod., 2011), marsikaj pa še vedno ni znano. Genetsko pestrost jedrne DNA rjavih medvedov so v Evropi začeli intenzivneje preučevati v zadnjem desetletju, v Skandinaviji (Waits in sod., 2000), Italiji in Romuniji (Lorenzini in sod., 2004; Zachos in sod., 2008) in v Kantabrijskem gorstvu (Perez in sod., 2008). Pri tem je še težava, da različni raziskovalci uporabljajo različne sisteme genetskih označevalcev, zaradi česar je pestrosti različnih populacij medveda pogosto težko primerjati (Swenson in sod., 2011). O genetski pestrosti medvedov pri nas ni bilo do naših raziskav objavljenega nič.

1.5 RAZISKOVALNE HIPOTEZE

a) Populacija medveda je bila v 19. in 20. stoletju zdesetkana (Huber in sod., 2009; Huber in Frković, 1993; Švigelj, 1961). Čeprav si je številčno opomogla, lahko pričakujemo, da je takšno pustošenje pustilo posledice, ki so še zdaj vidne v genetski sliki populacije.

a.1) Genetska pestrost populacije rjavega medveda v severnih Dinaridih je manjša kot v velikih naravnih populacijah. Dinaridi so eden izmed večjih strnjenih gozdnih kompleksov v Srednji Evropi in sklepamo, da celotna populacija, čeprav v zdesetkana in najverjetneje fragmentirana, ni bila nikoli dovolj dolgo tako majhna, da bi izguba genske pestrosti dosegla ravni, ki jo dosega v zelo majhnih in otoških populacijah (npr. populacija v Apeninih (Zachos in sod., 2008) ali na otoku Kodiak (Paetkau in sod., 1998). Kljub temu pa njena genetska pestrost ne dosega pestrosti velikih populacij iz Karpatov, Kanade ali Aljaske.

a.2) Efektivna velikost populacije medvedov v severnih Dinaridih je manjša od IUCN-ovega kriterija dolgoročne viabilnosti $N_e > 500$. Če uporabimo oceno, da je v celotni dinarski populaciji okrog 2800 medvedov (Zedrosser in sod., 2001), potem je glede na pričakovano razmerje N_e/N_c pričakovati efektivno velikost od 280 do 1064. Ker pa je velika verjetnost, da je populacija ločena v dva ali več demov (Swenson in sod., 2000), ker je bila populacija v preteklosti majhna in ker so ocene velikosti populacije v veliki meri bolj ali manj ekspertna mnenja, ki jim ni mogoče popolnoma zaupati, je lahko dejanska efektivna velikost populacije v severnih Dinaridih znatno manjša.

b). Populacijska rast v zadnjih dveh desetletjih (Jerina in sod., 2003; Jerina in sod., 2008) je povzročila rast efektivne velikosti populacije. Ob rasti števila medvedov raste tudi efektivna velikost populacije, pride pa lahko tudi do stika prej izoliranih demov, kar proces še pospeši (England in Luikart, 2010). Slovenija ima že vrsto let vzpostavljeno dobro evidentiranje smrtnosti medvedov, medvedom pa se določa tudi starost s pomočjo izbruskov zob (Jerina in Adamič, 2008a). Od leta 2003 dalje se vsem mrtvim medvedom vzame tudi vzorec tkiva za genetiko. Z novimi metodami za ocenjevanje N_e lahko s pomočjo teh podatkov spremljamo spreminjanje efektivne velikosti populacije skozi čas v okviru adaptivnega upravljanja, kar do naše raziskave po naših podatkih še ni bilo nikoli narejeno.

c). Številčnost medvedov v Sloveniji je nižja, kot se je dolga leta ocenjevalo.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

*2.1.1 Monitoring efektivne velikosti populacije rjavega medveda (*Ursus arctos*) z uporabo novih pristopov, ki potrebujejo en sam vzorec genotipov*

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Izvleček: Efektivna velikost populacije (N_e) bi lahko bila idealen parameter za monitoring populacij, ki potrebujejo aktivno varstvo, saj priročno povzema tako evolucijski potencial populacije kot njeno občutljivost na genetsko naključje. Na žalost pa je spremljanje tega parametra skozi čas pri naravnih populacijah zahtevno. Uporabili smo štiri nove metode za oceno N_e iz enega samega vzorca genotipov in tako spremljali spreminjanje N_e skozi čas pri medvedih v severnih Dinaridih. Genotipizirali smo 510 medvedov z uporabo 20 mikrosatelitskih lokusov in določili starost posameznih živali. Vzorce smo organizirali v kohorte glede na leto, v katerem so bile živali rojene in v letne vzorce s starostnimi kategorijami za vsako leto, v katerem so bile žive. Uporabili smo cenilko z določanjem starševstva (EPA) in z njo določili tako efektivno velikost populacije kot generacijski interval za vsak letni vzorec. Za vsako kohorto smo ocenili efektivno število živali, ki se parijo (N_b) z metodami vezavnega neravnovesja, določitvijo sorodstvenih povezav in približnega Bayesovega izračuna. Te ocene smo ekstrapolirali v N_e z uporabo generacijskega intervala. EPA ocena je bila 276 osebkov (183-350 95 % CI), kar zadostuje kriteriju za izogibanje parjenja v sorodstvu $N_e > 50$, je pa še vedno nižje od dolgoročnega kriterija za viabilno populacijo $N_e > 500$. Rezultati, ki smo jih dobili z drugimi metodami, dobro sovpadajo s tem rezultatom, vsi pa kažejo na hiter porast N_e, najverjetneje ob koncu 1990-ih in v zgodnjih 2000-ih. Novi pristopi za oceno N_e z enim samim vzorcem genotipov se lahko učinkovito uporabijo za vključevanje N_e v programe monitoringa in bodo v prihodnosti velikega pomena za upravljanje in varstvo.

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Monitoring the effective population size of a brown bear (*Ursus arctos*) population using new single-sample approaches

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Abstract

The effective population size (N_e) could be the ideal parameter for monitoring populations of conservation concern as it conveniently summarizes both the evolutionary potential of the population and its sensitivity to genetic stochasticity. However, tracing its change through time is difficult in natural populations. We applied four new methods for estimating N_e from a single sample of genotypes to trace temporal change in N_e for bears in the Northern Dinaric Mountains. We genotyped 510 bears using 20 microsatellite loci and determined their age. The samples were organized into cohorts with regard to the year when the animals were born and yearly samples with age categories for every year when they were alive. We used the Estimator by Parentage Assignment (EPA) to directly estimate both N_e and generation interval for each yearly sample. For cohorts, we estimated the effective number of breeders (N_b) using linkage disequilibrium, sibship assignment and approximate Bayesian computation methods and extrapolated these estimates to N_e using the generation interval. The N_e estimate by EPA is 276 (183–350 95% CI), meeting the inbreeding-avoidance criterion of $N_e > 50$ but short of the long-term minimum viable population goal of $N_e > 500$. The results obtained by the other methods are highly consistent with this result, and all indicate a rapid increase in N_e probably in the late 1990s and early 2000s. The new single-sample approaches to the estimation of N_e provide efficient means for including N_e in monitoring frameworks and will be of great importance for future management and conservation.

Keywords: conservation genetics, effective population size, genetic monitoring, population dynamics, population genetics—empirical, wildlife management

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Introduction

Effective population size (N_e) is arguably one of the most important parameters both in conservation (Frankham 2005) and evolutionary biology (Charlesworth 2009). Not to be mistaken with census population size, the number of individuals in the population, it is defined as the size of an idealized Wright–Fisher population (Fisher 1930; Wright 1931) that would lose genetic diversity or become inbred at the same rate as the actual population (Crow & Kimura 1970). It describes the rate of random genetic processes and can be understood as a direct measure of evolutionary potential and vulnerability of populations to genetic stochasticity. As such, it can be used as a basis for a predictive framework for the fate of small populations (Leberg 2005; Wang 2005; Palstra & Ruzzante 2008) and can be used for early detection of both population fragmentation (England *et al.* 2010) and population decline (Antao

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et al. 2011). Monitoring N_e, if feasible, would provide an excellent tool for monitoring the status of populations of conservation concern. Unfortunately, despite its conceptual simplicity, the effective population size is notoriously difficult to measure in natural populations (Leberg 2005; Wang 2005; Waples & Yokota 2007).

While there have been a number of studies dealing with estimations of effective population size of different species (see a recent review in Palstra & Ruzzante 2008), the estimates of changes of N_e through time are rare. There is in fact some confusion in the literature regarding the use of the term genetic monitoring (Schwartz *et al.* 2007), as it should by definition include detection of temporal change but has also been applied to single estimates (e.g. Tallmon *et al.* 2004a). There are cases in very small populations where monitoring of N_e was efficiently implemented using genetic-demographic data—genetic information was used to determine parentage and relatedness of all animals, which was then used to infer the effective population size (e.g. De Barba *et al.* 2010). However, by far the most frequently used genetic approach for estimating N_e has been the temporal method (Leberg 2005; Wang 2005; Luikart *et al.* 2010). It uses samples taken at different points in time and changes in allele frequencies produced by genetic drift as a signal for estimation of the harmonic mean of N_e over the period between the samples. This time period should be at least one generation, but in practice, it must be significantly longer to produce unbiased estimates, especially if generations overlap, making the concept very difficult to apply in a monitoring framework (Schwartz *et al.* 2007; Waples & Yokota 2007).

Promising tools became available with the development of methods enabling N_e estimation through analysis of a single sample of genotypes. The possibility to estimate N_e by analysing samples taken at a single point in time offers a considerable advantage and makes monitoring of a temporal change in N_e feasible. Until very recently, there were only two such methods available: one using heterozygote excess (Pudovkin *et al.* 1996) and the other using linkage disequilibrium (Hill 1981). While the former suffers from low power unless the actual N_e is very small (Schwartz *et al.* 1998; Leberg 2005; Wang 2005), an unbiased estimator for the later was developed only recently (Waples 2006). However, the field has seen considerable development over the last couple of years, and several new promising methods were introduced.

The goal of our study was to trace temporal change in N_e in a monitoring framework for the brown bear (*Ursus arctos*) population in the Northern Dinaric Region of the Western Balkans. The bears in Northern Dinarides belong to one of the few remaining natural populations in Europe. The entire population spans over 11 countries (including the edge of distribution in Italy and sporadic occurrences in Southern Austria) from the Alps in the north to Rodopi Mountains in the south and is estimated at 2800 individuals (Zedrosser *et al.* 2001; Fig. 1). The northern part of the population—Slovenia, Croatia and Bosnia and Herzegovina—is considered continuous, but the distribution further south in Northern Albania, Montenegro, Western Serbia and Kosovo may be fragmented (Zedrosser *et al.* 2001; Linnell *et al.* 2008), separating the northern part of the population from the second large block in the south (Greece, FYR Macedonia and Eastern Albania). Although the population is considered stable over most of its range, objective data at the population level are scarce and not much is known about its long-term viability. In its northern part, a substantial number of bears are harvested yearly, which can affect the population dynamics both directly and through changes in sex and age structure. Coordinated population-level management is critical for long-term survival and coexistence of these bears with humans (Linnell *et al.* 2008; Huber *et al.* 2009), but currently the population is spread across many countries with little common vision or cooperation. An important first step towards coordinated, transboundary management would be monitoring of a key population parameter like effective population size.

To trace the temporal change in the effective size of this population, we used the unbiased linkage disequilibrium (LDNe) estimator (Waples 2006), as well as three recently developed methods: a method utilizing approximate Bayesian computation (ONeSAMP, Tallmon *et al.* 2008), the sibship assignment (SA) method (Wang 2009) and the Estimator by Parentage Assignments (EPA) (Wang *et al.* 2010). We were able to apply these methods to a large empirical data set, obtain plausible estimates of N_e and its change through time and provide a starting point for genetic monitoring of the bears in Northern Dinarides.

Methods

Sample collection and analysis

We collected tissue samples for genetics from brown bear mortalities between 2003 and 2008 (n = 510) in the northernmost part of the population range, in Slovenia, with help from the Slovenia Forest Service (Fig. 1). A tooth was taken from each bear for age determination, and age determined using tooth cementum rings (Matson's Laboratory LLC, Milltown, MT, USA).

We extracted DNA from all samples using Sigma GeneElute™ Mammalian Genomic DNA Miniprep Kit,

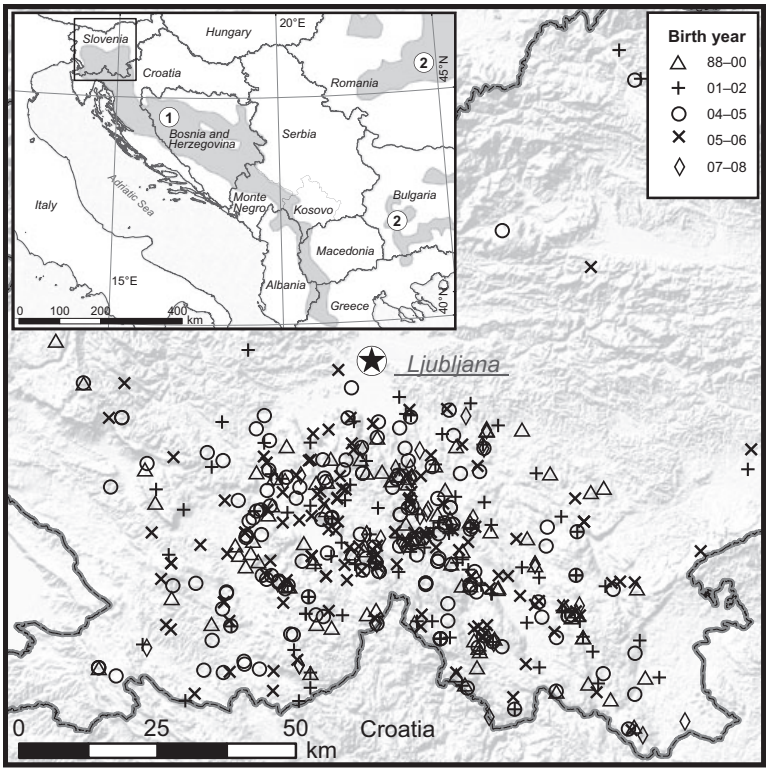


Fig. 1 Dinaric population of brown bears and study area (small map, after Zedrosser *et al.* (2001)), spatial and temporal distribution of bear samples (large map). 1 = Dinaric population; 2 = Carpathian population.

according to the manufacturer's instructions. The samples were genotyped at 22 microsatellite loci: G10X, G1A, G10C, G1D, G10J, G10M, G10B, G10H (Paetkau *et al.* 1998), G10P, Mu15, Mu09, Mu61, Mu05, Mu11, Mu26 (Taberlet *et al.* 1997), Mu10, Mu23, Mu50, Mu59, G10L, Mu51 (Bellemain & Taberlet 2004) and Cxx20 (Ostrander *et al.* 1993). Locus SRY (Bellemain & Taberlet 2004) was used to confirm field-based sex determination. All loci were amplified in three multiplex PCRs using Qiagen Multiplex PCR kit, run on an ABI 3130 × l Genetic Analyzer (Applied Biosystems) and analysed with GeneMapper software. All allele calls were re-checked independently by a second person. Liquid transfers were carried out using aerosol barrier pipette tips, with all critical pipetting steps being photographed and later rechecked to detect possible sample mixups. Negative controls were used at each step of the genotyping process. We randomly selected 10% of the samples (Pompanon *et al.* 2005) and repeated the genotyping process to determine error rates. We used the methods recommended by Broquet & Petit (2004) to estimate the frequency of allelic dropouts and false alleles. Details of the genotyping protocol are provided in T. Skrbinšek *et al.* (T. Skrbinšek, M. Jelenčič, H. Potočnik, I. Kos, L.P. Waits, P. Trontelj submitted).

Calculation of genetic diversity indices, tests for Hardy–Weinberg equilibrium and null alleles, selective neutrality of loci

To detect significant departures from Hardy–Weinberg equilibrium, we used the procedure described by Guo & Thompson (1992), as applied in program Arlequin (Excoffier *et al.* 2005), with 1 000 000 steps in the Markov chain and 100 000 dememorization steps. Holm–Bonferronni multiple test correction (Holm 1979) was used to correct for multiple testing, and $P = 0.05$ used as a significance threshold. Program Arlequin was also used to calculate allelic frequencies and standard diversity indices—observed heterozygosity (H_o), expected heterozygosity (H_e) and allelic diversity (A). To better understand the impact of rare alleles, the effective number of alleles (A_e) was calculated according to the formula in Frankham *et al.* (2002). Program Micro-Checker (Van Oosterhout *et al.* 2004) was used to check for presence of null alleles.

While there are a number of tests available to test for selective neutrality of genetic markers, they would be difficult to implement to our data set (single population, possible changes in population size). All loci we used were considered by their authors to be selectively neutral and have already been used in numerous studies [see reviews in Swenson *et al.* 2011, T. Skrbinšek,

M. Jelenčič, H. Potočnik, I. Kos, L.P. Waits, P. Trontelj (submitted)]. In these studies, the loci were either found to be in Hardy–Weinberg equilibrium or the departures were explainable by null alleles or demographic events. Considering this, we felt it is safe to assume their selective neutrality.

Estimation of the effective number of breeders (N_b)

While brown bears are a long-lived species with overlapping generations, most methods for N_e estimation assume discrete generations or even the Fisher–Wright model of an ideal population: a monoecious finite population of constant size with discrete generations (no generation overlap), random mating, equal contribution of individuals to the next generation and absence of selection or mutation (Table 1). Naively treating overlapping generations as if they were discrete can introduce substantial bias (Luikart *et al*. 2010). However, these methods can be used to estimate the effective number of breeders (N_b) in species with overlapping generations if a single cohort is sampled (Schwartz *et al*. 1998; Waples 2005; Beebee 2009). N_b is conceptually similar to N_e, with the important difference that only a single cohort is taken into account instead of the entire population.

We used three different single-sample approaches to the estimation of N_b: the unbiased linkage disequilibrium method (LDNe), the approximate Bayesian computation method (ONeSAMP) and the sibship method (SA). We estimated the effective number of breeders for cohorts of animals born within 3 years of each other. The cohorts were constructed using the age and time of death data.

Methods of estimating effective population size from linkage disequilibrium were developed over 20 years ago (Hill 1981) and use Weir's (1979) unbiased estimator of Burrows' Δ to estimate LD. A sample size bias correction (LDNe) has been derived recently by Waples (2006). The method builds on the expectation that in a finite population otherwise unlinked loci will drift out of linkage equilibrium as an effect of both random sampling of gametes during mating and random sampling of individuals in the study. The size of these random departures from equilibrium is expected to be inversely proportional with N_e and the number of samples analysed (S). The method, as modified by Waples (2006), has been extensively tested with simulated data and shown to be reasonably unbiased and precise at sample sizes $S \geq 30$ and S/N_e ratio > 0.1, even using a moderate number of microsatellite loci (10–20), if the effective size of the population is not very large (<300–500) (Waples 2006; Waples & Do 2010). The method seems to be robust to violations of some assumptions (see Table 1)—it performs well under uneven sex ratio and greater than random variance in reproductive success (Waples 2006). The estimate is sensitive to a violation of population closure and existence of population substructure, as this

Table 1 Assumptions of single-sample approaches to the estimation of the effective number of breeders (N_b) and effective population size (N_e) and comments regarding their application to the studied population

Assumption	Comments
Population is sampled at random	Only a part of the population range was sampled, so assuming a degree of site fidelity the animals can be more related than random expectation. Possibly offset by large sample sizes and high mobility of brown bears.
No subdivision of population	The habitat is continuous in the sampled area, and there are no reasons to suspect population subdivision.
Discrete generations	Single cohorts were analysed using LDNe, ONeSAMP and SA methods to estimate N_b. The EPA method relaxes this assumption, providing a direct estimate of N_e, as well as an estimate of the generation interval to connect N_b with N_e.
No immigration	Possibly violated to a certain degree, as the connectivity with the bears east of Bosnia is not known. However, there is no sign of deviations from Hardy–Weinberg expectations. The LDNe method should be robust to up to 10% levels of immigration (Waples 2010).
Stable population size	Historical records and population models show that the population has been growing through the second half of the 20th century (Jerina *et al*. 2003). However, the data in the 2000s show that the population may have been stable or even slightly declining (Jerina & Adamič 2008).
No mutation, no selection	Reasonable considering the short time intervals. All markers fit Hardy–Weinberg expectations.
Equal contribution of individuals to the next generation	Violated (uneven sex ratio, different contribution of different individuals and age categories). Relaxed in the EPA method (Wang *et al*. 2010). The LDNe method is largely robust to violation of this assumption (Waples 2006).

LDNe, linkage disequilibrium; ONeSAMP, approximate Bayesian computation; SA, sibship assignments; EPA, estimate by parentage assignments; GI, generation interval.

would also create a linkage disequilibrium signal. However, Waples & Smouse (1990) showed that even with substantial population mixing disequilibrium because of drift would dominate if population size was small, and Waples (2010) showed that the method is robust to equilibrium levels of migration as high as 10%. We applied this method to estimate the N_b in our study using program LDNe (Waples & Do 2008). As suggested by Waples & Do (2010), we excluded the alleles with frequencies below 0.01 when sample size was more than 100 and the alleles with frequencies below 0.02 with smaller sample sizes to avoid the bias caused by rare alleles but still keep precision high.

The approximate Bayesian computation (ONeSAMP) method uses an approximate Bayesian computation procedure to estimate N_e by comparing eight summary statistics that are a function of N_e (including linkage disequilibrium) for a large number of simulated populations to the same summary statistics in the studied population. It was originally developed for two-sample data sets (Tallmon *et al.* 2004b) but was recently adapted to single-sample microsatellite data (Tallmon *et al.* 2008). This method employs multiple N_e-related statistics, conferring increased accuracy and precision. However, the method has not been thoroughly evaluated (Luikart *et al.* 2010), and it is somewhat difficult to determine exactly what time period it applies to, what its assumptions are and how it behaves when they are violated. The main assumption is that the signal is only coming from genetic drift, and while some of the summary statistics it uses do apply to longer time frames, the result should be mostly influenced by the recent few generations (David A. Tallmon, personal communication). The method has been previously applied in species with overlapping generations to estimate the number of breeders in a single cohort (Beebee 2009), as well as N_e using samples containing several overlapping generations (Tallmon *et al.* 2008; Barker 2011; Phillipsen *et al.* 2011). In our study, we estimated the number of breeders in the 3-year birth cohorts and the long-term N_e using all collected samples. Program ONeSAMP (Tallmon *et al.* 2008) was used for estimation with 40 and 1000 as limits for a uniform prior on N_e.

The sibship assignment (SA) method proposed by Wang (2009) is a single-sample approach that is a hybrid between the demographic and the genetic methods for N_e estimation. It uses sibship assignments to determine full siblings and half-siblings in the sample and estimates N_e from frequencies of full- and half-sibling dyads. The method has been shown to perform well both with simulated and empirical data (Wang 2005; Beebee 2009; Barker 2011; Phillipsen *et al.* 2011). It assumes discrete generations, but relaxes assumptions of random mating and equal contribution of individuals to the next generation. The programme Colony 2 (Jones & Wang 2010) was used for estimation. We used parentage assignments to improve sibship inference (Wang 2009; Wang & Santure 2009). Animals born before the 3-year period of a cohort were treated as potential parents of the animals born during that period. Theoretical parent-offspring combinations in which the parents were 2 years old or younger when a particular offspring was born were excluded. We assumed polygamy for both sexes and used the full likelihood model with medium precision and a uniform prior for sibship size. Loci with null alleles or high error rates were excluded from the analysis, and observed error rates on other loci were included in the computation.

Estimation of the effective population size (N_e)

While monitoring N_b is useful and informative on its own, it is interesting to understand how it translates into N_e. The relationship between N_b and N_e is complex, but in general, it should apply that $N_b \leq N_e \leq N_b \times GI$, where GI is the generation interval (Wang 2009). A solution to the problem of estimating GI and directly estimating N_e using genetic data in species with overlapping generations has been proposed with a recently developed method, the Estimator by Parentage Assignments (EPA) (Wang *et al.* 2010). The method requires a single random sample (with respect to kinship) of the population, with multilocus genotypes, age, and sex data. It uses the observed parentage assignments among age classes and fits them in what is basically a mark-recapture framework to a genetic model to estimate a number of biologically interesting parameters, most notably N_e and generation interval, GI. In contrast to the SA method, the reliability of parentage assignments is much higher than reliability of sibship assignments given the same marker system (Blouin 2003; Wang *et al.* 2010), and simulation analyses demonstrated that eight highly polymorphic microsatellite loci produce accurate estimates when greater than 16% of the population is sampled (Wang *et al.* 2010). Simulations also showed that the method is robust to disproportional sampling and differential fertility in age classes but becomes biased if several age classes have no samples. The method is sensitive to the proportion of the population sampled, and the results become bimodally distributed when the sample size is small. The problem of bimodality seems to disappear when more than 8% of a population is sampled (Wang *et al.* 2010).

Construction of cohorts for the N_b methods and data preparation for direct estimates of N_e and generation interval

To construct samples for the three N_b methods (SA, LDNe, ONeSAMP), we organized animals into cohorts

according to their year of birth, determined by their age when they died and the recorded date of death. In each cohort, we pooled all animals born within a 3-year time window to get reasonable sample sizes. It is unlikely that a bear would produce offspring before the age of three (Swenson *et al*. 2000; Frković *et al*. 2001; De Barba *et al*. 2010), so our 3-year cohorts should not include any parents. The cohorts were then constructed using a sliding temporal window covering the entire monitoring period, so that all 3-year combinations yielding reasonable sample sizes were used for estimations of N_b. The cohorts obtained in this manner conform to the assumptions of Plan II sampling described by Waples (2005).

When constructing cohorts, the minimum sample size threshold was set to 30 animals per cohort, as simulations show the LDNe method to be reasonably unbiased above that threshold for populations with N_e (or, in this case N_b) < 300 (Waples 2006). In most cohorts, the sample size was considerably higher than this threshold. The same cohorts were also used to estimate N_b using the ONeSAMP and SA methods.

