Université de Montréal

La génétique au service de la conservation d'une espèce menacée endémique à Madagascar, la tortue radiée *Astrochelys radiata*

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La génétique au service de la conservation d'une espèce menacée endémique à Madagascar, la tortue radiée *Astrochelys radiata*

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Résumé

Les analyses génétiques peuvent fournir des informations intéressantes à propos de la démographie, de l'écologie ou de l'évolution des espèces, qui à leur tour peuvent jouer un rôle déterminant dans l'élaboration de plans de conservation appropriés. Le principal objectif de cette thèse était de réaliser une analyse détaillée de la génétique des populations d'une espèce menacée dans le but d'acquérir des connaissances cruciales à sa conservation. L'espèce étudiée était la tortue radiée, *Astrochelys radiata* (Chelonia : Testudinidae), endémique à la forêt épineuse semi-aride du sud de Madagascar, où, en plus de la destruction de l'habitat, elle est gravement menacée par la surexploitation pour approvisionner les marchés locaux et internationaux. D'un bout à l'autre de cette thèse, les données provenant de treize marqueurs microsatellites ont été analysées pour répondre à des questions qui concernaient, notamment, la structure génétique de l'espèce, l'impact de l'exploitation et de la fragmentation de l'habitat sur sa diversité génétique, et l'identification d'unités de conservation. Huit de ces marqueurs ont été spécifiquement développés pour *A. radiata* pendant cette étude, et plus de 330 échantillons recueillis sur l'ensemble de l'aire de répartition actuelle de l'espèce ont été analysés au total. Les résultats indiquent que la tortue radiée exhibe des niveaux modérés de différentiation, et deux populations génétiquement distinctes ont été principalement identifiées. Celles-ci compte chacune des sous-populations additionelles périphériques qui démontrent une différentiation faible mais significative en fonction de leurs fréquences alléliques. Une approche statistique basée sur la régression multiple de matrices de distances a mis en évidence le rôle de quelques fleuves en tant que barrières au flux génique entre populations de tortues, malgré le fait que ces cours d'eau soient saisonniers et complètement asséchés plusieurs mois par année. En combinaison avec des analyses de l'ADNmt, il a été trouvé que le fleuve Menarandra représente la plus importante barrière génétique historique et

contemporaine chez cette espèce. Des analyses de goulots d'étranglement ont suggéré que la diversité génétique plus faible des populations exploitées ne pouvait être attribuée à des activités anthropogéniques récentes, et des résultats de l'ADNmt indiquent plutôt que ce sont les tailles historiquement plus petites des populations situées à l'est du Menarandra, en raison de la nature fragmentée de l'habitat dans cette région, qui sont l'explication la plus probable. Des analyses morphométriques additionnelles ont trouvé des patrons divergents entre les variations génétiques et les variations de la forme de la carapace chez *A. radiata*, et l'exploitation humaine semble être le facteur expliquant les différences morphologiques. La méthodologie développée pour réaliser les analyses morphométriques pourrait devenir un outil utile pour la gestion des tortues confisquées auprès des braconniers et des trafiquants. Finalement, des analyses génétiques séparées pour les mâles et les femelles ont révélé que la dispersion est fortement biaisée en faveur des mâles chez *A. radiata*. Cette thèse fournit un exemple intéressant de la panoplie d'applications possibles de la génétique en conservation, et l'intérêt scientifique qu'a reçu la tortue radiée durant cette étude a été un catalyseur formidable pour amorcer une plus grande conscientisation face au déclin dramatique de cette espèce emblématique de Madagascar.

Mots-clés : barrières génétiques, dispersion inégale des sexes, différentiation des populations, exploitation, forêt épineuse de Madagascar, génétique de la conservation, microsatellites, morphométrie, unités de conservation, Testudinidae

Abstract

Genetic analyses can provide insightful information about the demography, ecology and evolution of species, which might in turn be highly relevant to launch appropriate conservation initiatives. The main objective of this thesis was to conduct a thorough population genetics assessment of an endangered species in order to gather crucial knowledge for its conservation. The species under study was the radiated tortoise, *Astrochelys radiata* (Chelonia: Testudinidae), endemic to the semi-arid spiny forest of southern Madagascar where, in addition to habitat destruction, it is severely threatened by overexploitation for both local and international markets. Throughout this thesis, data from thirteen microsatellite markers were analyzed to answer several questions concerning, among others, the genetic structure of the species, the impact of exploitation and habitat fragmentation on its genetic diversity, and the identification of conservation units. Eight of these markers were specifically developed for *A. radiata* in the course of this study, and a total of more than 330 samples collected across the extant range of the species were analyzed. Results indicated that the radiated tortoise harbors moderate levels of differentiation, and two major genetically distinct populations were uncovered. Within each of these, additional peripheral subpopulations showed low but significant differentiation based on allele frequencies. A statistical approach based on multiple regression on distance matrices emphasized the role of some rivers as barriers to gene flow between tortoise populations, despite the fact that these are seasonal rivers that are completely dry several months per year. In combination with mtDNA analyses, it was found that the Menarandra River represents the most important historical and contemporary genetic boundary in this species. Bottleneck analyses suggested that the lower genetic diversity in harvested populations may not be attributed to recent exploitation, and mtDNA results pointed towards historically lower sizes of populations located east of the Menarandra, due to the

fragmented nature of habitat in that region, as the most likely explanation. Additional morphometric analyses found contrasting patterns of genetic and shell shape variation in *A. radiata*, and human exploitation appears to be the force explaining morphometric differences. The methodology developed to perform morphometric analyses allowed for a high assignment success rate among three geographic groups of tortoise populations, so this method might become a helpful tool for the management of tortoises confiscated from poachers and smugglers. Finally, separate genetic analyses for male and female tortoises revealed that dispersal is strongly male-biased in *A. radiata*. This thesis provides an interesting example of the array of possible applications of genetics to conservation, and the scientific interest that the radiated tortoise received during this study was a great catalyst for raising awareness concerning the dramatic decline of this emblematic Malagasy species.

Keywords: conservation genetics, conservation units, exploitation, genetic barriers, Madagascar spiny forest, male-biased dispersal, microsatellites, morphometrics, population differentiation, Testudinidae.

Table des matières

Liste des tableaux

Liste des figures

Liste des abréviations et sigles

ACP	analyse en composantes principales
ADN	acide désoxyribonucléique
ADNmt	ADN mitochondrial
ADNn	ADN nucléaire
AFLP	amplified fragment length polymorphisms
AMOVA	analysis of molecular variance
ANGAP	Association Nationale pour la Gestion des Aires Protégées
ARN	acide ribonucléique
AUF	Agence Universitaire de la Francophonie
bp	base pair
cc	cubic centimeter
CEL	Centre Ecologique de Libanona
CI	confidence interval
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	centimètre, centimeter
CMH	complexe majeur d'histocompatibilité
CRF	Chelonian Research Foundation
cyt-*b*	cytochrome *b* mitochondrial gene
d-loop	région de contrôle de l'ADNmt, mitochondrial control region
df	degrees of freedom
DNA	desoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ESU	evolutionarily significant unit

FAO	Food and Agriculture Organization of the United Nations
FES	Faculté des Etudes Supérieures
FQRNT	Fonds Québécois de la Recherche sur la Nature et les Technologies
ha	hectare
HDZ	Henry Doorly Zoo
HKY85	Hasegawa-Kishino-Yano model of DNA sequence evolution
HPD	highest posterior density
HWE	Hardy-Weinberg equilibrium
IBD	isolation by distance
ICTE-MICET	Institute for Conservation of Tropical Environments Madagascar
IM	isolation with migration
IUCN	International Union for the Conservation of Nature
kb	kilobase
kg	kilogramme, kilogram
km	kilomètre, kilometer
LEMEE	Laboratoire d'Ecologie Moléculaire et Evolution
m	meter
MA	million d'années
MCMC	Markov chain Monte Carlo
MEF	Ministères des Eaux et Forêts de Madagascar
min	minute
mm	millimètre, millimeter
mM	millimolar
MP	maximum parsimony
mtDNA	mitochondrial DNA
MU	management unit
myr	million years

NAD4	sous-unité 4 de la NADH déhydrogénase; NADH dehydrogenase subunit 4
ng	nanogram
NGO	non-governmental organization
NJ	neighbor-joining
NSERC	National Science and Engineering Research Council of Canada
ONG	organisme non-gouvernemental
PBZT	Parc Botanique et Zoologique de Tsimbazaza
PCA	principal component analysis
PCR	polymerase chain reaction
pmol	picomol
PNA	Parc National d'Andohahela
RAPD	randomly amplified polymorphic DNA
RPC	réaction de la polymérase en chaîne
s	second
SMM	stepwise mutation model
SOPTOM	Société pour la Protection des Tortues du Monde
T_A	annealing temperature
TCF	Turtle Conservation Fund
TFTSG	Tortoise and Freshwater Turtle Specialist Group
TPM	two-phase model
UICN	Union Internationale pour la Conservation de la Nature
UT	Université de Tuléar
µL	microliter
µM	micromolar
WCS	Wildlife Conservation Society
WWF	World Wildlife Fund for Nature
yr	year

Liste des symboles

a	Rousset's genetic distance
α	seuil statistique
b	standardized regression coefficient, regression slope value
d	geographic distance
d_2H_2O	distilled water
D_{CE}	Cavalli-Sforza & Edwards's chord distance
Δ_g	average size of multi-step mutations in TPM
$\delta\mu^2$	distance génétique basée sur les tailles d'allèles
F_{IS}	coefficient de consanguinité
F_{ST}	mesure de différentiation génétique
Φ	analogue aux statistiques F, en AMOVA
H_E	expected heterozygosity
H_O	observed heterozygosity
K	subgroup, cluster
k, k_m	number of alleles, mean number of alleles per locus
M	ratio between number of alleles and size range of alleles
m_1, m_2	migration rate of population 1, population 2
μ	mutation rate
N, n	sample size
N_e	taille efficace de la population, effective population size
Nm	gene flow
p	probability
p_m	proportion of multi-step mutations in TPM
π	nucleotide diversity

r	correlation coefficient or size range of alleles (to calculate M)
r_m	Mantel correlation coefficient
R	relatedness coefficient
R^2	coefficient of determination
R_{ST}	analogue du F_{ST} basée sur SMM
R_t	allelic richness, mean allelic richness
ρ	average distance to the root in a haplotype network
σ^2	variance
t	divergence time, or value of Student's statistic (in t-test)
Taq	*Thermus aquaticus*
θ_1, θ_2	effective size of population 1, population 2 scaled by μ ($\theta = 4N_e \mu$)
θ_A	ancestral effective population size scaled by u
u	substitution rate

À la mémoire de John Behler

Qui a consacré sa vie et sa carrière à la conservation

des tortues, à Madagascar comme ailleurs

"[Comparing history with evolution] is a false analogy! Evolution is a matter of environment and chance, acting over millions of years. But history is a matter of environment and choice, acting within lifetimes, and sometimes within years, or months, or days! History is Lamarckian!"

Red Mars, Kim Stanley Robinson (1993)

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CHAPITRE 1

Introduction

Cette thèse de doctorat porte sur la génétique des populations et la conservation de la tortue radiée, une espèce que l'on retrouve uniquement dans le sud de Madagascar. En guise d'introduction, je présente ici brièvement le fantastique mais très menacé laboratoire naturel que constitue l'île de Madagascar. Je présente également les tortues en général, avant d'entrer dans les détails concernant la tortue radiée. Finalement, j'arbore le sujet de la génétique en conservation, en expliquant succintement les différentes applications de la génétique aux problématiques de conservation.

1.1 Présentation de Madagascar

1.1.1 Histoire tectonique

Jusqu'au milieu du Jurassique, Madagascar était profondément imbriqué au cœur du supercontinent appelé Gondwana, qui rassemblait toutes les masses continentales de l'hémisphère sud actuel (Amérique du Sud, Afrique, péninsule indienne, Australie et Antarctique). Il y a environ 160 millions d'années (MA), la séparation de Madagascar s'amorce par la perte du contact entre le complexe Madagascar/Inde/Australie/Antarctique et l'Afrique, due à la formation du bassin somalien qui générera éventuellement le canal du Mozambique (Rabinowitz *et al.* 1983). Au cours du Crétacé, la masse continentale formée de Madagascar et de l'Inde se déplace graduellement vers le nord-est et se scinde en deux autour de 90 MA (Storey *et al.* 1995). Alors que l'Inde poursuivra sa dérive dans la même direction jusqu'à sa collision avec le continent asiatique, Madagascar demeurera environ au même endroit, à quelques centaines de kilomètres de l'Afrique (Krause *et al.* 1997).

1.1.2 Endémisme

L'isolement très ancien de Madagascar est vraisemblablement le principal facteur qui explique la nature unique de la biodiversité malgache. Plusieurs dizaines de millions d'années d'évolution à l'écart des pressions sélectives du continent africain ont façonné une faune et une flore tout à fait exceptionnelles. La biodiversité de Madagascar est caractérisée à la fois par un débalancement important de sa composition taxonomique par rapport aux communautés animales et végétales africaines et par des niveaux d'endémisme pratiquement inégalés (Myers *et al.* 2000). Malgré une superficie relativement faible de 594150 km^2 (légèrement supérieure à celle de la France), on compte à Madagascar près de 10000 espèces de plantes vasculaires endémiques, ce qui représente un taux d'endémisme d'environ 85% pour ce groupe (Gautier & Goodman 2003). Cette proportion est encore plus élevée chez certains taxons d'animaux : 92% des 340 espèces de reptiles, 99% des 197 espèces d'amphibiens et la totalité des 101 espèces de mammifères terrestres sont endémiques à Madagascar (Goodman & Benstead 2005). De plus, le nombre d'espèces endémiques ne cesse de s'accroître depuis quelques années, à mesure que de nouvelles études révèlent une diversité spécifique qui demeurait insoupçonnée jusque là (Mittermeier *et al.* 2006; Glaw & Vences 2007). Au total, cela représente plus de 3% des espèces de plantes vasculaires et d'animaux vertébrés de la planète (Myers *et al.* 2000).

1.1.3 Conservation et contexte socio-économique

Or, Madagascar n'échappe pas à la crise que subie présentement la biodiversité à l'échelle mondiale. Les espèces s'éteignent à des rythmes de 100 à 1000 fois plus élevés que par le passé (Pimm *et al.* 1995), ce qui a poussé certains auteurs à décrire notre ère comme étant la sixième période d'extinctions massives (Leakey & Lewin 1995), faisant référence aux cinq périodes précédentes révélées par les relevés paléontologiques. Dans la majorité des cas, la destruction de l'habitat est le principal facteur responsable du déclin et

de l'extinction éventuelle des espèces. À Madagascar, à peine 10% du couvert végétal original persiste toujours (Ganzhorn *et al.* 2001). Cette déforestation accrue est intimement liée à des facteurs socio-économiques et démographiques particuliers, en l'occurrence une extrême pauvreté (les données de 2002 du Programme des Nations Unies pour le développement humain classent la nation malgache au 166^{e} rang des 175 pays listés en fonction du PIB par habitant), une croissance démographique parmi les plus élevées de la planète (~3%) et l'absence de ressources et de techniques pour améliorer l'efficacité de l'agriculture et de l'élevage (Sussman *et al.* 1994). Les projets entrepris pour la conservation sont largement handicapés par le déséquilibre entre la croissance démographique et la croissance économique du pays, l'inadéquation des services publiques (éducation, santé), l'inaptitude du gouvernement à instaurer et faire appliquer les lois et les décrets, et la vulnérabilité de la population à différentes crises (épidémies, famines, catastrophes naturelles; Durbin *et al.* 2003).

1.1.4 L'exemple des feux

Le problème des feux illustre bien les difficultés auxquelles sont confrontées de nouvelles initiatives en sol malgache. Malgré un siècle de répression et de législation anti-feux, les agriculteurs et les éleveurs font brûler près de la moitié de toutes les prairies et savanes herbeuses du pays à chaque année, de même que plusieurs milliers d'hectares de forêt, accidentellement ou non (Kull 2002). Puisque la saison sèche est longue dans plusieurs régions, les feux naturels étaient fréquents avant même l'arrivée de l'homme (tel qu'en témoigne l'analyse de sédiments; Burney 1997) mais représentent maintenant moins de 5% des ignitions (Kull 2003). Les raisons qui motivent cette pratique de feux sont complexes. Comme dans plusieurs régions du monde, la culture sur brûlis (*hatsake* en malgache) est une pratique ancestrale à Madagascar. Malheureusement, elle n'est pas aussi profitable au rendement agricole que ce qui est véhiculé dans les croyances locales. En réalité, la productivité est plutôt faible: dans le sud de Madagascar, par exemple, un lot d'un

hectare de culture de maïs produit quatre tonnes de grains lors de la première année d'exploitation, mais environ seulement le huitième de cela à la cinquième année (Seddon *et al.* 2000). Une telle terre est donc généralement abandonnée après trois à cinq ans, et un foyer Antandroy (peuple du sud de l'île) cultive en moyenne dix hectares (Seddon *et al.* 2000). Toutefois, les considérations agricoles ne sont pas les seules à l'origine des incendies. À l'époque coloniale, les occupants français ont tenté de prohiber cette pratique, et en réponse les paysans ont utilisé les feux pour protester contre cette décision (Kull 2002). Même dans le contexte post-colonial d'aujourd'hui, les feux demeurent une façon de défier l'autorité, d'attirer l'attention du gouvernement, et surtout de s'approprier des terres inoccupées. Ainsi, même si les ressources nécessaires à une agriculture plus efficace devenaient disponibles, le combat contre les feux ne serait pas gagné pour autant.

1.1.5 Madagascar: un hotspot prioritaire

Parmi les quelques 17 millions d'hectares de végétation primaire résiduelle à Madagascar (Dufils 2003), seulement 1,7 million d'hectares sont compris dans le réseau des 46 réserves et parcs nationaux (Randrianandianina *et al.* 2003). De plus, une étude récente a démontré que même les formations végétales comprises dans les aires protégées n'étaient pas totalement à l'abri de la déforestation (Ingram & Dawson 2005), ce qui n'est pas surprenant compte tenu de la perception très négative de certaines communautés locales envers les aires protégées (Marcus 2001). À cause de la dégradation aiguë des habitats et de la menace qui pèse sur ceux qui sont toujours intacts, Madagascar compte au-dessus de 300 espèces désignées vulnérables ou menacées selon la *Red List* de l'Union Internationale pour la Conservation de la Nature (UICN). En vertu de ses taux de richesse et d'endémisme, du rythme de la déforestation et des paramètres socio-économiques de l'île, Madagascar a été désigné comme l'un des cinq *hotspots* prioritaires pour la conservation de la biodiversité mondiale (Myers *et al.* 2000). L'identification des facteurs qui expliquent le déclin des

espèces à Madagascar met en évidence la nécessité de développer des plans de conservation adéquats dans un délai restreint.

Dans ce contexte d'urgence, l'intérêt de la communauté scientifique envers Madagascar s'est considérablement accru au cours des 20 dernières années. La récente publication de *The Natural History of Madagascar* (Goodman & Benstead 2003), une synthèse des études et des connaissances de plus de 300 experts de plusieurs disciplines, atteste de cet engouement croissant pour la biodiversité et les écosystèmes malgaches. La première étape vers la conservation d'une espèce est l'acquisition de connaissances sur celle-ci. Dans le cas de plusieurs taxons à Madagascar, l'état des données est encore très préliminaire, et il reste beaucoup de travail à faire pour les biologistes avant de pouvoir prétendre comprendre l'écologie, l'histoire naturelle et les autres aspects qui caractérisent les espèces malgaches dans leur milieu naturel. Ceci est particulièrement vrai pour les reptiles de Madagascar (Raxworthy 2003), dont la tortue radiée, *Geochelone radiata*, qui n'avait jamais fait l'objet d'études scientifiques dans la nature jusqu'à il y a quelques années (Leuteritz 2002; O'Brien 2002).