While the effects of sample size on the estimates produced by the ONeSAMP method are largely unexplored, it is known that for the SA method they are biased downwards when N_e is large, sample size much smaller than N_e and the number of loci is low (Wang 2009). We calculated how informative the markers we used in our study are for relatedness inference according to Wang (2006) and compared it with the marker system used for simulations in the original article describing the SA method (Wang 2009). Our marker system has RMSD-W = 68.41 and can be expected to perform between the 10 locus (RMSD-W = 49.92) and 20 locus system (RMSD-W = 98.38) used in simulations. Judging from the results of these simulations, the bias should become acceptably low when the sample size approaches the actual N_b. We applied the method to the same cohorts as the LD and ONeSAMP methods, but with an understanding that the results are possibly biased downwards for cohorts with small sample sizes.

A different approach was taken to construct samples for the EPA. For this method, we constructed yearly samples for each calendar year covered by our samples that included all sampled animals that were alive during that year. The age of the animals at the time of their death was used to calculate their age in each target year. It was possible to create samples for the period before our sample collection started in 2003, as many animals killed after 2003 were born before that year, but this only made sense for the year 2002 as the sample sizes for years prior to that rapidly became too small with several empty age classes. The 2002 sample was also comparatively smaller than the samples from 2003 onwards, but had a much more balanced age structure than the 2007 sample, making it sensible to include the estimate in the results. Animals were categorized into newborns (age 0) and eight 2-year age categories (category 1 = age 1 and 2, category 2 = age 3 and 4 etc.). The newborns and the first category, age 1–2 years, were considered non-reproductive. We considered only the yearly samples that had a maximum of one age category without samples. In running the Agestruct program (Wang *et al.* 2010), we used 95% reliability of parentage assignments, 0.5 as a prior probability of including a parent in the sampling, and 1000 bootstrap samples to calculate confidence intervals. As it is difficult to estimate the actual proportion of parents sampled, we tested different values of this parameter to check for sensitivity of the model.

While most other methods we used estimated the effective number of breeders, the EPA method estimated the effective population size. The EPA-estimated generation interval GI was also used to understand how the estimates of N_b obtained for the 3-year cohorts relate to N_e, as in general $GI \times N_b \approx N_e$ (Waples 2010). However, this relation between N_e and N_b is complex (Waples 2010), and as the generations in natural populations of brown bear overlap, the $GI \times N_b$ estimate should be the estimate of the upper limit of the 'true' N_e (Wang *et al*. 2010). For the generation interval, we used the average of the GI estimates. We used harmonic mean to average the EPA-obtained estimates over different years to understand the average effective population size in the studied period. Although the animals changed age categories between subsequent years and newborns entered into the sample, most animals were alive through several years and appeared in several yearly samples. As such, the yearly samples are not fully independent and have a significant overlap in the time period they apply to (Fig. 3). The confidence intervals were averaged between estimates.

We also attempted to estimate N_e directly with the ONeSAMP method, similar to Tallmon *et al*. (2008). For this purpose we used all samples for the estimate, regardless of the year when the animal was born or died.

Results

Description of loci

Locus Mu26 showed evidence of null alleles, and locus G10H proved difficult to genotype reliably. Both were excluded from further analysis. The genotyping error rates of the other loci were low (average allelic dropout 4.05E-4; SD = 5.85E-4). The loci met Hardy–Weinberg expectations at $P < 0.05$ (Holm–Bonferronni correction).

Average heterozygosities were 0.731 (H_e) and 0.738 (H_o). Average allelic diversity was 6.75 (SD = 1.77) alleles per locus, and average effective number of alleles 4.09 (SD = 1.12) alleles per locus. Detailed per-locus results are provided in T. Skrbinšek, M. Jelenčič, H. Potočnik, I. Kos, L.P. Waits, P. Trontelj (submitted).

Locus Mu23 had an irregular repeat pattern as two of eight alleles had a point deletion in the region flanking the $(CA)_n$ microsatellite, making their size a single base-pair different from the neighbouring alleles. While we were able to score the alleles reliably, we omitted the locus from the ONeSAMP method as it uses the M-ratio, which assumes the stepwise mutation model, as one of its summary statistics.

Estimates of N_b *and* N_e

All methods used for estimating N_b provided comparable results, although the confidence intervals differed (Table 3, Fig. 2). Some cohorts had comparatively small sample sizes.

The EPA method provided both estimates of the actual effective population size and the generation interval (Table 3). The 2007 and 2008 samples had relatively small sample sizes (172 and 79, respectively), a much lower number of animals in the young age classes, and several age classes without data. The last two issues make the estimates unreliable (Wang *et al.* 2010), so both were excluded from further analysis.

To test for sensitivity to the parents sampling proportions prior, we re-estimated N_e with this parameter set at 0.3 and 0.7. In all samples except the 2002 sample, the effects on final estimates were small (<0.02 of the estimate). For the 2002 sample, this effect was larger, at +0.15 of the estimate (222, 86–294 95% CI) when the parents sampling proportion was set at 0.3. The estimate was still well within bounds of the confidence interval. Generation interval estimates are highly consistent between samples, with average generation interval of 7.57 years and 6.68–8.51 years 95% confidence interval. The harmonic mean N_e is 276 (183–350 95% CI). The median long-term N_e estimated with the ONeSAMP method using all available samples was 305 (241–526 95% CI), close to the harmonic mean of the EPA estimates.

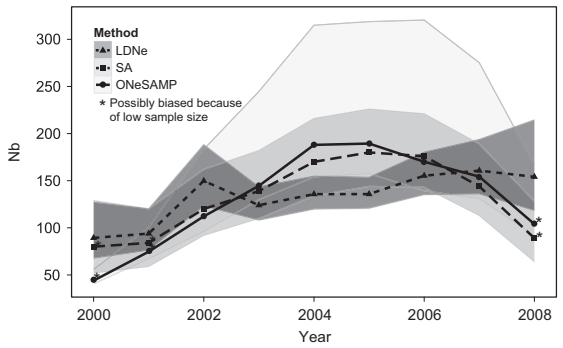


Fig. 2 Estimates of the effective number of breeders (N_b) estimated for the three-year cohorts (sliding window). The last year of each time window was used to draw the estimate (see Fig. 3). The filled polygons show the confidence intervals. LDNe = linkage disequilibrium, ONeSAMP = approximate Bayesian computation, SA = sibship assignments.

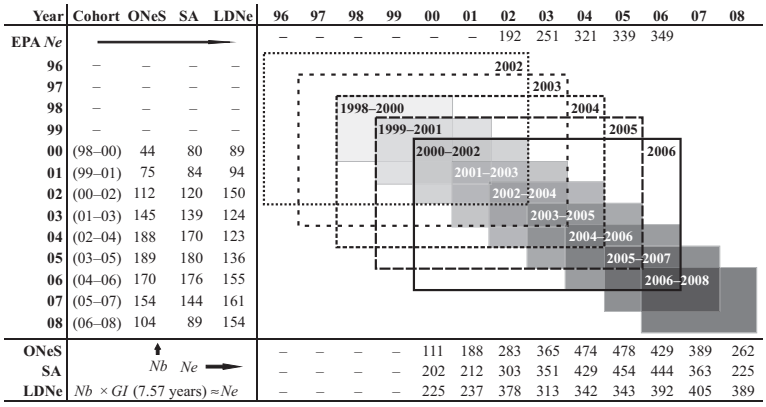
Overview of the estimates and the time periods they apply to is shown in Fig. 3. The time periods overlap with all methods used. We multiplied the N_b estimates by the average generation interval divided by the cohort interval (3 years) to extrapolate N_b to the estimation of the effective population size (Figs 3 and 4). When we consider the time periods the estimates apply to and that the EPA-estimated N_e should correspond to the harmonic mean of N_e within the generation interval that covers several cohorts, the results obtained by the single-cohort methods correspond closely with the EPA estimates.

The EPA estimates apply to much longer time periods than the estimates obtained by the N_b methods (6.7–8.5 vs. 3 years) and have consequently a higher degree of smoothing. They show an increasing trend in N_e (Fig. 4) and are in the beginning lower than the estimates obtained by the N_b methods, but start converging with them from 2004. This indicates a rapid increase in effective population size in 1990s and early 2000s.

Discussion

Using single-sample estimators of the effective population size for monitoring of populations of conservation concern

While the temporal method for estimating N_e has seen the most use, it has serious drawbacks when applied to monitoring scenarios. It becomes heavily biased when the assumption of discrete generations is violated (Waples & Yokota 2007; Waples 2010), as is the case for the majority of species, and even with discrete generations requires a minimum of two samples separated by a period of at least one generation for a single assessment of N_e, and at least three such samples to detect any temporal change (Schwartz *et al.* 2007). With overlapping generations, this period between samples must be significantly longer than a single generation to provide unbiased estimates (Leberg 2005; Waples & Yokota 2007). A possible solution is the method proposed by Jorde & Ryman (1995) that provides approximately unbiased results (Waples & Yokota 2007) but requires detailed demographic information and many loci (in the thousands) to measure drift accurately unless several



Year	Cohort	ONeS	SA	LDNe	96	97	98	99	00	01	02	03	04	05	06	07	08
EPA *Ne*					–	–	–	–	–	–	192	251	321	339	349		
96	–	–	–	–							2002						
97	–	–	–	–								2003					
98	–	–	–	–									2004				
99	–	–	–	–										2005			
00	(98–00)	44	80	89											2006		
01	(99–01)	75	84	94													
02	(00–02)	112	120	150													
03	(01–03)	145	139	124													
04	(02–04)	188	170	123													
05	(03–05)	189	180	136													
06	(04–06)	170	176	155													
07	(05–07)	154	144	161													
08	(06–08)	104	89	154													
ONeS					–	–	–	–	111	188	283	365	474	478	429	389	262
SA					–	–	–	–	202	212	303	351	429	454	444	363	225
LDNe					–	–	–	–	225	237	378	313	342	343	392	405	389

Nb *Ne*

Nb × *GI* (7.57 years) ≈ *Ne*

Fig. 3 N_e and N_b estimates and corresponding time periods. The filled rectangles show the time period for the single-cohort methods (ONeSAMP, SA, LDNe), and the empty rectangles show the time periods covered by the EPA estimates. In the corner of each rectangle is the year of the sample. The estimates of N_b obtained by ONeSAMP, SA and LDNe methods were multiplied by the average age generation interval (GI) divided by the cohort interval (3 years) to obtain the estimates of N_e comparable with the EPA estimates. However, because of the overlapping generations this N_b-derived estimates should act as an upper limit of N_e and are thus expected to be higher than the EPA estimates. LDNe = linkage disequilibrium, ONeS = ONeSAMP, approximate Bayesian computation, SA = sibship assignments, EPA = estimate by parentage assignments.

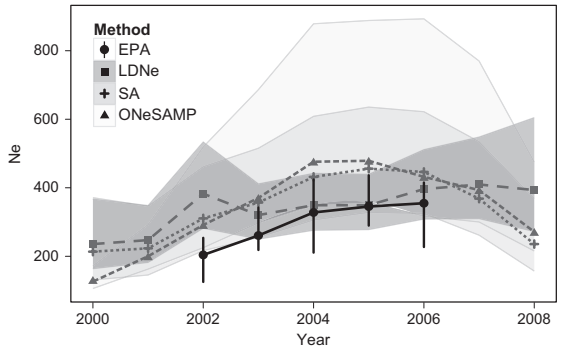


Fig. 4 Comparison of N_e estimates. The polygons (or handles in case of the EPA) show the confidence intervals. The estimates obtained by the ONeSAMP, LDNe and SA methods were multiplied by the average generation interval obtained by the EPA (7.57 years, 6.68–8.51 years averaged 95% CI) divided by the cohort period (3 years). The uncertainty of the generation interval estimate was included in graphing of the confidence interval for these methods. LDNe = linkage disequilibrium, ONeSAMP = approximate Bayesian computation, SA = sibship assignments, EPA = estimate by parentage assignments.

generations have passed between samples (Wang *et al*. 2010). This limits the usefulness of the temporal method in most wildlife populations to the exploration of historical change in N_e (e.g. Miller & Waits 2003) and is difficult to include in a monitoring framework useful for adaptive management and conservation.

On the other hand, the recently developed single-sample methods offer significant advantages but are currently underutilized (Waples 2010). It is easier to satisfy their assumptions, and their requirement of a sample taken at a single point in time makes them ideal for monitoring scenarios. While the ONeSAMP, LDNe and SA methods assume discrete generations, they can be used to estimate the effective number of breeders for a certain cohort in case of overlapping generations if there is a way to separate animals into cohorts. The relationship between N_e and N_b is complex, but N_b can be a useful, comparable measure which lends itself readily to monitoring of population change (Waples 2005).

The EPA method offers a more direct approach to estimate N_e. When the generation overlap and the time period to which the estimates obtained by different methods apply are considered, our EPA-obtained estimates are very close to the estimates obtained by other methods used in this study. But compared with the other methods, the EPA offers significant advantages. First of all, it is the only currently available method that can directly estimate N_e from a single sample of genotypes in species with overlapping generations (Wang *et al*. 2010). Moreover, it provides estimates of many other interesting population parameters. As such, the method has a considerable potential to be implemented in future monitoring frameworks for many wildlife species.

Additional strength of the EPA method when applied to monitoring is that it can be used complementary to the single-cohort methods. As the estimate applies to a generation interval, it will less readily detect sudden changes in N_e than the methods that estimate N_e (N_b)

over a single cohort. On the other hand, it will provide an understanding of how the N_b estimates relate to the actual parameter of interest, N_e. Also, if the sampling scheme is similar to what we used in this study, the sample size of each yearly sample grows as the animals alive in a specific year are sampled in later years, meaning that N_e can be estimated with some delay (in our case 2 years) even if the number of samples collected each year is not large enough for a direct estimate. The EPA estimates are important even when N_b estimates from other methods are available, as the generation interval can change rapidly with a change in the management strategy (e.g. a focus shift from culling of young animals with the intention of regulating population size towards trophy hunting of older animals), which would change the relationship between N_b and N_e. Use of both types of methods enables a rapid detection of important changes in the population, as well as an insight into what these changes actually signify.

A critical property of many natural populations, including those of brown bear, is generation overlap (also referred to as cross-generation mating or age structure of the population). It is notoriously difficult to take into account, and it effectively creates different classes of individuals with different vital rates, affecting (generally reducing) N_e in complex ways (Waples 2010). While the EPA method handles generation overlap directly, the other three methods required a lot of care during the analysis (i.e. construction of cohorts with regard to the species' biology) to keep violations of their assumptions reasonable. An important effect of the generation overlap in our case is that the N_e extrapolated from the N_b estimates using the generation interval should actually be the upper limit for N_e, not its direct estimate, which (among other things) explains why the N_b-derived estimates were consistently higher than the EPA estimates (Figs 3 and 4).

Another issue in species with overlapping generation is that genetic information alone is insufficient for an unbiased estimate of the contemporary N_e with the currently available methods (Waples & Yokota 2007; Beebee 2009; Wang *et al.* 2010; Waples 2010). In our case, we had age information available, which allowed us both to use the EPA method and to organize animals into cohorts for the other three methods. This requirement is a serious limitation for many populations of conservation concern, as it typically precludes the use of noninvasive samples. Interestingly, the result obtained for long-term N_e with the Bayesian method applied to all collected samples (305; 241–526 95% CI) agrees closely with the harmonic mean of the EPA estimates (276; 183–350 95% CI). This result is promising and warrants further research into the ONeSAMP method and its application to species with overlapping generations.

Technical considerations

While straightforward when applied to idealized populations, the methods used in this study become quite complex as one applies them to real populations. Any stratification of population, either spatial, by sex, or temporal through overlapping of generations, has an important effect on N_e (Waples 2010). And as a rule, it violates the assumptions of the estimation models, introducing unknown biases into the estimates. It is critical to understand the limitations of each method as well as the biology of the studied species and adjust sampling accordingly; however, it is also beneficial to use different methods for the estimate (Waples 2010). The methods used in this study differ considerably in their assumptions as well as in the signal they use to estimate N_e. Thus, the fact that we obtained very similar results across methods increases our confidence in our final estimates.

Another important challenge is determining the timeframe the estimates apply to, as it differs between methods. This issue is not straightforward and is crucial both for comparing the results obtained by different methods and for understanding the conservation implications of the estimates. This has been dealt with in a recent paper (Waples 2005), but that overview does not cover most of the new methods we used. For the LDNe and SA methods, the estimated N_b should apply to the 3-year period from which the samples were taken as the number of breeders that produced that cohort (Waples 2005; Beebee 2009). The time period for the ONeSAMP method as applied to the cohorts is less clear and while it should also be mostly influenced by the number of breeders producing the cohort, some of the metrics it uses (e.g. M-ratio) reflect population events further back (Beebee 2009; David Tallmon, personal communication). Conversely, the estimates of the EPA method should apply to the time period between the sample year and the sample year minus the generation interval, GI (Wang *et al.* 2010). Again, the time frame of the N_e estimated using the ONeSAMP method applied to all samples is not very clear but should be mostly influenced by the recent few generations (David A. Tallmon, personal communication). This gives the cohort-based estimates different time frames than those of the EPA and ONeSAMP using all samples, and the time frames of different estimates with each method overlapped (Table 2, Fig. 3).

An important assumption violated by our study is random sampling of the entire population. Samples were collected only in Slovenia, covering the northernmost part of the population range. Habitat characteristics show that the bears in the northern part of the Alps–Dinara–Pindos bear range possibly do not form a

Table 2 Overview of the methods applied

Method	Sampling strategy	Estimated parameters	Time period the estimate applies to	Extrapolation to obtain N_e
ONeSAMP (all samples)	All collected samples	Presumably N_e	Unknown, but should be mostly affected by N_e in the last few generations	None
ONeSAMP (cohort)	3-year sliding window, newborns cohort	N_b	Sampled 3-year period	$N_b \times GI^*$
LDNe	3-year sliding window, newborns cohort	N_b	Sampled 3-year period	$N_b \times GI^*$
SA	3-year sliding window, newborns cohort	N_b	Sampled 3-year period	$N_b \times GI^*$
EPA	Single year, all sampled animals alive that year	N_e, GI	Generation interval prior to the year of sampling	None

LDNe, linkage disequilibrium; ONeSAMP, approximate Bayesian computation; SA, sibship assignments; EPA, estimate by parentage assignments; GI, generation interval.
*In bears; $N_b \times GI$ is an estimate of the upper limit of N_e because of the generation overlap.

contiguous population with the bears in the south, but represent their own subpopulation or deme (Zedrosser *et al.* 2001; Linnell *et al.* 2008). Bears have large home ranges and large dispersal radii (Huber & Roth 1993; Dahle & Swenson 2003a,b; Zedrosser *et al.* 2007; Jerina & Adamič 2009). Most animals in our study were old enough to have dispersed from their maternal home ranges as at the time of death 75.9% of females and 73.6% of males were older than 1.5 years, which is the age when dispersal usually starts in this species (Støen *et al.* 2006). This makes it possible to assume that our estimates apply to a larger area than the area over which the samples were actually obtained. Assuming approximate panmixia and lack of isolation by distance within the Northern Dinarics subpopulation/deme the LDNe results should apply to that entire subpopulation/deme. The assumptions seems plausible considering the large movement distances of brown bears (Huber & Roth 1993; Krofel *et al.* 2010) and no detection of population structure in Croatia (Kocijan *et al.* 2011) or Slovenia [T. Skrbinšek, M. Jelenčič, H. Potočnik, I. Kos, L.P. Waits, P. Trontelj (submitted)]. The same might also apply for the ONeSAMP method, as it uses summary statistics that should be less sensitive to the localized sampling. However, the localized sampling introduces an unknown bias in the SA and EPA estimates, as it is more likely to obtain relatives of the animals born in the study area than those of the animals that dispersed there from elsewhere. As the results closely correspond to the results obtained by the other methods, we can assume this bias to be small.

The properties of the methods we use here are in many cases still insufficiently explored, especially their bias, precision and robustness to violations of assumptions (which are frequently difficult to satisfy), as well as the time period they apply to. Three recent multipopulation studies of different species report that SA and ONeSAMP methods produced less variable and more similar results than the LDNe method (Beebee 2009; Barker 2011; Phillipsen *et al.* 2011). While Phillipsen *et al.* (2011) suggest that the first two methods may be more reliable than the LDNe method, Beebee (2008) points out that such comparisons should not be generalized as the outcome may depend on the number of loci genotyped and individuals sampled, and on the life history of the species under study. As the 'true' N_e is unknown in natural populations, these properties are best explored by simulations. However, our study and some other recent studies (e.g. Beebee 2009; Barker 2011; Phillipsen *et al.* 2011) show that they these methods provide plausible results that make biological sense when correctly applied to real data.

Estimates of N_b *and* N_e

The results provided by different methods for estimating N_b were very similar (Table 3, Fig. 2) and indicated a possible growing trend of N_b. The estimates of the SA method and the ONeSAMP method were nearly identical in our study; however, the results obtained by ONeSAMP generally had wider confidence intervals than the results obtained by the other two methods. Also, a positive correlation between sample size and N_e estimates was reported for this method in certain data sets (Haag *et al.* 2010; Phillipsen *et al.* 2011). The counter-intuitive narrow confidence intervals obtained using the ONeSAMP method in the cohorts with small sample sizes, and strong association between estimates and the number of samples makes the results in these cohorts difficult to believe. The cohorts 1998–2000,

Table 3 N_b estimates for the 3-year cohorts (sliding window). Estimates are not independent, as time periods overlap

Cohort	1998–2000	1999–2001	2000–2002	2001–2003	2002–2004	2003–2005	2004–2006	2005–2007	2006–2008
N samples	41*	68*	117	170	218	236	216	160	88*
Approximate Bayesian computation (ONeSAMP)									
Median N_b	44	75	112	145	188	189	170	154	104
95% CI down	40	67	96	130	154	158	139	131	92
95% CI up	56	100	183	245	315	319	321	275	167
Sibship assignment (SA)									
N_b	80	84	120	139	170	180	176	144	89
95% CI down	52	59	92	110	134	144	143	113	64
95% CI up	129	120	162	182	216	226	221	189	128
Linkage disequilibrium (LDNe)									
N_b	89	94	150	124	136	136	155	161	154
95% CI down	68	76	123	108	120	121	135	136	118
95% CI up	128	120	189	144	155	154	181	194	215

*Possibly biased low with the sibship assignment method.

Table 4 Estimates of the effective population size (N_e) and generation interval (GI) obtained by the estimator by parentage assignments (EPA)

Sample year	Time period	*S*	N_e	N_e 95% CI	GI	GI 95% CI
2002	1996–2002	184	192	109–241	7.7	6.7–8.5
2003	1997–2003	254	251	204–337	7.2	6.3–7.9
2004	1998–2004	287	321	201–414	7.8	6.4–8.4
2005	1999–2005	295	339	280–434	7.6	6.9–8.6
2006	2000–2006	260	349	216–410	7.6	7.0–9.1

S, sample size.

1999–2001 and 2006–2008 have small sample sizes compared with the overall average and are possibly biased low in estimates with the SA method (Wang 2009). The LDNe method should be robust at these sample sizes.

The EPA method provided results that were to a large degree consistent with results of the cohort-based estimators when the difference in timeframe and the effects of the generation overlap are taken into account (Figures 3 and 4). The estimate of the generation interval critical for comparison of N_e and N_b estimates was highly consistent between samples (Table 4). The method also showed to be robust to different selection of priors.

Changes in the effective size of the brown bear population in Northern Dinarides

Our results show an interesting temporal pattern of a rapid growth of the effective population size. This could be a result of growth of the census size that was probably happening during this period (Jerina *et al.* 2003; Jerina & Adamič 2009). The results also show that the population of brown bears in Northern Dinarics is relatively large. The harmonic mean EPA-estimated N_e of 276 (183–350 95% CI) does meet the inbreeding-avoidance criterion of $N_e > 50$ but is short of the long-term minimum viable population goal of $N_e > 500$ (Franklin & Frankham 1998).

As all detected bear mortality in Slovenia is reported and individuals are aged and sampled for genetic analysis, it was straightforward to expand the existing monitoring framework to genetic monitoring of the effective population size. Nevertheless, N_e may just be the single most important metric the entire monitoring produces. It has important conservation and management implications not only at the national level, but also on the level of the entire Alps–Dinara–Pindos population. Administrative borders at the moment still present a serious obstacle to conducting population-level research, and there have been initiatives to overcome these limitations (Karamanlidis 2009). But until this happens, monitoring of the effective population size in a smaller area within national boundaries can still provide an indication of trends at a population-wide scale.

Conclusions

Monitoring of change in contemporary effective population size through time is a tempting idea that could, if feasible, provide a very powerful tool for management of

populations of conservation concern. Our study shows that it can be done, even with the complications posed by generation overlap and that it is at least for some species possible to include monitoring of N_e in routine population monitoring with minimal additional resources. While our study focuses on bears, it points out interesting possibilities that the recently developed methods offer for monitoring of N_e in other species that require active conservation effort. These methods also for the first time provide efficient means for including N_e in population monitoring frameworks for species with overlapping generations, and we expect them to be of great importance for management and conservation in the future.

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The research work of T.S. is in ecology, genetics and conservation of large carnivores, with additional interests in landscape genetics and mark-recapture analysis. M.J. is interested in conservation genetics of large carnivores. L.W. focuses her research on the molecular ecology and conservation genetics of a variety of animal taxa. The research interests of I.K. are structure and function of forest ecosystems. K.J.'s pursues research in wildlife ecology, management and conservation. The research of P.T. is focused on phylogenetics, phylogeography, evolution and conservation of various animal taxa.

Data accessibility

Data deposited in the Dryad repository: doi: 10.5061/dryad.22rm1728.

2.1.2 Uporaba referenčne populacije kot merila za kalibracijo in primerjavo genetskih pestrosti iz različnih študij: primer rjavega medveda.

Using a reference population yardstick to calibrate and compare genetic diversity reported in different studies: an example from the brown bear.

Tomaž Skrbinšek, Maja Jelenčič, Lisette Waits, Hubert Potočnik, Ivan Kos in Peter Trontelj

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Izvleček: Pri vrstah s široko geografsko razširjenostjo je lahko genetska pestrost različnih populacij dobro preučena, pri tem pa je zaradi razlik v uporabljenih lokusih in zaradi različnih velikosti vzorcev pogosto rezultate različnih študij težko medsebojno primerjati. Kljub temu pa so takšne primerjave pomembne za oceno varstvenega statusa populacij, ki potrebujejo aktivno varstvo. V članku predstavljamo enostaven pristop uporabe posamezne dobro preučene populacije kot »merila« za kalibracijo rezultatov različnih študij na isto skalo, kar omogoči primerjave. Pri tem uporabljamo dobro preučeno vrsto velike zveri, rjavega medveda (*Ursus arctos*), kot vzorčno študijo za predstavitev pristopa. Kot referenčno populacijo smo genotipizirali 513 rjavih medvedov iz Slovenije z uporabo 20 polimorfnih mikrosatelitskih lokusov. Te podatke smo uporabili za kalibracijo in primerjavo heterozigotnosti in alelske pestrosti 30 populacij rjavega medveda, preučevanih v 10 študijah po celotni globalni razširjenosti te vrste. Enostavnost primerjav z uporabo referenčne populacije dela metodo uporabno tudi za druge vrste in omogoča primerjavo genetske pestrosti med prej neprimerljivimi študijami in boljše razumevanje razporeditve genetske pestrosti posamezne vrste vzdolž njenega območja razširjenosti.

Heredity

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ORIGINAL ARTICLE

Using a reference population yardstick to calibrate and compare genetic diversity reported in different studies: an example from the brown bear

T Skrbinšek[1], M Jelenčič[1], LP Waits[2], H Potočnik[1], I Kos[1] and P Trontelj[1]

In species with large geographic ranges, genetic diversity of different populations may be well studied, but differences in loci and sample sizes can make the results of different studies difficult to compare. Yet, such comparisons are important for assessing the status of populations of conservation concern. We propose a simple approach of using a single well-studied reference population as a 'yardstick' to calibrate results of different studies to the same scale, enabling comparisons. We use a well-studied large carnivore, the brown bear (*Ursus arctos*), as a case study to demonstrate the approach. As a reference population, we genotyped 513 brown bears from Slovenia using 20 polymorphic microsatellite loci. We used this data set to calibrate and compare heterozygosity and allelic richness for 30 brown bear populations from 10 different studies across the global distribution of the species. The simplicity of the reference population approach makes it useful for other species, enabling comparisons of genetic diversity estimates between previously incompatible studies and improving our understanding of how genetic diversity is distributed throughout a species range.
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Keywords: genetic diversity; conservation; population comparison; genetics; inbreeding; *Ursus arctos*

INTRODUCTION

Loss of biodiversity is one of the critical challenges faced both by our planet and our species, as many plants and animals have been eradicated from human-dominated landscapes or remain in small populations that face a serious threat of extinction (UNEP, 1992). Conservation of these remaining populations may, in the long run, critically depend on genetic factors (Allendorf and Luikart, 2007; Frankham, 2009). Genetic diversity indicates a population's fitness and evolutionary potential, and consequently its adaptive potential and resilience to environmental change (Reed and Frankham, 2003; Allendorf and Luikart, 2007), which makes it a critical issue for conservation. Increased accessibility and decreasing costs are making the use of genetics in biodiversity conservation more attractive than ever, and increasingly large amounts of genetic data are available for species of conservation concern. Comparing these data between different populations along the range of a species would be useful for understanding and evaluating their genetic health and assessing the risk of inbreeding depression. However, genetic diversity of different populations is often evaluated using different methods and markers, making such comparisons difficult (see Swenson *et al.*, 2011).

We propose a simple approach for calibrating genetic diversity of different populations, reported by different studies, to the same scale relative to a reference population. By using this one well-studied population as a 'yardstick', we can perform large-scale comparisons of genetic diversity across a species range using the existing data. We demonstrate the utility of this concept using the brown bear (*Ursus arctos*), a widely distributed carnivore species that has been extensively studied using genetic methods.

Throughout most of its global range, the brown bear is suffering from habitat loss and overharvest, and more than 50% of its range and numbers have been lost since the mid-1800s (Servheen *et al.*, 1999). Large populations remain in Northeastern and Northwestern Russia, Alaska and Canada, but only smaller isolated populations remain in the rest of the bear's former range in Europe, the contiguous United States and the southern portions of the range in Asia. Although genetic diversity of different brown bear populations has been well documented, different studies typically use different types or panels of markers, making the results difficult to compare (Swenson *et al.*, 2011).

Centuries of persecution wiped out the bears from most of the Western Europe, and by the mid 20th century only a few isolated remnant populations remained in the Apennine Mountains, Italian Alps, Cantabrian Mountains and Pyrenees (Zedrosser *et al.*, 2001). Bears in Central, Eastern and Northern Europe fared somewhat better, with indigenous populations remaining in the Dinaric Mountains, the Carpathians and Northern Europe, but most of these populations were much smaller than today (Zedrosser *et al.*, 2001). This situation started to change in the second half of last century, when many remaining populations recovered and expanded as bears started making a comeback due to conservation and management efforts (Zedrosser *et al.*, 2001). The last decade of that century also marked the beginning of reintroductions of this species to Western Europe (Clark *et al.*, 2002). Although the overall situation is improving, many populations are still critically small (Linnell *et al.*, 2005). This makes understanding of genetic diversity both within and between European brown bear populations particularly important, as it can facilitate

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selection of the most appropriate source for reintroductions or population augmentations, as well as help identify the populations that need assistance.

We studied the Northern Dinaric bear population and used it as a reference population in this case study example. This population stretches from Slovenia through Croatia and Bosnia, and Herzegovina into Western Serbia and Montenegro (Zedrosser *et al.*, 2001), and has effective population size of approximately 280 bears (Skrbinšek *et al.*, 2012). It is a part of the larger Alps-Dinara-Pindos population, which spans over 11 countries, is thought to have approximately 2100–2500 individuals, and is considered stable over most of its range (Zedrosser *et al.*, 2001).

In this paper, we (1) introduce the reference population approach for calibrating and comparing genetic diversity reported by different studies of different populations, (2) survey the baseline genetic diversity data of the bears in Northern Dinaric Mountains and (3) use the reference population approach with the bears in Northern Dinaric Mountains as a reference population to calibrate and compare genetic diversity reported by different studies of bear populations across the range of the species.

MATERIALS AND METHODS

Comparing genetic diversity using the reference population approach

Different studies of genetic diversity typically vary in the number of samples and the sets of genetic markers they apply. Although this limits the degree to which the reported diversity indices are directly comparable, we can calculate the genetic diversity indices relative to the diversity indices of a single well-studied population (large sample size, a large number of loci) that we use as a 'yardstick' (the reference population).

For each pairwise comparison of a population with the reference, the genetic marker set of both the reference and the compared population is reduced to the loci they have in common. To correct for differences in sample size, individual genotypes from the larger sample size (typically the reference population) are randomly resampled with replacement many times ($\sim$1000) to the sample size of the smaller data set (Leberg, 2002). Average allelic richness, expected heterozygosity and their standard errors are then calculated over all random subsamples, thus correcting for differences in sample size. The standard errors are calculated as a mean of standard errors of each subsample.

Finally, a heterozygosity ratio (H_{er}) and allelic diversity ratio (A_{rt}) indices are calculated for the compared population as $H_{er} = H_e x / H_{es} R$ and $A_{rt} = Ax / A_s R$, where $H_e x$ and Ax are expected heterozygosity and allelic diversity for the compared population, and $H_{es} R$ and $A_s R$ the subsampling-corrected values of these indices in the reference population (assuming that the reference population had more samples). Standard errors of the H_{er} and A_{rt} indices are calculated as the standard error (s.e.) of division,

$$\text{s.e.}(H_{er}) = \sqrt{H_{er}^2 \cdot \left(\left(\frac{\text{s.e.}(H_e x)}{H_e x} \right)^2 + \left(\frac{\text{s.e.}(H_{es} R)}{H_{es} R} \right)^2 \right)},$$

and

$$\text{s.e.}(A_{rt}) = \sqrt{A_{rt}^2 \cdot \left(\left(\frac{\text{s.e.}(Ax)}{Ax} \right)^2 + \left(\frac{\text{s.e.}(A_s R)}{A_s R} \right)^2 \right)}$$

Genetic diversity of brown bears in Northern Dinaric Mountains—the reference population

Tissue and blood samples were collected from 2003 to 2008 from 505 dead bears and 8 bears captured for translocation (to France in 2006) or telemetry in the northernmost part of the Northern Dinaric population, in Slovenia (Figure 1). We analyzed 22 microsatellite loci for these 513 bears in three multiplex PCRs. Locus names, primer sequences, dyes, primer concentrations, analytic and quality assurance protocols used are detailed in Appendix 1. Further analytic protocols used for these loci are described in Skrbinšek *et al.* (2010). We randomly selected 10% of samples and repeated the genotyping to estimate error rates, as suggested by Pompanon *et al.* (2005). The actual number of repeats was considerably higher as the entire multiplex was repeated if the genotype at any locus was unclear. We used the methods recommended by Broquet and Petit (2004) to estimate the frequency of allelic dropouts and false alleles, and program Micro-Checker (Van Oosterhout *et al.*, 2004) to check the data for the presence of null alleles, and scoring errors due to stuttering and dropout of large alleles.

We used R statistical environment (R Development Core Team, 2011) and 'adegenet' package (Jombart, 2008) for data handling and calculation of genetic diversity indices—observed heterozygosity (H_o), expected heterozygosity (H_e) and allelic diversity (A). Probability of identity (PI) and probability of identity of siblings (PI_{sib}) were calculated according to Waits *et al.* (2001). We used the procedure described in Guo and Thompson (1992) with 1 000 000 steps in Markov chain and 10 000 dememorization steps to detect per-locus

Figure 1 Alps-Dinara-Pindos bear population and sampling area. Shaded areas show brown bear range. (**a**) Alps-Dinara-Pindos population NW, NW Dinaric Mountains; (**b**) Alps-Dinara-Pindos population SE; (2) Carpathian population (after Zedrosser *et al.*, 2001). Rectangle—sampling area.

significant departures from Hardy–Weinberg equilibrium using the program Arlequin (Excoffier and Lischer, 2010). Holm–Bonferronni multiple test correction with $\alpha = 0.05$ threshold was used to correct for multiple testing.

Using the reference population approach to explore differences in genetic diversity of brown bear populations across species range

We compared genetic diversity of different brown bear populations across the species range using the bears in Northern Dinaric Mountains as the reference. The details of the included studies are presented in the Appendix 2. The marker set we used for the reference population included the majority or all markers used in any other study, allowing for a large panel of loci for most comparisons. As our data set also included several times the number of samples analyzed in any other study, we always used it as the larger data set for resampling. We made 1000 random subsamples for each comparison. Finally, we calculated the H_{er} and A_{rt} indices, and used these to compare genetic diversity of bear populations across the species range.

The R code required to run comparisons between populations using the reference population approach (in the form of an R package with user manual and a user-friendly vignette), as well as the genetic data from the Dinaric bear population used for this study, are accessible in the Dryad repository (doi:10.5061/dryad.qt3j5).

RESULTS

Genotyping

No loci showed evidence of long allele dropout or scoring errors due to stuttering. Locus Mu26 had null alleles (estimated frequency using detected null homozygotes = 0.117), and was excluded from downstream analyses. Locus G10H did not provide reliable genotyping results and was also excluded. Locus Mu23 had an irregular repeat pattern, as two out of the eight alleles had a single base deletion in the region flanking the $(CA)_n$ microsatellite, making their size a single base pair different from the neighbouring alleles. We were able to score the alleles reliably, so we can include this locus in the analyses. However, as the other studies may have missed this, or used primers that did not include the region with this single base polymorphism, using this locus for the reference population could bias the genetic diversity estimates for the reference population high. Considering this, we decided to exclude this locus from the reference population data.

On average 66% of per-locus genotype analyses were repeated more than once (varies between multiplexes: A = 69%, B = 71%, C = 51%). Median allelic dropout rate was 0.19% (0.00–0.70%). We detected false alleles only on locus G10P (0.19%). Taking into account the number of loci, per-locus error rates, the number of samples genotyped and the number of times analyses of each sample were repeated, we can expect that there are still approximately 10 (9.6) single-locus errors in the data set. This makes the estimated remaining per-locus error rate in the entire data set 9.36×10^{-4}.

Genetic diversity of bears in Northern Dinaric Mountains (Slovenia)

Average heterozygosities using the 20 remaining loci were 0.731 (H_e) and 0.738 (H_o). All these loci fit Hardy–Weinberg expectations after Holm–Bonferronni multiple test correction at $P = 0.05$. Average allelic diversity was 6.75 (s.d. = 1.77). Per-locus results are summarized in Table 1.

Comparison of genetic diversity of brown bear populations across the range of the species

The results of the range-wide comparison of genetic diversity in brown bears are summarized in Table 2, and show considerable differences between populations. On one extreme, the most diverse is the Carpathian population in Romania, followed by large populations in Canada and Alaska. At the other extreme, the lowest levels of diversity are observed for island populations and very small populations of high conservation concern (Gobi Desert, Cantabrian Mountains—Spain, Kodiak Island—Alaska).

Table 1 Genetic diversity indices for brown bears in Northern Dinaric Mountains

Marker	A	H_o	H_e	*PI*	PI_{sib}	$P_{(HW)}$	*Size*
Cxx20	5	0.77	0.76	0.10	0.40		121–141
G10B	8	0.73	0.71	0.11	0.42	0.010	130–154
G10C	9	0.76	0.74	0.11	0.41		93–115
G10D	7	0.80	0.79	0.08	0.37		168–182
G10J	5	0.65	0.69	0.16	0.45		78–92
G10L	5	0.63	0.64	0.20	0.48	0.054	153–163
G10M	6	0.76	0.76	0.09	0.39		204–218
G10P	8	0.78	0.78	0.07	0.38		147–175
G10X	10	0.82	0.84	0.04	0.34	0.007	132–154
G1A	4	0.66	0.65	0.19	0.47		180–190
Mu05	7	0.62	0.66	0.16	0.46		127–141
Mu09	9	0.69	0.72	0.11	0.42		174–206
Mu10	4	0.69	0.68	0.17	0.45		112–126
Mu11	7	0.72	0.74	0.11	0.41		80–94
Mu15	6	0.78	0.77	0.09	0.39		117–131
Mu23[a]	8	0.79	0.81	0.06	0.36		142–156
Mu26[b]	—	—	—	—	—	**<0.001**	182–200
Mu50	7	0.80	0.80	0.07	0.37		79–103
Mu51	5	0.56	0.59	0.26	0.52	0.036	115–127
Mu59	9	0.86	0.85	0.04	0.34		97–121
Mu61	6	0.76	0.78	0.08	0.38		141–153
Mean	**6.75**	**0.73**	**0.74**	**0.12**	**0.41**		
s.d.	1.77	0.08	0.07	0.06	0.05		
s.e.	0.40	0.02	0.02	0.01	0.01		

Abbreviations: *A*, number of observed alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; $P_{(HW)}$, probability of deviation from Hardy–Weinberg equilibrium; PI, probability of identity; PI_{sib}, probability of identity for siblings; s.d., standard deviation; s.e., standard error; Size, size range of alleles.
Values in bold under $P_{(HW)}$ remained statistically significant after a Holm–Bonferroni multiple test correction (corrected $\alpha = 0.05$).
[a]Locus Mu23 has a single base pair deletion in this population and consequently an irregular repeat pattern. The locus was included in the calculation of averages in this table, but excluded from the reference population.
[b]Locus Mu26 has null alleles, and has been excluded from the calculation of averages.

DISCUSSION

The reference population approach provides a simple and easy to implement method of comparing genetic diversity between different populations of a species that were analysed in different studies using different loci, while collecting no or only minimal additional data. We demonstrate the application of this approach by evaluating the global distribution of genetic diversity of brown bears. Typically, there are two obstacles to comparing genetic diversity reported by different studies of the same species: different panels of genetic markers used and differences in sample sizes. The standard approach to addressing this problem is to shrink the genetic marker set to the largest common denominator of all studies, and use the smallest sample size in any population to correct for unequal sampling (El Mousadik and Petit, 1996; Leberg, 2002). This approach works only if similar sets of markers were used to study all populations or if marker sets are very large, which is often not the case. Also, by using a very small sample size to correct for unequal sampling, the power to detect differences

Table 2 Comparison of genetic diversity between bear populations using bears in NW Dinaric Mountains (Slovenia, population Alps-Dinara-Pindos NW in bold face) as a reference to correct for different panels of loci and sample sizes

			Compared population		*Reference pop. (resampled)*		*Ratio*	
Population	N	*Study*	A *(s.e.)*	H_e *(s.e.)*	A *(s.e.)*	H_e *(s.e.)*	A_{rt} *(s.e.)*	H_{er} *(s.e.)*
Carpathians–Romania (1)	16	5	7.78 (0.81)	0.81 (0.010)	5.15 (0.56)	0.70 (0.030)	1.51 (0.23)	1.16 (0.05)
Carpathians–Romania (2)	109	10	8.46 (0.57)	0.80 (0.014)	6.33 (0.54)	0.73 (0.023)	1.34 (0.15)	1.09 (0.04)
Alaska Range, Alaska	28	1	—	0.78 (—)	5.84 (0.68)	0.72 (0.026)	—	1.08(—)
Kluane, Yukon	50	1,2	7.38 (0.56)	0.76 (0.025)	6.12 (0.70)	0.73 (0.026)	1.21 (0.17)	1.04 (0.05)
Richardson Mountains, NWT	119	2	7.50 (0.63)	0.76 (0.030)	6.48 (0.72)	0.73 (0.025)	1.16 (0.16)	1.03 (0.05)
Brooks Range, Alaska	148	2	7.63 (0.50)	0.75 (0.019)	6.56 (0.72)	0.74 (0.025)	1.16 (0.15)	1.02 (0.04)
Croatia (Alps-Dinara-Pindos NW)	156	9	7.58 (0.54)	0.74 (0.028)	6.48 (0.60)	0.73 (0.025)	1.17 (0.14)	1.01 (0.05)
Slovenia (Alps-Dinara-Pindos NW)	**513**	**REF[a]**	**6.68 (0.41)**	**0.73 (0.020)**	—	—	**1.00 (0.06)**	**1.00 (0.03)**
Greece (Alps-Dinara-Pindos s.e.)	49	8	6.33 (0.42)	0.76 (0.020)	6.55 (0.52)	0.77 (0.023)	0.97 (0.10)	0.99 (0.04)
Carphatians–Northern Slovakia	71	10	6.08 (0.29)	0.71 (0.025)	6.20 (0.54)	0.73 (0.023)	0.98 (0.10)	0.97 (0.05)
Scandinavia–NN	29	3	5.59 (0.40)	0.68 (0.024)	5.59 (0.42)	0.72 (0.020)	1.00 (0.10)	0.96 (0.04)
Flathead River, BC/MT	40	2	6.50 (0.71)	0.69 (0.027)	6.01 (0.69)	0.73 (0.026)	1.08 (0.17)	0.95 (0.05)
Carpathians–Central Slovakia	96	10	6.00 (0.25)	0.70 (0.031)	6.30 (0.54)	0.73 (0.023)	0.95 (0.09)	0.95 (0.05)
Scandinavia–NS	108	3	6.18 (0.35)	0.69 (0.027)	6.10 (0.44)	0.73 (0.019)	1.01 (0.09)	0.95 (0.04)
West Slope, Alberta	41	2	6.38 (0.56)	0.68 (0.036)	6.03 (0.69)	0.73 (0.026)	1.06 (0.15)	0.93 (0.06)
Kuskoskwim Range, Alaska	55	1,2	6.13 (0.44)	0.68 (0.026)	6.15 (0.71)	0.73 (0.025)	1.00 (0.14)	0.93 (0.05)
Scandinavia–M	88	3	5.94 (0.40)	0.68 (0.022)	6.02 (0.44)	0.73 (0.019)	0.99 (0.10)	0.93 (0.04)
Scandinavia–S	155	3	5.47 (0.33)	0.68 (0.020)	6.20 (0.44)	0.73 (0.019)	0.88 (0.08)	0.93 (0.04)
East Slope, Alberta	45	2	7.00 (0.82)	0.67 (0.062)	6.07 (0.70)	0.73 (0.026)	1.15 (0.19)	0.92 (0.09)
Carpathians–Eastern Slovakia	16	10	5.23 (0.22)	0.65 (0.028)	5.47 (0.49)	0.72 (0.025)	0.96 (0.09)	0.91 (0.05)
Paulatuk Alaska	58	2	5.75 (0.88)	0.65 (0.650)	6.18 (0.71)	0.73 (0.026)	0.93 (0.18)	0.89 (0.89)
Admiralty Island, Alaska	30	1	—	0.63 (—)	5.88 (0.68)	0.73 (0.026)	—	0.87 (—)
Coppermine, NWT	36	2	5.75 (1.03)	0.61 (0.073)	5.96 (0.69)	0.73 (0.026)	0.96 (0.21)	0.84 (0.10)
Pakistan	28	4	3.92 (0.38)	0.58 (0.043)	5.45 (0.53)	0.72 (0.025)	0.72 (0.10)	0.81 (0.07)
Yellowstone, MT/WY	57	2	4.38 (0.60)	0.55 (0.081)	6.17 (0.7)	0.73 (0.025)	0.71 (0.13)	0.75 (0.11)
Cantabrian (Spain)–W	39	7	3.44 (0.30)	0.48 (0.050)	5.73 (0.49)	0.71 (0.022)	0.6 (0.07)	0.67 (0.07)
Baranof and Chicgagof Is, Alaska	35	1	—	0.49 (—)	5.96 (0.69)	0.73 (0.026)	—	0.67 (—)
Apennines	17	5	2.44 (0.24)	0.44 (0.069)	5.19 (0.56)	0.70 (0.030)	0.47 (0.07)	0.63 (0.10)
Gobi (Mongolia)	8	6	2.00 (—)	0.29 (—)	4.59 (0.62)	0.68 (0.038)	0.44 (—)	0.43 (—)
Cantabrian (Spain)–E	8	7	1.75 (0.17)	0.28 (0.062)	4.56 (0.38)	0.68 (0.026)	0.38 (0.05)	0.41 (0.09)
Kodiak Island, Alaska	34	1,2	2.13 (0.35)	0.27 (0.098)	5.94 (0.69)	0.73 (0.026)	0.36 (0.07)	0.37 (0.14)

Abbreviations: A, allelic richness; A_{rt}, allelic richness ratio between the compared population/resampling-corrected, marker-set specific values for bears in NW Dinaric Mountains; H_e, expected heterozygosity; H_{er}, heterozygosity ratio; N, number of samples; s.e., standard error.
'Reference pop. (resampled)' column shows the multiple subsampling corrected values from the reference population used for calculating H_{er} and A_{rt} ratios. The studies referenced in the 'Study' column are detailed in the Appendix 2.
[a]Reference population. Values of some parameters in certain populations are missing as they were not available in the published data.

in allelic richness is greatly reduced decreasing the power of all comparisons (Leberg, 2002).

The reference population approach overcomes many of these issues with a simple solution of scaling the genetic diversity of each considered population relative to the genetic diversity of a single well-studied population, effectively using this reference population as a calibration 'yardstick'. Its main advantage is the ability to compare studies that would be otherwise impossible to compare—for example, studies that have no common genetic markers—if the markers they used are also used in the study of the reference population. The problem of low power of comparison will still remain when a study with a small sample size is compared, but this would not affect the power of pairwise comparisons of other populations.

Technical considerations, application and limitations of the reference population approach

Application of this method requires a reference population with a large sample size and a large number of genotyped loci. It is beneficial if a large population with high genetic diversity is used as a reference. If a study is designed specifically to provide reference population data, the panel of loci chosen should cover all or the majority of the loci used in other populations of interest. As more journals require genotype-level data to be deposited in online data repositories, reference population data should be increasingly easy to obtain. When suitable reference population data are available, it is straightforward to compare genetic diversity estimated in any new study of the same species with the existing data, provided that a large enough proportion of the marker set matches the marker set of the reference population.

We used multiple subsampling (Leberg, 2002) to correct for unequal sample sizes in different studies. Although it is argued that allelic diversity is a better predictor of a population's evolutionary potential than heterozygosity (Allendorf, 1986), it is also much more sensitive to sample size, and corrections for unequal sampling must be applied to calculate allelic richness if studies with different sample sizes are being compared (El Mousadik and Petit, 1996; Leberg, 2002). The most commonly used method is the rarefaction approach suggested by El Mousadik and Petit (1996). Simulations done by Leberg (2002) suggest that the multiple subsampling approach we

used provides marginally better precision, but both methods perform adequately and without bias.

There was considerable variation in resampled allelic richness for the reference population (Table 2). This is a consequence of both subsampling to a smaller sample size, as rare alleles will get missed (see Leberg, 2002), as well as of the differences in locus panels that were subsampled to match the panels in the compared populations. The related standard error shows the standard error of allelic richness at the subsample size, providing the basis for comparison with the population of interest. Comparing calibrated expected heterozygosity to the values reported in original studies, it is clear that we would draw similar inferences using either the reported H_e or the calibrated indices (Table 2). Brown bears are studied with a relatively standard set of microsatellite markers, so all the studies included in this comparison had considerable overlap in markers. Although the reference population approach provides a formal framework for the bear case study, it should be even more useful in a species studied with a more diverse set of markers.