1.2 Introduction aux chéloniens

1.2.1 Situation dans le monde

Les tortues (ordre: Testudines) forment un groupe très ancien, apparu au Permien (200 MA), qui compte actuellement environ 320 espèces, dont 46% sont considérées menacées ou vulnérables (IUCN 2004). Ainsi, la moitié des espèces de chéloniens risquent de s'éteindre avant 2100 si les tendances actuelles se maintiennent. La destruction et la fragmentation de l'habitat demeurent une cause importante du déclin des tortues à travers le monde (Mitchell & Klemens 2000). Plusieurs espèces de tortues utilisent une grande

variété d'habitats pour compléter leur cycle annuel : ruisseaux, rivières, rives, plaines inondées associées aux cours d'eau et milieu forestier jusqu'à plusieurs centaines de mètres de l'eau. Ainsi, elles sont particulièrement vulnérables à la fragmentation de l'habitat.

Toutefois, un grand nombre d'espèces sont également victimes d'une exploitation humaine très soutenue, et la collecte d'individus pour la consommation est considérée comme le facteur principal menaçant les espèces de chéloniens, étant impliquée dans le déclin de 66% des espèces inscrites à la *Red List* (Klemens & Thorbjarnarson 1995). Malgré la protection dont bénéficient plusieurs espèces, l'exploitation des tortues ne cesse de s'accroître; pour la consommation d'une part, mais également pour la confection de produits médicinaux avec leur carapace ou d'huiles avec leur foie, de même que pour les vendre sur le marché des animaux de compagnie (Thorbjarnarson *et al.* 2000). La plupart des espèces figurent à l'annexe I ou II de la CITES (*Convention on International Trade of Endangered Species*), mais cela n'a pas empêché le commerce illégal de tortues de s'amplifier au cours des dernières décennies. Par exemple, ce qui était autrefois un simple commerce domestique en Asie du sud-est est maintenant devenu un marché à grande échelle impliquant entre autres la Chine, l'Indonésie, le Vietnam, la Thaïlande et plusieurs milliers de tonnes de tortues annuellement (Thorbjarnarson *et al.* 2000).

1.2.2 Vulnérabilité à l'exploitation

Il est à noter que les traits de l'histoire naturelle de tous les chéloniens les rendent particulièrement vulnérables à la surexploitation. Leur démographie est caractérisée par un taux de mortalité très élevé des œufs et des nouveaux nés, un taux de survie des juvéniles et des adultes très élevé et une maturité sexuelle tardive (Gibbs & Amato 2000). Des études ont démontré que le maintien des populations n'était pas substantiellement affecté par des changements dans le taux de survie des œufs ou de la fécondité, mais qu'un accroissement de la mortalité des adultes pouvait provoquer de sérieux déclins (Congdon *et al.* 1993;

1994). Or, l'exploitation des tortues cible surtout les adultes, ce qui a poussé certains auteurs à formuler la crainte que l'idée d'une exploitation soutenable des populations de tortues soit un oxymore (Congdon *et al.* 1993).

1.2.3 Le cas des tortues terrestres

Les tortues strictement terrestres sont regroupées dans la famille des Testudinidae (sous-ordre Cryptodira), dont l'âge est assez récent et remonte probablement à 55-35 MA (Auffenberg 1974). Cette famille est d'ailleurs terminale dans le clade des tortues et son taxon frère est la famille des Emydidae, c'est-à-dire les tortues aquatiques telles que *Glyptemys, Emys* et *Graptemys* (Shaffer *et al.* 1997). Les Testudinidae comprennent 11 genres et environ 42 espèces (Bonin *et al.* 1998). Parmi celles-ci, les tortues du genre *Geochelone* (*sensus lato*; voir section 1.8 pour une précision taxonomique) sont assurément les plus connues, parmi lesquelles on compte les tortues géantes de l'archipel des Galápagos (*G. nigra*) et de l'atoll d'Aldabra (*G. gigantea*). Les 13 espèces actuelles de ce genre sont distribuées sur tous les continents de l'hémisphère sud, à l'exception de l'Australie et de l'Antarctique. Historiquement, ces espèces ont toujours été victimes d'une exploitation sévère (McDougal 2000). Leur grande taille et l'extrême facilité à les capturer en font des cibles de choix et une source de viande très appréciée, d'autant plus que le gibier est absent des îles où plusieurs d'entre elles se retrouvent. Du 17^{e} au 19^{e} siècle, la surexploitation des tortues géantes par les navigateurs et colons européens a provoqué l'extinction d'espèces aux Galápagos (MacFarland *et al.* 1974), aux Seychelles (Arnold 1979) et aux Mascareignes (Stoddart & Peake 1979; Arnold 1980).

Contrairement aux îles précédentes que l'homme n'avait jamais atteintes avant l'ère coloniale européenne, Madagascar est peuplé depuis un peu plus de 2000 ans (Dewar 1997). Les données subfossiles ont révélé que pratiquement toute la mégafaune de Madagascar était disparue après l'arrivée de l'homme sur l'île. Hippopotames nains

(*Hippopotamus lemerlei* et *H. madagascariensis*), oiseaux-éléphants (*Aepyornis* sp. et *Mullerornis* sp.) et au moins une dizaine de lémuriens de grande taille sont parmi les espèces qui se sont éteintes au cours des deux derniers millénaires (MacPhee & Marx 1997; Burney *et al.* 2004). Les chéloniens n'y ont pas échappé, puisque deux espèces de tortues géantes s'y sont déjà éteintes, *G. grandidieri* et *G. abrupta*, probablement à cause de leur surexploitation (Arnold 1979; Gerlach 2004; Pedrono 2008). Ces deux espèces étaient sympatriques avec la tortue radiée, mais cette dernière ne fut pas exploitée avec autant d'ardeur (du moins jusqu'à plus récemment), probablement en raison de sa taille beaucoup plus modeste.

1.3 La tortue radiée (*Geochelone radiata*)

1.3.1 Tortues malgaches

La tortue radiée est l'une de quatre espèces de Testudinidae endémiques à Madagascar. Deux de celles-ci, la tortue à queue plate (*Pyxis planicauda*) et la tortue à soc (*Geochelone yniphora*), figurent à la liste des 25 espèces de chéloniens les plus menacées du monde (TCF 2002). La seconde, la *angonoka*, est la tortue terrestre la plus rare du monde, les quelques 600 individus qui persistent étant confinés à une petite région autour de la Baie de Baly, dans le nord-ouest de l'île (Pedrono *et al.* 2004). La dernière espèce, la tortue araignée (*Pyxis arachnoides*), est partiellement sympatrique avec la tortue radiée dans le sud-ouest de Madagascar. Une étude moléculaire suggère que la forme ancestrale de *Geochelone* serait arrivée à Madagascar, probablement par dispersion océanique en provenance du continent africain (Austin *et al.* 2003), il y a environ 20 MA, et que *G. radiata* aurait divergé de son espèce sœur *G. yniphora* il y a 12-8 MA (Caccone *et al.* 1999*a*; voir la section 1.8 pour des précisions phylogénétiques et taxonomiques sur les Testudinidae). Les Testudinidae représentent probablement un des meilleurs exemples de colonisation de Madagascar par dispersion océanique: les tortues terrestres sont

particulièrement reconnues pour leur capacité à coloniser des îles (Quammen 1996), notamment à cause de leur flottabilité positive, de la position de leurs poumons près du haut de leur carapace leur assurant un maintien stable dans l'eau sans effort, et de leur long cou leur permettant de garder la tête hors de l'eau (Austin *et al.* 2003). De plus, de nombreux récits de matelots relatent que des tortues terrestres isolées pendant plusieurs mois sur des bateaux sans eau ni nourriture n'avaient peine à survivre (MacFarland *et al.* 1974), et des exemples contemporains de tortues ayant flotté sur des centaines de kilomètres dans l'Océan Indien sont documentés (Gerlach *et al.* 2007). Il est intéressant de noter que la situation n'est cependant pas la même pour tous les groupes qui ont colonisé Madagascar, même au sein des tortues. Ainsi, les espèces les plus apparentées à *Erymnochelys madagascariensis* (sous-ordre Pleurodira, famille des Podocnemididae), la seule espèce endémique malgache de tortue d'eau douce (qui figure également parmi les espèces les plus menacées du monde; TCF 2002), se retrouvent en Amérique du Sud, ce qui est attribuable à la vicariance reliée à la dérive des continents (Noonan 2000). Chez les autres groupes constituant l'herpétofaune malgache, la dispersion océanique est prédominante et inclut le cas des caméléons (Raxworthy *et al.* 2002), des geckos (Austin *et al.* 2004), et des grenouilles (Vences *et al.* 2003), mais la vicariance est invoquée pour les boas et les iguanes, qui, comme *E. madagascariensis*, partagent une ancestralité commune avec des espèces sud-américaines (Noonan & Chippindale 2006). La situation varie également parmi les autres groupes de vertébrés de Madagascar (Vences 2004; Masters *et al.* 2006; Yoder & Nowak 2006).

1.3.2 La forêt épineuse

La tortue radiée, appelée sokake en malgache, est restreinte à la forêt épineuse du sud-ouest. Ce biome renferme les plus hauts taux d'endémisme de l'île (90% pour les plantes vasculaires; Gautier & Goodman 2003). Il est caractérisé par l'abondance d'espèces de la famille endémique Didiereaceae (*Didierea* sp. et *Alluaudia* sp.), toutes couvertes

d'épines, d'où l'appellation de cette formation végétale. On y retrouve également les emblématiques baobabs (*Adansonia* sp.), de même qu'une vaste diversité d'espèces d'Euphorbiaceae et de Leguminosae (par exemple *Delonix* sp. et *Acacia* sp.) adaptées à la longue saison sèche et au caractère hautement sporadique des précipitations (Labat & Moat 2003). Il y tombe en moyenne entre 350 et 700 mm de pluie annuellement, presque exclusivement entre octobre et mars, et les températures y varient de 15 à 33 °C, ce qui est le plus grand écart de variation intra-annuelle de l'île (Donque 1972). A la végétation indigène se rajoutent les espèces de cactus du genre *Opuntia*. Natifs d'Amérique mais introduits pendant la période coloniale pour protéger des positions militaires, ces cactus ont rapidement envahi les paysages du sud de Madagascar et constituent maintenant une source d'eau et de nourriture indispensable aux communautés locales pendant la saison sèche (Kaufmann 2004).

La forêt épineuse, désignée comme une *Écorégion exceptionnelle* à l'échelle mondiale (Olson & Dinerstein 1998), est gravement menacée par la déforestation, et son pouvoir de régénération très faible la rend excessivement vulnérable aux pressions anthropiques. De plus, parmi tous les biomes qui contiennent encore une fraction substantielle de végétation primaire, la forêt épineuse est de loin celui dont la proportion incluse dans le réseau d'aires protégées est la plus faible (moins de 2%; Du Puy & Moat, 2003). Les facteurs responsables de la déforestation dans cette région sont la production de charbon de bois et la collecte de bois de chauffage, l'expansion des terres agricoles (incluant le *hatsake*) et l'expansion des pâturages pour les bovins et les ovins (Casse *et al.* 2004). Le problème du charbon, produit fondamental à l'économie locale, est particulièrement inquiétant. Les feux ciblent indistinctement toutes les espèces, et sa valeur est si faible que 30 arbres matures brûlés sont nécessaires pour produire l'équivalent de 1$ de charbon (Seddon *et al.* 2000).

1.3.3 Exploitation de la sokake et tabou

La destruction et la fragmentation de l'habitat ne sont pas les facteurs prédominants qui compromettent la survie à long terme de la tortue radiée. Malgré qu'elle soit nationalement protégée depuis 1961 (décret 60126) et listée à l'annexe I de la CITES depuis 1975, la demande pour cette tortue et ses produits dérivés sur le marché noir international s'est dramatiquement accrue au cours des dernières années. Par exemple, des saisies considérables ont été effectuées sur l'île avoisinante de La Réunion, où 1098 tortues ont été confisquées d'un bateau en 2002 (Szkaradek 2002), et plus de 1000 additionnelles lors d'une autre saisie en février 2003 (M. Emonot, communication personelle). En raison de l'éloignement des centres urbains, et de la répartition et de la densité très clairsemées des habitants dans le sud de Madagascar (Hoerner 1981), l'application des lois y est spécialement difficile. L'exploitation de cette espèce autrefois abondante est inquiétante (Behler 2002), d'autant plus qu'au commerce international s'ajoute la collecte d'individus pour la consommation locale.

L'aire de répartition de la tortue radiée est représentée à la figure 1.1. Celle-ci a diminué d'approximativement 20% au cours des 30 demières années (Juvik 1975; O'Brien 2002). L'extinction locale de la sokake à certains endroits et sa persistance au cœur de son aire de distribution sont intimement liées à la culture des différents peuples de cette région. En effet, ce n'est pas un hasard si la quasi-totalité de la distribution de la tortue radiée est comprise dans le territoire des Antandroy et des Mahafaly. Comme plusieurs aspects de la vie quotidienne à Madagascar, la relation de ces gens avec la tortue est dictée par un tabou (fady) ancestral. L'origine de ce tabou est nébuleuse, mais selon une légende, un ancêtre aurait un jour fait cuire une tortue dans un pot de terre cuite, mais celle-ci se serait débattue assez vigoureusement pour faire éclater le pot en morceaux. Depuis ce temps, les Antandroy et les Mahafaly refuseraient de consommer les sokake (Leuteritz 2002). L'intensité de ce tabou se manifeste à différents degrés, allant de la prohibition de tuer ou

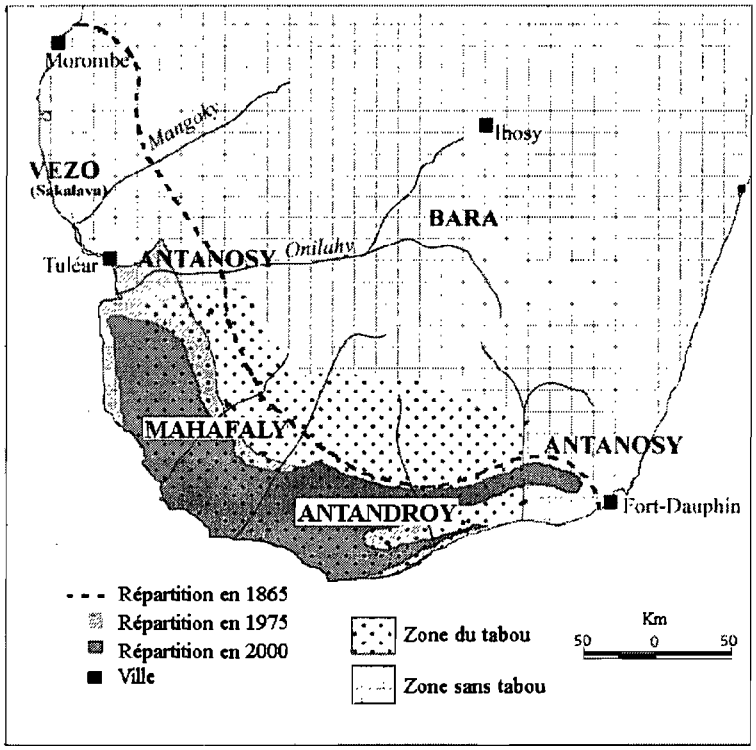

Figure 1.1 Répartition passée et actuelle de *Geochelone radiata* dans le sud de Madagascar. Les mots en lettres majuscules indiquent l'affiliation ethnique des communautés locales. Modifiée à partir de O'Brien (2002).

de manger les tortues chez certains clans, jusqu'à l'interdiction de les toucher ou de s'en approcher. Où la distribution de l'espèce atteignait auparavant les territoires des Sakalava, des Vezo et des Antanosy (des peuples qui n'observent pas le fady envers la tortue), celle-ci est maintenant disparue. Pour ces gens qui habitent un milieu très pauvre, la tortue radiée représente une source de viande non négligeable, et la demande suit l'accroissement de la population. Il est estimé qu'une famille consomme en moyenne deux tortues par semaine à Tuléar, où le prix d'une tortue adulte (1$) est semblable à celui d'une volaille (alors qu'il peut atteindre plus de 1000$ sur le marché international) (O'Brien 2002). Les braconniers, pourvus de charrettes et de pirogues, sont de plus en plus nombreux en provenance de l'extérieur de la zone du tabou et l'immigration au sein des groupes ethniques entraîne

l'érosion du fady (Lingard *et al.* 2003). Ainsi, des déclins significatifs ont été remarqués même aux endroits où le tabou protégeait jadis la tortue radiée (O'Brien *et al.* 2003). La situation s'est aggravée à un point tel récemment qu'un rapport publié vers la fin de ce projet de doctorat soutient que l'espèce s'éteindra dans la nature au cours des 45 prochaines années, soit une seule génération de tortue, si les tendances actuelles ne sont pas freinées (Randriamahazo *et al.* 2007).

1.3.4 Histoire naturelle et écologie

La tortue radiée peut atteindre une longueur de 40 cm et peser 12 kg. Elle est herbivore, se nourrissant d'herbes, de fruits et de plantes succulentes. Elle apprécie particulièrement les cactus du genre *Opuntia*, qui représentent une proportion importante de sa diète (Leuteritz *et al.* 2005). D'ailleurs, ces plantes introduites jouent un double rôle auprès de la tortue radiée, puisqu'il semble que les femelles optent souvent pour l'ombre de ces cactus lorsqu'elles choisissent l'emplacement de leur nid (36% des nids observés; Leuteritz 2002). Dans la nature, les femelles peuvent pondre jusqu'à trois fois par année, entre février et mai, et chaque nid peut abriter jusqu'à cinq œufs (Leuteritz & Ravolanaivo 2005). Les tortues radiées sont actives pendant les périodes les moins chaudes de la journée, soit tôt le matin et tardivement en après-midi, et sont plutôt inactives durant la saison sèche (de mai à octobre), les juvéniles atteignant même un état de dormance, à l'abri du soleil sous la végétation (Pedrono & Smith 2003). Une des adpatations de *G. radiata* au climat aride de la forêt épineuse est sa capacité à boire par ses narines, ce qui lui permet de s'abreuver à partir d'accumulations d'eau très peu profondes qui ne permettent pas la submersion de la bouche (Leuteritz 2003). La maturité sexuelle est atteinte autour de 15-19 ans. La longévité de la tortue radiée est impressionnante, et malgré que les données dans la nature soient inexistantes, elle peut probablement atteindre l'âge de 150 ans dans la nature (Pedrono 2008). Le record absolu appartient à une tortue radiée donnée à la reine de Tonga

en 1777 par le capitaine James Cook qui mourut en 1966, donc à l'âge vénérable d'au moins 189 ans (Pedrono & Smith 2003).

1.4 La génétique en conservation

L'objectif principal de cette étude est d'acquérir des informations fondamentales sur les paramètres génétiques des populations de tortues radiées afin d'élaborer des plans de conservation adéquats. Avant d'entrer dans les détails des objectifs, il est approprié ici d'expliquer les rôles que peuvent jouer des études génétiques dans une perspective de conservation d'une espèce.