A logical precondition of the reference population approach is that it assumes the same type of genetic markers used in all studies that are to be compared. We implemented the approach using microsatellite data; however, the general idea of using a 'yardstick' reference population could be transferred to other types of markers suitable for measuring genetic diversity (for example, single-nucleotide polymorphisms). Another potential problem for application is that sometimes only summary genetic diversity data are reported for a population, without any estimate of standard errors. Although such data are still useful, testing hypotheses about statistical significance of the observed differences between populations is impossible. This shows the importance of publishing standard error estimates in all genetic diversity studies, even if only a single population was studied. However, with recent changes to published data accessibility policies such cases should become increasingly rare.

The brown bear case study

The dramatic range of genetic diversity in brown bears that was observed by Paetkau *et al.* (1998b) in North America is also evident at the global scale (Table 2). Most of the observed patterns are expected—high genetic diversity in large populations (Alaska, Canada, Carpathians, Dinaric Mountains) and very low levels of genetic diversity in populations that have been isolated for a long time or have passed through severe demographic bottlenecks. The demographic history of many of these populations shows a large decline and a questionable future: the Gobi population in Mongolia (McCarthy *et al.*, 2009), Cantabrian population in Spain (Perez *et al.*, 2009) and the population in the Apennines in Italy (Ciucci and Boitani, 2008).

However, the genetic diversity in these populations is higher than the diversity of Kodiak Island bears in Alaska. This latter population is relatively large (>2500) and healthy, with low genetic diversity attributed to a long period of isolation from the bears on the continent (Paetkau *et al.*, 1998a, 1998b). On the other hand, the demographic history of the other populations with low genetic diversity is presumed to be one of a recent contraction and isolation. For example, the Apennine population is estimated at around 50 remaining animals (Gervasi *et al.*, 2008) and has been isolated for at least 400–600 years (Ciucci and Boitani, 2008). The story is similar with the Cantabrian bears in Spain, where the population suffered a dramatic decline in recent centuries and is now threatened with extinction (Perez *et al.*, 2009).

Despite evidence from Kodiak bears that a brown bear population can exist and even prosper at very low levels of genetic diversity measured at neutral markers, this should not be generalized to the small populations that live in human-dominated landscapes. An island population may stabilize in a mutation-drift equilibrium at very low levels of genetic diversity, but it is possible that these bears survived against all odds through many generations of reduced fitness, all the time purging strongly deleterious alleles (Peatkau *et al.*, 1998b). Although this may be a plausible scenario in Alaskan wilderness with favourable habitat and low human densities, the risk of inbreeding depression is likely to increase due to increased stress in degraded and human-dominated landscapes (Armbruster and Reed, 2005). For these populations, it is quite possible that they will need genetic rescue or restoration (Tallmon *et al.*, 2004; Hedrick, 2005), or face extinction.

The highest genetic diversity levels were observed in the Carpathian brown bears. The population is relatively large, estimated to number around 8100 animals (Zedrosser *et al.*, 2001), which may explain the high diversity. Another possible explanation for such high diversity might be historical mixing of animals from Eastern and Western glacial refugia as suggested by mitochondrial DNA data (Zachos *et al.*, 2008). It would be interesting to compare genetic diversity levels of large bear populations in Russian Far East, but unfortunately there is no published research that would enable these comparisons.

CONCLUSIONS

Genetic diversity is a key component of long-term population viability (Allendorf and Ryman, 2002; Keller and Waller, 2002; O'Grady *et al.*, 2006). By calibrating previously incompatible studies through comparisons with a reference population, we were able to directly compare neutral genetic diversity of brown bears from all previously studied populations. This method can easily be applied to other species and to test hypotheses about variables that influence genetic diversity across the range of a species. The method will also be helpful for identifying populations with low levels of diversity that have the greatest need for direct conservation actions, and can aid in providing the scientific justification needed to gain management and public support. The simplicity of the reference population approach should make it useful in future comparisons of genetic diversity estimates between previously incompatible studies and in improving our understanding of how genetic diversity is distributed along a species range.

DATA ARCHIVING

Data and all R code have been deposited at Dryad: doi:10.5061/dryad.qt3j5.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX 1

Sample collection, laboratory analysis and quality assurance protocols used for genotyping of brown bear tissue samples

About a cubic centimeter of muscle, liver and/or skin tissue was taken from each dead animal, stored in 96% ethanol and kept at −20 °C. Blood from live captures was stored in Vacutainer tubes with anticoagulant (BD Diagnostics, Franklin Lakes, NJ, USA), also at −20 °C. We extracted DNA using GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's recommendation. The extracted DNA was then kept at −20 °C until analysis.

PCR setup was performed in a pressurized PCR hood using dedicated pipettes. We implemented a strict one-way workflow, where PCR cycling and post-PCR sample handling was done in a different room and no PCR products were returned to the lab where DNA extraction and PCR setup took place. Aerosol barrier pipette tips were used for all liquid transfers. All critical pipetting steps were photographed and later rechecked to detect possible sample mixups.

Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) was used for all PCR. We prepared 10 µl reactions—5 µl of Qiagen Mastermix, 1 µl of Q solution, 1 µl of template DNA and 3 µl of water and primers mixture to obtain the appropriate concentration in the final solution.

PCR conditions of different multiplexes differ only in the annealing temperature. The initial 10 min denaturation step at 95 °C is followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at the multiplex-specific temperature for 90 s and elongation at 72 °C for 60 s. Annealing temperatures were 58 °C for multiplexes M1 and M3, and 49.6 °C for multiplex M2. PCR was concluded with a 30-min final elongation step at 60 °C designed to add +A to all fragments and minimize the problem of split peaks.

Fragment analysis was done on an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA). A mixture of 1 µl of the PCR product, 0.25 µl of GS500LIZ size standard (Applied Biosystems, Foster City, CA, USA) and 8.75 µl of formamide was loaded on the sequencer. We analyzed the output with GeneMapper software (version 4.0, Applied Biosystems).

Each electrophoregram was independently checked by two persons. If the genotype at any locus was unclear, the PCR and analysis of the entire multiplex were repeated.

Table A: Locus names, dyes, primer sequences, primer concentrations and PCR multiplexes (MP) used for genotyping of brown bear tissue samples

Locus	*5′ Primer*	*3′ Primer*	*MP*	*Primer C [μM]*
G10C[2]	VIC-AAAGCAGAAGGCCTTGATTTCCTG	GGGACATAAACACCGAGACAGC	M1	0.07
G10P[3]	TACATAGGAGGAAGAAAGATGG	VIC-AAAAGGCCTAAGCTACATCG	M1	0.09
G10X[2,3]	6FAM-CCCTGGTAACCACAAATCTCT	TCAGTTATCTGTGAAATCAAAA	M1	0.27
G1D[2]	ATCTGTGGGTTTATAGGTTACA	6FAM-CTACTCTTCCTACTCTTTAAGAG	M1	0.18
Mu10[4]	ATTCAGATTTCATCAGTTTGACA	6FAM-TCAGCATAGTTACACAAATCTCC	M1	0.16
Mu15[3]	PET-CTGAATTATGCAATTAAACAGC	AAATAAGGGAGGCTTGGGT	M1	0.25
Mu23[4]	NED-TAGACCACCAAGGCATCAG	GCCTGTGTGCTATTTTATCC	M1	0.11
Mu50[4]	GTCTCTGTCATTTCCCCATC	6FAM-AACCTGGAACAAAAATTAACAC	M1	0.10
Mu59[4]	GCTCCTTTGGGACATTGTAA	NED-TGACTGTCACCAGCAGGAG	M1	0.20
Cxx20[1]	AGCAACCCCTCCCATTTACT	NED-TTGTCTGAATAGTCCTCTGCC	M2	0.30
G10J[2]	NED-GATCAGATATTTTCAGCTTT	AACCCCTCACACTCCACTTC	M2	0.10
G10M[2]	6FAM-TTCCCCTCATCGTAGGTTGTA	GATCATGTGTTTCCAAATAAT	M2	0.40
Mu09[3]	AGCCACTTTGTAAGGAGTAGT	VIC-ATATAGCAGCATATTTTTGGCT	M2	0.07
Mu61[3]	6FAM-TCCACTGGAGGGAAAATC	CTGCTACCTTTCATCAGCAT	M2	0.10
G10B[2]	GCCTTTTAATGTTCTGTTGAATTTG	6FAM-GACAAATCACAGAAACCTCCATCC	M3	0.01
G10H[2]	6FAM-CAACAAGAAGACCACTGTAA	AGAGACCACCAAGTAGGATA	M3	0.10
G10L[4]	PET-ACTGATTTTATTCACATTTCCC	GATACAGAAACCTACCCATGCG	M3	0.10
G1A[2]	VIC-GACCCTGCATACTCTCCTCTGATG	GCACTGTCCTGCGTAGAAGTGAC	M3	0.08
Mu05[3]	6FAM-AATCTTTTCACTTATGCCCA	GAAACTTGTTATGGGAACCA	M3	0.13
Mu11[3]	VIC-AAGTAATTGGTGAAATGACAGG	GAACCCTTCACCGAAAATC	M3	0.20
Mu26[3]	6FAM-GCCTCAAATGACAAGATTTC	TCAATTAAAATAGGAAGCAGC	M3	0.08
Mu51[4]	AGCCAGAATCCTAAGAGACCT	PET-AAAGAGAAGGGACAGGAGGTA	M3	0.09

[1]Ostrander *et al*., 1993; [2]Paetkau *et al*., 1998a, 1998b; [3]Taberlet *et al*., 1997; [4]Bellemain and Taberlet, 2004.

APPENDIX 2
Studies included in comparison of genetic diversity of brown bears along the species range

No.	*Reference*	*Geographic area*	*Aim of the study*	*NP*	*Loc*
1*	Paetkau *et al*., 1998a	Alaska, North America	Exploration of gene flow between coastal and interior populations of brown bears in Alaska	7	8/8
2	Paetkau *et al*., 1998b	North America	Exploration of variation in genetic diversity across the range North American brown bears	11	8/8
3	Waits *et al*., 2000	Scandinavia, Europe	Study of genetic diversity and gene flow in the Scandinavian brown bear, comparison with the North American populations	4	19/18
4	Bellemain *et al*., 2006	Northern Pakistan, Asia	Conservation and management of a small and endangered Himalayan brown bear population	1	15/13
5	Zachos *et al*., 2008	Romania and Italy (Apennines), Europe	Phylogeography and genetic diversity comparison of two bear populations, implications for conservation	2	9/9
6	McCarthy *et al*., 2009	Mongolia, Asia	Determination of population size and genetic diversity for the critically endangered Gobi brown bear population	1	6/6
7	Perez *et al*., 2009	Spain, Europe	Genetic diversity and population substructure data for the critically endangered Cantabrian brown bear population	1(2)	18/16
8	Karamanlidis *et al*., 2010	Greece, Europe	Pilot study, a test of new method for collection of brown bear non-invasive samples	1	6/6
9	Kocijan *et al*., 2011	Croatia, Europe	Genetic diversity of brown bears in Northern Dinaric Mountains	1	12/12
10	Straka *et al*., 2012	Carpathians, Europe	Population substructure, demographic history and genetic diversity of brown bears in Carpathians	1(2)	13/13

Abbreviations: Loc, number of microsatellite loci used in the study/number of loci common with the reference population; NP, number of populations (subpopulations) included in the study.
*The data from this study was taken as summarized in Waits *et al.* (2000), as the original paper did not include sufficient level of detail for comparison.

2.1.3 Visoko učinkovit sočasen PCR večih genetskih markerjev (multipleksni PCR) iz neinvazivnih genetskih vzorcev ne potrebuje predpomnoževanja DNA

Highly efficient multiplex PCR of noninvasive DNA does not require preamplification

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Izvleček: Ena od ključnih zahtev za uspešnost študij, ki uporabljajo molekularno-genetska orodja za monitoring prostoživečih živali, je dovolj velik nabor visoko informativnih genetskih markerjev in zanesljiva, poceni metoda za njihovo analizo. Čeprav za ljudi in domače živali obstajajo optimizirani komercialni genotipizacijski kiti, so takšni protokoli za prostoživeče živalske vrste redki. Razvili smo visoko optimiziran multipleksni PCR protokol, s katerim je mogoče amplificirati 12 mikrosatelitskih lokusov in lokus za določitev spola v enem samem multipleksnem PCR in eni sami analizi na sekvenatorju. Ta protokol smo uporabili za genotipizacijo 1053 vzorcev iztrebkov medvedov iz dinarske populacije in dobili uporabne genotipe pri 88 % vzorcev, kar je zelo visoka uspešnost. Protokol je bolje deloval od multipleksnega PCR protokola s prepomnoževanjem DNA, ki smo ga uporabili v prejšnji študiji na 473 vzorcih iztrebkov z uspešnostjo 78,4 %. Na podvzorcu 182 vzorcev smo neposredno primerjali učinek obeh pristopov in nismo zaznali nobenih prednosti predpomnoževanja. Čeprav lahko protokoli s predpomnoževanjem DNA izboljšajo uspešnost PCR in zanesljivost pri majhnem delu vzorcev nizke kvalitete, višji stroški in povečan obseg dela ne opravičuje njihove uporabe pri analizi razmeroma svežega neinvazivnega materiala. Ob tem visoko število multipleksiranih lokusov v novem protokolu naredi le-tega primerljivega s komercialnimi genotipizacijskimi kiti, razvitimi za domače živali in ljudi.

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TECHNICAL ADVANCES

Highly efficient multiplex PCR of noninvasive DNA does not require pre-amplification

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Abstract

Among the key issues determining success of a study employing molecular genetics tools in wildlife monitoring or research is a large enough set of highly informative genetic markers and a reliable, cost effective method for their analysis. While optimized commercial genotyping kits have been developed for humans and domestic animals, such protocols are rare in wildlife research. We developed a highly optimized multiplex PCR that genotypes 12 microsatellite loci and a sex determination locus in brown bear (*Ursus arctos*) faecal samples in a single multiplex PCR and a single sequencer run. We used this protocol to genotype 1053 faecal samples of bears from the Dinaric population, and obtained useful genotypes for 88% of the samples, a very high success rate. The new protocol outperformed the multiplex pre-amplification strategy used in a previous study of 473 faecal samples with a 78.4% success rate. On a subset of 182 samples we directly compared the performance of both approaches, and found no advantage of the multiplex pre-amplification. While pre-amplification protocols might still improve PCR success and reliability on a small fraction of low-quality samples, the higher costs and workload do not justify their use when analysing reasonably fresh noninvasive material. Moreover, the high number of multiplexed loci in the new protocol makes it comparable to commercially developed genotyping kits developed for domestic animals and humans.

Keywords: faecal samples, genetic tagging, NGS, PCR optimization, pre-amplification, *Ursus arctos*

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Introduction

Molecular genetics is increasingly becoming one of the most important tools for research and monitoring of wildlife species. Molecular tools can provide information relevant to both ecological and evolutionary questions, while costing less and being more sensitive and reliable than traditional monitoring approaches (Schwartz *et al*. 2007). The usefulness of these tools became even more pronounced with the introduction of noninvasive sampling that enables biologists to collect critical data about wildlife populations without handling, capturing or even observing individual animals (Taberlet *et al*. 1999; Waits & Paetkau 2005). Genetic tools have been applied to a variety of wildlife species and problems (DeYoung & Brennan 2005a; DeYoung & Honeycutt 2005b; Waits & Paetkau 2005; Schwartz *et al*. 2007).

Among the key issues determining success of a study employing molecular genetics tools in wildlife monitoring and research are a large enough set of highly informative genetic markers and reliable, cost effective methods for their analysis. Multiplex PCR procedures, which co-amplify several loci in the same reaction, decrease workload, costs and enable a more efficient use of the template DNA. Although a certain degree of multiplexing is often used, high numbers of loci (about 10 or more) in a single multiplex typically provided by commercial genotyping kits have, to our knowledge, not yet been reported for noninvasive wildlife samples.

Genotyping can be problematic when using noninvasive samples, which are frequently plagued by low success rates, contamination concerns and high genotyping

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error rates (Taberlet *et al*. 1996, 1997, 1999; Waits & Paetkau 2005). One of the suggested solutions was a 'multiplex pre-amplification' PCR strategy that uses a two-stage PCR with semi-nested primers to obtain lower error and higher success rates (Piggott *et al*. 2004a). Several other authors confirmed its advantages (Bellemain & Taberlet 2004b; Hedmark & Ellegren 2006; Arandjelovic *et al*. 2008). Although the approach requires more steps in the analysis and increases per-reaction workload and costs, it is argued that this is compensated by a higher overall amplification success and decrease in the allelic dropout rate.

We developed a protocol for analysis of 12 microsatellite loci and a sex specific locus from noninvasive samples of brown bears (*Ursus arctos*) in a single multiplex PCR and a single run on an automated DNA sequencer. We used the protocol to analyse a large number of brown bear faecal samples, and we thoroughly tested its efficiency and reliability. We compared the performance of this new protocol for field-collected faecal samples with the results from a multiplex pre-amplification strategy (Bellemain & Taberlet 2004b). The work was conducted within the framework of a conservation genetics study of brown bears in Slovenia.

Material and methods

Experimental design

Faecal samples were collected in two studies: a smaller pilot study between 2004 and 2007, and a large-scale study in 2007 and 2008. In the pilot study the samples were collected over two relatively small sampling areas (170 and 240 km^2, respectively) in one spring and two autumn sessions in 2004 and 2005. In the large-scale study, the samples were collected over the entire bear range in Slovenia (approximately 6500 km^2), in a single intensive sampling session between the beginning of September and the end of November 2007. In the pilot study, the samples were amplified using the multiplex pre-amplification approach that amplified 6 microsatellite loci and a sex determination locus (Bellemain & Taberlet 2004b). While the success rate was high, the multiplex pre-amplification increased the costs and labour requirements of the analyses. Preliminary tests didn't show significant benefits of the pre-amplification, and a new, single reaction multiplex PCR protocol was developed and used exclusively for the large-scale study. The panel of loci used for this new protocol was an extension of the panel used previously with the multiplex pre-amplification protocol. Locus Mu51 was dropped, as it was not very informative in the Dinaric bear population and the product size made it difficult to include in the multiplex. Additional seven microsatellite loci were added to provide a total of 13 loci (12 microsatellite loci + 1 sex determination locus) amplified in a single PCR reaction.

To determine the efficiency of the new protocol and assess the actual benefits of the pre-amplification, we compared the samples from the large scale study (Large Scale) that were analysed with the new single reaction protocol with all the samples analysed with the pre-amplification protocol in the pilot study (Pilot All). Additionally, we compared the large scale study samples with the subset of the pilot study samples collected during the same season as the samples in the large scale study (Pilot Sep-Dec). The success rates, quality indices and allelic dropout rates were calculated using the entire panels of loci, as we were interested in the overall performance of each approach. The names in brackets refer to the summarized results in Table 1.

To provide compatibility between both studies, as well as a direct test of the benefits of the multiplex pre-amplification approach, a subset of 182 samples analysed with the pre-amplification protocol in the pilot study was reanalysed with the new single reaction protocol (Pilot Subsample). The samples were analysed using the entire panels of loci with each approach, however the direct comparison was performed with only the five microsatellite loci amplified using both approaches. For these samples, consensus genotypes were constructed with results from both protocols and used to determine error rates and quality indices.

Sample collection, storage and extraction of DNA

In both studies the samples were collected by volunteers, mostly hunters of the Slovenian Hunting Association, Slovenia Forest Service personnel and university students. All participants received detailed written

Table 1 Success rates (SR), global quality indices (QI) and allelic dropout rates (ADO) obtained with the single PCR protocol (Single) and with the multiplex pre-amplification approach (Preamp)

Sample set	Protocol	*n*	SR (%)	QI	ADO
Large Scale	Single	1053	88.2	0.923	0.129
Pilot All	Preamp	473	78.4	0.889	0.178
Pilot Sep-Dec	Preamp	176	84.9	0.913	0.161
Pilot Subsample	Preamp	182	92.9	0.801	0.230
Pilot Subsample	Single	182	88.5	0.802	0.201

QI and ADO are calculated only for successfully genotyped samples. Pilot = samples from the pilot study, Large Scale = samples from the large scale study. *n* = number of samples. The Pilot Subsamples are the samples from the pilot study that were analysed using both protocols, indices are for the five loci they had in common.

instructions for sample collection, including guidelines for estimation of the scat's age. These guidelines were not precise, but provided helpful pointers for participants to tell old scats from the fresh ones (contents-specific smell, visual appearance, presence of mucous and insect larvae). A scat's age estimated in this manner is highly subjective and may not directly indicate the actual age of the scat, but provides an 'index' of the scat's appearance that might be even more important for the ultimate PCR success. We have observed a clear association between the estimated scat age and the actual PCR success (Skrbinšek T., unpublished data). Only samples judged to be not more than 5 days old were collected. The samples were stored in 50 mL screw-cap tubes with 96% ethanol. The participants were instructed to keep the collected samples in a cool and dark place until delivery to the laboratory. When delivered (usually within 1 month), the samples were kept at −20 °C in a dedicated noninvasive laboratory until analysis.

We used Qiagen QIAmp™ DNA Stool Mini Kit for DNA extraction. A part of each faecal sample was taken out of the storage tube, spread over the surface of a disposable Petri dish and left for a few minutes for the ethanol to evaporate. Large particles (large parts of leaves, hair, corn seeds etc.) were separated, and the remaining fine material with a large surface to volume ratio was used for the extraction. The rest of the extraction was performed according to the manufacturer's instructions, with the exception that we extended duration of some extraction steps: vortexing with the ASL buffer was done for 20–45 min, vortexing with the InhibitEX® tablet was done for 5–10 min, and the digestion with Proteinase K was done for 20–30 min. Extracted DNA was kept at −20 °C in the noninvasive laboratory.

We used a dedicated laboratory for DNA extraction and PCR setup from noninvasive samples, enforced strict rules regarding movement of personnel, equipment and material between laboratories to prevent contamination, and applied rigorous cleaning and decontamination regimes. Pipette tips with aerosol barriers were used for all liquid transfers. A negative control extraction was performed with each batch of 11–23 samples and later analysed downstream with the samples. Three negative controls were used on each 96-well PCR plate to detect possible contamination. We minimized manual entry of data to avoid typing errors. In the large-scale study, we used bar codes to track samples and photo documented and later rechecked all critical steps where a sample mix-up could have occurred.

Single reaction 13-plex PCR protocol

Qiagen Multiplex PCR kit was used for all PCRs. We prepared 10 μL reactions – 5 μL of Qiagen Mastermix, 1 μL of Q solution, 2 μL of template DNA and 2 μL of water and primers to obtain the appropriate concentration in the final solution (Table 2). When nested primers were available [primers developed by Taberlet *et al*. (1997) and Bellemain & Taberlet (2004b)], the internal primer providing a shorter PCR product was used. An exception was the locus G10X, where forward and reverse primers developed by different authors were used to provide the

Table 2 Locus names, dyes, primer sequences and primer concentrations for the single-step multiplex PCR for genotyping of brown bear faecal samples

Locus	5′ primer	3′ primer	Primer C [μM]	Allelic range
Mu10[B]	ATTCAGATTTCATCAGTTTGACA	6FAM-TCAGCATAGTTACACAAATCTCC	0.19	114–130
G10X[TP]	6FAM-CCCTGGTAACCACAAATCTCT	TCAGTTATCTGTGAAATCAAAA	0.40	132–154
G1D[P]	ATCTGTGGGTTTATAGGTTACA	6FAM-CTACTCTTCCTACTCTTTAAGAG	0.25	168–182
G10H[P]	6FAM-CAACAAGAAGACCACTGTAA	AGAGACCACCAAGTAGGATA	0.20	221–257
Mu50[B]	GTCTCTGTCATTTCCCCATC	6FAM-AACCTGGAACAAAAATTAACAC	0.06	79–103
G10P[T]	TACATAGGAGGAAGAAAGATGG	VIC-AAAAGGCCTAAGCTACATCG	0.09	122–150
Mu09[T]	AGCCACTTTGTAAGGAGTAGT	VIC-ATATAGCAGCATATTTTTGGCT	0.07	174–206
G10C[P]	VIC-AAAGCAGAAGGCCTTGATTTCCTG	GGGACATAAACACCGAGACAGC	0.05	97–116
SRY[B]	GAACGCATTCTTGGTGTGGTC	PET-TGATCTCTGAGTTTTGCATTTG	0.06	75
Mu15[T]	PET-CTGAATTATGCAATTAAACAGC	AAATAAGGGAGGCTTGGG T	0.15	117–131
G10L[B]	PET-ACTGATTTTATTCACATTTCCC	GATACAGAAACCTACCCATGCG	0.10	156–166
Mu59[B]	GCTCCTTTGGGACATTGTAA	NED-TGACTGTCACCAGCAGGAG	0.15	97–121
Mu23[B]	NED-TAGACCACCAAGGCATCAG	TTGCTTGCCTAGACCACC	0.07	142–156

[O]Ostrander *et al*. (1993).
[P]Paetkau *et al*. (1998).
[T]Taberlet *et al*. (1997).
[B]Bellemain & Taberlet (2004b).

appropriate size product. All primers were premixed in a primer mastermix for easier pipetting. The cycling regime was a 15-min initial denaturation at 95 °C, followed by 38 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s and elongation at 72 °C for 60 s. PCR was completed with a 30 min final elongation step at 60 °C. A mixture of 1 μL of the PCR product, 0.25 μL of GS500LIZ size standard (Applied Biosystems) and 8.75 μL of formamide was loaded on an Applied Biosystems ABI 3130xl automated sequencer for fragment analysis.

Pre-amplification PCR protocol

We used a similar protocol as described by Bellemain & Taberlet (2004b), and their primers, for the samples collected in the 2004–2007 study. This protocol uses one multiplex pre-amplification with external primers, and three parallel second-stage PCRs with one of the external primers replaced with a nested internal primer to amplify six microsatellite loci and the SRY locus for sex determination. We used the published primers, annealing temperatures and primer concentrations, but modified some PCR conditions to use the Qiagen Multiplex PCR kit, and changed the composition of the second stage multiplexes.

Pre-amplification was performed in a 20 μL reaction – 10 μL of Qiagen Multiplex Mastermix, 2 μL of Q-solution, 5 μL of template DNA and 3 μL of water and primers to obtain 0.01 μM concentration of each primer in the final solution. The cycling regime was a 15-min initial denaturation at 95 °C followed by eight touchdown cycles of denaturation at 94 °C for 30 s, annealing at a decreasing temperature for 180 s, and elongation at 72 °C for 60 s. The starting annealing temperature for the touchdown was 62.4 °C, and was decreased by 0.3 °C in each cycle. This was followed by 21 regular PCR cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 180 s and elongation at 72 °C for 60 s.

The second-stage amplification was in three multiplex PCRs using internal primers as described by Bellemain & Taberlet (2004b), but with different combinations of loci and different dyes than originally published, and with optimized primer concentrations to obtain balanced peak sizes (Table 3). The PCRs were performed in 7 μL reactions – 3.5 μL of Qiagen Multiplex Mastermix, 0.7 μL of the Q-solution, 1.1 μL of template DNA, and 1.3 μL of water and primers to obtain the appropriate primer concentration in the final solution. We used the same cycling regime for all multiplexes: a 15-min initial denaturation at 95 °C followed by 12 touchdown cycles of denaturation at 94 °C for 30 s, annealing at a decreasing temperature for 90 s, and elongation at 72 °C for 60 s. The starting annealing temperature was 62.2 °C, and was decreased by 0.2 °C in each cycle. This was followed by 27 regular PCR cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s and elongation at 72 °C for 60 s. PCR was completed with a 30 min final elongation step at 60 °C. PCR products were then mixed in the ratio M1 : M2 : M3 = 2 μL : 3 μL : 4 μL. A 0.5 μL of this solution was then mixed with 9.3 μL of formamide and 0.2 μL of GS350ROX size standard (Applied Biosystems) and loaded on an Applied Biosystems ABI 3130xl automatic sequencer.

Table 3 Multiplexes, dyes and primer concentrations for the second stage of the pre-amplification protocol

Locus	Primer C [μM]	Dye	Multiplex
Mu10	0.5	6FAM	M1
Mu50	0.5	6FAM	M1
Mu23	0.5	NED	M2
Mu59	0.5	NED	M2
Mu51	0.5	HEX	M3
G10L	0.4	HEX	M3
SRY	0.2	HEX	M3

Genotype reliability, error checking and statistical analysis

We performed the fragment analysis on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The output was analysed using the GeneMapper software (versions 3.7 and 4.0, Applied Biosystems). Each electropherogram of the large-scale study samples and the samples used for comparison of both genotyping protocols was checked independently by two persons. We used a multitube-based genotyping procedure (Taberlet *et al.* 1996), similar to the one proposed by Frantz *et al.* (2003) and modified by Adams & Waits (2007), to decide when to accept a genotype or discard a sample. We modified the Adams & Waits (2007) procedure to accept a genotype if it was matching a genotype of an already reliably genotyped reference sample, with a constraint that the maximum likelihood estimated reliability using program Reliotype (Miller *et al.* 2002) of the reference sample must have been at least 0.95. For samples that didn't match any other sample, this threshold was increased to 0.99. Analysis of each sample was repeated at least twice, and up to eight times with regard to the estimated reliability. We used the methods recommended by Broquet & Petit (2004) to estimate the frequency of allelic dropouts and false alleles, and calculated a quality index for each sample following the method described by Miquel *et al.* (2006). Samples with the quality index below 0.4 that did not match any other sample were discarded.

We used R version 2.8.1 (R Development Core Team 2008) for data handling and statistical tests.

Results

In the large-scale study, we collected 1053 faecal samples that we genotyped using the new single multiplex PCR protocol. The median estimated age of the fecal samples was 2 days (n = 1001, not all samples had the age estimate recorded). We were able to genotype 929 (88.2%) samples successfully. The average allelic dropout rate in successfully genotyped samples across all loci was 0.129 (SD = 0.015). The average false allele rate was 0.004 (SD = 0.002). The global quality index of the successfully genotyped samples was 0.923. Electropherograms for all loci except G10H were easy to interpret and reasonably free of PCR artifacts. G10H proved difficult to interpret and was, despite similar estimated error rates (0.143 allelic dropout rate, 0.004 false allele rate), considered unreliable and later discarded in the downstream analyses.

In the pilot study we collected 473 faecal samples, of which 371 (78.4%) were successfully genotyped using the pre-amplification protocol. This success rate is lower than what was achieved using the new single step protocol, but the differences are only marginally significant (Pearson's Chi Squared test, $\chi^2 = 3.53$, $P = 0.060$). The median estimated age of the scat samples was 2 days (n = 419, not all samples had the age estimate recorded). The global quality index was 0.889, lower than the quality index obtained in the large scale study with the single reaction protocol (Wilcoxon–Mann–Whitney test, $W = 158738$, $P = 0.009$). We found that the genotyping success is affected by the month of sampling (Skrbinšek T., unpublished data), and thus used the genotype data of a subset of 176 samples from the pilot study that were collected during the same months as the samples in the large-scale study (between September and December) for a better comparison of both protocols. Median estimated age of these samples was also 2 days (n = 144). We were able to genotype 146 (84.9%) of these samples successfully. Average observed allelic dropout rate in successfully genotyped samples across all loci was 0.161 (SD = 0.039). Average false allele rate was 0.003 (SD = 0.004). The global quality index of all successfully genotyped samples was 0.913, similar to that of the new single step protocol (Wilcoxon–Mann–Whitney test, $W = 67733$, $P = 0.977$). Electropherograms were easy to interpret for all loci; however, the two longest alleles at Mu59 consistently provided much weaker amplifications than the shorter alleles.

We further genotyped a subset of 182 samples from the pilot study using both PCR protocols and used the five loci amplified under the both regimes to compare them directly. All these samples provided specific PCR products when amplified with the pre-amplification protocol. The extracted DNA from these samples was amplified using the pre-amplification protocol soon after the extraction, but was stored at −20 °C for approximately 3 years before it was re-amplified using the new single multiplex protocol. The genotype data obtained through both PCR protocols were pooled to construct consensus genotypes. With the pre-amplification protocol, fewer samples (n = 13, 7.1%) were below the 0.4 quality index threshold compared with the single reaction protocol (n = 21, 11.5%), but the difference was not statistically significant (Pearson's Chi Squared test, $\chi^2 = 1.59$, $P = 0.207$). The global quality index was very similar when using the single reaction protocol, 0.802, compared to 0.801 when using pre-amplification (dependent 2-group Wilcoxon Signed Rank Test, $V = 6246$, $P = 0.118$). We found a high allelic dropout rate on locus Mu59 when using the pre-amplification protocol (0.508), largely because of a dropout of the two longest alleles (119 and 121 bp). The allelic dropout was lower and not allele specific when the single reaction protocol was used (0.164). If the Mu59 locus was omitted from the analysis, the global quality index was higher with the pre-amplification – 0.878, compared to 0.817 obtained with the single amplification protocol (dependent 2-group Wilcoxon Signed Rank Test, $V = 4882$, $P < 0.001$).

Apart from the faecal samples, we also applied the new PCR protocol to hair samples, both field-collected (n = 12) and taken directly from a live animal (n = 9), and it performed well (successful amplification of 75% of field collected samples and 100% of samples taken from a live animal). We also found that the protocol works very well for tissue samples if the number of PCR cycles is decreased to 29, and the amount of template DNA is decreased to 1 μL.

Discussion

The developed protocol efficiently genotypes a large number of brown bear microsatellite loci in a single PCR and a single sequencer run, and is comparable to commercial genotyping kits developed for domestic animals and humans (e.g. Applied Biosystems StockMarks® or AmpFlSTR® Profiler Plus®). It was used for identification of individual bears in a large scale study, and provided a very high genotyping success rate of 88.7% from field-collected faecal samples. This success rate is higher than the 75.7% average success rate for herbivores and omnivores reported by Broquet *et al.* (2007) in a review of 30 studies that employed both field-collected samples as well as fresh samples collected from animals in captivity. In the reviewed studies that employed field collected samples (n = 11), the average success rate was 59.9%, with the highest reported success rate of 82% (Bradley

et al. 2000). Other large-scale studies of brown bears that used faecal samples reported success rates between 46% and 80% (Bellemain & Taberlet 2004b; Bellemain *et al*. 2004a, 2006).

Multiplex pre-amplification strategies, as opposed to direct amplification of target loci, have been reported to improve genotyping success rates and decrease error rates in studies that use noninvasive or historical samples by several authors (Bellemain & Taberlet 2004b; Piggott *et al*. 2004b; Hedmark & Ellegren 2006; Arandjelovic *et al*. 2008). Conversely, we did not observe significant advantages of the multiplex pre-amplification that would justify its higher costs and workload. A similar observation was recently obtained by De Barba (2009) using brown bear faeces and hair samples. Although the quality index was higher with the pre-amplification when the same samples were amplified using both protocols and the outlying Mu59 locus was omitted, the actual genotyping success was similar. It must also be noted that the extracted DNA was kept at −20 °C for 3 years before it was re-amplified with the new single reaction protocol, and might have undergone some degradation, causing the lower quality index values. When we compared samples collected in different studies that were amplified soon after DNA extraction, the new single reaction protocol performed at least as well as the multiplex pre-amplification. Furthermore, the new single reaction protocol provided more data in a single low-volume PCR.

It is necessary to discuss why previous studies found the pre-amplification technique to be advantageous, contrary to the results reported here. Only samples considered reasonably fresh were collected in our studies, so we can expect that the quality of DNA in them was still high, as the largest drop in success rate occurs during the first few days after faeces deposition (Murphy *et al*. 2007). It is possible that in more limiting conditions, e.g. with older non-invasive samples or museum samples, the multiplex pre-amplification strategy would prove to be advantageous. It is also possible that multiplexing at the second stage of the multiplex pre-amplification strategy could have reduced the overall amplification success, and that we could have achieved better results with the pre-amplification strategy if singleplexes were used. Multiplexing at that stage was performed by Bellemain & Taberlet (2004b), who also reported advantages of the pre-amplification approach, but not by some other authors, who used singleplex PCRs at the second stage of the procedure (Piggott *et al*. 2004b; Hedmark & Ellegren 2006; Arandjelovic *et al*. 2008).

A high number of analysed loci are not necessarily a good thing for noninvasive samples when the goal is individual identification for mark-recapture analysis. Analysis of such material carries an inherent high rate of genotyping errors, and the possibility of a multilocus genotype containing an error grows with the number of loci genotyped (Paetkau 2005). When used naively for mark-recapture, such genotypes can lead to completely erroneous conclusions (Waits & Leberg 2000; Roon *et al*. 2005). Approaches for dealing with these errors, apart from the now standard multiple tube approach to genotyping, are to limit the number of analysed loci to the necessary minimum (Paetkau 2005; Waits & Paetkau 2005), or to search for problematic samples examining bimodality or difference in capture history (McKelvey & Schwartz 2004). Our multiplexing protocol for faecal samples provides flexibility to a researcher tackling noninvasive genetic sampling of brown bears to use either of these approaches with practically no additional costs or workload and also to have a higher number of informative loci available for relatedness, parentage or population genetic studies.

The new genotyping protocol we developed for brown bear faecal samples demonstrates the value of a thorough optimization of laboratory procedures. A well optimized genotyping protocol typically multiplexes 4–5 loci in a single PCR. Our protocol effectively decreases genotyping material costs and workload to one third of what would be required if such 4–5 locus multiplexing protocols were used, and 1/12 of what would be required with no multiplexing at all. While the effort required for such optimization is probably not justified in small studies, in large scale studies it can have a major impact on cost and efficiency.

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2.1.4 Ilegalno ubijanje kot ovira ponovnemu naseljevanju rjavega medveda v vzhodne Alpe

Illegal killings may hamper brown bear recovery in the Eastern Alps.

Petra Kaczensky, Klemen Jerina, Marko Jonozovič, Miha Krofel, Tomaž Skrbinšek, Georg Rauer, Ivan Kos, Bernhard Gutleb

Ursus (2011) 22 (1):37-46.

Izvleček: Ilegalno ubijanje je globalno ena izmed najpomembnejših groženj divjim živalim. Boj proti ilegalnemu ubijanju in razumevanje motivov zanj je med glavnimi izzivi varstva kontroverznih vrst kot so velike zveri. V Evropi so vzhodne Alpe ciljno območje večih aktivnih projektov za varstvo in ponovno naselitev rjavega medveda (*Ursus arctos*). Čeprav ima širša javnost običajno do medvedov in njihove ponovne naselitve pozitiven odnos, se zdijo nekateri lovci in živinorejci medvedu manj naklonjeni. Kako daleč lahko to nasprotovanje pride je bilo prikazano na dobro dokumentiranem primeru ilegalno ubitega medveda v trikotniku med tremi državami, Slovenijo, Italijo in Avstrijo, v juniju 2009. V tem članku podrobneje predstavimo ozadje in diskutiramo ta primer v kontekstu omejevanja širjenja Dinarsko-pindske populacije medvedov proti severu in propadle ponovne naselitve medvedov v osrednjo Avstrijo.

Illegal killings may hamper brown bear recovery in the Eastern Alps

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Abstract: Illegal killings are a major threat to wildlife conservation worldwide. Combating illegal killings and understanding the motives behind them are among the top challenges for the conservation of controversial species such as large carnivores. In Europe, the Eastern Alps are a focal area for many active brown bear (*Ursus arctos*) conservation and restoration projects. The wider public generally has a positive attitude toward bears and bear restoration, but some hunters and farmers seem less supportive. The extent this opposition can reach was demonstrated by the well documented illegal killing of a bear in the three-country triangle of Slovenia, Italy, and Austria in June 2009. We provide detailed background information and discuss this case within the context of the lack of a northward expansion of the Dinaric–Pindos bear population and the failed bear re-introduction in central Austria.

Key words: Austria, brown bear, Eastern Alps, illegal killing, Italy, poaching, re-colonization, Slovenia, *Ursus arctos*, wildlife crime

Ursus 22(1):37–46 (2011)

Illegal killings and legal implications

Illegal killings are a major threat to wildlife conservation worldwide (Manel et al. 2002). There is a large body of literature about poaching for economic reasons, either to gain benefits (e.g. Milner-Gulland and Leader-Williams 1992) or to avoid losses (e.g. Jackson and Wangchuk 2004). The extent and motives of illegal killings without obvious economic benefits are much less documented and understood (Muth and Bowe 1998; Eliason 1999, 2003). In Europe, illegal killings have been identified as one of the most important sources of mortality for recovering Eurasian lynx (*Lynx lynx*; Breitenmoser and Breitenmoser-Würsten 2008, Breitenmoser et al. 2010), grey wolf (*Canis lupus*; Marucco et al. 2009), and brown bear (*Ursus arctos*; Ciucci and Boitani 2008) populations. It is clear that any large carnivore population in Europe has to be robust enough to sustain a certain level of hunting, be it legal or illegal. Motives of poachers seem to be primarily driven by hatred toward large carnivores (Caniglia et al. 2010), perceived threat to self and property (Muth and Bowe 1998), and resistance to regulations imposed by a society from which certain groups feel marginalized (Skogen and Krange 2003, Skogen et al. 2006, Bell et al. 2007).

Although incidents of illegal killings of large carnivores in Europe are readily covered by the media, detailed descriptions of case studies are largely restricted to grey literature (Ceza et al. 2001, Linnell 2004, Liberg et al. 2008). Furthermore, few cases of large carnivore poaching make it to court, and even fewer result in convictions (Caniglia et al. 2010). Success in uncovering and persecuting cases of illegal killings are hindered by administrative and legal fragmentation (Ciucci and Boitani 2008), insufficient capacity and training of state control organs (Anderson 1999), and a romanticised image in which poaching is regarded as being a minor or folk crime (Muth 1998, Reiter et al. 2005). Consequently, there is little experience with detecting, understanding, and combating the illegal killing of large carnivores, and the problems illegal killings generate for large carnivore restoration programs in Europe tend to be underestimated as the result of occasional exceptions.

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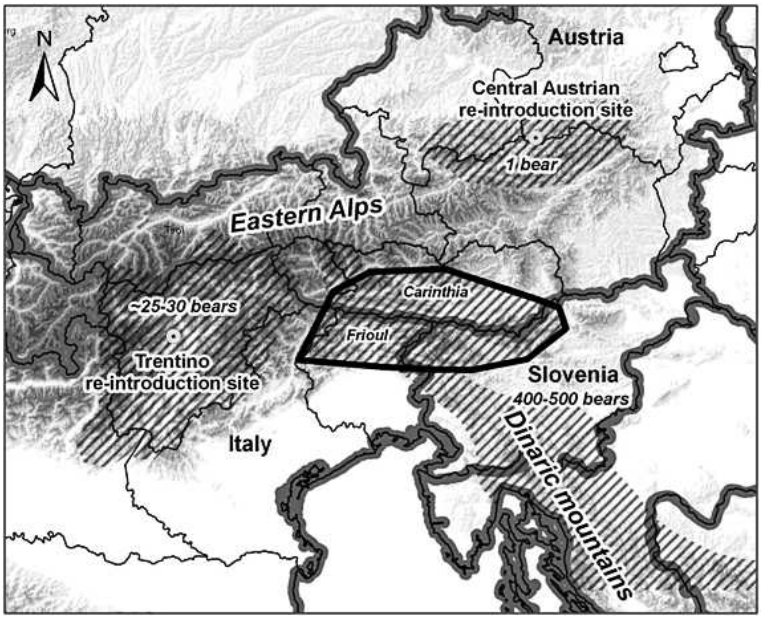

Fig. 1. Brown bear occurrence in the Eastern Alps, 2009. The black polygon encompasses the 3-country triangle of Slovenia, Austria's province of Carinthia, and Italy's province of Frioul. Population estimates area based on genetic and conventional monitoring (Skrbinšek et al. 2008, Groff et al. 2009, Kruckenhauser et al. 2009).

Brown bears in the Eastern Alps

The Eastern Alps include parts of Austria, Italy, Switzerland, Germany, Slovenia, and Lichtenstein (in decreasing order), and are a focal area for brown bear conservation and restoration projects (Fig. 1). A remnant bear population survived in the Trentino region of northern Italy (Roth 1980), and occasional dispersers from the bear population of Slovenia and Croatia regularly reached the Alps in the three-country triangle (3CT) of Slovenia, Italy, and Austria (Jerina et al. 2003, B. Gutleb et al., 1999, Recent changes in the distribution of brown bear (*Ursus arctos*) in the Southeastern Alps, unpublished report), but rarely beyond. However, because Slovenian policy has discouraged the expansion of its vital bear population into the Slovenian part of the Alps (Jerina et al. 2003) and dispersal in bears is heavily sex-biased (Støen et al. 2006, Zedrosser et al. 2007), female bears have been rare in this area (Jerina and Adamič 2008, Skrbinšek et al. 2008).

In 1972 a single male bear ventured beyond the 3CT and settled in the northern limestone Alps of central Austria. In 1989 the first reintroduction of free-ranging brown bears worldwide was initiated in this area to re-establish a breeding population (Zedrosser et al. 1999). In 1999, another reintroduction program was launched in the Italian Alps to avert bear extinction in Trentino (Groff et al. 2008, 2009, 2010). However, recovery success in the eastern Alps has been moderate to date. Only the bear population in Trentino is increasing (Groff et al. 2010), whereas the population in 3CT is stagnating (Jerina and Adamič 2008), and the Austrian reintroduction attempt has failed (Kruckenhauser et al. 2009, G. Rauer unpublished data). Furthermore, in Slovenia high bear damage decreased human tolerance in the Alpine region. Between 1994–2002, bear damage in the Alpine and sub-Alpine parts of Slovenia accounted for 67% of all compensation payments for bear damage in the country, even though fewer than 5% of the country's bears were estimated to live there. This led to increased harvests within the entire country, apparently halting further northwest expansion (Jonozovič and Adamič 2002, Kryštufek and Griffiths 2003, Jerina and Adamič 2008).

A 2007 intensive non-invasive mark–recapture study coupled with conventional methods estimated the Slovenian population at 400–500 bears, 21 (19–23 95% CI, approximately 70% males) of which

occupied Western Slovenia (Skrbinšek et al. 2008). Even though genetic samples in the Slovenian part of 3CT had only been collected opportunistically, 4 out of 7 bears detected this way in 2008 and 2009 in that area were already known from either the 2007 systematic sampling effort in Western Slovenia or the 2004 systematic sampling efforts in northeastern Italy (S. Filacorda, University of Udine, Italy, personal communication, 2009). Thus, genetic results, as well as recent telemetry data (Krofel et al. 2010), suggest that bears in 3CT occur at low densities and that they roam widely.

Although the failure of the Austrian re-introduction has been widely attributed "to a sustained, but low number, of illegal killings, which had a significant effect because the original number of bears was so low that the population was especially vulnerable to stochastic effects" (International Union for Conservation of Nature and WWF Workshop, 2009, Summary protocol of workshop: Towards a strategy for brown bear conservation in the Austrian Alps, 24 November 2009, Vienna, Austria), hard evidence of an illegal killing has so far only been obtained in one case (Krukenhauser et al. 2009, G. Rauer unpublished data). There is as of yet no hard evidence of illegal killings of bears in the Slovenian and Italian Alps.

Although opinion polls of the general public in the eastern Alps suggest that the wider public have a positive attitude toward bears and bear restoration (Kaczensky et al. 2004; Genovesi 2005; Wechselberger and Leizinger 2005; W. Beutelmeyer, Market Institut Survey 2007–08, Linz, Austria, unpublished data), hunters or farmers may be less supportive (Zeiler et al. 1999, Kryštufek and Griffiths 2003). The extent this opposition can reach was demonstrated by the well-documented illegal killing of a bear in 3CT in the summer of 2009.

The case of the bear Rožnik

On 16 April 2009, a bear wandered in the main city park of Ljubljana, Slovenia's capital, from the adjacent forest. The animal, a subadult male of 94 kg, was immobilized and equipped with a GPS-GSM (global system for mobile communications)-VHF radiocollar (GPS PLUS-3 Collar TARIC, 850g; Vectronic Aerospace GmbH, Berlin, Germany) by the Slovenian bear emergency team. The collar was scheduled to attempt a GPS fix every hour and attempt to send them via text message every 6 hours. The bear, who was named Rožnik, had not shown aggressive behavior toward people in the park and was relocated into prime bear habitat on Snežnik plateau in the core bear area in southwest Slovenia, about 50 km from the capture location (Fig. 2).

Age estimation in the field suggested that Rožnik was 3 years old when captured, thus born in the winter 2006–07. His genotype matched that of 2 fecal samples in the database at the Biotechnical Faculty of the University of Ljubljana: one had been collected on 9 October 2007 within the area of permanent bear presence and one collected opportunistically on 3 April 2009 in the pre-Alps (Fig. 2). Parentage analysis of Rožnik's genotype against the invasive sample database identified a likely father from the area of permanent bear presence. Thus it can be assumed that Rožnik originated from the area southeast of Ljubljana, but had already left the area of permanent bear presence before venturing into the capital (Fig. 2).

Rožnik traveled extensively after his release on Snežnik plateau. Unlike most bears (Kaczensky et al. 2006, K. Jerina et al. unpublished data) Rožnik frequently moved during the day and was regularly observed by local people, showing little fear but also no aggression. Despite his habituated behavior, Rožnik caused little damage, only destroying 3 beehives and killing one goat (M. Jonozovič, unpublished data). Rožnik crossed 2 main highways 4 times, and, highlighting the fact that bears have little concept of national borders, moved to and from Croatia shortly after his release, before crossing into Austria on 27 May 2009 (Fig. 2). Notified about the arrival of a conspicuous bear by the Slovenian bear monitoring team, the Austrian bear monitoring team informed the authorities in Carinthia, Southern Austria, who officially welcomed Rožnik to Austria in the press (Austria Presse Agentur 2009). During the following 3 days, he was observed at least 6 times, and the GPS signals showed he moved back and forth between Austria and Slovenia at least 3 times (Fig. 2). The last text message with GPS fixes was received at 1705 on 30 May 2009. The last GPS fixes were all in Austria, as were 2 later visual observations on the same day, one from 1610 to 1620 and one at 1800 (give or take 30 min). Thereafter, no text messages or observations were recorded on either side of the border and a search flight failed to pick up his VHF signal.