1.4.1 Introduction à la discipline

La première mention de la contribution de facteurs génétiques au déclin des espèces remonte à il y a près de 25 ans (Frankel & Soulé 1981), mais la discipline de la « génétique de la conservation » (*conservation genetics*) a vraiment connu son essor au cours des cinq à dix dernières années. Les avancées techniques dans le domaine de la biologie moléculaire, dont le progrès de la réaction de la polymérase en chaîne (RPC) et le développement de plusieurs nouveaux marqueurs génétiques, de même que dans le domaine de l'écologie, tel que le perfectionnement des méthodes non invasives d'échantillonnage d'ADN, ont permis à ce domaine de prendre une place importante au sein de la vaste discipline de la biologie de la conservation (DeSalle & Amato 2004). Essentiellement, cette discipline emprunte des techniques et des méthodes à la génétique des populations et à la systématique moléculaire et les applique à des problématiques de conservation. Le tableau 1.1 résume les principaux rôles des études génétiques en conservation.

Puisque plusieurs des aspects mentionnés dans le tableau 1.1 seront appliqués au cas de la tortue radiée dans le cadre de ma thèse, il est de mise à ce point de résumer les

fondements théoriques de ceux-ci et leur pertinence en conservation. Concrètement, les quatre premiers rôles de la liste seront brièvement expliqués.

Tableau 1.1 Liste des principaux rôles de la génétique en conservation.*

Rôle en conservation
Évaluation du degré de consanguinité et de la perte de variabilité génétique
Résolution de la structure génétique des populations
Définition des unités de conservation au sein des espèces
Assignation d'individus à leur population d'origine
Détection de l'hybridation (pollution génétique)
Identification de sites et de génotypes pour les réintroductions
Résolution d'incertitudes taxonomiques
Identification spécifique d'individus (*barcoding*)
Estimation de la taille efficace et du ratio mâles:femelles des populations
Analyse des liens de parenté (généalogie ou *pedigree*)
Compréhension de la connectivité entre populations (*landscape genetics*)

*modifié à partir de DeSalle & Amato (2004)

1.4.2 Évaluation du degré de consanguinité et de la perte de variabilité génétique

1.4.2.1 Stochasticité génétique: la dérive génique

Il existe deux paradigmes en biologie de la conservation: celui qui s'intéresse à la persistance des petites populations et celui qui traite des causes du déclin des grandes populations (Caughley 1994). Malgré que le second soit plus déterminant pour la plupart des problématiques actuelles, le premier a suscité davantage de réflexions théoriques parce que tous les phénomènes qui régissent la viabilité des populations de petite taille peuvent être facilement résumés par une caractéristique commune: ce sont tous des processus stochastiques (Caughley 1994). Parmi ceux-ci, la dérive génique est le processus par lequel

la composition génétique d'une petite population varie dans le temps en raison de la transmission aléatoire des allèles d'une génération à la suivante. Ceci est d'abord dû au fait que tous les individus d'une population n'ont pas la même probabilité de contribuer à la génération suivante. Cette constatation a mené à la définition du concept de taille efficace d'une population (N_e; Wright 1931) qui réfère à la taille d'une population « idéale » qui aurait les mêmes propriétés (telle que la variation intergénérationnelle des fréquences alléliques due à la dérive) que la population en question. Cette valeur est généralement inférieure à la taille réelle de la population (Frankham 1995), et plusieurs causes y sont attribuables, dont une différence du succès reproducteur entre individus ou un ratio inégal des deux sexes dans la population (Whitlock & Burger 2004). Toutefois, même si les tailles réelle et effective d'une population convergeaient vers la même valeur, la dérive génique agirait tout de même, puisque les parents ne lèguent jamais à leur descendance que la moitié de leur génome, déterminée aléatoirement lors de la méiose. Les effets stochastiques de la dérive génique sont évidemment de plus grande amplitude dans les petites populations, c'est-à-dire les populations de petite taille efficace (Lande 1988). Les allèles rares sont plus susceptibles de disparaître et la perte de diversité génétique est plus rapide.

1.4.2.2 Perte de variabilité génétique

Les conséquences de la dérive ont des impacts à deux échelles temporelles distinctes. À long terme (échelle évolutive), la perte de diversité génétique d'une population réduit sa possibilité d'adaptation, puisque cette dernière dépend de la variabilité génétique (Milligan *et al.* 1994). Par conséquent, les populations génétiquement diversifiées sont moins vulnérables à des perturbations de leur milieu (Lande 1993). À court terme (quelques générations), la diminution de la diversité peut mener à une réduction de l'aptitude (*fitness*) des individus, mais les cas sont plutôt rares et concernent des situations de sélection balancée (avantage des hétérozygotes ou sélection dépendante de la fréquence) (Frankham *et al.* 2004). Les exemples classiques de telles situations sont le maintien de l'auto-incompatibilité chez les plantes (Charlesworth & Awadalla 1998) et le polymorphisme du

complexe majeur d'histocompatibilité (CMH), impliqué dans la réponse immunitaire chez les vertébrés (Hedrick & Kim 2000).

1.4.2.3 Consanguinité

Par contre, un autre processus influence rapidement l'aptitude des individus d'une population, surtout lorsque cette dernière est de petite taille. La perte de diversité génétique entraîne l'accroissement de la proportion d'individus homozygotes. En addition, la probabilité que des individus apparentés se reproduisent ensemble (consanguinité [*inbreeding*]) est plus élevée dans une petite population que dans une grande. Or, la consanguinité (le terme juste est plutôt endogamie) contribue également à l'augmentation de l'homozygotie, et cela réduit l'aptitude des individus dans pratiquement tous les cas étudiés de plantes et d'animaux (Frankham *et al.* 2004). Par exemple, Ralls & Ballou (1983) ont montré que le taux de mortalité juvénile était supérieur chez presque toutes les populations consanguines de mammifères étudiées. Cette baisse d'aptitude est généralement appelée dépression de consanguinité, et est due à l'expression d'allèles récessifs délétères rares dans la population, mais dont la probabilité de se retrouver à l'état homozygote est accrue à cause de la consanguinité. Il est intéressant de noter que l'interaction des effets des croisements consanguins et de la dérive génique ne fait qu'accroître la rapidité avec laquelle la perte de diversité génétique s'effectue.

1.4.2.4 Goulot d'étranglement

Il existe un phénomène démographique additionnel qui accentue l'impact de la dérive génique. Si une population subit une diminution drastique de son effectif (suite à une catastrophe naturelle ou une épidémie par exemple), la diversité génétique de la population sera par la suite entièrement déterminée par les allèles des quelques individus qui auront survécu. Ce phénomène est nommé goulot d'étranglement (*bottleneck*) et son intensité est

proportionnelle à la réduction de la taille de la population, et également au temps pendant lequel l'effectif de la population demeure faible.

1.4.2.5 En pratique

À l'aide de données provenant de marqueurs génétiques (voir section 1.5), il est possible de déterminer la variabilité génétique des populations, leur niveau de consanguinité (F_{IS} ; Weir & Cockerham 1984) ou la présence d'un goulot d'étranglement dans leur histoire (Piry *et al.* 1999; Garza & Williamson 2001). Ces informations permettent d'élaborer des plans de conservation qui assureront le maintien de la diversité ou réduiront l'impact de la consanguinité, par des programmes de translocation ou de reproduction en captivité, par exemple. Dans le cas de goulots d'étranglement, en fonction des marqueurs choisis, il est possible de détecter des contractions très lointaines (telles qu'une éruption volcanique qui a décimé une population de tortues géantes aux Galápagos il y a 90000 ans; Beheregaray *et al.* 2003) ou plutôt récentes (telles que la surexploitation qui a poussé au bord de l'extinction la tortue malgache *G. yniphora* au cours des 400 dernières années; Mandimbihasina 2004). Ces données peuvent avoir un impact sur les décisions prises, notamment en ce qui concerne les quotas pour la chasse (Lubick 2003; Roman & Palumbi 2003).

1.4.3 Résolution de la structure génétique des populations

La dispersion des individus d'une espèce est limitée par des traits du paysage, des barrières physiographiques et les capacités intrinsèques de cette espèce à la dispersion. Cela engendre l'isolement démographique et génétique de populations sur l'aire de répartition de l'espèce, un patron de répartition globalement désigné sous le terme de structure génétique (Milligan *et al.* 1994). Une vision différente de ce concept peut être résumée ainsi: la diversité génétique d'une espèce se répartit en deux niveaux fondamentaux (Allendorf 1983). La variation entre individus d'une même population

représente le premier, alors que le second est la variation qui existe entre différentes populations. Déterminer la manière dont se distribue la diversité génétique entre ces deux paliers équivaut à définir la structure génétique de l'espèce. La compréhension de cette structure est une préoccupation importante pour les biologistes dans plusieurs cas, ne serait-ce que pour choisir une échelle spatiale appropriée à laquelle les efforts de conservation devraient être consacrés. Dans le même sens, établir la distinction entre un ensemble de populations génétiquement isolées et une métapopulation régie par des événements de migration, d'extinctions locales et de recolonisations (Hanski & Gilpin 1991) est primordial.

Les méthodes les plus répandues pour investiguer la structure génétique des espèces sont basées sur les statistiques *F* introduites il y a plus d'un demi-siècle par Wright (1951) et leurs variantes (par exemple, Weir & Cockerham 1984; Slatkin 1985; 1993; 1995; Excoffier *et al.* 1992; Goldstein *et al.* 1995; Michalakis & Excoffier 1996; Weir & Hill 2002). L'exactitude de ces méthodes a récemment été remise en question puisqu'elles sont fondées sur des modèles idéaux de migration, de paramètres démographiques et d'évolution qui ne sont pas nécessairement réalistes (Whitlock & McCauley 1999) et qui ne sont probablement pas retrouvés dans les populations naturelles (Pearse & Crandall 2004). Toutefois, elles demeurent des outils intéressants pour quantifier la structure des populations (Neigel 2002), mais leurs lacunes ont stimulé le développement de nombreuses nouvelles méthodes, qui reposent sur des approches de maximum de vraisemblance et d'inférence bayésienne (voir Pearse & Crandall 2004 pour une revue des méthodes). Une des plus utilisées jusqu'à ce jour permet de déterminer, à partir d'un jeu de données moléculaires, le nombre de groupes d'individus génétiquement isolés par rapport aux autres (Pritchard *et al.* 2000), ce qui est très utile pour décider du nombre d'unités de conservation retrouvées au sein d'une espèce.

1.4.4 Définition des unités de conservation au sein des espèces

L'essor des études génétiques en conservation a mené à la formulation du concept d'unité de conservation. Puisque la plupart des espèces exhibent une certaine structure génétique, la gestion de celles-ci devraient s'assurer de considérer les groupes génétiquement et démographiquement isolés de façon distincte. Le maintien de l'intégrité des processus évolutifs, ou la présence d'adaptations locales chez ces populations, sont parmi les raisons invoquées pour justifier la reconnaissance de telles unités (Frankham *et al.* 2004). Deux types d'unités de conservation sont généralement reconnus (Moritz 1994). Le premier est issu des principes de la phylogéographie (Avise 1989) et est nommé « unité évolutivement significative » (*evolutionarily significant unit*; ESU). Une ESU est une population ou un groupe de populations historiquement isolé par rapport aux autres de l'espèce, avec une prédominance des processus ancestraux sur les adaptations actuelles. La théorie liée à ce concept suggère un critère génétique pour la reconnaissance des ESU: celles-ci devraient être réciproquement monophylétiques pour des locus mitochondriaux et démontrer une divergence significative dans leurs fréquences alléliques à des locus nucléaires (Moritz 1994).

Le second type d'unités de conservation est nommé « unité de gestion » (*management unit*; MU) et est plutôt basé sur la reconnaissance de processus récents et actuels. Si deux populations présentent des différences considérables dans leur composition génétique contemporaine, cela dénote que les niveaux de flux géniques (migration) sont si faibles entre ces deux unités qu'elles sont démographiquement et écologiquement indépendantes, d'où l'intérêt de les gérer séparément (Moritz 1994). En pratique, les MU sont reconnues comme des populations qui montrent des divergences significatives de leurs fréquences alléliques à des locus nucléaires ou mitochondriaux, sans se soucier de la phylogénie des allèles. Un des exemples célèbres de la reconnaissance des ESU et des MU pour une espèce menacée est le cas de la tortue marine *Chelonia mydas*, pour laquelle deux

ESU ont été trouvées (clade Indo-Pacifique et clade Atlantique-Méditerranée), chacune constituée de plusieurs MU (Bowen *et al.* 1992; Karl *et al.* 1992).

1.4.5 Assignation d'individus à leur population d'origine

Parmi les applications de la génétique en conservation, la possibilité d'assigner des individus à leur population d'origine est sûrement l'une des plus attrayantes pour les gestionnaires de la nature. Il existe une panoplie de méthodes qui permettent d'identifier la population d'origine d'un individu dont on connaît le génotype (pour plusieurs locus microsatellites par exemple) parmi un ensemble de populations définies *a priori*. Certaines méthodes sont très simples, telles que le calcul de probabilités à partir des fréquences alléliques des populations sources (Paetkau *et al.* 1995; Banks & Eichert 2000), alors que d'autres sont plus complexes, étant basées entre autres sur des algorithmes bayésiens (Rannala & Mountain 1997; Cornuet *et al.* 1999). Si des données suffisantes sont récoltées au sein des populations naturelles, l'identification de la provenance d'individus capturés sur le marché noir devient alors possible, ce qui permettrait de localiser les sites où le braconnage est important ou de relâcher les individus dans leur population d'origine (Manel *et al.* 2002).

1.4.6 Autres applications

Les autres rôles de la génétique de la conservation listés au tableau 1 ne seront pas décrits exhaustivement, puisque leur application concerne des cas particuliers qui ne seront pas abordés directement dans le cadre ma thèse. Les problématiques associées à ces applications n'en demeurent pas moins captivantes. Avec les perturbations provoquées par l'homme et l'introduction d'espèces exotiques, l'hybridation (avec ou sans introgression) menace maintenant la persistance de plusieurs espèces dans le monde (Allendorf *et al.* 2001) et les marqueurs génétiques permettent d'identifier et de suivre la progression de ce

phénomène. Par ailleurs, l'analyse des généalogies est devenue une composante capitale de plusieurs programmes de reproduction en captivité (Russello & Amato 2004). L'identification des espèces à partir d'un simple échantillon de tissu (*DNA barcoding*) permet de contrôler le commerce de produits animaux. Par exemple, il a été découvert que de la viande d'espèces de tortues protégées était vendue sous d'autres noms dans les marchés du sud des Etats-Unis (Roman & Bowen 2000). Finalement, la génétique du paysage (*landscape genetics*) connaît ses premiers balbutiements et s'intéresse à la connectivité et au flux génique à une échelle spatiale très fine (Manel *et al.* 2003), mais les tortues, avec leur temps de génération très long, se prêtent mal à ce genre d'études.

1.5 Marqueurs génétiques utilisés

Les microsatellites seront les marqueurs génétiques de prédilection dans cette étude, puisqu'ils permettent d'étudier la structure génétique d'une espèce à très haute résolution. Toutefois, l'utilisation de séquences mitochondriales n'est pas exclue, et pourrait venir compléter l'étude de la structure de la tortue radiée si les microsatellites en révélaient la présence. Les prochaines sections sont consacrées à la définition de ces deux classes de marqueurs génétiques.

1.5.1 Microsatellites

Les premiers marqueurs génétiques largement répandus qui ont pris pleinement avantage de la technologie de la RPC sont les microsatellites (Jarne & Lagoda 1996; Schlötterer 2004). Ils consistent en de courtes séquences de deux à quatre nucléotides répétées en tandem un grand nombre de fois ($GT_{(24)}$ par exemple). Leur popularité auprès des généticiens est attribuable à plusieurs de leurs caractéristiques : ils sont distribués partout dans le génome nucléaire des organismes eucaryotes, se retrouvent dans des régions non codantes de l'ADN (marqueurs neutres), démontrent des niveaux de polymorphisme

très élevés et sont des marqueurs co-dominants, c'est-à-dire qu'ils obéissent aux lois de l'hérédité mendélienne (Zhang & Hewitt 2003). Cette dernière caractéristique rend l'inférence de processus génétiques (tels que le flux génique) beaucoup plus facile avec des microsatellites qu'avec des marqueurs dominants tels que les RAPD (*randomly amplified polymorphic DNA*) ou les AFLP (*amplified fragment length polymorphisms*). De plus, quoique leur développement initial soit fastidieux et coûteux, et que les amorces utilisées soient souvent propres à une seule espèce, les protocoles de laboratoires sont rapides et faciles une fois les marqueurs mis au point, et le faible coût des analyses permet le traitement d'un grand nombre d'individus pour des sommes raisonnables.

Le polymorphisme des microsatellites est associé à des variations du nombre de répétitions du motif di-, tri-, ou tétranucléotidique entre les différents allèles. Le mécanisme de mutation qui explique ce changement vers un nombre inférieur ou supérieur de répétitions est probablement, en accord avec la plupart des études récentes, un glissement lors de la réplication de l'ADN et un mauvais appariement des brins homologues (Figure 1.2). Classiquement, un modèle de mutation nommé SMM (*stepwise mutation model*; Kimura & Ohta 1978) était utilisé pour illustrer l'évolution des microsatellites. Dans le modèle SMM, chaque événement mutationnel correspond à un gain ou une perte d'une seule répétition. Plusieurs mesures de différentiation génétique ont été conçues pour incorporer ce modèle dans le calcul d'une valeur qui tient compte de la longueur des allèles et non seulement de leurs fréquences relatives telles que les statistiques F (R_{ST} de Slatkin 1995; $\delta\mu^2$ de Goldstein *et al.* 1995). Toutefois, plusieurs études semblent montrer que ce modèle ne concorde pas avec la réalité dans bien des cas. Par exemple, 63% des mutations impliquaient plusieurs répétitions dans une étude sur l'homme (Huang *et al.* 2002) et 74% chez de la tortue verte *C. mydas* (FitzSimmons 1998). Ces résultats suggèrent qu'un modèle TPM (*two phase model*; Di Rienzo *et al.* 1994) est peut-être plus approprié. Dans ce modèle, les mutations d'une seule répétition et celles en impliquant plusieurs se produisent

selon une proportion variable. Il faut donc être prudent dans l'interprétation des mesures de différentiation génétique calculées à partir d'un modèle SMM.