On 11 June 2009 the body of a bear was found by a villager near the town of Solčava in Slovenia. The

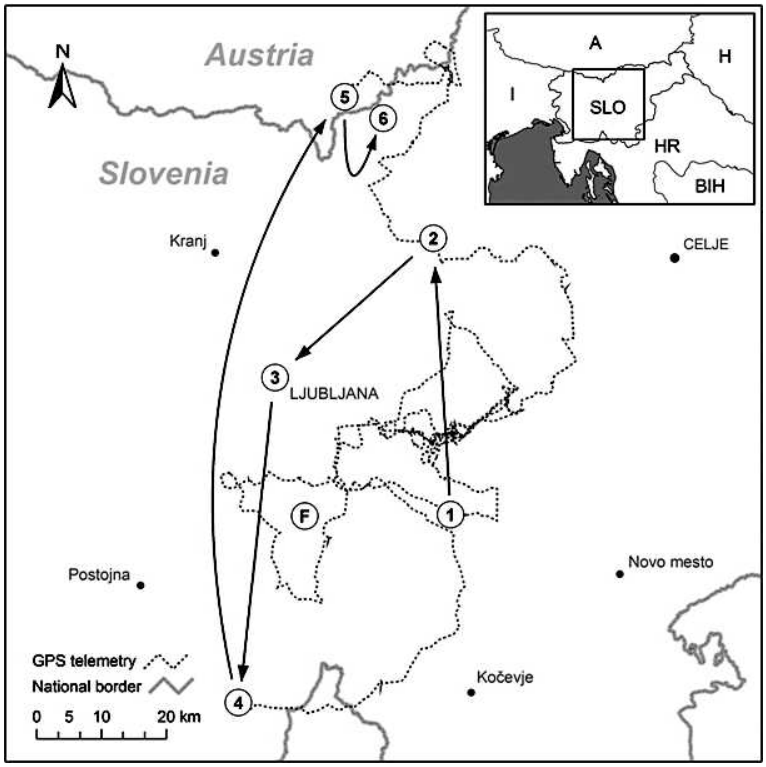

Fig. 2. Map and chronology of events around bear Rožnik: F – Legally shot presumed father of Rožnik, 26 Mar 2007; 1 – Scat found, 9 Oct 2007; 2 – Scat found, 3 Apr 2009; 3 – Rožnik captured in city park in Ljubljana, 16 Apr 2009; 4 – Rožnik translocated to bear core area, 16 Apr 2009; 5 – Last GPS fix; 6 – Body found, 11 Jun 2009. Dotted line represents GPS tracking between 16 Apr and 30 May 2009. Countries on map are Slovenia (SLO), Italy (I), Austria (A), Croatia (HR), Hungary (H), Bosnia and Herzegovina (BIH).

location was 3 km straight line distance from the Austrian border and 11.5 km by road from the closest border crossing at Pavličevo sedlo (Paulitsch-sattel, Fig. 2). The body was lying in Jurčef creek and was clearly visible from the edge of the road, suggesting that whoever dumped the carcass did not intend to hide the offence. The carcass was skinned and decapitated, and the distal phalanges with claws were removed from all paws. A hole in the chest suggested the impact of a bullet, and post-mortem cuts through the heels suggested the bear had been hung for skinning. Judging from the level of decay, the body likely had been dumped several days before. A blanket and a plastic garbage bag, both with adhering bear hair, were found a few meters from the road in the forest and taken for forensic analysis by the police. No collar was found. The autopsy report by the veterinary faculty of the University of Ljubljana confirmed the observations made by the investigative team, and genetic analysis at the Biotechnical Faculty of the University of Ljubljana confirmed the carcass was Rožnik's.

Because GSM coverage in the border area is good, the collar was probably destroyed within 6 hours after the reception of the last text message at 1705 on May 30; the next message download was scheduled for 23:05. Because the bear was seen at 1800 (within 30 min) on that day, we conclude it was shot after 1730. Since the use of artificial light for hunting is strictly prohibited in most of Europe, including in Slovenia and Austria, the killer is also unlikely to have been able to aim after civil dusk, which on May 30 was at 2125 and certainly not after nautical dusk which was at 2216. Thus Rožnik was likely killed between 1730 and 2130 and had a maximum of 4 hours to move after his last observation. Given his previous movement patterns, it is unlikely that he covered more than 4–6 km during those final hours

Table 1. Probability estimates for distances covered during a 6 hour, 4 hour, and 2 hour interval following the last SMS message received at 1700.

	Maximum distance traveled in a given time[a]		
Probability estimates	**1700–2300 ($n = 83$)**	**1900–2300 ($n = 98$)**	**2100–2300 ($n = 93$)**
0.50	2,842	2,081	732
0.75	4,794	3,805	2,109
0.90	6,613	5,631	3,043
1.00	13,685	9,673	5,014

[a]To increase the sample size and make the estimates less sensitive to circadian differences, values for each time interval were derived for the exact interval and the interval plus and minus 1 hours (e.g. for interval 1700–2300, the distances between locations recorded at 15–21, 16–22, and 17–23 were used).

of his life (Table 1). This suggests he was shot on any of several small hunting units on the Austrian side of the border.

Given the cross-border whereabouts of Rožnik, police investigations were immediately launched in Slovenia and the Austrian province of Carinthia. The media also picked up the case, and politicians, non-govermental organizations (NGO), area residents, and hunters in both countries openly condemned the illegal killing. Because circumstances suggest that the bear was killed in Austria and dumped in Slovenia, the Austrian NGO Vier Pfoten, the Carinthian Hunters Association, and the Carinthian Nature Conservation Administration together offered a reward of 10,000 euros for information that would result in the identification of the culprit.

Police investigations were not completed before July 2010, but resulted in a charge against a suspected local hunter. Based on the evidence that led to the charge, the Carinthian Hunters Association expelled the suspect for life. The court case was opened on 7 October 2010, but adjourned to include further witnesses and inspect the relevant locations.

Discussion

There is little evidence that Rožnik was killed primarily for economic reasons. Although he behaved rather conspicuously, he caused little damage to livestock or property (M. Jonozovič unpublished data). Purely economic reasons seem to be of generally minor importance for the illegal killing of large carnivores in Europe. There is no evidence of large carnivore trophies or body parts entering a wildlife market, and most European countries prevent or mediate economic consequences of bear damage through well-established prevention schemes (Linnell et al. 1996) and damage compensation mechanisms (Fourli 1999). Several countries also allow a legal harvest, and yet illegal killings of large carnivores are still widespread (Andren et al. 2006, Liberg et al. 2008). Experiences from other parts of the world further support that compensation schemes (Naughton-Treves et al. 2003) or the possibility of hunting large carnivores (Treves 2009) does not automatically result in positive attitudes.

Apart from livestock depredation (Kaczensky 1999), conflicts over hunting seem to be the second most important conflict with large carnivores in Europe (Andren et al. 2006, Bisi et al. 2007, Luikkonen et al. 2009, Breitenmoser et al. 2010). Whereas there is a large toolkit of potential mitigation measures to combat livestock depredation (Linnell et al. 1996), predation on wild ungulates is a biological necessity that cannot be mitigated. Furthermore, there is no legal ownership of wild animals, as they are either state property or unowned, which makes compensation claims legally impossible. Even though hunters only become the legal owners of game animals after they have killed them, they can feel they also own the living animals, especially in the small scale territorial hunting system (Revierjagdsystem, Bubenik 1989) which prevails in Austria, Germany, and eastern Switzerland (also see Internet platform MALME). In these regions the perception of ownership is further enhanced by a tradition of caring for the game through intensive feeding and selective removal of weak animals.

Wolves, lynx, and bears challenge the hunters' exclusive claim on game and game management. Hunters in Austria complain that, compared with human hunters, large carnivores kill too many game animals, have differing selection criteria (e.g. kill trophy males), do not adhere to the moral norms set out by the hunters (e.g. spare pregnant females or young), scare the game, make the game less predictable, and expose the hunter to the risk of

being attacked (G. Rauer, unpublished data). The presence of top predators also seems to challenge the self-concept of hunters and the justification of hunting in general (Breitenmoser et al. 2010). Although bears in southern latitudes rarely prey on wild ungulates, they are attracted to bait- and feeding sites for deer and wild boar (*Sus scrofa*; Große et al. 2003). In the northern limestone Alps of Austria, the destruction of ungulate feeding sites by bears to access ungulate feed has been a common source of conflict (Zedrosser et al. 1999). The presence of a bear at bait or feeding sites also reduces the opportunity a hunter has to see or shoot wild ungulates, and exposes the hunter to the risk of a close encounter. Furthermore, bears might find and claim a shot animal before the hunter is able to find or retrieve the carcass (G. Rauer and M. Krofel, personal observation).

Because very few cases of illegal large carnivore killings are discovered and even fewer are resolved, little qualitative and quantitative data on the motives of poachers actually shooting large carnivores are available. Exposing large carnivore carcasses or parts of carcasses (e.g. severed paws or heads) in public occasionally occurs (Ceza et al. 2001, Breitenmoser and Breitenmoser-Würsten 2008) and suggests an element of defiance against authorities. The conspicuous dumping of Rožnik's carcass points to a similar motive, possibly coupled with an attempt to lay a false track. There are possibly also strong neutralization mechanisms in place (Eliason 2003), which may help poachers feel justified in killing large carnivores. Our speculation is that the poacher who killed Rožnik may have felt justified by the lack of fear the bear showed toward people (e.g. ''if I had not killed the bear, sooner or later it would have harmed somebody'' or ''the animal behaved very abnormally and thus needed to be removed''). Thus the perceived motive of the poacher might well have fallen into the protection of self and property typology defined by Muth and Bowe (1998). The removal of body parts that have trophy value indicates an additional motive of the killing.

A study of opposition to protected areas in Germany suggests that there are ''powerful emotional and cultural drivers that divide nature conservationists and local land users and residents into two camps, maintained by stereotyping and group bonding'' (Stoll-Kleemann 2001:369). Likely, similar mechanisms also hold true for the opposition toward large carnivores, which often seems to reflect an underlying urban–rural conflict (Breitenmoser 1998). Consequently, hunters often close ranks around any of their members who commit illegal acts, not because they agree with the action, but rather because they feel accused as a group. To openly discuss the issue of illegal killing of wildlife requires tact and trust. Hunters need to be assured that the aim is not to condemn hunters or hunting, but rather to cooperate to combat illegal actions that are harmful both for nature conservation and public perception of hunters and hunting. In this respect, the case of Rožnik was encouraging because both nature conservation and hunting organizations immediately condemned the illegal killing and even offered a reward for information that would result in the identification of the culprit. The Carinthian Hunters Association went even further and expelled the suspect for life from their organization, a step that had never been taken for any hunting offence.

Although illegal killing of large carnivores is widespread, it is certainly not as widespread as other forms of illegal hunting (out of season, over the bag limit, without valid licence, and others; Bell et al. 2007). Large carnivores range over large areas and, as Rožnik showed, regularly cross regional and international borders, exposing themselves to a large number of land users. In Austria, hunting districts are particularly small, and the home range of a single bear may intersect >100 hunting units, increasing the chances of a bear encountering the occasional hunter willing to illegally pull the trigger. Illegal killings of large carnivores by just a few hunters can have serious consequences for conservation and need to be exposed for the criminal acts they are. In small or re-colonizing populations, the loss of a single individual may dramatically slow (e.g. wolf or lynx recovery in the Alps; Marucco et al. 2009), stall (e.g. the brown bear population in the Abruzzo region, central Italy, Ciucci and Boitani 2008; the Cantabrian Mountains, northwestern Spain, Naves et al. 2003), or even prevent population re-establishment (Krukenhauser et al. 2009).

Illegal killings by nature are difficult to detect and measure (Gavin et al. 2010). The case of Rožnik shows how much monitoring effort is needed to document even a single illegal killing, especially in a cross-border population. Consequently the absence of hard evidence is a poor guarantee that illegal killings do not occur. Without telemetry and the genetic reference database, no link toward a possible Austrian involvement would have been possible.

The outcome of the court case likely will also have far-reaching consequences for brown bear recovery in the Eastern Alps. Slovenia's present management policy of maintaining the country's bear population at a constant level has been the subject of heavy criticism, in particular by Austrian nature conservation NGOs. Managing large carnivores on a population level and maintaining connectivity is also a key recommendation of the recently developed *Guidelines for population level management plans for large carnivores in Europe* (Linnell et al. 2007). However, if the illegal killing of the bear Rožnik cannot be resolved or is treated as a minor offence, Slovenian authorities and managers will have an even harder time getting broader public consensus for allowing bear expansion into the Alps. The Rožnik incident fuelled the ongoing public and political debate which strongly questions why Slovenia — which is already home to 400–500 bears within the continuous Dinaric–Pindos population — should shoulder the burden of bear recovery in an area with high conflict potential, particularly when the illegal killing of bears in neighbouring Austria goes without consequences. The ongoing court case and the reaction of the Carinthian Hunters Association is an encouraging sign that the authorities take bear recovery seriously. Fully resolving the case will help discourage further illegal removals of bears in the Eastern Alps (Keane et al. 2008).

Management recommendations

To facilitate access to the scarce information on illegal killings of large carnivores, it is desirable to document each case in detail and compile all cases in a common database. This database could be made accessible through the website of the Large Carnivore Initiative for Europe (LCIE, a specialist group within the Species Survival Commission of the World Conservation Union).

To raise awareness about the magnitude and effect of illegal killings of large carnivores in Europe, it should be clearly stipulated as a criminal act, rather than a trivial or civil offence. This could be done by compiling and widely communicating the setbacks and costs caused by the illegal killing of large carnivores on the regional, national, and European level.

To understand the motives behind the apparently prevailing negative attitudes of some hunters and farmers toward brown bears in the Eastern Alps — well beyond the purely economic context — we recommend initiating a study focused on the social, economic, and political issues surrounding large carnivore conservation in this area.

To help understand the apparently stagnant bear situation and the scarcity of long-distance dispersers in the 3-country triangle of Slovenia–Italy–Austria and to provide possible forensic evidence, we recommend collaring additional bears with GPS–GSM collars and establishing a permanent genetic monitoring system that can be incorporated into the existing reference databases in Slovenia, Italy, and Austria.

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Ocena velikosti populacije rjavega medveda v Sloveniji z uporabo neinvazivnega genetskega vzorčenja in mreže prostovoljcev

Estimation of brown bear population size in Slovenia using noninvasive genetic sampling and a network of volunteers

Tomaž Skrbinšek, Maja Jelenčič, Hubert Potočnik, Ivan Kos, Franc Kljun, Peter Trontelj

Ta del besedila je namenjen povezavi doktorske naloge v zaključeno celoto in v tukaj predstavljeni obliki ne bo objavljen v znanstvenem tisku. Besedilo je podlaga širšemu znanstvenemu članku, ki je v pripravi, in je s tem namenom napisano v angleščini.

Izvleček: Hiter razvoj molekularne genetike je ekologom in upravljavcem priskrbel nabor zelo uporabnih orodij za preučevanje in spremljanje prostoživečih živali. Ta orodja smo uporabili za oceno velikosti populacije rjavega medveda v Sloveniji. Načrtovali in izpeljali smo obsežno neinvazivno genetsko vzorčenje rjavih medvedov po območju te vrste v Sloveniji, s pomočjo mreže prostovoljcev ter z modeli označevanja in ponovnega ulova ocenili velikost populacije medveda. V intenzivnem trimesečnem vzorčenju jeseni 2007 smo zbrali 1057 neinvazivnih vzorcev rjavih medvedov. 931 vzorcev (88 %) smo uspešno genotipizirali in določili 354 različnih genotipov (osebkov). S pomočjo modeliranja ulova – ponovnega ulova, ob upoštevanju učinka roba zaradi medvedov, ki se prihajajo in odhajajo iz območja vzorčenja preko meje s Hrvaško in z upoštevanjem zaznane smrtnosti, smo ocenili »zimsko« velikost populacije (po celoletni smrtnosti, pred reprodukcijo) na 424 medvedov, s 95 % intervalom zaupanja od 383 do 458. Opazili smo tudi premaknjeno spolno razmerje, 40.5 % samcev in 59.5 % samic. Ocena je prva robustna ocena velikosti populacije te vrste v Sloveniji in ena redkih takšnih ocen v svetu in daje trdno podlago upravljanju z medvedom v naši državi.

Estimation of brown bear population size in Slovenia using noninvasive genetic sampling and a network of volunteers

Tomaž Skrbinšek, Maja Jelenčič, Hubert Potočnik, Ivan Kos, Franc Kljun, Peter Trontelj

Abstract

Rapid development of molecular genetics has provided ecologists and wildlife managers with a powerful set of tools for studying and monitoring of wildlife. We applied these tools to estimate the size of the brown bear population in Slovenia. We designed and executed a large-scale noninvasive genetic sampling of brown bears across the range of this species in this area with a network of volunteers and estimated the size of the brown bear population in Slovenia using mark-recapture modelling. In a highly intensive three-month sampling in autumn 2007 we collected 1057 noninvasive samples of brown bears. 931 samples (88 %) were successfully genotyped, and we found 354 different genotypes (individuals). Through mark-recapture modelling, correcting for the edge effect caused by bears moving in and out of the sampling area across Croatian border and accounting for detected mortality, we estimated the "winter" population size (after annual mortality, before reproduction) at 424, with 95 % confidence interval of 383 to 458. We also observed an uneven sex ratio of 40.5 % males and 59.5 % females. This estimate is the first robust population size estimate of this species in Slovenia, and one of the few such estimates in the world, providing a sound basis for bear management in our country.

Introduction

Rapid development of molecular genetics has provided ecologists and wildlife managers with a powerful set of tools for studying and monitoring of wildlife populations (Schwartz, Luikart & Waples 2007). This is especially true for noninvasive genetic sampling (Waits & Paetkau 2005), which is increasingly becoming the method of choice for estimation of census population size in many species (Waits 2004; Schwartz, Luikart & Waples 2007). However, even though there are several examples of successful implementation of these methods (e.g. Bellemain *et al.* 2004; Kendall *et al.* 2008; Karamanlidis *et al.* 2010), there is rarely a follow-up describing the actual application of the results and the impact this had had for the population in the wild.

One of the flagship species for application of noninvasive genetic sampling for population size estimation has been the brown bear (*Ursus arctos*). A large, powerful animal, the bear tickles imagination and holds an important place in lore of many cultures (Lescureux *et al.* 2011). On the other hand, bears can cause considerable damage to property and can under certain circumstances be dangerous to people (Herrero 2002). In any case, the bear seldom leaves people indifferent. Even though a part of the public literally worships this

charismatic large carnivore, it is also often met with considerable opposition from other interest groups, and management for bear-human coexistence is frequently a very fine line to be walked (Huber *et al.* 2009; Majić *et al.* 2011).

Centuries of persecution wiped brown bears from most of Western Europe where only a handful of bears still remain in the wild (Zedrosser *et al.* 2001). Large populations remain in Northern and Eastern Europe. One of these remaining populations is the Alps-Dinara-Pindos population that stretches along the Adriatic coast from borders of Italy and Austria in the north and all the way Greece in the south. Bears in Slovenia represent a north-western edge of this large population. While the total area of the bear range in Slovenia is relatively small, the bear population density seems very high (Jerina *et al.* 2013). The importance of these bears is also disproportionate to their numbers as they form the only "bridge" for the much-coveted natural recolonization of the Alps by this species, and have been the source of animals for bear reintroductions to Austria, Italy and France (Clark, Huber & Servheen 2002). However, for over a decade, bears have been a source of controversy in Slovenia. Relatively high number of conflicts with humans and very high official estimates of this species' abundance have nearly doubled the cull quotas in the early 2000's from what they used to be in the 1990's (Kryštufek *et al.* 2003). Sustainability of these quotas has been questioned by experts (Reynolds 2002), and a need for a credible, science-based estimate was recognized.

In this work we're describing what we're hoping to be the beginning of rigorous population-size monitoring in Slovenia and the effects it had on management of the bear population. We 1) designed and executed a large-scale, cost-effective noninvasive sampling of brown bears across the range of this species in Slovenia with a network of volunteers, 2) genetically tagged a large number of bears and 3) estimated the size of the brown bear population in Slovenia using mark-recapture modelling. As such, we are providing an account of how a well-designed population monitoring scheme using non-invasive genetics can be implemented rapidly, with relatively low costs and a high level of precision.

Methods

We used noninvasive genetic sampling and mark-recapture modelling to estimate the number of bears that live in Slovenia.

Study area

We sampled the entire area of permanent bear presence in Slovenia, covering approximately 6000 km^2 (see Figure 2). The main part of the study area is in the Dinaric Mountains, which span the length of the Adriatic coast to form one of the largest

continuous forest complexes in Europe. Density of human population is relatively low for European standards. Human residence is in most cases limited to valleys, leaving large, continuous patches of dense forests that expand across the border with Croatia for wildlife. The most common forest plant community is the Dinaric beech-fir forest, Abieti-Fagetum. A small part of the study area to the south belongs to the Mediterranean biogeographic region, and the western part assumes pre-Alpine characteristics. While there are bears present in the Julian Alps at the border with Italy, these are just few individuals and sampling these areas wouldn't have an effect on the total estimate. However, we did sample in these areas opportunistically.

Study design and power analysis

In a pilot study using non-invasive genetic sampling, which we performed in two relatively small study areas in Slovenia in years 2003-2006, we obtained the highest amplification success rate from scat samples in autumn and early winter (Skrbinšek *et al.* 2007a; Skrbinšek *et al.* 2010). In the same study we also found that the population behaved as an approximately closed population even in small study areas (175 or 240 km^2, respectively) if samples were collected within a three-month timeframe (Skrbinšek *et al.* 2007a). This was the basis for the decision to plan for an intensive, three month long non-invasive sampling session in autumn 2007, from 7th of September until 30th of November.

To understand the sampling effort required to obtain a reasonable confidence interval of the mark-recapture estimate, we performed a power analysis using a simulation study in program MARK (White & Burnham 1999). We used 600 animals as the best-guess upper limit population size, a pessimistic 70 % expected genotyping success rate and a simple p=c(.) Huggins' model (Huggins 1989) as both the simulation and the estimation model. We simulated sampling and successfully genotyping 1000, 800 and 600 samples (1429, 1143 and 857 samples taking the expected genotyping success rate into account) to understand the width of confidence interval that would be obtained in the ideal circumstances. We simulated six sampling sessions and used 1000 iterations in each simulation run. The results were used to scale the sampling effort.

Motivating and managing a network of volunteers, and providing feedback

Initial estimates showed that our budget would not support organizing field crews that would go around and look for samples, and the only viable option remained in recruiting a large number of volunteers. We organized such network of volunteers with help of Slovenian Hunters Association and Slovenia Forest Service. We prepared sampling material (three sampling tubes and an instruction booklet for each participant) and distributed it through hunting clubs, regional Forest Service offices and special purpose hunting reserves (state-owned hunting areas managed by Slovenia Forest Service). In an

attempt to maintain approximately the same sampling effort throughout the study area, we used GIS (ArcGIS, ESRI, Redlands, CA, USA) and CORINE landcover data to calculate the amount of forest in the area covered by each hunting club or hunting reserve. We also used relative local bear population densities estimated from annual bear counting from high hunting hides (Jerina *et al.* 2013) and the best-guess upper limit of the bear population size (600) to estimate the highest expected population density. We used this estimated highest population density as a guideline for the amount of sampling material required per area unit for every bear to have a fair chance of being included in sampling even at the highest population density. We then distributed the material proportionally to the area of forest cover in a hunting ground regardless of the presumed population density of bears in each area to obtain approximately equal sampling effort.

To motivate the people to participate, we published three comprehensive articles in the main Slovenian hunting magazine "Lovec" (which all of Slovenia's 22,000 hunters get as a part of their Hunting Association membership) prior to sampling, describing the project and asking people to participate (Skrbinšek *et al.* 2007b; Skrbinšek *et al.* 2007c; Skrbinšek *et al.* 2007d). We gave 10 lectures at different locations around the bear range presenting the project to employees of hunting reserves and representatives of all participating hunting clubs. We went out of our way to make participation as simple as possible for anyone wishing to provide samples by delivering material to the participating organizations and organizing sample pickup. During the sampling we made telephone calls to leaders of hunting clubs, asking about possible problems and suggestions, and we made rounds around hunting clubs to collect samples and provide additional sampling material if needed. After the project we provided feedback to participants by publishing two articles in the "Lovec" hunting magazine, by publishing all results and project reports at the project webpage (http://www.medvedi.si), and by producing personalized tables and maps for each hunting club detailing where each sample was collected, who collected it and what the results were for the samples they collected (e.g. the sex of the bear, a map of other places where its samples were found). At the end of the project we also organized a press conference where we presented the results, gave a number of interviews for national television and main newspapers, and provided the final report of the project to all key people involved in management of bears in Slovenia.

Sample collection, storage, tracking, genotyping, and quality assurance

Both scat and tissue samples were collected in 96 % non-denatured ethanol. The participants in sample collection were asked to keep the samples in a cool, dark place. Upon arrival in the laboratory they were stored at -20°C until analysis.

Each sample tube was fitted with a printed label that contained a form for field data entry to keep the data with the sample. At the time of collection the location of the sample was

recorded either by GPS or in a 1×1 km grid the hunters routinely use for monitoring of hunting species' harvest (Jerina K., personal communication). Subjectively estimated age of each scat was also recorded, and the participants were instructed not to collect samples they subjectively considered older than 5 days (Skrbinšek *et al.* 2010). We used a dedicated laboratory for DNA extraction from noninvasive samples where we enforced strict rules regarding movement of personnel, equipment and material to prevent contamination, and used negative controls throughout. Upon entry in the laboratory the data about a sample was entered into a relational database, and barcodes were used to track samples through the genotyping process and eliminate manual data entry. We used a single reaction 13-plex PCR protocol to amplify 12 polymorphic microsatellite loci and a sex determination locus in a single reaction and a single sequencer run. We used a modified multi-tube approach (Taberlet *et al.* 1996; Adams & Waits 2007) with up to 8 re-amplifications of each sample according to the sample's quality and matching with other samples. We used the maximum likelihood approach for estimating genotype reliability, and set reliability thresholds for accepting a genotype to 0.95 for samples that matched other samples and 0.99 for samples that didn't match any other sample. The full analysis and genotype quality assurance protocols for noninvasive samples are detailed in (Skrbinšek *et al.* 2010). Analysis protocols for tissue samples are detailed in (Skrbinšek *et al.* 2012b).

Matching of samples with the same genotype and assigning individuals to samples

Although discovering samples that have the same genotype (and should in principle belong to the same individual) seems straightforward, this is not necessarily the case. Incorrect matching either "merges" the actual individuals if the information in analysed loci is too low, or creates "new" virtual individuals if the samples are erroneously considered to have different genotypes because of genotyping errors. The first problem decreases with increasing the number of loci used, however this exacerbates the second problem. Genotyping errors, even with the most strict quality assurance protocols, are unavoidable in noninvasive samples (Taberlet, Waits & Luikart 1999; Waits & Paetkau 2005). Incorrect matching can cause considerable biases in mark-recapture estimates (Roon, Waits & Kendall 2005). A solution has been proposed to analyse the minimum number of loci that still provide enough resolution to reliable identify individual animals, minimizing the error (Paetkau 2005). While this does make intuitive sense, the problem is that in noninvasive samples an odd locus will not amplify reliably in a sample, and even with low number of loci analysed the errors caused by allelic dropout remain a significant issue. In such case a large number of samples will get discarded, loosing data, limiting the number of recaptures and decreasing the chances of a study's success, while much of the problem of incorrectly assigning individuals to samples will still remain. Also, some samples won't reach the genotype reliability criteria with any sensible amount of repeats, but may provide a reliable

multi-locus genotype match with another, reliably genotyped sample. Another problem that we have not yet seen mentioned in the literature, but becomes very real when a large number of animals is included in the study, is the multiple-testing problem. Some measure of probability of identity between two animals (Waits, Luikart & Taberlet 2001) is typically considered to determine the number of loci required to obtain enough resolution to discern between animals, however such PID or PIDsib is valid only for a single comparison. In a study there are N*(N-1)/2 comparisons (where N is the number of individuals included in the study), so an appropriate multiple testing correction should be used to correct the PID and PIDsib values for the study. When N gets large, the resolution of a modest set of loci quickly becomes inadequate.

We took another approach of analysing a large number of loci and allowing for mismatches resembling allelic dropout (a non-amplifying allele, which is the most common genotyping error in noninvasive samples - see Broquet & Petit (2004)). We used a large dataset of brown bears from the same population genotyped using tissue samples with a very low error rate (Skrbinšek *et al.* 2012b) to explore distribution of mismatches, and used this mismatch distribution to set thresholds for allowable genotype mismatch. If the observed mismatches couldn't be caused by allelic dropout (e.g. 3 or 4 different alleles at the same locus in both samples) the samples were either considered to belong to different animals or additional evidence was collected through further repetitions of the genotyping procedure.

Mark-recapture analysis

We used several mark-recapture modelling approaches. We used the Capwire approach (Miller, Joyce & Waits 2005) with the R-package R-Capwire (Pennell *et al.* 2013). We also used the generalized linear model approach with the information-theoretic model selection (Burnham & Anderson 2002), as applied in program MARK (White & Burnham 1999). To provide a robust validation of the final results, we used the Chao's Mh model (Chao, Lee & Jeng 1992), which has lower statistical power, but should also be robust to capture heterogeneity. Separate models were done for males and females, and for both sexes together.

The Capwire model assumes continuous sampling, which fits with how our data has been collected. An additional advantage of this model is that it's reasonably robust to capture heterogeneity. We used likelihood-ratio test to select between the even capture rate model (ECM) and the two innate rates model (TIRM).

While the MARK approach requires discrete sampling sessions, this wasn't a case in our study. However, we considered MARK for analysis of our data because of its well-developed model selection procedures and flexibility to include additional information

about individuals, or groups of individuals, directly in the models. To fit this requirement, we considered the data collected within a certain time interval (sampling interval) as a single sampling session. This has the additional benefit that as the data gets aggregated into a smaller number of discrete sampling intervals, all captures of an individual animal within an interval will get aggregated into a single capture, lowering the capture heterogeneity and increasing robustness of the analysis. On the other hand, aggregation into sampling intervals invariably means loss of data (Petit & Valiere 2006). To find the ideal limits of each sampling interval, we programmed a recursive optimization routine in R programming language (Team 2010) which iterated through all possible combinations of uneven interval durations for a given number of intervals and found a solution with the minimal data loss and the maximum number of animals captured in each interval. The duration of each sampling interval (in days) was included in the modelling as a linear covariate. We corrected the sampling date of a sample with the estimated age of a scat to get an estimate of the actual time the sample was deposited and minimize interdependence between sampling sessions.

We used the Huggins model (Huggins 1989), including the newer derivations that allow for heterogeneity and misidentification (Lukacs & Burnham 2005; Cooch & White 2007), to construct an a-priori model set using the biological knowledge of the species and data/study characteristics (Burnham & Anderson 2002). Since these models use the same likelihood, they can be compared in the same model set using the information-theoretic approach. We used the median C-hat method to estimate goodness of fit of the most parameterized model (Cooch & White 2007). As there is no reason to expect behavioural response in detection of scat samples, we equalled the probability of first captures with the probability of recaptures. In the maximum model we grouped the animals by sex, included variation in capture probability between sampling intervals, and included the number of samples collected in a sampling interval (not the same as the number of captures as multiple captures of the same individual during the same interval get aggregated, but an indicator of sampling intensity) as a linear covariate of capture probability. We used Akaike's second-order information criterion (AICc) (Akaike 1974; Sugiura 1978) for model selection, constructed a confidence set of models with $\Delta AICc < 3$ and considered model averaging using Akaike's weights for the final parameter estimation (Burnham & Anderson 2002).

All the models we used assume a demographically closed population. Since sampling was relatively short and before reproduction and the majority of mortality recorded and included into the dataset (as mortality on capture), we assumed that the sampled population should behave as demographically closed. We tested this assumption using the Pradel model (Pradel 1996) with recruitment parameterization (Boulanger *et al.* 2002).

The population is demographically completely open towards Croatia and the national border crosses bear habitat without providing any significant physical obstacles to bear movement, so we expected some edge effect – inclusion of the animals that had only part of their homerange in our study area in our sampling. This meant that our estimate would actually estimate a "superpopulation". We used the correction proposed by (Wilson & Anderson 1985) to correct for the edge effect and estimate of the "moment" population size for Slovenia (the number of bears expected to be present in a certain moment in our study area), which is the parameter required for management purposes. We used detected pairwise distances between locations of samples of the same animal to calculate *W*, the width of the strip of Croatian territory bordering our study area from where the animals would have a non-negligible probability of being included in our sampling. Because of expected differences in habitat use, *W* was calculated separately for each sex. To obtain the moment population size estimate, we used the A_s/A_t as the correction factor for our superpopulation estimate, where A_s is the surface area being sampled, and A_t the total area including the edge strip in Croatia.

Results

Power analysis simulation study

The simulation study showed that in a population numbering 600 bears and with 70 % genotyping success rate, we would in ideal circumstances need 1429 samples to obtain 8 % confidence interval and 857 samples to obtain 18 % confidence interval (Table 1). Since we considered 20 % to be the maximum acceptable confidence interval and understood that the actual data would be considerably noisier than the ideal simulated data, we scaled the study with the aim to collect at least 900 – 1000 samples.

Table 1: Power analysis of mark-recapture effort and expected confidence intervals in idealized circumstances. *Ns* – number of genotyped samples; N_{sc} – number of collected samples assuming 70 % success rate; *p* – simulated capture probability in each of 6 sampling sessions; *N*^ - estimated number of individuals; SE(*N*^) – standard error of *N*^; CI – 95 % confidence interval, as absolute numbers and as percentage of *N*^.

N_s	N_{sc}	*p*	*N*^	SE(*N*^)	CI	CI(%)
1000	1429	0.28	600.46	12.7	576-625	8 %
800	1143	0.22	600.59	18.37	565-637	12 %
600	857	0.17	601.57	27.84	547-646	18 %

Sample collection and genotyping

Following the power analysis, we started a very intensive campaign to get sampling intensity high enough to produce useful results. There were 105 hunting clubs, four special purpose hunting reserves, and 6 regional offices of Slovenia Forest Service participating in the sampling, plus additional volunteers that contacted us directly. We distributed 5613 sampling tubes to over 1000 people. We received 1057 scat samples collected by 391 different people for analysis. All samples were relatively fresh, with the mean estimated scat age 2.17 days (SD = 1.51). We also collected tissue samples of 26 bears that were killed (legally shot or traffic mortality) during the sampling.

We managed to successfully genotype 931 samples (88 %). We recorded 10.05 % average allelic dropout and 0.38 % average false allele rate. On average we performed 3.61 amplifications per sample.

Although we made an effort to keep the network of volunteers active through direct contact by telephone throughout the sampling, we could see some variability in sampling intensity (Figure 1). Sampling intensity was very high in the beginning but started decreasing until we re-visited all participating hunting clubs after 8 weeks of sampling. After the visit the intensity increased again and gradually dropped until the end of sampling.

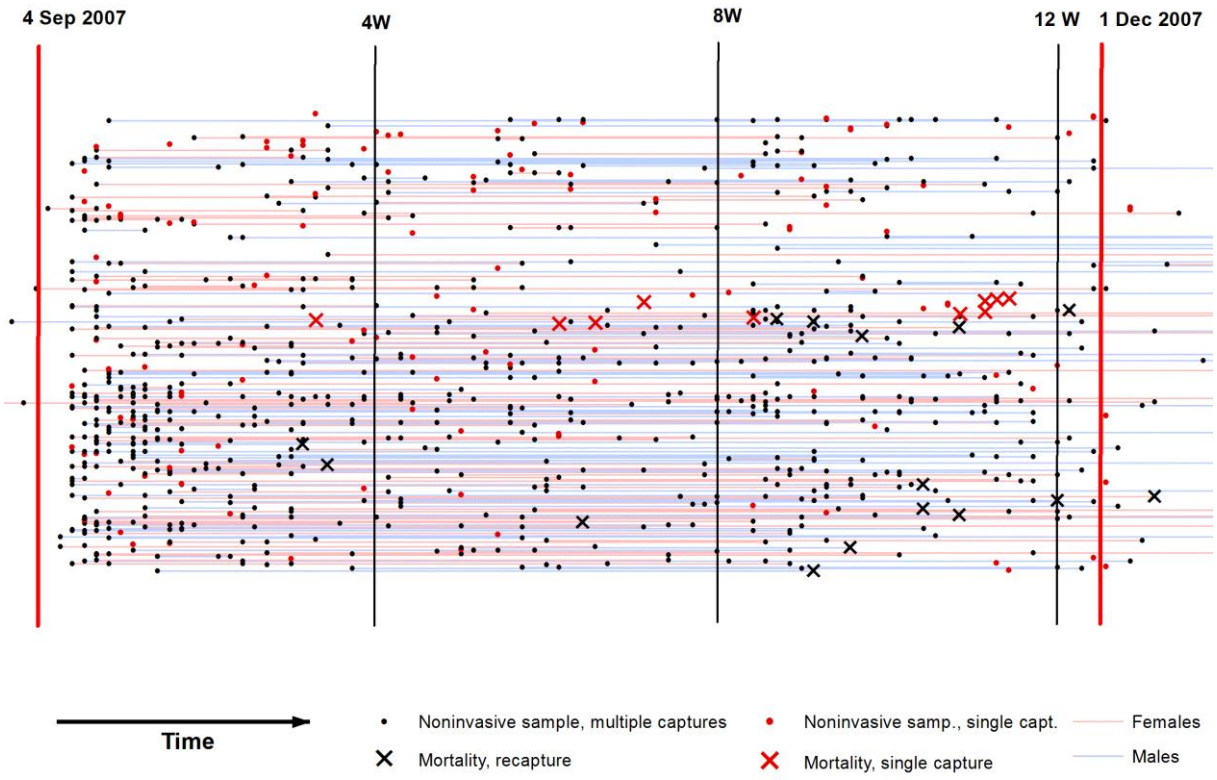

Figure 1: Graph of the mark-recapture process. Time increases from left to right, each symbol is a sample, lines connect samples of the same individual. We can see the peaks of sampling intensity in the first two weeks of sampling and in the two weeks following the re-visits of the hunting clubs in week 8.

We found 354 different genotypes, 159 (45 %) males and 195 (55 %) females. This provided a mean recapture rate of 2.70.

Mark-recapture modelling

The results of the Pradel model with recruitment parameterization were survival 0.994 and immigration 0.006, very close to the expectations in the closed population (survival = 1 and immigration = 0), supporting the assumption of population closure.

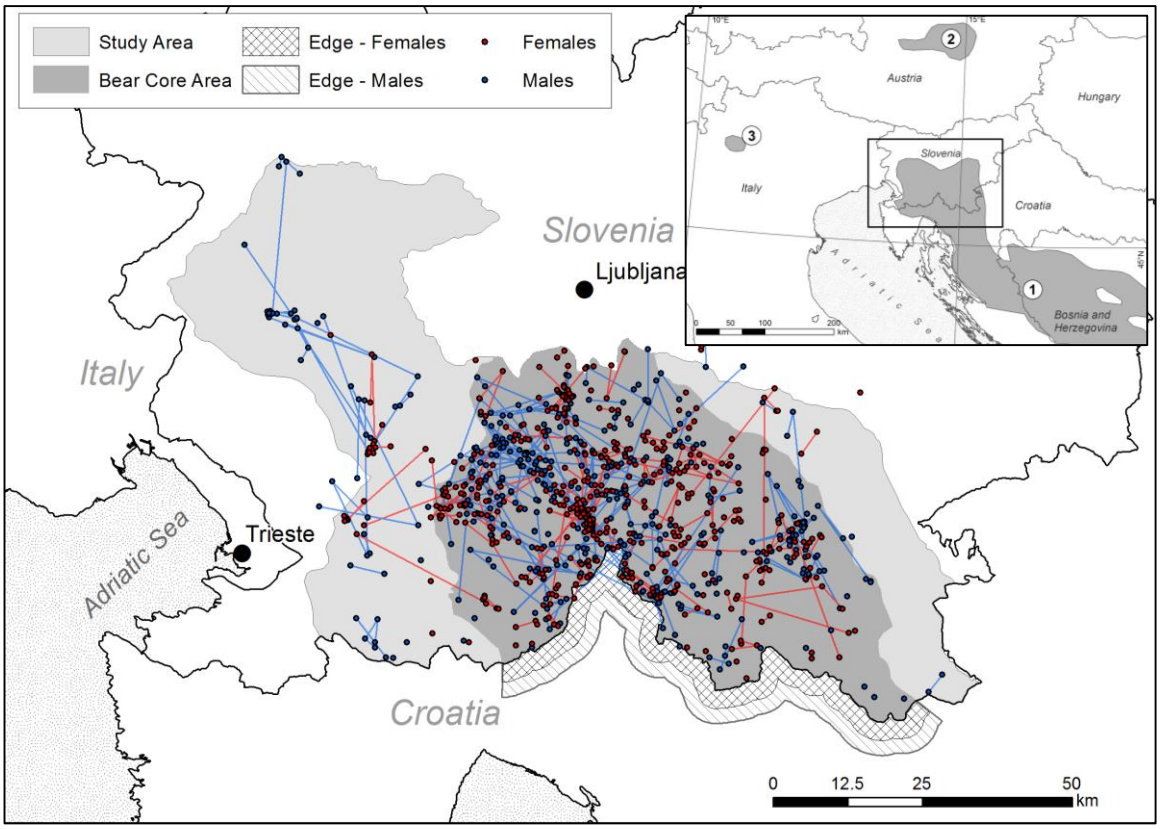

Figure 1: Study area and locations of successfully genotyped samples, with the core bear range and the buffer zones in Croatia for edge effect correction. Lines chronologically connect samples from the same individual.

Using Capwire, the TIRM model provided a better fit to the data than the ECM, indicating some capture heterogeneity in the data.

In the Markov chain optimization routine designed to optimize the sampling intervals for MARK, we aimed for approximately 0.3 capture probability in each capture interval. Following the optimization for minimal data loss we obtained 7 capture intervals covering the entire sampling period. Median C-hat goodness of fit test for the most parametrized model (Huggins c=p(sex.t)) showed a very reasonable model fit (C-hat = 0.352, s.e. = 0.022).

In Huggins models in MARK, the most parsimonious (best) model included the sex of the animals, heterogeneity modelled as two finite mixtures, and the number of samples collected per sampling interval as a linear covariate. A similar model that included misidentification had similar support in the data (ΔAICc = 0.85), however as the misidentification coefficients for both groups were very close to 1, the model is more complex, and the abundance estimates were nearly identical to the best model, we decided not to include it in the confidence set. Some support in the data was also for the model that

included the duration of each sampling interval instead of the number of samples as a linear covariate (ΔAICc = 2.18), and this was the only other model besides the best model included in the confidence set. The models that didn't include either linear covariate of the sampling interval had low support in the data (ΔAICc = 6.92), and the models that didn't account for heterogeneity had practically no support in the data (ΔAICc = 35.05). We didn't use model averaging, but used the best model for estimation of abundance since the only other model with a meaningful Akaike's weight produced nearly the same estimate.

We obtained very similar results using all three modelling approaches (Table 2, Figure 3), but the Capwire provided the narrowest confidence intervals. The estimates of models done separately for males fit very well with the estimate for both sexes.

Table 2: Brown bear abundance estimates and their 95 % confidence intervals (in brackets) obtained by different mark-recapture models for the superpopulation of the sampled area in Slovenia.

Model	**Males**	**Females**	**Total**
Huggins	212 (196-238)	297 (249-395)	509 (445-633)
Capwire	221 (185-243)	290 (257-320)	511 (470-545)
Mh(Chao)	206 (185-246)	283 (248-341)	489 (433-587)

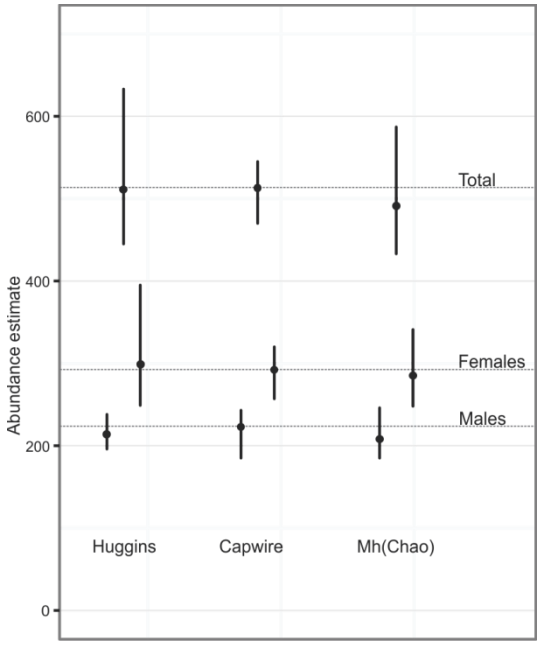

Figure 3: Superpopulation size estimate of bears in Slovenia (no correction for edge effect) using three different modeling approaches. While the Capwire model provided the narrowest confidence intervals, the results obtained by different models are nearly identical.

Edge effect correction

An implicit assumption in the edge effect correction we used is that of a constant population density. The number of different animals found was high in the "core" bear range in the Dinaric mountains (N=318), and low in the border areas (N=36, see Figure 2), indicating considerable variability in population density (this issue has been explored further with additional independent data in (Jerina *et al.* 2013)). Since we put a lot of effort into that during study design and sampling, we believe that the sampling effort was reasonably consistent across the study area. Since the core bear range lies along the Croatian border, we excluded the low population density areas separated by linear barriers (highways, main roads) or dense human settlements and the bears detected there in calculation of the edge effect correction factors (Figure 2). As the number of animals for the correction, we estimated the number of animals in the core bear range by multiplying the mark-recapture estimate with the proportion of the number of individuals of each sex detected there (females = 0.933; males = 0.855; both sexes = 0.898). There was a considerable difference between males and females in the maximum observed pairwise distances between locations of samples of the same animal - 6167 m for females, and 11 165 m for males, so we calculated a different edge effect correction factor for the core bear range for each sex (females = 0.893; males = 0.828; both sexes, weighted mean = 0.866).

Table 3: Sex-specific and total estimates obtained by Capwire, corrected for edge effect (Ncorr). In the brackets is the 95 % confidence interval. After excluding mortality during sampling, the final (winter) estimate (Nf) represents the annual minimum population size, after finished cull and before reproduction. Since mortality of 108 brown bears was detected in 2007, the maximum (spring) estimate should include these animals. However, doing this might produce an overestimate if the high cull rates created a source-sink dynamics with the bears in Croatia. Sex structure was calculated for the winter estimate, the spring estimate should produce less skew since mortality is greater in males.

	Ncorr	**Mortality**	**Nf**	**Sex structure**
Males	188(152-210)	17	171(135-193)	40.5 %
Females	261(228-291)	9	252(219-282)	59.5 %
Total	450(409-484)	26	424(383-458)	100 %

Discussion

Population size is always presented as a critical parameter in any management and conservation efforts, especially if a species is managed by hunting. On the other hand, estimating size of a wildlife population is demanding, and has been difficult to implement robustly in a species like the brown bear prior to development of noninvasive genetic sampling (Bellemain *et al.* 2004; DeYoung & Honeycutt 2005).

There were several population size estimates which varied considerably. While the official estimate for the territory of Slovenia has been 500-700 bears between 2002 and 2007, the other estimates based on different methodologies provided different results. Neither of the methodologies used could be defended as reliable, which provided considerable room for speculation. The problem was exacerbated by the media and some NGO's with misinterpretation of the data that either inflated the population size to unreasonable numbers or presented the population as being threatened, depending on the personal position or agenda of the author. This produced a volatile atmosphere for brown bear conservation with vocal supporters of both positions. The numbers of killed bears, mainly through hunting, have more than doubled since 2002 from what they were in 1990s (mean annual mortality 1990-2001 = 41.2 individuals; 2002-2007 = 99.5 individuals), causing concern among experts (Reynolds 2002), especially since they were based on the official population size estimates that many experts regarded as excessive.

One of our goals was to stop the speculations and provide a defendable, scientifically sound population size estimate of the number of bears in Slovenia, and we feel that we succeeded. We made the results public in 2008, and received considerable media attention. Since the methods we used for the estimate were improved from what was originally reported in the project report, there is a minimal difference (2.4 %) between the estimate we provided for management purposes in 2008 and the estimate published here. The difference is insignificant for any practical purposes.

The estimate has been included in official bear management documents as one of the management parameters. Additional benefit was that one of the most critical interests groups, the hunters, was directly involved in the study and the hunting organization took the estimate for their own (e.g. the Slovenian Hunters Association organized a press conference and presented the results). We took care to provide feedback to hunters, and published two comprehensive articles in the national hunting magazine "Lovec" that is distributed to all hunters in Slovenia as a part of their membership fee. Credibility of the estimate was never questioned even by the most vocal proponents of bear population size reduction.

Our study confirmed that it is possible to provide robust, reliable population size estimates of a difficult to monitor species in a rapid, cost-effective manner. The net cost of our study was 90 000€, which is very reasonable for monitoring of a large carnivore species at a national level. We provided results in one year since beginning of sampling, but with minimal laboratory upgrades and the gained experience the results could be provided in half of this time, in time to support the next management decision. There are also additional benefits of molecular genetics approaches, e.g. monitoring of effective population size (Skrbinšek *et al.* 2012a), genetic diversity and population dynamics, to name but a few, which could with some careful planning be achieved with minimal additional costs.

Although the potential of molecular tools for wildlife management and conservation has been frequently pointed out (e.g. DeYoung & Brennan 2005; Waits & Paetkau 2005; Schwartz, Luikart & Waples 2007), the managers still need time to adjust to this new situation to utilize these new tools to their full potential. At the moment we feel that the value molecular genetics for wildlife management and conservation, especially in longitudinal studies, is still considerably underappreciated.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Molekularno genetska orodja vedno bolj prihajajo iz domene čiste znanosti v rutinsko uporabo in vse bolj vplivajo na različne vidike naše znanosti, družbe in kulture (Avise, 2004). Vedno cenejši, hitrejši in zanesljivejši analitični pristopi nam tudi v ekologiji in varstvu narave omogočajo iz dneva v dan boljše razumevanje dogajanj v naravi na ravni, ki si je še pred nekaj desetletji nismo mogli niti predstavljati (DeYoung in Brennan, 2005; Waits in Paetkau, 2005). V svojem delu sem skupaj s kolegi te metode tudi praktično vpeljal v raziskovanje, varstvo in upravljanje karizmatične in varstveno zelo zahtevne živalske vrste, rjavega medveda. Pri tem sem uporabil najsodobnejše pristope, ki jih ponuja današnja znanost, razvili pa smo tudi nekatere nove metodološke pristope. Večina dela je že objavljena v obliki člankov, ki so sestavni del tega doktorskega dela, v vodilnih znanstvenih revijah na področju molekularne ekologije in varstvene genetike.

Izhodišče naloge je bilo aplikativno. V začetku stoletja je Slovenija dobivala iz tujine kar nekaj kritik in neprijetnih vprašanj na račun upravljanja z medvedom (Reynolds, 2002), vprašanja o ustreznosti spremljanja populacije in upravljanja z njo pa so se pojavljala tudi pri domačih strokovnjakih (Kryštufek in sod., 2003). Na Oddelku za biologijo Biotehniške fakultete Univerze v Ljubljani smo tako leta 2004 predlagali uporabo molekularno-genetskih metod za spremljanje številčnosti in genetskega statusa populacije kot podporo upravljavskim odločitvam. Zgodba se je razvijala preko več nacionalnih in enega mednarodnega projekta, rezultat pa je, da imamo danes, slabo desetletje kasneje, globalno eno najbolje raziskanih populacij rjavega medveda v svetu in odličen temelj za upravljanje in varovanje te naravne dediščine.