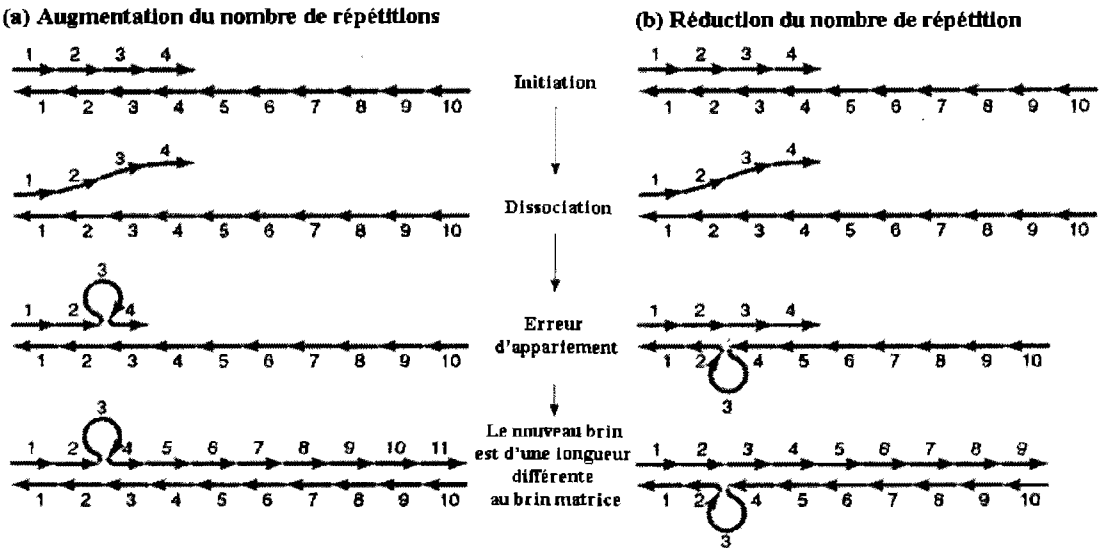

Figure 1.2 Schématisation de l'accroissement (a) ou de la diminution (b) du nombre de répétitions d'un microsatellite pendant la réplication. Les unités répétées en tandem sont symbolisées par les flèches. D'après Ellegren (2000*a*).

D'autres interrogations ont été avancées quant à la compréhension des modalités de l'évolution des microsatellites et, par conséquent, de leur applicabilité. L'homoplasie, c'est-à-dire le fait que deux allèles de la même taille soient d'une ancestralité différente, est un phénomène qui pourrait influencer les résultats obtenus (Angers & Bernatchez 1997), mais son impact est souvent contrebalancé par des niveaux si élevés de polymorphisme qu'il ne semble pas que ce soit un problème pour des études à l'échelle des populations (Estoup *et al.* 2002). La neutralité des microsatellites a également été remise en question (Zhang & Hewitt 2003). En outre, des études appuient la conservation de certains locus sur une échelle de temps étonnamment longue (FitzSimmons *et al.* 1995), ce qui s'oppose à la présumée neutralité sélective de ces marqueurs. Finalement, le taux de mutation des

microsatellites, habituellement estimé autour de 10^{-3} à 10^{-4} mutation par génération, semble être très hétérogène entre espèces (Ellegren 2000*a*) et même entre locus (Ellegren 2000*b*). Il est important d'être conscient de ces limitations des marqueurs microsatellites, mais malgré tout, ils demeurent certainement un des outils les plus puissants à ce jour pour étudier la structure génétique des populations naturelles.

La méthodologie associée à l'analyse des microsatellites est très simple: des amorces dessinées à partir des régions flanquantes du motif répété sont utilisées pour amplifier la séquence d'ADN répété par RPC. Par la suite, les fragments amplifiés peuvent être séparés sur un gel d'électrophorèse, ou bien leur taille peut être déterminée à l'aide d'un séquenceur automatique si les amorces ont été marquées en fluorescence. Comme l'étendue de variation de la longueur des allèles varie considérablement entre locus et qu'il existe plusieurs types de marqueurs fluorescents, il est possible de combiner plusieurs microsatellites pour les analyser simultanément sur le séquenceur, ce qui réduit considérablement les coûts associés à l'analyse d'un grand nombre d'individus.

1.5.2 ADN mitochondrial

Les séquences d'ADN mtochondrial (ADNmt) sont grandement utilisées dans les domaines de l'analyse phylogénétique et de la systématique moléculaire, mais sont également utiles pour des problématiques intra-spécifiques, telles que la phylogéographie des espèces. Le génome mitochondrial des vertébrés (15-20 kilobases) est constitué de 37 gènes codant 22 ARN de transfert, 2 ARN ribosomaux et 13 protéines impliquées dans le transport membranaire et la phosphorylation oxydative (Avise 2004). Une région non codante (1 kb), appelée région de contrôle (*D-loop*), est également présente et est à l'origine de la réplication et de la transcription.

Or, le génome mitochondrial possède certaines caractéristiques qui expliquent son utilisation très répandue. Tout d'abord, il est haploïde, et est hérité strictement de façon maternelle (dans la plupart des cas du moins). Ainsi, pour chacune des copies du génome mitochondrial qui sont passées à la génération suivante, quatre copies de l'ADN nucléaire (ADNn) autosomique sont transmises. Par conséquent, la taille efficace des marqueurs mitochondriaux est équivalente au quart seulement de la taille efficace de marqueurs nucléaires. La dérive génique est donc environ quatre fois plus rapide pour l'ADNmt, et les allèles y sont fixés (la fixation signifiant qu'un allèle atteint une fréquence relative de 100% dans la population) quatre fois plus vite (Ballard & Whitlock 2004). De plus, les taux de mutation de l'ADNmt sont beaucoup plus rapides que pour l'ADNn, fort probablement en raison de l'inefficacité des mécanismes de réparation de l'ADN dans les mitochondries (Ballard & Whitlock 2004). Finalement, l'ADNmt se comporte comme une seule molécule liée qui ne subit pratiquement pas de recombinaison chez les animaux (ce n'est pas le cas chez les plantes et les mycètes) (Birky 2001). Cela signifie que des phénomènes « d'auto-stop génétique » (*hitchhiking*) peuvent survenir via un balayage sélectif (*selective sweep*) ou une sélection négative (*background selection*) envers un des locus de l'ADNmt, ce qui accentue le rythme apparent de la dérive génique (Figure 1.3). Toutes ces caractéristiques font des séquences d'ADNmt des marqueurs idéaux pour étudier l'historique des populations d'une espèce (et identifier les ESU), à une échelle temporelle plus ancienne que celle révélée par les microsatellites. La région de contrôle accumule les mutations encore plus rapidement que les autres locus mitochondriaux, ce qui la désigne comme un marqueur génétique de choix.

Comme les microsatellites, les séquences d'ADNmt peuvent être amplifiées par RPC, mais contrairement à ceux-ci, les séquences des amorces sont généralement très bien conservées entre taxons (Kocher *et al.* 1989), de sorte qu'il n'est habituellement pas nécessaire de développer de nouvelles amorces lorsque l'on travaille sur une espèce pour la première fois. Notons par contre que la prémisse selon laquelle l'ADNmt des animaux ne

subit pas de recombinaison est sérieusement remise en question par plusieurs études récentes, même chez les vertébrés supérieurs (Tsaousis *et al.* 2005; Ujvari *et al.* 2007), de sorte qu'il soit possible que l'ADNmt ne se comporte pas exactement tel qu'on l'assume généralement (Ballard & Whitlock 2004).

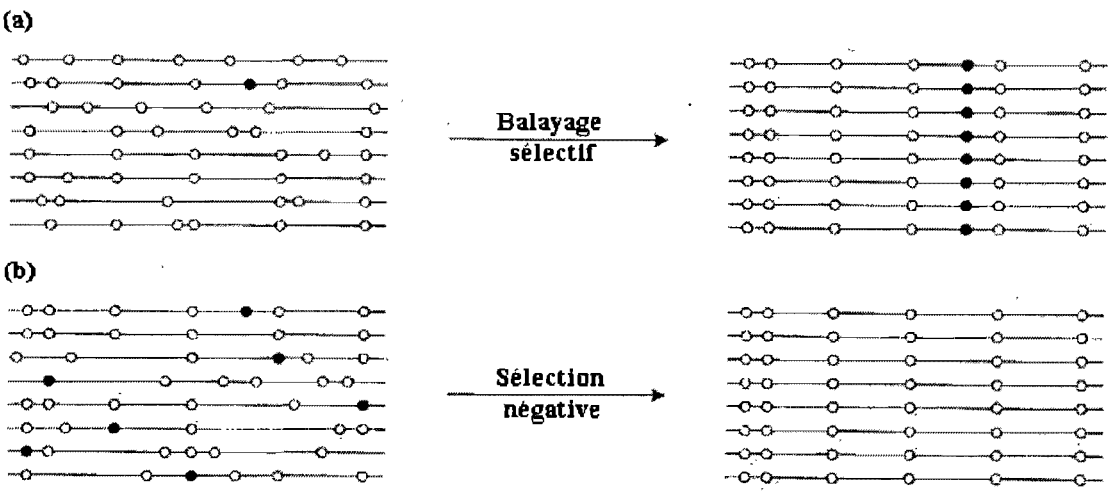

Figure 1.3 Effets de la sélection sur des marqueurs liés. Chaque diagramme représente des locus liés (tels que l'ADNmt), où les cercles vides représentent des allèles neutres et les cercles noirs des allèles sous sélection. En (a), l'allèle noir procure un avantage sélectif, alors qu'en (b) il est délétère. Suite aux processus sélectifs indiqués, un allèle est fixé à tous les locus, ce qui pourrait être confondu avec un effet de la dérive génique. D'après Avise (2004).

1.6 Objectifs de la thèse

Tel que mentionné précédemment, l'objectif global de cette thèse est de contribuer à la conservation de la tortue radiée grâce à l'acquisition d'informations génétiques sur les populations de l'espèce. Cet objectif général englobe plusieurs objectifs secondaires, décrits aux sections suivantes.

1.6.1 Développer des marqueurs génétiques pour la tortue radiée

Au début de ce projet, aucun marqueur microsatellite n'existait pour la tortue radiée. La première étape du projet était donc de développer ces marqueurs. En addition aux marqueurs développés spécifiques pour *G. radiata*, des microsatellites développés à partir de *G. yniphora* (Mandimbihasina 2004), et d'autres marqueurs propres à des espèces de tortues d'eau douce (King & Julian 2004) ont été testés. Par ailleurs, les amorces requises pour l'amplification de gènes mitochondriaux des tortues du genre *Geochelone* étaient déjà disponibles (Caccone *et al.* 1999*b*).

1.6.2 Déterminer la structure génétique de la tortue radiée sur l'ensemble de sa distribution

Les lémuriens sont sans contredit les taxons les plus étudiés à Madagascar, et des études phylogéographiques ont montré qu'ils n'exhibaient pratiquement aucune structure dans le sud-ouest de Madagascar, du moins pour des marqueurs mitochondriaux, aux échelles spécifiques et subspécifiques (Pastorini *et al.* 2003 ; Mittermeier *et al.* 2006). De plus, lors d'une analyse préliminaire de la structure génétique de *G. radiata* sur une fraction de sa distribution, le long de la côte sud-ouest de l'île, Leuteritz *et al.* (2005) n'ont trouvé chez 14 individus que deux haplotypes différents, distingués par une seule mutation, pour une séquence de 491 paires de bases du gène mitochondrial ND4. Les auteurs ont conclu qu'il n'y avait pas de barrières physiographiques, historiques ou actuelles, qui pouvaient expliquer la présence d'une structure chez cette espèce.

Toutefois, je crois que l'utilisation de microsatellites devrait révéler l'existence d'une certaine structure génétique entre les populations de tortues radiées. Il n'existe pas de sous-espèces reconnues de tortues radiées, mais la plus petite *Pyxis arachnoides*, sympatrique avec *G. radiata*, est subdivisée en trois sous-espèces (Pedrono 2008), ce qui

suggère la présence de barrières dans cette région. Il y a quatre fleuves importants dans le sud de Madagascar (Linta, Menarandra, Manambovo et Mandrare, de l'ouest vers l'est), et je crois qu'ils peuvent potentiellement constituer des barrières à la dispersion des tortues radiées. En effet, le lit de ses fleuves est à sec pendant la majeure partie de l'année (Battistini 1964), mais cette période coïncide avec le moment de l'année où les tortues radiées sont surtout inactives, les températures extrêmes les décourageant de franchir de grandes distances. De plus, même lors de la saison des pluies, les tortues radiées effectuent des déplacements très restreints, et parmi sept femelles suivies par télémétrie entre les mois d'octobre 2000 et mars 2001, le plus grand déplacement net enregistré fut de seulement 64 mètres (O'Brien 2002). Il semble donc peu probable que les tortues franchissent les fleuves, d'une largeur de 550 à 700 mètres (Battistini 1964), même lorsqu'ils sont à sec, ce qui sera vérifié à l'aide de l'analyse de microsatellites pour des populations réparties de part et d'autre de ces cours d'eau. Par conséquent, l'hypothèse à tester est qu'il existe de la différentiation génétique entre populations de tortues séparées par des rivières, l'hypothèse nulle étant l'absence de structure génétique.

Récemment, Yoder *et al.* (2005) ont mis l'emphase sur la nécessité de décrire les patrons de diversité génétique et de phylogéographie à Madagascar, si on aspire à conserver l'intégrité de la biodiversité de cette île. Ma thèse est la première étude qui s'intéresse à la structure génétique d'une espèce endémique à la forêt épineuse.

1.6.3 Déterminer les impacts de la fragmentation de l'habitat

Il a été discuté précédemment de l'étendue de la déforestation dans la forêt épineuse. Une des conséquences de cela est la fragmentation de l'habitat, qui se traduit par la persistance de parcelles de forêt isolées dans une matrice de savanes herbeuses anthropogéniques peu propices à la survie des tortues radiées. Le réseau des populations qui se retrouvent ainsi dispersées les unes par rapport aux autres peut être comparable au

concept de métapopulation (Hanski & Gilpin 1991). Or, une métapopulation a typiquement une taille efficace inférieure à celle d'une seule population homogène de même taille (Pannell & Charlesworth 1999), et les conséquences de cela ont été vues dans une section précédente (1.4.2). De plus, si l'impact de la fragmentation est assez important pour empêcher la migration des individus, les populations deviendront complètement isolées et seront sujettes aux menaces qui guettent les petites populations, dont la consanguinité. Idéalement, l'objectif des études génétiques qui portent sur la fragmentation de l'habitat est de quantifier à quel degré la fragmentation anthropogénique a modifié les paramètres génétiques des populations (Colgan *et al.* 2002). Toutefois, dans le cas de la tortue radiée, il est peu probable que des changements soient observés, compte tenu de l'échelle temporelle considérée et du temps de génération très long de cette espèce.

Néanmoins, certaines populations de tortues radiées vivent dans un milieu naturellement fragmenté, dans la partie est de la distribution de l'espèce. La composition géomorphologique hétérogène de cette région, dominée par des roches mères basaltiques et des sols sableux peu propices aux arbres de la forêt épineuse (Du Puy & Moat 1996), explique cette fragmentation. Ainsi, il sera possible de comparer les paramètres génétiques de populations de cette région avec ceux de populations qui vivent dans un milieu homogène, afin d'évaluer les impacts que la fragmentation anthropogénique pourrait avoir sur ces populations de grande taille. L'hypothèse testée sera que la diversité génétique des populations fragmentées de l'Androy est inférieure à celle des populations des territoires Mahafaly, à l'ouest.

1.6.4 Déterminer les impacts de l'exploitation humaine

Plusieurs études récentes suggèrent que les contraintes que l'homme impose aux populations naturelles peuvent entraîner leur évolution de façon très rapide (Reznick & Ghalambor 2002). Dans le cas de la tortue radiée, O'Brien (2002) a remarqué que plusieurs

caractéristiques démographiques et individuelles de la tortue radiée varient entre les zones où l'espèce est exploitée par l'homme et celles où la collecte d'individus n'est pas effectuée. Par exemple, les densités d'individus sont beaucoup plus faibles, les tortues adultes ont des tailles inférieures et le ratio mâles:femelles est biaisé contre les mâles dans les populations exploitées. On ignore pour le moment si la réduction de la taille de ces populations et la sélection systématique contre les grands individus ont des répercussions sur leurs paramètres génétiques. Puisque quelques rares populations qui persistent toujours en périphérie des territoires Antanosy sont possiblement exploitées depuis quelques centaines d'années par les tribus locales, la comparaison de celles-ci avec des populations préservées grâce au fady permetttra de quantifier l'impact de cette exploitation. Je m'attends à observer une diversité génétique réduite chez ces populations, à condition bien sûr que l'exploitation soit suffisamment ancienne, ce qui est difficile à déterminer d'après le peu de données qui existent sur l'historique de l'exploitation des tortues radiées. L'hypothèse testée est que les populations exploitées démontrent une diversité génétique inférieure à celle des populations qui demeurent non-exploitées.

1.6.5 Identifier des unités de conservation

L'utilisation de marqueurs microsatellites permettra d'identifier des MU parmi les populations de tortues radiées. Il est également possible que des ESU soit identifiés si la structure de l'espèce est suffisamment ancestrale. Le discernement de telles unités de conservation pourrait, par exemple, fournir des arguments pour le choix de l'emplacement de nouvelles aires protégées dans le sud malgache, pour prendre en considération l'ensemble de la diversité génétique de l'espèce.

1.6.6 Établir une méthode permettant d'assigner des individus à leur population d'origine

Tel que décrit à la section 1.4.5, plusieurs méthodes existent pour permettre d'identifier la population d'origine d'un individu à partir de son génotype multi-locus. C'est un outil qui serait particulièrement intéressant pour gérer le commerce illégal de la tortue radiée et réintroduire les individus confisqués. Toutefois, l'accès à des outils génétiques à Madagascar est plutôt restreint, voire impossible, ce qui réduit considérablement l'intérêt de telles méthodes si elles sont destinées à être utilisées directement sur le terrain. Pour cette raison, une analyse de la variation morphométrique des tortues radiées sera réalisée. La carapace et le plastron des tortues sont des structures biologiques qui se prêtent bien à l'analyse statistique des formes, de sorte que plusieurs études leur ont été consacrées, et ce depuis bien longtemps (par exemple, Jolicoeur & Mosimann 1960). À l'aide de méthodes statistiques multivariées telles que l'analyse en composantes principales (ACP) et l'analyse discriminante (Legendre & Legendre 1998), il est possible de discerner des groupes d'individus morphologiquement semblables. De plus, certaines corrections de ces méthodes classiques permettent de distinguer efficacement l'effet de la taille des individus de l'effet de leur forme comme telle (Somers 1986). Ces méthodes ont été utilisées à plusieurs reprises pour des études de taxonomie à l'échelle des espèces ou des sous-espèces de tortues, mais plutôt rarement au niveau des populations, puisqu'une différentiation est souvent impossible à observer (Iverson 1977; Reynolds & Seidel 1983). Toutefois, ces études utilisent généralement un faible nombre de caractères. Ici, près de 40 mesures relevées sur la carapace et le plastron des tortues échantillonnées seront utilisées. L'objectif est évidemment de mettre au point une méthodologie qui permette l'identification approximative de l'origine géographique des individus strictement à partir de données morphométriques.

1.7 Organisation de la thèse

Chacun des chapitres suivants constitue un article publié ou qui sera soumis pour publication prochainement, qui s'intéresse à un ou plusieurs des objectifs décrits dans la section précédente. Le chapitre 2 est une brève note technique qui décrit le développement de marqueurs microsatellites chez *G. radiata*. Le chapitre 3 relate les premiers résultats des analyses des données microsatellites chez six populations de tortues radiées, et met en évidence le rôle de certaines rivières dans la structure de l'espèce. Le chapitre 4 traite de la variation morphométrique entre populations de tortues radiées et du développement d'une méthodologie pour identifier le site d'origine des individus. Le chapitre 5 est le plus important chapitre de la thèse; avec un échantillonnage exhaustif et des données provenant autant de marqueurs microsatellites que d'ADNmt, il complète l'analyse de la structure de l'espèce amorcée au chapitre 3, analyse les impacts potentiels de l'exploitation et décrit l'histoire évolutive récente de l'espèce. Dans le chapitre 6, des résultats surprenant révélant la fidélité aux sites de ponte des tortues radiées femelles sont présentés. Le chapitre 7 présente la caractérisation de la seule population de tortues radiées qui persiste dans le sud-est de Madagascar et expose mes observations et recommandations relatives au déclin dramatique de l'espèce depuis quelques années. Finalement, le chapitre 8 (conclusion) est une synthèse concise des principaux résultats de mes travaux de doctorat et des aspects qui demeurent inexplorés et qui mériteront davantage d'attention dans le futur.