3.1.1 Neinvazivno vzorčenje in številčnost medvedov v Sloveniji

Prvo vprašanje, iz katerega so raziskave pravzaprav izhajale, je bilo vprašanje o številčnosti medvedov pri nas. Problematika ocenjevanja števila prostoživečih živali v naravi je že zelo dolgo en od ključnih problemov v ekologiji, naravovarstvu in upravljanju z naravnimi viri (Amstrup in sod., 2005). Če citiram Johna Shepherda, enega vidnejših raziskovalcev, ki so se ukvarjali s tem problemom: »Štetje rib je podobno kot štetje dreves, s to razliko, da jih ne vidimo in da se premikajo«. Štetje medvedov gre v veliki meri v isto kategorijo, preboj na tem področju pa so naredila prav molekularno genetska orodja, ki nam omogočajo genetsko »označevanje« živali (Luikart in sod., 2010; Waits in Paetkau, 2005). Pri razvoju genetskih orodij je bil najpomembnejši korak razumevanje analitike neinvazivnih genetskih vzorcev – genetskega materiala, ki ga žival pusti v okolju (iztrebki, urin, dlaka itd.)(Waits in Paetkau, 2005), ob tem pa smo v zadnjih dveh desetletjih tudi priča razcvetu metod označevanja in ponovnega ulova in programskih orodij za njihovo

uporabo (Amstrup in sod., 2005; Cooch in White, 2007). V pilotskem projektu (L1-6484) med leti 2004 in 2007 smo opremili ustrezen laboratorij za obdelavo neinvazivnih genetskih vzorcev, vpeljali zelo stroge protokole za preprečevanje kontaminacije in pridobili ustrezno znanje, da smo lahko analize takšnih vzorcev korektno izpeljali (Skrbinšek, 2007; Skrbinšek in sod., 2007c). Leta 2007 smo v sodelovanju z Lovsko zvezo Slovenije in Zavodom za gozdove Slovenije izpeljali intenzivno vzorčenje celotnega območja stalne prisotnosti medveda pri nas (poglavje 2.2.1). Uporabili smo simulacijsko študijo, kjer smo simulirali proces označevanja in ponovnega ulova in tako ocenili intenzivnost vzorčenja, ki bi zagotovila zadovoljivo natančnost ocene velikosti populacije (Skrbinšek in sod., 2008); poglavje 2.2.1). Z intenzivno promocijsko akcijo, sodelovanjem z Lovsko zvezo in ZGS ter z objavami v reviji Lovec (Skrbinšek in sod., 2007a; Skrbinšek in sod., 2007b; Skrbinšek in sod., 2007d) smo k sodelovanju pritegnili po naših ocenah več kot 1000 ljudi, ki so nam pri zbiranju vzorcev pomagali. Vzorčenje je uspelo nad pričakovanji, saj smo v analize dobili 1057 neinvazivnih vzorcev medvedov. Javnost, zlasti lovci, so izkazali velik interes za sodelovanje in pokazali, da lahko dobro organizirana mreža prostovoljcev zelo učinkovito prispeva k zbiranju podatkov za upravljanje z vrstami, ki potrebujejo aktivno upravljanje in varovanje.

Ker smo bili s sredstvi za analize tako velikega števila analitično zahtevnih vzorcev zelo omejeni, smo protokole optimizirali in tako za več kot štirikrat pocenili in pospešili laboratorijske analize. Izsledke in optimiziran analitični protokol, ki omogoča analizo dvanajstih mikrosatelitskih markerjev in lokusa za določitev spola v eni sami PCR in eni analizi na avtomatskem sekvenatorju, smo objavili v Skrbinšek in sod. (2010). Dobili smo tudi eno od najvišjih uspešnosti genotipizacije iz neinvazivnih vzorcev (88 %) v do danes objavljenih študijah, ki so uporabljale tak material. Sklepamo, da je tako visoka uspešnost pogojena s tem, da smo zbirali samo svež material in prostovoljcem dali dovolj natančna navodila, kako na terenu oceniti starost iztrebka medveda (Skrbinšek in sod., 2010). V istem članku smo tudi pokazali, da multipleksna preamplifikacija, ki naj bi glede na literaturne vire izboljšala uspešnost analize neinvazivnih vzorcev (Hedmark in Ellegren, 2006; Piggott in sod., 2004), nima bistvenega vpliva na uspešnost analiz in ne opravičuje znatno višjih stroškov in napora.

Z modeli označevanja in ponovnega ulova smo pokazali, da se populacija obnaša ustrezno predpostavkam o zaprti populaciji (brez imigracije/rodnosti in emigracije/smrtnosti), kar je ena od pomembnih predpostavk statističnih modelov za ocenjevanje številčnosti. Številčnost smo ocenili s široko paleto modelov, ki imajo različne lastnosti in so različno občutljivi na kršenje predpostavk. Prvotne ocene (Skrbinšek in sod., 2008) smo nekoliko modificirali z uporabo kasneje razvitih metod (poglavje 2.2.1), na splošno pa so vse metode podale zelo podobne ocene številčnosti z velikim prekrivanjem intervalov zaupanja, kar vliva dodatno zaupanje v rezultate.

S pomočjo modeliranja označevanja in ponovnega ulova, ob upoštevanju učinka roba zaradi medvedov, ki prihajajo in odhajajo iz območja vzorčenja preko meje s Hrvaško in z upoštevanjem zaznane smrtnosti, smo ocenili »zimsko« velikost populacije (po celoletni smrtnosti, pred reprodukcijo) na 424 medvedov, s 95 % intervalom zaupanja od 383 do 458. Opazili smo tudi premaknjeno spolno razmerje, 40.5 % samcev in 59.5 % samic. Ocena je prva robustna ocena velikosti populacije te vrste v Sloveniji in ena redkih takšnih ocen v svetu in daje trdno podlago upravljanju z medvedom v naši državi.

3.1.2 Razvoj nove metode za primerjavo genetske pestrosti različnih populacij in analiza globalne distribucije genetske pestrosti medvedov

Čeprav uživajo genetske raziskave medvedov veliko pozornosti in lahko rjavega medveda smatramo med z genetskega vidika bolje raziskane živalske vrste, je primerjava genetske pestrosti med populacijami zaradi različnih sistemov genetskih markerjev in različnih velikosti vzorcev težko izvedljiva (Swenson in sod., 2011). Nova metoda, ki smo jo objavili v Skrbinšek in sod. (2012a) vpeljuje pojem referenčne populacije, ki se uporabi kot »merilo« za primerjavo genetske pestrosti drugih populacij. Ker imamo eno od najbolj temeljito vzorčenih populacij rjavega medveda v svetu in analize narejene z zelo široko paleto genetskih markerjev, smo medvede iz Slovenije uporabili kot referenčno populacijo in tako z meta analizo prvič pogledali globalno distribucijo genetske pestrosti rjavega medveda. Vse študije dinarske populacije medvedov so pokazale podobno genetsko pestrost, nekoliko nižjo kot pri velikih populacijah v Karpatih in v severni Ameriki, vendar veliko višjo kot pri majhnih in ogroženih populacijah kot je tista v Apeninih in Kantabrijska populacija v Španiji (za opis populacij medveda po Evropi glej Zedrosser in sod. (2001). Ob velikem pomenu raziskave za globalno razumevanje genetskih vidikov ogroženosti različnih populacij rjavega medveda pa ima metoda sama tudi širšo uporabnost pri drugih živalskih in rastlinskih vrstah, saj omogoča korektno primerjavo genetske pestrosti med različnimi, prej neprimerljivimi študijami iste vrste.

3.1.3 Spremljanje efektivne velikosti populacije medvedov v severnih Dinaridih

Efektivna velikost populacije (N_e) je verjetno idealen parameter za spremljanje tako evolucijskega potenciala populacije kot njene občutljivosti na naključne genetske procese (Charlesworth, 2009). Preko spremljanja N_e lahko zaznamo tudi druge procese v populaciji, ki so z ostalimi metodami težko zaznavni, lahko pa so ključnega pomena za varstvo in upravljanje: fragmentacijo (England in sod., 2010) in učinke sprememb v varstvenem ali upravljavskem režimu. Spremljanje N_e skozi čas je zahtevno, zlasti pri vrstah, pri katerih se generacije prekrivajo (Schwartz in sod., 2007). V članku Skrbinšek in sod. (2012b) smo kot prvi pokazali, da je to izvedljivo tudi pri velikih sesalcih v prosti naravi in da je mogoče spremljanje N_e vključiti v rutinske programe monitoringa.

Izsledki pri naših medvedih kažejo, da je populacija sicer velika, da pa še vedno ne dosega kriterijev za dolgoročno ohranitev evolucijskega potenciala. Zato bo medvede v severnih Dinaridih treba še naprej varovati, pozornost bi pa morali posvetiti tudi preprečevanju fragmentacije življenjskega prostora in ohranjanju oziroma vzpostavljanju genetskega pretoka med populacijami medvedov v južni in srednji Evropi.

Ob pomenu za znanost ima raziskava precejšen pomen za razumevanje in spremljanje varstvenega stanja medvedov v Sloveniji, pa tudi širše v Severnih Dinaridih. Ker se tkivni vzorci medvedov in zobje za določanje starosti rutinsko jemljejo vsem odstreljenim ali drugače poginulim medvedom pri nas, lahko s spremljanjem efektivne velikosti populacije nadaljujemo tudi v prihodnje. Tkivne vzorce zbirajo tudi na Hrvaškem in jih po istih metodah analiziramo v našem laboratoriju, kar je velikega pomena, saj gre za isto populacijo medveda. Tako se spremljanje varstvenega stanja te vrste pri nas dejansko seli po eni strani na temeljno, molekularno-evolucijsko raven, po drugi strani pa na raven populacije, kar je tudi izpostavljeno kot en izmed prioritetnih ciljev pri varovanju velikih zveri v evropskem prostoru (Linnell in sod., 2008). Pomen raziskave je širši od same problematike medveda, saj smo z njo odprli nova vrata in postavili nove standarde za spremljanje varstvenega stanja populacij redkih in ogroženih vrst.

3.1.4 Forenzična genetika prostoživečih živali – primer krivolova medveda "Rožnika"

Čeprav raziskav v osnovi nismo zastavili s ciljem obravnave forenzične tematike, se je med delom začelo pojavljati vse več primerov, kjer so bila molekularna orodja edini način, da pridemo do zadovoljivega odgovora. En od teh primerov, primer krivolova medveda »Rožnika«, je bil dovolj zanimiv in izpostavljen, da smo ga objavili tudi v obliki znanstvenega članka (Kaczensky in sod., 2011).

Naš ključen prispevek k primeru je bil, da smo odrto in obglavljeno truplo medveda, najdeno na severu Slovenije, lahko nedvoumno povezali z medvedom Rožnikom. Sicer do obsodbe storilca zaradi pomanjkljivih dokazov ni prišlo, je pa dobra dokumentiranost primera sprožila pomembno diskusijo o razlogih za izumrtje ponovno naseljene populacije medveda v Avstriji in razlogih, da ne prihaja do naravne rekolonizacije medvedov iz Slovenije v avstrijski prostor. Rožnik, ki je v Avstriji »trajal« manj kot teden dni, preden ga je krivolovec ustrelil, je močno podkrepil predvidevanja, da je prav krivolov ena izmed ključnih ovir obstoju medveda v Avstriji. Ker je krivolov na splošno tabu tema, zlasti za lovce, je bila genetska potrditev identitete mrtvega medveda velikega pomena in je dala celotnemu primeru znatno večjo težo, kot bi jo imel brez nje.

4 SKLEPI

Molekularno-genetska orodja vedno bolj prihajajo iz domene čiste znanosti v rutinsko uporabo in vse bolj vplivajo na različne vidike naše znanosti, družbe in kulture (Avise, 2004). Vedno cenejši, hitrejši in zanesljivejši analitični pristopi nam tudi v ekologiji in varstvu narave omogočajo iz dneva v dan boljše razumevanje dogajanja v narave na ravni, ki si je še pred nekaj desetletji nismo mogli niti predstavljati (DeYoung in Brennan, 2005; Waits in Paetkau, 2005).

S pomočjo neinvazivnega genetskega vzorčenja in modeliranja označevanja in ponovnega ulova, ob upoštevanju učinka roba zaradi medvedov, ki prihajajo in odhajajo iz območja vzorčenja preko meje s Hrvaško in z upoštevanjem zaznane smrtnosti, smo ocenili »zimsko« velikost populacije (po celoletni smrtnosti, pred reprodukcijo) na 424 medvedov, s 95 % intervalom zaupanja od 383 do 458. Opazili smo tudi premaknjeno spolno razmerje, 40.5 % samcev in 59.5 % samic. Ocena je prva robustna ocena številčnosti te vrste v Sloveniji in ena redkih takšnih ocen v svetu in daje trdno podlago upravljanju z medvedom v naši državi.

Razvili in objavili smo novo metodo za nepristransko primerjavo genetske pestrosti med različnimi, prej neprimerljivimi študijami iste vrste s pomočjo referenčne populacije (Skrbinšek in sod., 2012a). V članku smo kot referenčno populacijo uporabili medvede iz Slovenije in tako v meta analizi prvič obravnavali globalno distribucijo genetske pestrosti rjavega medveda. Vse študije dinarske populacije medvedov so pokazale podobno genetsko pestrost, nekoliko nižjo kot pri velikih populacijah v Karpatih in v severni Ameriki, vendar veliko višjo kot pri majhnih in ogroženih populacijah, kot je tista v Apeninih in Kantabrijska populacija v Španiji. Ob velikem pomenu raziskave za globalno razumevanje genetskih vidikov ogroženosti različnih populacij rjavega medveda pa ima metoda sama tudi širšo uporabnost pri drugih živalskih in rastlinskih vrstah, saj omogoča korektno primerjavo genetske pestrosti med različnimi, prej neprimerljivimi študijami iste vrste.

V članku (Skrbinšek in sod., 2012b) smo kot prvi v svetu pokazali, da mogoče spremljati efektivno velikost populacije (N_e) tudi pri velikih sesalcih v prosti naravi in da je mogoče spremljanje N_e vključiti v rutinske programe monitoringa. Izsledki pri medvedih v severnih Dinaridih kažejo, da je populacija sicer velika, da pa še vedno ne dosega kriterijev za dolgoročno ohranitev evolucijskega potenciala. Zato bo to populacijo treba še naprej varovati, pozornost bi pa morali posvetiti tudi preprečevanju fragmentacije življenjskega prostora in ohranjanju oziroma vzpostavljanju genetskega pretoka med populacijami medvedov v južni in srednji Evropi. Pomen raziskave je širši od problematike medveda, saj smo z njo odprli nova vrata in postavili nove standarde za spremljanje varstvenega stanja populacij redkih in ogroženih vrst.

V članku (Kaczensky in sod., 2011) smo sprožili pomembno diskusijo o razlogih za izumrtje ponovno naseljene populacije medveda v Avstriji in razlogih, da ne prihaja do naravne rekolonizacije medvedov iz Slovenije v avstrijski prostor. Z dobro dokumentacijo krivolova z GPS ovratnico spremljanega medveda »Rožnika« smo podkrepili predvidevanja, da je prav krivolov ena izmed ključnih ovir obstoju medveda v Avstriji.

O rjavem medvedu pri nas vemo danes več kot kadarkoli prej. Z delom, opisanim v tej doktorski disertaciji, smo postavili temelje za slovenski prostor novemu principu spremljanja statusa vrst, ki potrebujejo aktivno varstvo in upravljanje. Vse kaže, da se bodo tukaj opisane raziskave razširile v trajen genetski monitoring varstvenega stanja populacije medveda, s podobnimi metodami pa že preučujemo varstveno stanje populacij risa (*Lynx lynx*) (Sindičić in sod., 2013) in volkov (*Canis lupus*). S tem v Sloveniji začenjamo slediti globalnim trendom upravljanja in varovanja redkih in ogroženih vrst, marsikje pa gremo tudi korak dlje.

5 POVZETEK

Karizmatične vrste, kot to medved nedvomno je, so pogosto v žarišču javnega interesa, njihovo upravljanje in varovanje pa zahtevna in občutljiva tema. Ključno vlogo pri odločanju mora imeti znanost, saj lahko samo znanstveno preverjeni, verodostojni podatki omogočijo suverene odločitve, učinkovite v realnem svetu. Pri tem postaja vedno pomembnejša vloga varstvene genetike. Molekularno-genetska orodja zapuščajo domeno čiste znanosti in prihajajo v rutinsko uporabo ter tako vse bolj vplivajo na različne vidike naše znanosti, družbe in kulture (Avise, 2004). Vedno cenejši, hitrejši in zanesljivejši analitični pristopi nam tudi v ekologiji in varstvu narave omogočajo iz dneva v dan boljše razumevanje dogajanja v naravi na ravni, ki si je še pred nekaj desetletji nismo mogli niti predstavljati (DeYoung in Brennan, 2005; Waits in Paetkau, 2005). Te metode sem v svojem delu skupaj s kolegi tudi praktično vpeljal v raziskovanje, varstvo in upravljanje karizmatične in varstveno zelo zahtevne živalske vrste, rjavega medveda.

Prvo vprašanje, iz katerega so raziskave pravzaprav izhajale, je bilo vprašanje o številčnosti medvedov pri nas. Problematika ocenjevanja števila prostoživečih živali v naravi je že zelo dolgo en od ključnih problemov v ekologiji, naravovarstvu in upravljanju z naravnimi viri (Amstrup in sod., 2005). Leta 2007 smo v sodelovanju z Lovsko zvezo Slovenije in Zavodom za gozdove Slovenije izpeljali intenzivno neinvazivno genetsko vzorčenje celotnega območja stalne prisotnosti medveda pri nas (Skrbinšek in sod., 2008) (poglavje 2.2.1). Ker smo bili s sredstvi za analize tako velikega števila analitično zahtevnih vzorcev zelo omejeni, smo protokole optimizirali in tako za več kot štirikrat pocenili in pospešili laboratorijske analize (Skrbinšek in sod., 2010). V intenzivnem trimesečnem vzorčenju smo zbrali 1057 neinvazivnih genetskih vzorcev rjavih medvedov. 931 vzorcev (88 %) smo uspešno genotipizirali in določili 354 različnih genotipov (osebkov). S pomočjo modeliranja označevanja in ponovnega ulova, ob upoštevanju učinka roba zaradi medvedov, ki se prihajajo in odhajajo iz območja vzorčenja preko meje s Hrvaško in z upoštevanjem zaznane smrtnosti, smo ocenili »zimsko« velikost populacije (po celoletni smrtnosti, pred reprodukcijo) na 424 medvedov, s 95 % intervalom zaupanja od 383 do 458. Opazili smo tudi premaknjeno spolno strukturo, 40.5 % samcev in 59.5 % samic.

Genetska pestrost je temelj fitnesa in evolucijskega potenciala populacije, posledično pa tudi njene sposobnosti za prilagajanje na spremembe v okolju (Allendorf in Luikart 2007; Frankham in sod., 2002; Reed in Frankham 2003). Čeprav uživajo genetske raziskave medvedov veliko pozornosti in lahko rjavega medveda smatramo med z genetskega vidika bolje raziskane živalske vrste, je primerjava genetske pestrosti med populacijami zaradi različnih sistemov genetskih markerjev in različnih velikosti vzorcev težko izvedljiva (Swenson in sod., 2011). Nova metoda, ki smo jo objavili v Skrbinšek in sod. (2012a), vpeljuje pojem referenčne populacije, ki se uporabi kot »merilo« za primerjavo genetske

pestrosti drugih populacij. V istem članku smo s to metodo v meta analizi prvič raziskali in predstavili tudi globalno distribucijo genetske pestrosti rjavega medveda.

Efektivna velikost populacije (N_e) je verjetno idealen parameter za spremljanje tako evolucijskega potenciala populacije kot njene občutljivosti na naključne genetske procese (Charlesworth, 2009). V članku Skrbinšek in sod. (2012b) smo kot prvi pokazali, da je to izvedljivo tudi pri velikih sesalcih v prosti naravi in da je mogoče spremljanje N_e vključiti v rutinske monitoring programe. Izsledki pri naših medvedih kažejo, da je populacija sicer velika, da pa še vedno ne dosega kriterijev za dolgoročno ohranitev evolucijskega potenciala. Zato bo medvede v severnih Dinaridih potrebno še naprej varovati, pozornost bi pa morali posvetiti tudi preprečevanju fragmentacije življenjskega prostora in ohranjanju oziroma vzpostavljanju genetskega pretoka med populacijami medvedov v južni in srednji Evropi.

Čeprav moja naloga v začetku ni bila zastavljena s ciljem obravnave forenzične tematike, se je med delom začelo pojavljati vse več primerov, kjer so bila molekularna orodja edini način, da pridemo do zadovoljivega odgovora. En od teh primerov, primer krivolova z GPS telemetrijo spremljanega medveda »Rožnika«, je bil dovolj zanimiv in izpostavljen, da smo ga objavili tudi v obliki znanstvenega članka (Kaczensky in sod., 2011). Naš ključen prispevek k primeru je bil, da smo odrto in obglavljeno truplo medveda, najdeno na severu Slovenije, lahko nedvoumno povezali z medvedom Rožnikom. Sicer do obsodbe storilca zaradi pomanjkljivih dokazov ni prišlo, je pa dobra dokumentiranost primera sprožila pomembno diskusijo o pomenu krivolova za nedavno izumrtje ponovno naseljene populacije medveda v Avstriji in razlogih, da ne prihaja do naravne rekolonizacije medvedov iz Slovenije v avstrijski prostor. Ker je krivolov na splošno tabu tema, zlasti za lovce, je bila genetska potrditev identitete mrtvega medveda velikega pomena in je dala celotnemu primeru znatno večjo težo, kot bi jo imel brez nje.

O rjavem medvedu pri nas vemo danes več kot kadarkoli prej. Z delom, opisanim v tej doktorski disertaciji, smo postavili temelje za slovenski prostor novemu principu spremljanja statusa vrst, ki potrebujejo aktivno varstvo in upravljanje. Vse kaže, da se bodo tukaj opisane raziskave razširile v trajen genetski monitoring varstvenega stanja populacije medveda, s podobnimi metodami pa že preučujemo varstveno stanje populacij risa (*Lynx lynx*) (Sindičić in sod., 2013) in volkov (*Canis lupus*). S tem v Sloveniji začenjamo slediti globalnim trendom upravljanja in varovanja redkih in ogroženih vrst, v marsičem pa gremo tudi korak dlje.

6 SUMMARY

Charismatic species like the brown bear are frequently the focus of public interest, which makes their management and conservation a challenging prospect. The key role in this task should be played by science, since confident decisions that work in the real world can only be made when they are based on scientifically sound, hard data. In support of this process, the value of conservation genetics is being increasingly recognized. Molecular genetics tools are rapidly shifting from the domain of pure science into routine use, more and more affecting every aspect of our science, society and culture (Avise, 2004). Increasingly cost effective, rapid and reliable analytical approaches are also in ecology and nature conservation providing insights and understanding of processes in nature at the level unimaginable even a few decades ago (DeYoung and Brennan, 2005; Waits and Paetkau, 2005). Together with my colleagues, I introduced these methods into practical research, conservation and management of a charismatic, and from the conservation perspective very challenging animal species, the brown bear.

The first question that actually initiated this research was the question of bear abundance in Slovenia. The issue of population censuses in natural populations has for a long time been one of the key problems in ecology, nature conservation and natural resources management (Amstrup et al., 2005). In 2007, we implemented an intensive noninvasive genetic sampling of the entire bear range in Slovenia in collaboration with Slovenia Forest Service and Hunters Association of Slovenia (Chapter 2.2.1). Since we had limited funding for analysis of such a large number of samples, we optimized the protocols to achieve a four-fold increase in analysis speed, and decrease in costs (Skrbinšek et al., 2010). In a highly intensive three-month sampling in autumn 2007 we collected 1057 noninvasive samples of brown bears. 931 samples (88 %) were successfully genotyped, and we found 354 different genotypes (individuals). Through mark-recapture modelling, correcting for the edge effect caused by bears moving in and out of the sampling area across Croatian border and accounting for detected mortality, we estimated the "winter" population size (after annual mortality, before reproduction) at 424, with 95 % confidence interval of 383 to 458. We also observed an uneven sex ratio of 40.5 % males and 59.5 % females.

Genetic diversity is the basis for a population's fitness and evolutionary potential, and consequently for its capability to adapt to environmental change (Allendorf and Luikart, 2007; Frankham et al., 2002; Reed and Frankham, 2003). Although genetic research of brown bears is receiving considerable attention and we can consider the species as one of the better researched from the genetic perspective, different systems of genetic markers and different sample sizes make any comparison of genetic diversity between populations challenging (Swenson et al., 2011). The new method that we published in Skrbinšek et al. (2012a) introduces the concept of a reference population that is used as a "yardstick" for

comparisons of genetic diversity between other populations. In the same paper we also for the first time looked at global distribution of genetic diversity in brown bear.

Effective population size (N_e) is possibly the ideal parameter for monitoring both the evolutionary potential of a population, as well as its sensitivity to genetic stochasticity (Charlesworth, 2009). In our paper (Skrbinšek et al., 2012b) we were the first to show that this can be done in a natural population of a large mammal, and that monitoring of N_e can be included into routine monitoring programs. The results for bears in northern Dinaric Mts. show that although the effective population size is relatively large, it still doesn't reach the threshold for long-term conservation of the evolutionary potential. This means that the bears in northern Dinaric Mts. will require further protection, and attention should be given to prevention of fragmentation and maintenance/establishment of geneflow between brown bear populations in southern and central Europe.

Although my research was primarily not aimed at tackling forensic issues, several such cases appeared during our work where molecular tools were the only way to get a satisfactory answer. One of these cases, the poaching of GPS-tracked bear "Rožnik", received a lot of public attention and was interesting enough that we published it as a scientific paper (Kaczensky et al., 2011). Our main contribution to the case was that we proved that a skinned, headless bear carcass found in northern Slovenia was indeed the bear "Rožnik". The case made it to court, but here was not enough evidence to get a conviction of the suspect. Nevertheless, the high profile of the case started an important discussion about the importance of poaching for the extinction of the reintroduced bear population in Austria and about the reasons underlying the slow natural recolonisation of Austria by bears from Slovenia. Since poaching is generally a taboo topic, especially among hunters, our genetic confirmation of the dead bear's identity provided considerable weight to the entire case.

We know more about our bears today than we ever knew before. Through the work described in this doctoral dissertation, we laid the foundations in our country for a new principle of monitoring of conservation status for the species that require active conservation and management. There is a good chance that the research described here will be expanded into long-term genetic monitoring of the brown bear conservation status, and we're already using the same methods to study the conservation status of lynx (*Lynx lynx*) (Sindičić et al., 2013) and wolf (*Canis lupus*). With this we're starting to follow the global trends in management and conservation of rare and threatened species, but in many ways we're also taking a step beyond.

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9 PRILOGE

9.1 PRILOGA A: DOVOLJENJA ZALOŽNIKOV ZA UPORABO ČLANKOV V TISKANI IN ELEKTRONSKI VERZIJI DOKTORSKE DISERTACIJE

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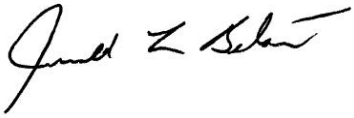

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