1.8 *Geochelone* ou *Astrochelys*?

Avant de conclure cette introduction, une précision concernant la nomenclature de la tortue radiée (et des tortues terrestres en général) est de mise. Au début de mes études doctorales, le nom le plus fréquemment utilisé pour la tortue radiée était *Geochelone radiata*. Par contre, au fil des années, la tendance s'est graduellement tournée vers l'utilisation de *Astrochelys radiata* (et *A. yniphora* pour la tortue à soc plutôt que *G.*

yniphora). L'acquisition continuelle de nouvelles données morphologiques et moléculaires qui influencent les hypothèses sur l'histoire évolutive de ces organismes a inévitablement pour conséquence d'entraîner des modifications taxonomiques, afin de respecter les principes d'une classification phylogénétique (DeQueiroz & Gauthier 1990).

Or, la taxonomie des Testudinidae a toujours été un ardent débat en herpétologie, particulièrement au niveau du genre *Geochelone* (Bour 1980; Gerlach 2001). Les nombreux cas de parallélisme et la très grande plasticité morphologique au sein de ces tortues ont souvent été invoqués en tant que sources d'erreurs importantes lors des tentatives de retracer la phylogénie de ce groupe (Auffenberg 1974; Pritchard 1994; Claude *et al.* 2003; Fritz *et al.* 2007). C'est ainsi que *Pyxis* et *Astrochelys*, les deux genres malgaches actuels, ont parfois été associés à des groupes très différents, plusieurs études plaçant *Pyxis* avec d'autres genres de tortues terrestres de petite taille tels que *Psammobates* ou *Homopus*, tandis qu'*Astrochelys* était associé aux autres espèces de grande taille du genre *Geochelone* (par exemple, Crumly 1984; Meylan & Sterrer 2000). Par contre, une analyse de l'ensemble des données génétiques disponibles (séquences mitochondriales et nucléaires) a permis de formuler une hypothèse solide concernant l'évolution des Testudinidae (Le *et al.* 2006), représentée à la figure 1.4. Plus spécifiquement, un consensus concernant les tortues terrestres de l'Océan Indien a émergé: une forme unique ancestrale aurait d'abord colonisé Madagascar. Elle serait à l'origine d'un groupe comptant bien sûr les genres malgaches actuels *Astrochelys* et *Pyxis*, mais également toutes les tortues géantes de l'Océan Indien, c'est-à-dire *Dipsochelys* à Madagascar, Aldabra, et les Seychelles granitiques, et *Cylindraspis* à la Réunion, Maurice et Rodrigues (Gerlach 2004). Ce scénario est d'ailleurs celui que j'ai présenté à la section 1.3.1. Il est à noter que *Cylindraspis* est absent des analyses de Le *et al.* (2006), mais d'autres travaux suggèrent qu'il représente le taxon frère à toutes les autres tortues terrestres de l'Océan Indien (Austin & Arnold 2001).

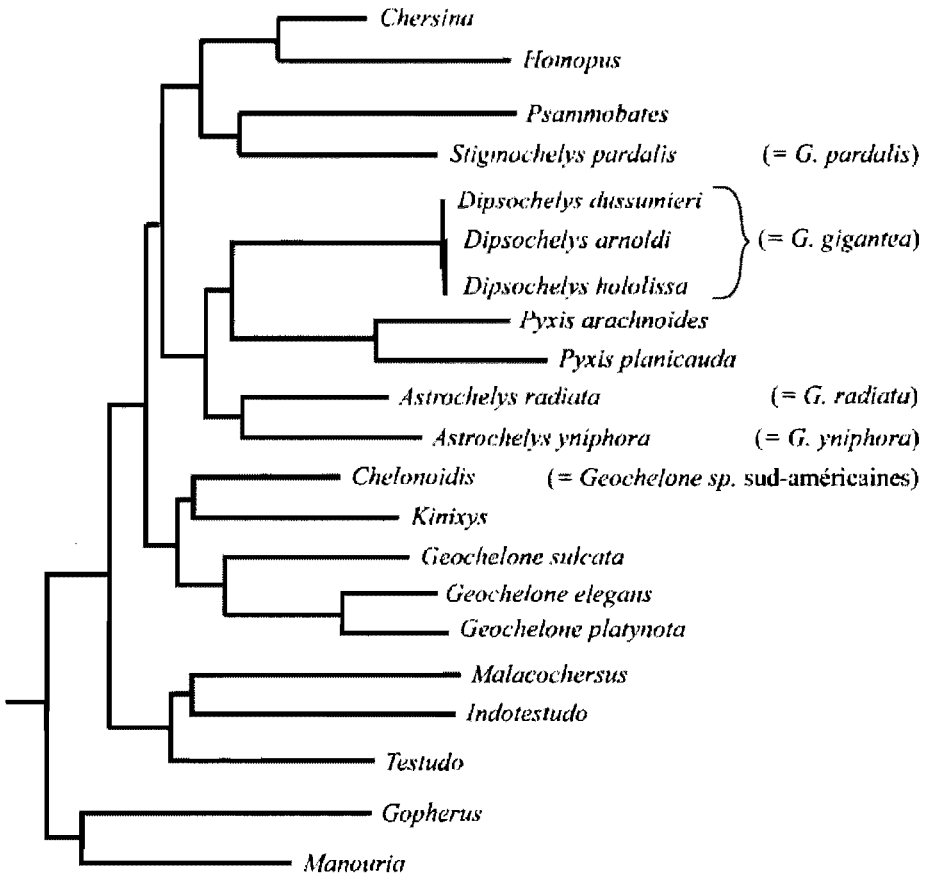

Figure 1.4 Relations phylogénétiques au sein de la famille des Testudinidae, mettant en évidence la paraphylie du genre *Geochelone* lorsqu'il comprenait, en plus des trois espèces actuelles, *G. radiata, G. yniphora, G. gigantea, G. pardalis,* et quatre espèces sud-américaines. (Modifié à partir de Le *et al.* 2006).

Il ne fait donc aucun doute que le genre *Geochelone*, lorsqu'il regroupait *G. radiata, G. yniphora, G. gigantea* (= *Dipsochelys*, Seychelles et Aldabra), *G. pardalis* (Afrique de l'Est) et les quatre espèces d'Amérique du Sud (*G. nigra, G. denticulata, G. carbonaria* et *G. chilensis*), était hautement paraphylétique. En effet, ce genre était paraphylétique non seulement par rapport à *Pyxis*, mais également par rapport à *Chersina, Psammobates, Homopus* et *Kinixys* (Fig. 1.4). Les changements qui s'imposaient furent graduellement appliqués à la nomenclature de ces tortues, ne conservant que trois espèces au sein du genre

Geochelone, soit *G. sulcata, G. elegans* et *G. platynota*. L'utilisation du genre *Astrochelys* est devenue très répandue au cours des dernières années, et la tortue radiée et la tortue à soc sont dorénavant listées sous ce genre dans la plus récente édition de la *Liste Rouge* de l'UICN.

Toutefois, j'ai choisi de conserver le genre *Geochelone* pour cette introduction, ainsi que pour les chapitres 2 à 4. Premièrement, les chapitres 2, 3, et 4 ont été publiés avec ce nom de genre, qui était toujours d'usage courant au moment de la soumission des manuscrits. De plus, pour tous les herpétologistes, le nom *Geochelone* évoque la grande taille et la magnificence de ces tortues emblématiques, et le genre lui-même est étroitement associé à l'émergence de la théorie évolutive, puisque ce sont, en partie, les tortues géantes des Galápagos, désignées pendant près de deux siècles par le nom *Geochelone nigra*, qui inspirèrent à Charles Darwin ses réflexions sur la théorie de la sélection naturelle.

Toutefois, malgré la valeur historique du genre *Geochelone*, je me suis résigné, sous le poids accablant des résultats phylogénétiques, à employer la nouvelle nomenclature dans la seconde moitié de ma thèse (chapitres 5 à 8, écrits plus récemment) et ainsi rejoindre la tendance actuelle. Ce compromis a aussi une signification symbolique reliée au sujet de cette thèse. En effet, les réelles inquiétudes de la communauté internationale envers la conservation de la tortue radiée ont vraiment commencé à se manifester, par coïncidence, lorsque *Geochelone radiata* devint *Astrochelys radiata*, et que cette thèse était déjà à moitié achevée. J'espère que la transition taxonomique ne causera pas de confusion auprès des lecteurs.

CHAPITRE 2

Characterization of polymorphic microsatellite markers for the endangered Malagasy radiated tortoise (*Geochelone radiata*)[1]

2.1 Résumé

La tortue radiée (*Geochelone radiata*) est une espèce menacée endémique à Madagascar, où elle habite la forêt épineuse semi-aride de l'extrême sud de l'île. La destruction de l'habitat et la chasse illicite menacent sérieusement cette espèce, tel qu'en démontre la réduction importante de son aire de répartition au cours des 30 dernières années. Afin de planifier des actions appropriées pour sa conservation, il est essentiel d'acquérir des connaissances sur sa structure génétique. Ici, 145 échantillons sanguins ont été collectés parmi trois populations de tortues radiées dans le sud-ouest de Madagascar. Huit marqueurs microsatellites polymorphes ont été trouvés, et la diversité allélique et l'hétérozygosie observée étaient élevées pour tous les marqueurs.

2.2 Abstract

The radiated tortoise *(Geochelone radiata*) is an endangered endemic species from Madagascar that inhabits the semi-arid spiny forest of the southern part of the island. Habitat destruction and illegal harvesting greatly threaten this species, as attested by the significant reduction of its distribution area in the past 30 years. In order to undertake appropriate conservation actions, it is essential to acquire a better knowledge of its genetic structure. For this study, 145 blood samples were collected from three populations of

[1] Rioux Paquette S., Shore G.D., Behncke S.M., Lapointe F.-J. & Louis, Jr. E.E. 2005. Characterization of polymorphic microsatellite markers for the endangered Malagasy radiated tortoise (*Geochelone radiata*). *Molecular Ecology Notes* **5**: 527-530.

radiated tortoises in southwestern Madagascar. Eight microsatellites loci were found to be polymorphic, and allelic diversity and observed heterozygosity were high for all markers.

2.3 Primer note

The status of Testudines across the world is preoccupying, some of their natural history traits (i.e., late sexual maturity, very low egg and hatchling survivorship) rendering their conservation particularly difficult (Gibbs & Amato 2000). The radiated tortoise (*Geochelone radiata*) is one of five chelonian species endemic to the island of Madagascar, all of which are threatened. Although this species has been protected nationally since 1961 and listed on the Appendix I of the CITES since 1975, its distribution area has been reduced by over 20% in the last 30 years (Juvik 1975; O'Brien *et al.* 2003). The radiated tortoise inhabits the semi-arid spiny forests of the south (dominated by Euphorbiaceae and Didiereaceae species), an ecosystem heavily affected by anthropic pressures such as slash-and-burn agriculture and charcoal production (Seddon *et al.* 2000). These activities have been considerably intensified in recent years to supply for increasing demands of a booming human population on the island (FAO 1998). Moreover, aside from the threat caused by habitat loss and fragmentation, demand for Malagasy tortoises and their derived products has dramatically increased on the international market, and harvesting of this once very abundant species is worrying (Behler 2002). It has been estimated that over 45,000 individuals transit every year via Tulear (O'Brien *et al.* 2003), the biggest city in southern Madagascar and a major trading point for tortoises. Consequently, densities vary from 20 individuals per ha where traditional beliefs prohibit tortoise consumption or collection to 0.6 individuals per ha in areas where it is harvested (Lingard *et al.* 2003).

There has been no study using nuclear DNA markers on *Geochelone radiata* and no species-specific microsatellite primers are available. Thus, to investigate population genetic structure and gene flow across the range of this endangered species, eight polymorphic

microsatellite markers were developed and are presented in this paper. The methodology followed to isolate microsatellites is based on a modification of Kandpal *et al.* (1994) in Moraga-Amador *et al.* (2001), and was utilized previously in the laboratory (An *et al.* 2004). It comprises a method for enriching the selection of recombinants containing specific microsatellite repeats. Genomic DNA was extracted from a blood sample drawn from a free-ranging individual from the Toliara province in southern Madagascar. Procedures for DNA digestion with restriction enzyme *Sau*3AI, ligation with *Sau*3AI linkers, hybridization with the biotinylated probe (5'-$(CA)_{15}$TATAAGATA-Biotin), ligation to pCR TOPO vectors (Invitrogen, San Diego, CA) and transformation into *Escherichia coli* are described in details in An *et al.* (2004). Identification of plasmids containing repeat inserts was carried with a Quick-light Hybridization Kit by Lifecodes Corporation (Stamford, CT), following the directions of the manufacturer. Among the 3072 colonies screened, 396 positives were found and sequenced. DNA was isolated from plasmids according to protocol 15 in Hillis *et al.* (1996). Cycle sequence reactions were carried out using an ABI 480 thermocycler (Applied Biosystems Inc., Foster City, CA) in a 10.5 µl volume with 2.5 µl of sequencing buffer, 0.5 µl of M13 forward or reverse primer (reactions were carried for both), 1.0 µl of BigDye, 6 µl of d_2H_2O and 0.5 µl of template DNA. Cleaned products were sequenced on an ABI 3100 automated sequencer (Applied Biosystems Inc., Foster City, CA). Fourteen primer pairs were designed from the flanking regions of microsatellites using the MACVECTOR 6.5.3 software package (Oxford Molecular Group, Campbell, CA), and eight of these were found to be polymorphic, after eight individuals were sequenced.

Blood samples were collected from radiated tortoises sampled in three regions across the southwestern part of the species' range, relatively close to the coast. These three populations, designated as Itampolo, Androka and Androy, were respectively represented by 26, 100 and 19 individuals. DNA was extracted from these samples according to standard procedures (Sambrook *et al.* 1989). PCR amplification was carried out in a 25 µl

reaction volume with approximately 50 ng of genomic DNA as template. Final amplification conditions consisted of 12.5 pmol of unlabeled reverse primer, 12.5 pmol of fluorescently labeled forward primer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, and 0.5 unit of *Taq* DNA polymerase (Promega, Madison, WI). The thermal profile for amplification consisted of 34 cycles of 30 s at 95°C, 45 s at a primer-specific annealing temperature (Table 1) and 45 s at 72°C, followed by a final extension of 10 min at 72°C. Allele sizes were determined by analysis on a 16-capillary ABI 3100 automated sequencer and fragment length was assigned with GeneMapper (Applied Biosystems Inc.) using the ROX500(-250) size standard. Observed and expected heterozygosity values and tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were carried out with GENEPOP (Raymond & Rousset 1995). When computing tests, probabilities were corrected according to Bonferroni procedures (Rice 1989). Primer sequences, annealing temperature, repeat motif, dye used to label the forward primer and Genbank accession number for each locus are presented in Table 2.1. Table 2.2 lists sample sizes, number and size range of the observed alleles along with the observed and expected heterozygosity values for the three studied populations.

No significant linkage disequilibrium was observed among all pairs of loci, and most of the loci were in agreement with HWE expectations. The presence of null alleles could be responsible for deviations from HWE, particularly in the case of RAD313. For this marker, the PCR success was considerably lower than for other primers (approximately 83%) and this could result from non-amplifying homozygotes. Further investigation is required to determine whether these deviations are due to null alleles or demographic processes (the Androka population is out of HWE for three markers out of eight). In all cases, the markers exhibit a large allelic diversity, the total number of alleles for the species ranging from 10 for RAD573 to 23 for RAD313. They should prove valuable for the study of population genetics of *G. radiata* and are currently used in a conservation genetics study on this endangered species.

Table 2.1 Primer sequences and characteristics of eight *Geochelone radiata* microsatellites

Primer	Sequence	Repeat motif	T_A (°C)	Dye	Genbank accession no.
RAD14 F	5'- GAT CCC CAA CTG TCA CCA C -3'	$(CT)_{12}(AC)_{14}$	56	HEX	AY900651
RAD14 R	5'- AAA ATG TTG CTC TCC TAA ATG C -3'				
RAD27 F	5'- AAA ATC TAC CAA GGT CTG CAA AG -3'	$(TG)_7TA(TG)_{16}$	56	FAM	AY900652
RAD27 R	5'- TTA CAG AGC ATC AGC AAG GC -3'				
RAD284 F	5'- GTG CTG AAC AGA GGC TGA TG -3'	$(GT)_{22}$	58	HEX	AY900653
RAD284 R	5'- CAC ACA CAC AGA CAG AAG ATT ATT -3'				
RAD313 F	5'- AGT TGT TTT CCC ACC CCC -3'	$(GT)_{12}GAG(GT)_3(GA)_6(GT)_5(GA)_{11}$	60	HEX	AY900654
RAD313 R	5'- TCC CCA AGA CAC CTG CTG -3'				
RAD542 F	5'- TCC TGT GAT TGT TTC ATA GAA CG -3'	$(CA)_{13}$	54	FAM	AY900655
RAD542 R	5'- TCT GCT CCT TCC TGT GTG C -3'				
RAD573 F	5'- TGA ACA GAA CGA TCC TCC CC -3'	$(CA)_6(TGCA)_2(CA)_2CG(CA)_4$	60	HEX	AY900656
RAD573 R	5'- GGG AAA GCC AGG GCA CTA G -3'				
RAD891 F	5'- TAT TCA CCC ACG AAA GCT CA -3'	$(CT)_{12}(CA)_6CG(CA)_9$	58	HEX	AY900657
RAD891 R	5'- GGT TGT TGG AGA AAG GAG GA -3'				
RAD932 F	5'- GGT AGA TAG TTC CTT CAG CCT TG -3'	$(GT)_{15}$	60	FAM	AY900658
RAD932 R	5'- TCC CCT CTT TTT CTG TCT CAT AG -3'				

Table 2.2 Sample size (N), number (k) and size range of alleles, observed (H_O) and expected (H_E) heterozygosity values for three populations of *Geochelone radiata*

Locus	Population	N	k	Size range (bp)	H_O	H_E
RAD14				218 - 262		
	Itampolo	26	15		0.769	0.907
	Androka	94	20		0.819	0.923
	Androy	18	10		0.722	0.870
RAD27				230 - 270		
	Itampolo	26	10		0.731	0.737
	Androka	99	13		0.768	0.770
	Androy	19	4		0.579	0.624
RAD284				209 - 243		
	Itampolo	26	11		0.654	0.826
	Androka	99	14		0.727	0.815
	Androy	19	8		0.737	0.754
RAD313				220 - 292		
	Itampolo	21	12		0.429*	0.830
	Androka	82	18		0.512*	0.829
	Androy	18	4		0.444	0.668
RAD542				148 - 196		
	Itampolo	26	13		0.885	0.890
	Androka	100	18		0.790	0.884
	Androy	19	10		0.895	0.842
RAD573				199 – 225		
	Itampolo	26	9		0.692	0.797
	Androka	99	10		0.747	0.794
	Androy	19	6		0.632	0.700
RAD891				194 - 242		
	Itampolo	26	10		0.500*	0.799
	Androka	100	14		0.560*	0.829
	Androy	19	9		0.421*	0.812
RAD932				152 - 204		
	Itampolo	25	18		0.640	0.900

Locus	Population	N	k	Size range (bp)	H_O	H_E
RAD932	Androka	100	20		0.600*	0.869
	Androy	18	7		0.778	0.765

* = significant deviation from HWE at $\alpha = 0.05$

2.4 Acknowledgements

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CHAPITRE 3

Riverbeds demarcate distinct conservation units of the radiated tortoise (*Geochelone radiata*) in southern Madagascar[1]

3.1 Résumé

La tortue radiée (*Geochelone radiata*) est une espèce menacée endémique à Madagascar. Elle y habite la forêt épineuse semi-aride du sud de l'île, un écosystème lourdement affecté par la destruction de l'habitat. De plus, la collecte illicite menace sérieusement cette espèce. L'objectif principal de notre étude était d'établir la structure génétique de la tortue radiée, afin d'entreprendre des actions appropriées pour la gestion de cette espèce, telles que la réintroduction d'individus confisqués par les autorités. Notre hypothèse était que certains cours d'eau constituent de réelles barrières à la dispersion des tortues radiées, et ce malgré le fait qu'ils soient complètement asséchés plusieurs mois par année. Nous avons utilisé 13 marqueurs microsatellites polymorphes pour comparer des échantillons provenant de six populations réparties à travers l'aire de répartition de l'espèce. Toutes les analyses (tests exacts de Fisher, valeurs de F_{ST}, AMOVA) ont indiqué que la tortue radiée démontre une structure génétique modérée sur son aire de répartition. Additionnellement, nous avons employé une approche de régression multiple qui a révélé l'importance des fleuves pour expliquer la structure observée. Cette analyse a démontré le rôle des fleuves Menarandra et Manambovo en tant que barrières à la dispersion des tortues radiées, mais des simulations utilisant des chaînes de Markov de Monte Carlo ont suggéré que de faibles niveaux de flux génique récurrent entre les populations expliquent probablement pourquoi les valeurs de F_{ST} ne sont pas plus élevées. Nous avons identifié trois unités de conservation distinctes caractérisées par un taux d'assignation génétique

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[1] Rioux Paquette, S., Benhcke, S.M., O'Brien, S.H., Brenneman, R.A., Louis Jr., E.E. & Lapointe, F.-J. 2007. Riverbeds demarcate distinct conservation units of the radiated tortoise (*Geochelone radiata*) in southern Madagascar. *Conservation Genetics* **8**: 797-807.

relativement élevé (87%), ce qui devrait s'avérer utile pour la gestin de cette espèce. Ceci est la première étude qui décrit la structure génétique d'une espèce répartie d'un bout à l'autre de la forêt épineuse de Madagascar.

3.2 Abstract

The radiated tortoise (*Geochelone radiata*) is an endangered species endemic to Madagascar. It inhabits the semiarid spiny forest of the southern part of the island, an ecosystem heavily affected by habitat destruction. Furthermore, illegal harvesting greatly threatens this species. The main objective of our study was to acquire better knowledge of its genetic structure, in order to take appropriate management decisions concerning, for instance, the reintroduction of confiscated individuals. Our hypothesis was that rivers represent effective barriers to tortoise dispersal despite the fact that they are dry most of the year. We used 13 polymorphic microsatellite markers to compare samples from six populations across the range of the species. All analyses (Fisher's exact tests, F_{ST} values, AMOVA) indicated that the radiated tortoise exhibits moderate levels of genetic structure throughout its range. In addition, we used a multiple regression approach that revealed the importance of rivers to explain the observed structure. This analysis supported the role of the Menarandra and Manambovo Rivers as major barriers to the dispersal of most radiated tortoises, but Markov chain Monte Carlo simulations revealed that low levels of recurrent gene flow may explain why F_{ST} values were not higher. We identified three distinct conservation units with relatively high assignments rates (87%), which should be valuable for the management of the species. This is the first study to report the genetic structure of a species sampled throughout the Malagasy spiny forest.

3.3 Introduction

The radiated tortoise, *Geochelone radiata* (Shaw 1802), called sokake in Malagasy, is endemic to southern Madagascar, where it inhabits the unique Didiereaceae-dominated

spiny forest. It is one of five chelonian species endemic to the island, all of which are threatened. The semiarid spiny forest is one of the world's critically endangered ecoregions (Olson & Dinerstein 1998). It is heavily affected by anthropogenic pressures such as slash-and-burn agriculture and livestock grazing, as well as further deforestation for fuel wood and charcoal production (Seddon *et al.* 2000). These activities have been considerably intensified in recent years to supply for increasing demands of a booming human population which has increased from around 8 million in 1975 to over 16 million in 2001 (United Nations database). Consequently, habitat loss and fragmentation greatly jeopardize the long term persistence of the radiated tortoise at many locations, and has contributed to the reduction of its distribution area by over 20% in the last 30 years (Juvik 1975; O'Brien *et al.* 2003).

Although the radiated tortoise has been protected nationally since 1961 and listed on the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1975, demand for this Malagasy tortoise and its derived products has dramatically increased recently. Significant seizures have occurred on the nearby island of La Réunion, where 1098 tortoises were confiscated from one ship in 2002 and over a thousand more from another in February 2003 (M. Emonot, personal communication). Due to the sparse population density of Malagasy people in the south (Hoerner 1981), the maintenance of adequate levels of law enforcement is a serious problem, and harvesting of this once very abundant species for both local food consumption and foreign markets is worrisome (Nussbaum & Raxworthy 2000; Behler 2002). The majority of the range of the radiated tortoise (Fig. 3.1) is now restricted to the Antandroy and Mahafaly territories, which are inhabited by local people with an ancestral taboo (fady) prohibiting tortoise collection or consumption. Where the range originally reached Vezo and Antanosy regions (which are inhabited by people who do not observe the *fady*), the sokake is now locally extinct. People residing outside the current tortoise range regularly collect tortoises using pirogues or oxcarts and immigration among local people is causing

the erosion of the fady (Lingard *et al.* 2003). Thus, important declines have been noticed even where the fady once protected the tortoise populations (O'Brien *et al.* 2003).

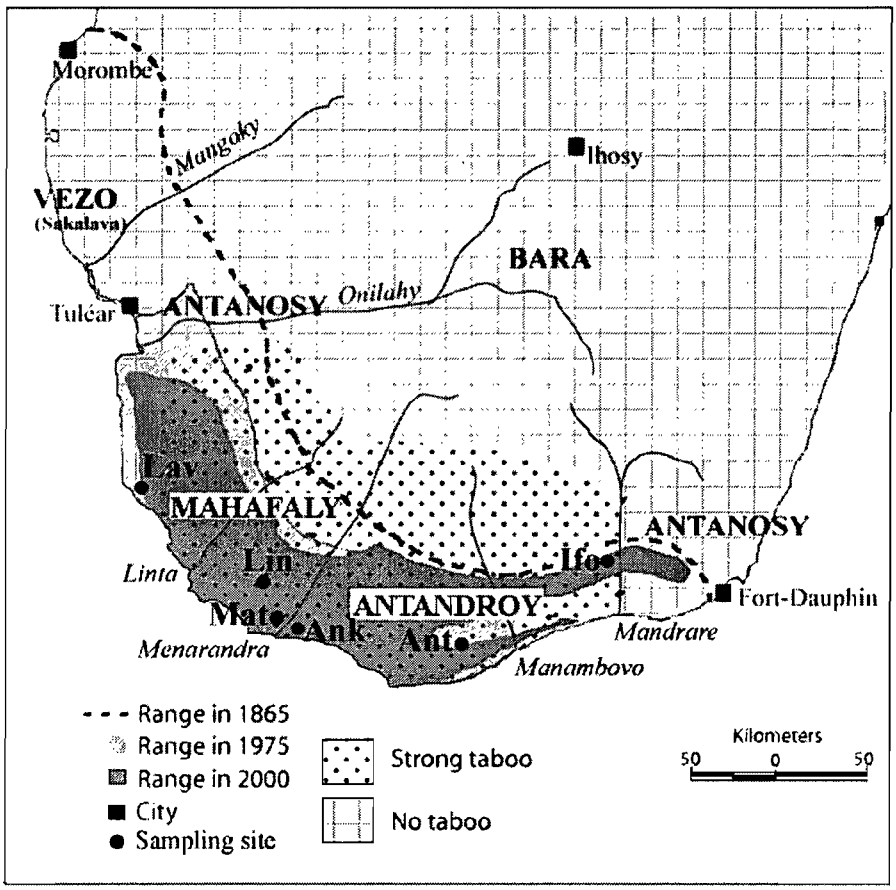

Figure 3.1 Map of southern Madagascar showing the distribution of *Geochelone radiata* and the location of populations sampled in this study (Lav, Lavavolo; Lin, Lintsa; Mat, Matsandry; Ank, Ankaboa; Ant, Antreaky; Ifo, Ifotaka). All-cap words indicate the ethnic affiliation of the people living in the region and italic words indicate rivers (Modified from O'Brien et al. 2003).

Aside from local reports, the radiated tortoise has not been the subject of scientific interest in the wild until very recently. These studies mainly provided insights on the distribution, population dynamics and reproductive biology of this species (Leuteritz 2002; O'Brien 2002; O'Brien *et al.* 2003; Leuteritz *et al.* 2005; Leuteritz & Ravolanaivo 2005).

For conservation purposes, it is necessary to assimilate data to estimate population genetic parameters of the sokake, in order to assess the level of genetic differentiation of populations. Furthermore, the urgency of documenting and describing natural phylogeographic patterns in Madagascar has been emphasized recently (Yoder et al. 2005), and patterns of reptile distribution in the southwestern part of the island have never been described (Raxworthy 2003). The main objective of our study was to acquire information on the genetic structure of the radiated tortoise throughout its range, using microsatellite markers. To our knowledge, this is the first study to describe the genetic structure of a species across the Malagasy spiny forest.

The first underlying secondary objective of this study was to assess the importance of rivers on the genetic structure of *G. radiata*. There are four major rivers in southern Madagascar (Linta, Menarandra, Manambovo, and Mandrare Rivers, from west to east) potentially representing effective barriers to tortoise dispersal. During the dry season, these riverbeds are completely dry (Battistini 1964), which coincides with the moment when radiated tortoises are mostly inactive (Lewis 1995). This season is accompanied by extreme temperatures that discourage them from traveling great distances. Thus, we believe that these rivers promote the genetic differentiation of sokake populations. The other secondary purpose of this work was the identification of conservation units for the radiated tortoise. Future management strategies would benefit from the identification of management units (MUs), which are conservation units defined as demographically and ecologically independent populations, revealed by significant divergence of allelic frequencies at nuclear or mitochondrial loci (Moritz 1994). For instance, it is important to genetically characterize the species so that the large numbers of tortoises that are confiscated from smugglers can be returned to their source MU. If radiated tortoises exhibit a strong genetic structure, translocation of individuals (frequent in protected areas because individuals caught from poachers are often released in parks and reserves) could theoretically

contribute to the erosion of the genetic diversity of the species, which is assumed to reflect its evolutionary potential (Frankham 2005).

3.4 Methods

3.4.1 Sample collection

A total of 141 blood samples were collected from six radiated tortoise populations in southern Madagascar during the months of November and December 2000, and an additional 10 skin samples were scraped from the front limbs of tortoises in 2004 to complete the sampling for the Ifotaka site (Fig. 3.1; see Table 3.1 for details on sample sizes). Blood samples were stored in blood storage buffer (Longmire *et al.* 1992) and tissue samples in 95% ethanol (Tessier & Lapointe 2003) until DNA was extracted.

Table 3.1 Sample size (n), mean number of alleles per locus (k), allelic richness (R_t), and observed (H_O) and expected (H_E) heterozygosity for six radiated tortoise populations in southern Madagascar.

Population	n	k	R_t	H_O	H_E
Lavavolo	23	12.7	8.5	0.733	0.848
Lintsa	20	12.1	9.5	0.711	0.841
Matsandry	34	12.8	8.9	0.728	0.853
Ankaboa	46	11.8	7.7	0.725	0.820
Antreaky	12	6.5	6.4	0.703	0.758
Ifotaka	16	6.3	5.7	0.688	0.751

3.4.2 DNA extraction and amplification

We performed total DNA extraction from blood samples according to standard phenol chloroform procedures (Sambrook *et al.* 1989). For skin samples, DNA extraction

was done with QIAGEN DNeasy extraction columns (QIAGEN, Valencia, CA 91355) following the directions of the manufacturer. We used 13 microsatellite markers to assess the genetic structure of radiated tortoises. Eight of them (RAD14, RAD27, RAD284, RAD313, RAD542, RAD573, RAD891 and RAD932) had been specifically designed for this species and polymerase chain reaction (PCR) amplifications were carried out as described in Chapitre 2 (Rioux Paquette *et al.* 2005). Locus GMuD87 (King & Julian 2004) was the only polymorphic marker among eight Emydidae microsatellites tested, whereas loci YNIP14 (Genbank accession number: AY596205), YNIP239 (AY596208), YNIP405 (AY596212) and YNIP604 (AY596213) were originally designed for the Malagasy plowshare tortoise *Geochelone yniphora*. PCR for these five loci were carried out in a 25-µl reaction volume with approximately 50 ng of genomic DNA (or 3 µl of suspended DNA for DNeasy extractions) as template. Final PCR conditions consisted of 1X reaction buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 12.5 pmol of unlabeled reverse primer, 12.5 pmol of fluorescently labeled forward primer and 0.5 unit of *Taq* DNA polymerase. Amplification reactions were performed in either ABI 480 or ABI 9700 thermocyclers (Applied Biosystems) programmed with 34 cycles of 30 s at 95°C, 45 s at 58°C (GMuD87, YNIP604) or 60°C (YNIP14, YNIP239, YNIP405) and 45 s at 72°C, followed by a final extension step of 10 min at 72°C. Allele sizes were determined by analysis on an ABI 3100 automated sequencer and fragment length was assigned with GeneScan version 1.0 (Applied Biosystems) using the ROX 500 size standard.

3.4.3 Data analysis

We computed parameters of within-population genetic diversity with the online version of GENEPOP 3.3 (Raymond & Rousset 1995), except for allelic richness (R_t), a measure of the number of alleles per locus corrected for different sample sizes, which was computed in FSTAT (Goudet 1995). Observed (H_O) and expected (H_E) heterozygosity, tests of linkage disequilibrium and tests of departures from Hardy-Weinberg equilibrium (HWE)

were performed separately for each microsatellite locus as well as across all thirteen loci, and significance levels were tested with the Markov chain method implemented in GENEPOP (Guo & Thompson 1992) with 5000 permutations, 5000 dememorization steps and 1000 batches. Microsatellite data were analyzed with the software MICRO-CHECKER (van Oosterhout *et al.* 2004) to detect genotyping artifacts that could interfere with the analysis of genetic parameters, i.e. null alleles, large allele dropout and stutter bands. If null alleles were detected, we estimated their frequency with the method of Kalinowski & Taper (2006). To assess among-population variability, we performed Fisher's exact tests for pairwise comparisons of allele frequencies at each locus (Raymond & Rousset 1995). The genetic differentiation of populations was also evaluated by calculating F_{ST} values in Arlequin 2.0 (Schneider *et al.* 2000). We tested the significance of this fixation index with 1000 permutations, for α=0.05. For all statistical tests, in order to minimize the error introduced when computing multiple comparisons, we adjusted probability values with a sequential Bonferroni correction (Rice 1989). We also calculated bootstrap confidence intervals (CI) on F_{ST} values by resampling loci 1000 times for every pairwise comparison with the software GDA (Lewis & Zaykin 2001).

To further investigate the genetic structure of *G. radiata*, we used the program STRUCTURE (Pritchard *et al.* 2000) to determine the most likely number of genetically distinct clusters (K) of populations. To do so, we computed different hypotheses representing one to six clusters, with 10 iterations for each solution and 1 100,000 Markov Chain Monte Carlo (MCMC) replicates (burn-in length: 100,000) for each iteration. The mean likelihood was calculated for each solution in order to determine the most appropriate number of clusters. To further test the significance of genetic variance observed between suggested clusters of populations, we computed an AMOVA (Michalakis & Excoffier 1996) in Arlequin and we tested the significance of the Φ indices (analogous to F statistics) by performing 1000 permutations of multilocus haplotypes within and among groups.

We employed a multiple regression approach on distance matrices (Permute! program; Legendre *et al.* 1994) to assess the relative contribution of geographical distances (i.e. isolation-by-distance) and rivers on the genetic structure of the species. The matrix of F_{ST} values was considered as the dependent variable in these computations. The dependent variable was compared to two matrices (independent variables), one of geographical distances between populations (calculated from GPS coordinates) and the other containing the number of rivers separating pairs of populations instead. The standardized regression coefficients (b), which are equal to the partial Mantel correlation coefficients (r), and the coefficient of determination (R^2) of the model were tested with the same permutational procedure (with 1000 permutations) as the Mantel test (Mantel 1967). We used a backward elimination procedure (p-to-remove: 0.05) to enter in the regression only the variables that contributed significantly to the model when accounting for collinearity between independent variables (Legendre *et al.* 1994). We computed a second regression to compare the contribution of the three rivers (Linta, Menarandra and Manambovo Rivers) to the explanation of the dependent variable. For this model, we constructed a binary matrix for each river (with the value 0 for pairs of populations on the same side and 1 for populations on opposite sides) and these three matrices were used as the independent variables.

Assignments of individuals to populations were carried out in GENECLASS2 (Piry *et al.* 2004) with the Bayesian algorithm of Rannala & Moutain (1997), since it has been demonstrated that Bayesian methods perform better than distance or frequency based methods (Cornuet *et al.* 1999). We performed the tests several times, at first by assigning individuals to the six original populations, and then by merging them according to the different clusters suggested by the previous analyses. For each test, the individual to be assigned was excluded from the computations to prevent bias towards its population of origin. The standard error of the assignment rate was calculated by resampling allele frequencies with the software WHICHLOCI (Banks *et al.* 2003) to estimate its 95% CI. Detection of recent migrants (Rannala & Mountain 1997) was also performed with

GENECLASS2, and for each individual, the probability of being a recent migrant was computed based on the [likelihood of the population of origin] / [maximum likelihood] ratio.

The number of migrants ($p < 0.05$) exchanged between populations was considered as a direct estimate of migration among populations (Cegelski *et al.* 2003). Indirect estimates of gene flow (*Nm*) were also calculated. We employed Wright's formula (1943) $Nm = \{1- F_{ST}\}/ 4F_{ST}$ and the private allele method (Barton & Slatkin 1986) implemented in GENEPOP to obtain estimates for comparison with direct estimates.

We used the program IM (Nielsen & Wakeley 2001) to determine if moderate differentiation among populations was due to recurrent gene flow or recent divergence. The isolation with migration model (IM) has been the object of several studies recently (e.g. Hey & Nielsen 2004; Hey *et al.* 2004; Hey 2005; Won & Hey 2005). Given a molecular dataset for two populations, this coalescence model evaluates the relative importance of six parameters scaled by the mutation rate of loci: θ_A, the ancestral population effective size; θ_1 and θ_2, the effective size of populations 1 and 2; t, the time since splitting; and m_1 and m_2, the migration rate from population 1 to population 2 (which, in a backwards coalescent framework, is equivalent to a movement of genes from 2 to 1; Hey 2005) and vice versa. Preliminary MCMC simulations were conducted to determine appropriate maximum values for uniform priors. The final chain consisted of an initial burn-in period of 500,000 cycles followed by 5 500,000 cycles with the following maximum parameter values: 10 for θ_1 and θ_2; 500 for θ_A; 2000 for t; and 15 for m_1 and m_2. The results were considered as strong evidence for a model with recurrent gene flow if 0 was not included in the 90% highest posterior density (HPD) intervals (a type of Bayesian CI, see Won & Hey 2005) of m_1 and m_2.

3.5 Results

All thirteen microsatellite loci were very polymorphic and all populations showed high degrees of genetic diversity (Table 3.1). A total of 239 different alleles were obtained across all loci, with the number of alleles per locus ranging from 10 (RAD573) to 27 (YNIP239). Within each of the six populations, the absolute number of alleles ranged from 82 (Ifotaka) to 166 (Matsandry). Observed heterozygosity values were very similar across all populations, ranging from 0.688 to 0.733. No significant linkage disequilibrium was detected among all pairwise comparisons of loci across all populations, following a Bonferroni correction, thus confirming the independence of loci. For five loci (YNIP14, YNIP239, RAD313, RAD891 and RAD932), two to four populations were out of HWE. When exploring the data for genotyping artifacts, analyses revealed that five loci were likely to contain null alleles. These are the same loci for which HWE deviations were noticed. The estimated frequency of null alleles for these loci ranged from 0.105 (YNIP239) to 0.248 (RAD313). The eight other loci all had null allele frequencies below 0.05.

Significant differences in allele distributions among populations were detected in most of pairwise comparisons with Fisher's exact tests (Table 3.2). These tendencies were also obtained with the F_{ST} values (Table 3.2), since the only values that were not significant were those involving pairs of populations for which none of the exact tests were significant. The Ifotaka population was the most distinct, with Ankaboa and Antreaky also exhibiting significant differences with all other populations, except with each other.

Table 3.2 Results of Fisher's exact tests (below the diagonal) and F_{ST} values (above the diagonal, with 95% CI in parentheses) among six radiated tortoise populations from southern Madagascar. The number of significant results out of 13 loci is given for Fisher's exact tests and significant F_{ST} values are indicated by asterisks (following a sequential Bonferroni correction for α=0.05).

	Lavavolo	Lintsa	Matsandry	Ankaboa	Antreaky	Ifotaka
Lavavolo	-	0.001	0.001	0.034*	0.062*	0.063*
		(-0.004 ~ 0.002)	(-0.004 ~ 0.006)	(0.024 ~ 0.045)	(0.042 ~ 0.080)	(0.045 ~ 0.081)
Lintsa	0/13	-	0.002	0.034*	0.056*	0.048*
			(-0.002 ~ 0.006)	(0.019 ~ 0.052)	(0.040 ~ 0.073)	(0.037 ~ 0.058)
Matsandry	0/13	0/13	-	0.021*	0.039*	0.045*
				(0.012 ~ 0.030)	(0.025 ~ 0.054)	(0.029 ~ 0.061)
Ankaboa	10/13	10/13	8/13	-	0.008	0.023*
					(0.000 ~ 0.017)	(0.014 ~ 0.033)
Antreaky	6/13	5/13	3/13	0/13	-	0.038*
						(0.013 ~ 0.061)
Ifotaka	8/13	8/13	7/13	3/13	3/13	-

Analyses in STRUCTURE suggested that the optimal solution was to group populations in two genetically homogenous clusters (ln likelihood of posterior probabilities (p) = -8153) closely followed by a three-cluster solution (ln(p) = -8183). All other solutions had much lower results (ln(p) = -8461 for K=1, the solution with the third highest likelihood). When testing the optimal solution with AMOVA, genetic variation between the two groups was not significant, while a three-group hypothesis (Lavavolo/Linsta/Matsandry, Ankaboa/Antreaky and Ifotaka) resulted in significant among-group comparisons (Table 3.3). These groupings are in agreement with results from Table 3.2.

Table 3.3 Results of the AMOVA for a two-group hypothesis (the three populations on both sides of the Menarandra River clustered together) and a three-group hypothesis (Ifotaka separated from the other eastern populations).

	Level of variance	Variance	% variance	Φ	p
2-group hypothesis	Among groups	0.1469	2.79	Φ_{CT} = 0.0279	0.106
	Among populations within groups	0.0548	1.04	Φ_{SC} = 0.0107	0.001*
	Within populations	5.0573	96.17	Φ_{ST} = 0.0383	0.001*
3-group hypothesis	Among groups	0.1651	3.15	Φ_{CT} = 0.0315	0.014*
	Among populations within groups	0.0265	0.50	Φ_{SC} = 0.0052	0.184
	Within populations	5.0573	96.35	Φ_{ST} = 0.0365	0.001*

Isolation by distance was significant among the six populations, i.e. pairwise genetic and geographical distances were correlated (r = 0.597; p = 0.003). When computing a multiple regression to explain the F_{ST} matrix, the only independent variable kept in the model following a backward elimination was the number of rivers between populations (b = 0.785, p = 0.012). This regression model explained 61.6% (p = 0.012) of the variation of the dependent variable. Geographical distance (b = 0.141, p = 0.301 in the first-step regression) was not kept. Furthermore, when explaining the dependent variable with the

three binary river variables, the Menarandra River (b = 0.812, p = 0.016) and the Manambovo River (b =0.429, p = 0.006) were significant in this model that explained 82.2% (p = 0.023) of the variation. The Linta River was not a significant explanatory variable (b = 0.123, p = 0.247).

The success rate of assignments, when considering each population separately, reached only 53.0% on average (Table 3.4). Once the populations were merged according to the F_{ST} and AMOVA results, this number was raised to 86.8% (95% CI: 86.4 ~ 87.1), with only 13 individuals wrongly assigned to their respective side of the Menarandra River and 7 individuals wrongly assigned with respect to the Manambovo River. These were also erroneously assigned by the STRUCTURE algorithm. The migrant detection analysis further revealed that these 20 individuals, and only these, had a $p < 0.05$ (p values ranging from < 0.001 to 0.03), which supports the hypothesis that they were indeed recent migrants. Indirect Nm estimates and IM simulations were computed only between the two larger groups of populations, located on both sides of the Menarandra River (Lavavolo/Lintsa/ Matsandry and Ankaboa/Antreaky). An estimate of 7.7 migrants per generation was obtained with Wright's formula and 4.8 with the private allele method. The IM coalescence analyses strongly pointed towards a model of persistent gene flow (fig. 2). The highest probability value for m_1 was 1.25 (90%HPD: 0.65 ~ 1.53) and 2.03 (1.29 ~ 9.69) for m_2. The other parameters in the model also had well-defined posterior probability distributions with complete tails, and five subsequent Markov chains converged towards the same values.

Table 3.4 Results of assignment tests, showing to which population (columns) individuals from each original population (rows) were assigned based on their multilocus genotype, along with assignment success rates (%). Two models are shown, first by considering the six populations as distinct units, and the second, by merging the populations in three clusters, as suggested by other analyses (Tables 3.2 and 3.3).

	Lavavolo	Lintsa	Matsandry	Ankaboa	Antreaky	Ifotaka	%
Lavavolo	**7**	5	11	0	0	0	30.4
Lintsa	6	**6**	5	3	0	0	30.0
Matsandry	8	4	**19**	2	1	0	55.9
Ankaboa	0	1	6	**30**	5	4	65.2
Antreaky	0	0	0	7	**5**	0	41.7
Ifotaka	0	0	0	3	0	**13**	81.3

		Lav/Lin/Mat	Ank/Ant	Ifotaka	%
	Lav/Lin/Mat[a]	**70**	7	0	90.9
	Ank/Ant[b]	6	**48**	4	82.8
	Ifotaka	0	3	**13**	81.3

[a]Lavavolo, Lintsa and Matsandry populations clustered together

[b]Ankaboa and Antreaky populations clustered together

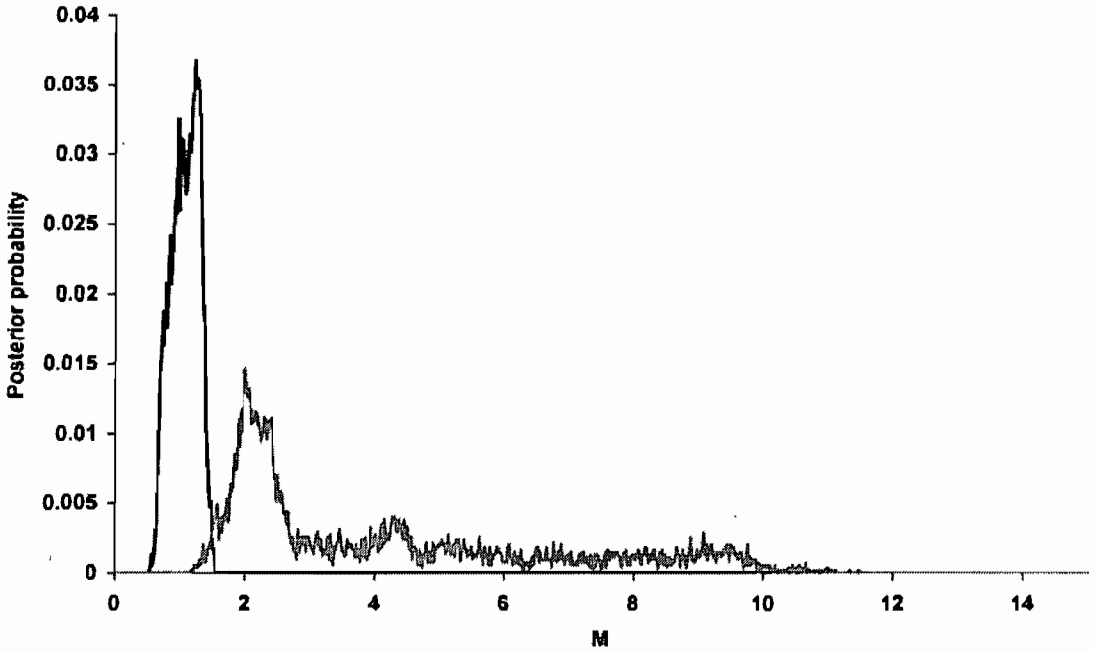

Figure 3.2 The posterior probability distribution of m_1 (migration rate from Ankaboa/Antreaky to Lavavolo/Lintsa/Matsandry; black line) and m_2 (gray line) between two groups of *G. radiata* populations. This distribution was generated by a Markov chain consisting of 6 million iterations, where the first 500,000 iterations were considered as burn-in. See text for information on assumed priors for parameters in the model.

3.6 Discussion

3.6.1 Intra-population diversity

The analysis of 13 microsatellite loci allowed us to detect particularly high levels of genetic diversity within all six populations of radiated tortoises included in this study. This is consistent with other studies on terrestrial and freshwater testudines (e.g. Sites *et al.* 1999; Ciofi *et al.* 2002; Cunningham *et al.* 2002; Tessier *et al.* 2005), and allele diversity and heterozygosity values were generally high within each sampled population. Some HWE departures were observed, but these heterozygote deficiencies were all associated with the presence of null alleles. In the case of the locus RAD313, suspicions about the presence of null alleles have been expressed before, because of the considerably lower success of amplification (85 %) for this locus, suggesting the presence of nonamplifying homozygotes (Chapitre 2). Another aspect of the data interpretation to consider when estimating null allele frequencies is the excess of homozygotes for rare alleles (Kalinowski & Taper 2006). This is a strong evidence for the presence of null alleles, and in our case homozygote excesses were significantly higher than predicted values for most rare alleles in loci YNIP14, YNIP239, RAD891 and RAD932. There is no hint of HWE departures among the other eight loci, suggesting that the sampled populations are indeed in equilibrium. The exclusion of the five markers with null alleles did not affect the outcome of the among-population analyses. We computed pairwise F_{ST} across loci with null alleles and loci without null alleles, and no significant differences were noticed among mean values (results not shown). Thus, we decided to retain all 13 markers in the analyses.

3.6.2 Riverbeds as barriers to dispersal

In an introductory survey of genetic diversity, Leuteritz *et al.* (2005) identified only two mtDNA haplotypes in a 491-bp ND4 fragment in 14 individuals sampled from

southwestern coastal populations, and suggested that there was no subspecific differentiation in the species. However, our multilocus genotype data demonstrate that the radiated tortoise populations we sampled harbor quite acceptable levels of genetic diversity and a relatively strong genetic structure across their present range. Leuteritz *et al.* (2005) argued that there were no historical or current physiographic barriers to gene flow that could explain the presence of structure in *G. radiata*. To the contrary, our results indicate that both the Menarandra and the Manambovo Rivers do in fact provide those boundaries. The results of the multiple regression models particularly highlight the importance of rivers to explain the genetic structure of the sokake. The presence of the Menarandra and Manambovo Rivers alone explains the majority (82%) of the genetic differentiation between populations across the range of the radiated tortoise. Given our sampling site locations, the Menarandra River is a significant barrier between the Lavavolo/Linta/ Matsandry and the Ankaboa/Antreaky/Ifotaka clusters, with the three populations from each side being clearly separated in all analyses. The Manambovo River does provide a secondary barrier between the Ankaboa/Antreaky group and the Ifotaka population, supported by the AMOVA and multiple regression. The Linta River, however, does not appear to be a significant barrier on the west. Although the width of southern riverbeds are similar (550 m for the Menarandra River at Matsandry and 600 m for the Linta River near Androka), their water regime is different. In the Linta River, only the most important floods reach the sea. The riverbed is completely dry for at least eight months a year, even inside the Mahafaly plateau. Water levels are higher and water persists a few months longer in the Menarandra River (Battistini 1964). This difference may explain why populations from opposite sides of the Menarandra River are genetically distinct while those from across the Linta River do not maintain significant genetic differentiation. The multiple regression results also emphasize the need to incorporate putative barriers to gene flow in analyses of isolation by distance. The correlation between genetic and geographical distances alone was highly significant prior to conducting partial tests, which could lead to erroneous interpretations of the processes that really promote population differentiation.

Although the sampled populations exhibit a significant genetic structure, the extent of differentiation (F_{ST}) is quite low. Two different hypotheses can explain this type of genetic structure: there may be recurrent gene flow between the distinct groups, or alternatively, they may have diverged too recently to discern higher degrees of differentiation (Palsboll *et al.* 2004). Our results suggest that a model incorporating low levels of current gene flow is likely to explain the observed genetic structure. The addition of mtDNA sequences in further studies will certainly be helpful in understanding the evolutionary history of this species. Nonetheless, our results can further be interpreted in relation with many studies that have provided faunal and geomorphological evidence of a progressive aridification of the southwestern part of Madagascar since the late Pleistocene or early Holocene, i.e. over the past 12,000 years (Battistini 1971; Mahé & Sourdat 1972; Burney 1993; Burney 1997). The presence of dry riverbeds that seem to have carried large amounts of water in the past, points towards a drastic climatic change that probably occurred in recent millennia (Burney 1997). At that time, rivers probably represented greater barriers to the dispersal of tortoises, and their progressive drying may have contributed towards the establishment of the current structure among *G. radiata* populations. The ecological traits of the species may attenuate the contemporary rate of migration across rivers, because tortoises are mostly inactive during the driest season of the year (Lewis 1995). Thus, the current genetic structure is likely to reflect equilibrium among populations with respect to recurrent gene flow, genetic drift and mutation.

Our study shows that southwestern Madagascar is not a single zoogeographic zone with respect to *G. radiata* populations. Yoder *et al.* (2005) noticed that the Onilahy River separates two cryptic species of cordylid lizards and fine-scale patterns of plant endemism around the Tulear region have also been found recently (P.J. Rakotomalaza, personal communication). As other species from the spiny forest are adequately sampled, this model should be tested to determine whether or not additional taxa exhibit similar genetic

structures, in order to identify zones of evolutionary importance. The spiny forest has the smallest representation of all major Malagasy biomes in the national network of protected areas (Du Puy & Moat 2003), and these findings should be considered as future decisions concerning the location of reserves and parks are made to preserve the integrity of the Malagasy herpetofauna.

3.6.3 Conservation units

While most debates concerning the identification of conservation units have been directed at the concept of evolutionarily significant units (ESUs; e.g. Waples 1991; Moritz 1994; Vogler & DeSalle 1994; Pennock & Dimmick 1997; Crandall *et al.* 2000; Fraser & Bernatchez 2001), Moritz (1999) points out that ESUs and MUs are equally important since MUs are the ecological components that must be managed to conserve the larger ESUs. The criterion of significant differences in allele frequencies originally described by Moritz (1994) to distinguish MUs is still widely applied (e.g. Knapen *et al.* 2003; Pabijan *et al.* 2005). With respect to this criterion, the three groups of populations that we have identified qualify for the designation of MUs. These units are: the three populations of the west side of the Menarandra River (Lavavolo/Lintsa/Matsandry), the two populations between the Menarandra and Manambovo (Ankaboa/Antreaky) Rivers, and the isolated Ifotaka population. However, Taylor & Dizon (1999) have emphasized that the designation of MUs requires more information than genetic data only, such as anthropogenic risks facing populations. Recently, this idea was improved by Green (2005). In his reasoning, this author highlights the importance of considering extinction risks when identifying conservation units. We believe this to be extremely relevant for the management of the radiated tortoise, and it supports our delineation of MUs. The high abundance of tortoises at the core of the species range (Lewis 1995; Leuteritz *et al.* 2005) has overshadowed the need of conserving peripheral populations in the past. For instance, the Ifotaka population shows aggravated risks of extinction considering its proximity to Antanosy territories and recent

trends in local extinctions (O'Brien *et al.* 2003). Given our results, managing this population as a distinct unit is thus of utter importance if we are to preserve the integrity of the species as a whole, even if the genetic differentiation of this population is not particularly pronounced.

An intriguing aspect of our results is the high direct estimate of migrants detected between distinct units across the species' range. In the case of the Menarandra River, 13 individuals were likely migrants wrongly assigned to their respective side of the river. However, such a high proportion of migration (9.6 % of sampled individuals) would not enable the populations to maintain the observed levels of differentiation, and indirect estimates of gene flow rather suggest that these two MUs exchange 5-8 individuals per generation. Several of these individuals may be statistical outliers with uncharacteristic genotypes, but an alternate explanation to the discrepancy between direct and indirect measures of gene flow may be that some individuals were moved by humans. Poachers are known to transport great numbers of live tortoises by oxcart to take them to cities (Nussbaum & Raxworthy 2000; O'Brien *et al.* 2003), and there may be occasions when tortoises are either released to avoid arrest or accidentally dropped. Another explanation could be related to the release of smuggled tortoises confiscated from authorities, although tortoises are mostly released in parks and reserves and the selected sampling sites were relatively far from any protected area to prevent these biases in genetic analyses. In a study of gopher tortoise (*Gopherus polyphemus*) population genetics, Schwartz & Karl (2005) also concluded that several individuals detected as recent migrants might have been the result of human translocations. Aside from genetic evidence, their assertion was supported by the fact that releases of alien tortoises often occur in reserves and that gopher tortoises do not move over long distances, which is also true for radiated tortoises (O'Brien 2002). In any case, although more exhaustive sampling would be required to assess the extent of this, human translocation of radiated tortoises may contribute to the genetic homogenization of the species across its range.

Because radiated tortoises occur at impressively high densities in the wild and their habitat in southwestern Madagascar is so remote, very few conservation efforts had been directed towards the species until recently. In light of our study revealing the importance of rivers on the genetic structure of the radiated tortoise, the local extinctions of the sokake north of the Onilahy River and east of the Mandrare River (the only known remaining population is in Andohahela National Park but is vastly composed of reintroduced individuals; Leuteritz *et al.* 2005) may already represent a considerable loss of the species diversity. It has been pointed out that the most encouraging aspect of the conservation of the radiated tortoise was the high numbers of individuals still left in the wild (Leuteritz *et al.* 2005). Thus, we recommend, while it is still possible, to take into consideration the different MUs of the species in future management plans, in order to preserve its genetic variability. From our multilocus genotype analyses, assignment rates were relatively high between the three distinct MUs and such genetic assignment capabilities would be helpful to the Malagasy government and NGOs for accurately reintroducing confiscated tortoises to their approximate home ranges. Ongoing research is currently looking at the possibility that this genetic differentiation could be detected by morphometric differences across these three conservation units.

3.7 Acknowledgements

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CHAPITRE 4

The use of shell morphometrics for the management of the endangered Malagasy radiated tortoise (*Geochelone radiata*)[1]

4.1 Résumé

La tortue radiée (*Geochelone radiata*), espèce endémique à la forêt épineuse du sud de Madagascar, est gravement menacée par la collecte illicite, à la fois pour la consommation locale et le marché international. De grandes quantités d'individus vivants sont régulièrement confisquées par les autorités, et leur population d'origine est souvent inconnue. Des travaux précédents ont identifié trois unités génétiquement distinctes à travers l'aire de répartition de l'espèce. L'objectif de la présente étude était de développer une méthode pour assigner des individus *G. radiata* à leur population d'origine grâce à l'analyse de caractères morphométriques. Nous avons mesuré 39 variables sur la carapace et le plastron de tortues capturées à sept localités réparties au sein de l'aire de distribution de l'espèce afin d'évaluer sa variabilité morphométrique. Nous avons employé une méthode basée sur l'ACP pour analyser les données morphométriques, et les analyses ont révélé la présence de trois groupes en fonction de la variation de la forme de la carapace. Nous avons obtenu des fonctions discriminantes permettant la bonne classification de 83% des individus parmi ces trois groupes. L'abondance de tortues aux différents sites, une variable étroitement reliée au niveau d'exploitation de la population, était corrélée à la différentiation morphométrique des populations. Nos résultats suggèrent que l'homme ait pu jouer un rôle dans l'évolution morphologique récente des tortues radiées. La méthodologie décrite a le potentiel de devenir un outil intéressant pour la gestion des tortues radiées, et pourrait théoriquement être appliquée à toute espèce de chélonien.

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[1] Rioux Paquette, S., & Lapointe, F.-J. 2007. The use of shell morphometrics for the management of the endangered Malagasy radiated tortoise (*Geochelone radiata*). *Biological Conservation* **134**: 31-39.

4.2 Abstract

The radiated tortoise (*Geochelone radiata*) is an endangered species endemic to the spiny forest of southern Madagascar. The main threat that this species faces is illegal harvesting for both local consumption and foreign markets. Large numbers of live individuals are often confiscated and their population of origin is in most cases unknown. Previous work has identified three genetically distinct units across the current distribution of the species. The objective of the present study was to develop a method of assigning *G. radiata* individuals to their original population based on the analysis of morphometric characters. We measured 39 variables on the shell of tortoises sampled at seven locations across the species range in order to assess differentiation. We employed a PCA-based method to analyze morphometric data and found three distinct groups of populations based solely on shape variation. Discriminant functions allowed for the correct classification of 83% of individuals among these groups. The abundance of tortoises, a variable strongly linked to the level of human exploitation at each site, was correlated with the morphometric differentiation of populations. Our results thus suggest that humans may have played a role in the recent evolution of radiated tortoises. The outlined methodology could become a useful tool for the management of radiated tortoises, and could theoretically be applied to any chelonian species.

4.3 Introduction

The radiated tortoise (*Geochelone radiata*) is one of five chelonian species endemic to the island of Madagascar, all of which are threatened. This tortoise species, called sokake in Malagasy, is restricted to the semiarid spiny forest of the southwestern part of the island, a biome characterized by the abundance of spine-covered plant species from the endemic family Didiereaceae (*Didierea* sp. and *Alluaudia* sp.). This unique ecosystem, considered as one of Earth's most valuable ecoregions (Olson and Dinerstein 1998), is highly threatened

by deforestation, and its low rate of regeneration makes it excessively vulnerable to anthropic pressures. Major factors explaining deforestation in southern Madagascar include the production of charcoal and fuelwood, cropland expansion, and development of grazing pastures (Seddon *et al.* 2000; Casse *et al.* 2004). However, as intense as habitat destruction may be in some areas, the main threat to the persistence of the radiated tortoise remains overexploitation (O'Brien 2002; O'Brien *et al.* 2003). Although the species has been nationally protected since 1961 and listed on the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1975, demands for the radiated tortoise and its derived products for both local and foreign markets are still significant. This is reflected by recent seizures of several hundred individuals in Madagascar and on the nearby island of La Réunion, and supported by studies and observations on the current harvest of the sokake (Nussbaum & Raxworthy 2000; Behler 2002; O'Brien 2002; O'Brien *et al.* 2003).

The distribution of the radiated tortoise has undergone a reduction of more than 20% over the last 30 years (Juvik 1975; O'Brien *et al.* 2003), and this situation is closely linked to the patterns of exploitation by the local people with different ancestral beliefs (Figure 4.1). The near totality of the current species range is now restricted to Antandroy and Mahafaly territories, which are inhabited by people with a cultural taboo (fady) prohibiting tortoise collection or consumption. In regions inhabited by Vezo and Antanosy people (who do not observe the taboo), the sokake has already gone extinct. Furthermore, poachers from outside the tortoise's range regularly venture inside Antandroy and Mahafaly lands nowadays to collect large numbers of tortoises, and immigration among local people is causing the erosion of the ancestral fady (Lingard *et al.* 2003). Thus, important declines have been noticed even in regions where taboos once protected the radiated tortoise (O'Brien *et al.* 2003).

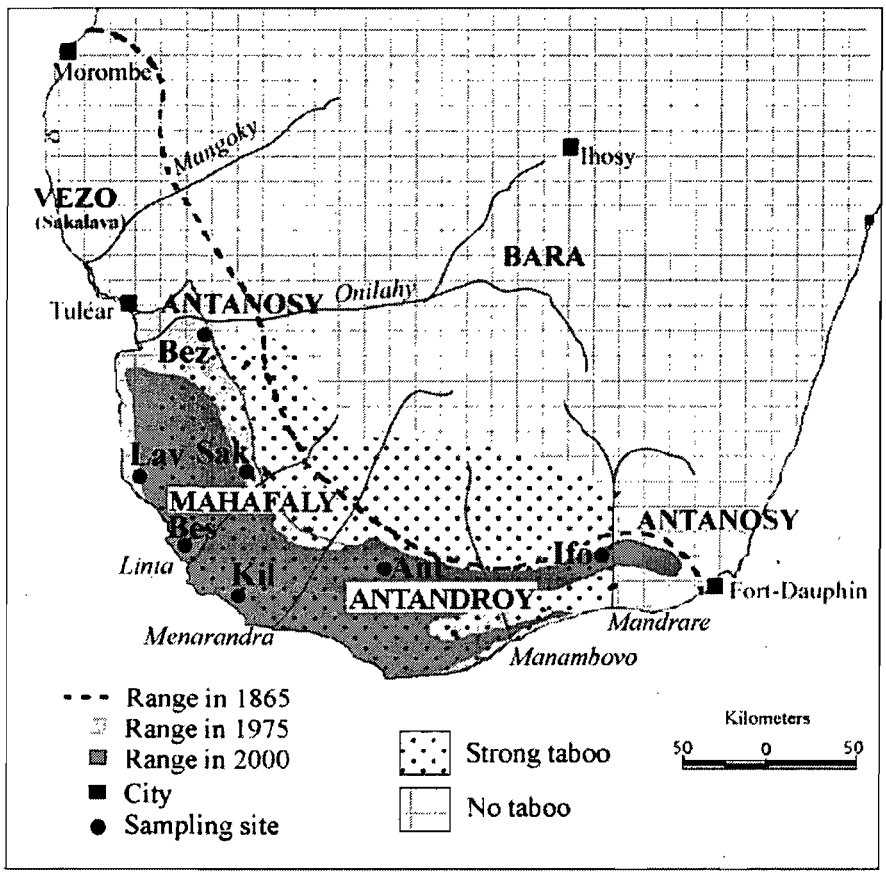

Figure 4.1 Map of southern Madagascar illustrating the past and current range of *G. radiata* and the location of the seven populations sampled in this study (modified from O'Brien *et al.*, 2003). The ethnic affiliation of the people living in the region is given in majuscule and italic words indicate rivers. (Population name abbreviations: Bez, Bezaha-Mahafaly; Sak, Sakotoavo; Lav, Lavavolo; Bes, Besily; Kil, Kilibory; Ant, Antsifitse; Ifo, Ifotaka).

When creating conservation management plans for endangered species, it is important to ensure that the genetic integrity of distinct populations be preserved to maintain the adaptive potential of species (Frankham 2005). Three genetically distinct conservation units have recently been identified within *G. radiata*, and unplanned

reintroductions of high numbers of individuals may contribute to the homogenization of the genetic diversity of the species (Chapitre 3; Rioux Paquette *et al.* 2007). In that study, approximately 90% of sampled tortoises were correctly assigned to their respective conservation unit using molecular markers. However, even though molecular assignment methods can be extremely useful in some cases (Manel *et al.* 2002), such techniques are inaccessible to wildlife managers in southern Madagascar, and it is an expensive and involved undertaking for local non-governmental organizations (NGOs) to collect DNA samples and entrust genetic analyses to foreign laboratories.

The chelonian shell is especially well suited for morphometric studies. It contains connective tissue sutures normally found only in the skull (Sarnat & McNabb 1981), which allows the direct measurement of numerous osteological characters. Shell morphometrics have been used to investigate taxonomy and adaptive ecology in Testudines (Germano 1993; Bonnet *et al.* 2001; Claude *et al.* 2003; Willemsen & Hailey 2003), to describe the distinction between closely-related species or subspecies (Seidel & Lucchino 1981; Lamb 1983; Lamb & Jovich 1990; Yasukawa *et al.* 1996; Ernst & Laemmerzahl 2002) and to assess the variation among and within populations (Iverson 1977; Reynolds & Seidel 1983; Stickel & Bunck 1989; Van Heezik *et al.* 1994; Tucker *et al.* 1998; van Dam & Diez 1998; McLuckie *et al.* 1999; Carretero *et al.* 2005). However, with the increasing popularity of molecular methods, morphological approaches have been overshadowed by population and conservation genetics studies. Although advances in multivariate methods and geometric morphometrics have improved the ability to discriminate subtle morphological differences, very few studies have tried to determine the extent to which genetic differentiation is associated with morphometric distinction. It is thus of particular interest to assess the variation in shell morphology across the radiated tortoise's range, in conjunction with the distinct genetic units that have already been identified.

The intensity of illegal activities that target the radiated tortoise confronts wildlife managers with a considerable problem. Tortoises are frequently confiscated and decisions have to be made regarding the management of these confiscated individuals. They are often kept in captivity, where many die due to inadequate conditions (S.R.P., pers. obs.). The objective of the current research was to develop a method of determining the geographic origin of radiated tortoises using multivariate analysis of morphometric characters. Not only would this facilitate the establishment of effective reintroduction programs, it would also enable the identification of regions where poachers are particularly active in order to concentrate law enforcement actions in strategic sites. The methodology used here could also be applied to the management of other chelonian species, a taxonomic group particularly threatened, with almost half of the 300 species of turtles and tortoises listed as vulnerable or endangered (IUCN 2004). To achieve our objective, we relied on relatively simple multivariate analysis methods to ensure the straight forward applicability of the methodology.

4.4 Materials and methods

We visited seven sites selected across the range of the radiated tortoise in March and April 2004 (Figure 4.1), and we sampled a total of 158 individuals. For each tortoise, we visually determined the sex when possible, and body mass was measured with a spring scale (precision: 0.01 kg). We measured 39 characters (Figure 4.2) from the carapace and plastron with a 20 cm vernier caliper (precision: 0.1 mm), a 100 cm tree caliper (precision: 1.0 mm), or a 100 cm flexible ruler tape (precision: 1.0 mm) for curvilinear carapace length.

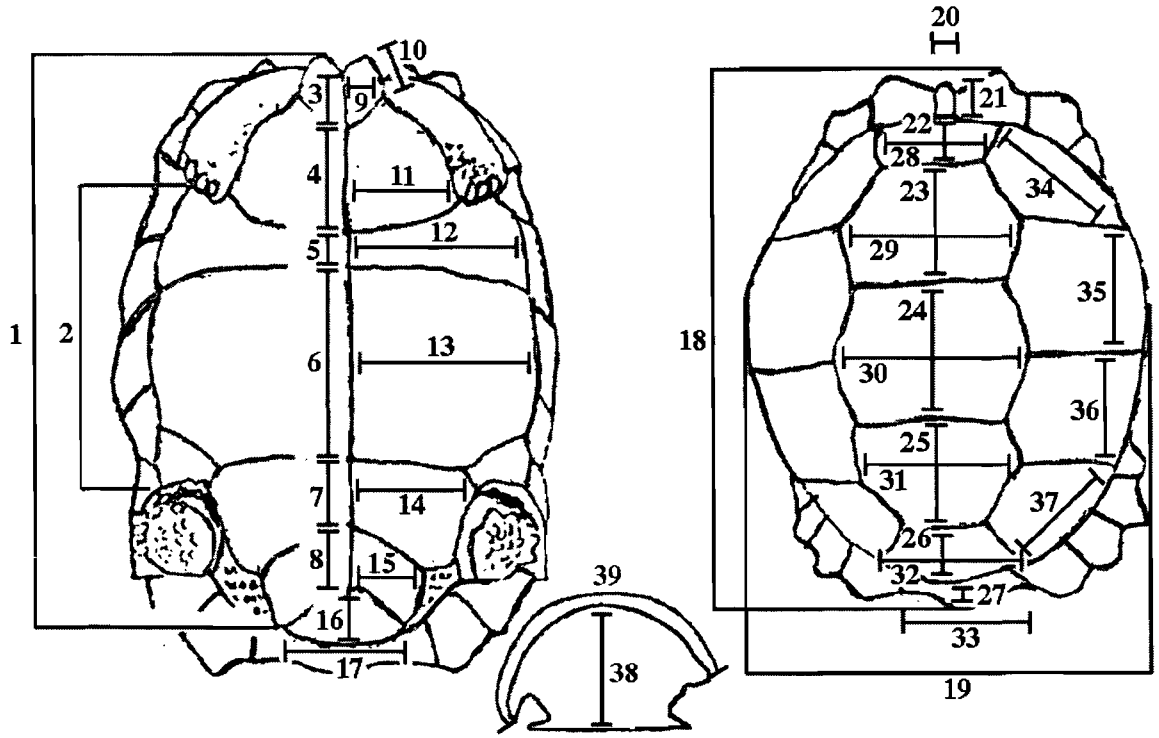

Figure 4.2 Shell characters used in this study (redrawn from Leuteritz 2002). 1. plastron total length; 2. bridge length; 3. gular scute median length; 4. humeral scute median length; 5. pectoral scute median length; 6. abdominal scute median length; 7. femoral scute median length; 8. anal scute median length; 9. left gular scute width; 10. left gular scute external length; 11. left humeral scute width; 12. left pectoral scute width; 13. left abdominal scute width; 14. left femoral scute width; 15. left anal scute width; 16. anal fork length; 17. anal fork width; 18. linear carapace length; 19. carapace width; 20. nuchal scute width; 21. nuchal scute length; 22. first central scute median length; 23. second central scute median length; 24. third central scute median length; 25. fourth central scute median length; 26. fifth central scute median length; 27. supracaudal scute median length; 28. first central scute width; 29. second central scute width; 30. third central scute width; 31. fourth central scute width; 32. fifth central scute width; 33. supracaudal scute width; 34. first right costal scute length; 35. second right costal scute length; 36. third right costal scute length; 37. fourth right costal scute length; 38. carapace height; 39. curvilinear carapace length.

Shell growth in chelonians is allometrical, i.e. shape (or ratios between different measures) varies during the growth of individuals (Mosimann 1958). Because poaching in southern Madagascar mainly targets mature individuals, and because the objective was to develop an assignment method of confiscated tortoises, juveniles were excluded from the analyses. Sexual dimorphism is important in *G. radiata*, with males exhibiting a concave plastron and a wider anal fork (Leuteritz 2002). The sex of mature individuals can thus be easily identified in the field, and individuals that did not show convincing sexual traits were considered juveniles. After eliminating juveniles, 121 tortoises distributed among the seven populations were used in the analyses (Bezaha-Mahafaly, 13 individuals; Lavovolo, 31 individuals, Sakotoavo, 20; Besily, 11; Kilibory, 19; Antsifistse, 16; Ifotaka, 11). Moreover, to prevent any bias associated with sexual dimorphism in the analysis of morphometric variation, we computed the mean length for each of the 39 characters and we centered the mean values for both sexes on the global mean. This correction thus eliminates the discrepancies between the two sexes without affecting the variation among populations. We logarithmically transformed all data before subsequent analyses.

For 13 individuals, we could not measure some of the 39 variables (0.4% of the total data). In regions where the fady prohibits killing tortoises but not touching them, local cultivators often grab tortoises that venture in their fields and throw them out (S.R.P., pers. obs.). This causes some tortoises to suffer fractures and these injuries may preclude the measurement of some shell characters. In order to retain these individuals in the analyses, we estimated missing values using a method proposed in Gauthier *et al.* (2003). Considering all individuals of the same sex, we computed linear regression models for each pair of variables, with the character with missing data designated as the dependent variable. Hence, 38 regressions were computed for each character, and we used the model that provided the greatest R^2 to estimate the missing values.

We calculated a size-constrained PCA (Somers 1986) to describe the morphometric variation of the dataset and discriminate the size- from the shape-related sources of variation. This method constrains the size variation to be incorporated solely into the first principal component. An isometric size vector (Jolicoeur 1963) is computed and used to calculate the first eigenvalue, and the residual matrix is analyzed with a standard eigenanalysis procedure. The other components can thus be interpreted as representing shape variation only. The algebra of Somers (1986) was integrated in a function written in R language (R Foundation for Statistical Computing, Vienna, Austria) to compute the size-constrained PCA. We used the resulting matrix of corrected coordinates instead of raw data for all subsequent analysis, eliminating the first principal component to conserve strictly the shape-related coordinates. We computed Euclidean distances between the 121 objects in the 38-dimension ordination space using the R Package version 4.0 (Casgrain & Legendre 2000).

To assess whether morphometric distances between individuals within populations were significantly lower than distances between individuals among different populations, we computed a Mantel test (Mantel 1967). To do so, we generated a theoretical matrix for comparison with the matrix of Euclidean distances among individuals. This binary matrix contained the value 0 for pairs of individuals from the same population, and the value 1 for individuals from different populations. The Mantel test was computed with the R Package, and we tested the significance of the correlation coefficient (r) with 1000 permutations. A significant correlation would indicate that, indeed, within-population distances are lower than among-population distances. To further investigate the subdivision of individuals in morphometrically distinct groups, we applied K-means partitioning (Lance & Williams 1967; see Legendre & Legendre 1998) to the matrix of Euclidian distances, with the R Package, using K values ranging from 2 to 7.

To evaluate the accuracy of the morphometric characters to correctly classify individuals, we performed discriminant analysis on the matrix of coordinates representing the 38 shape components. We computed discriminant functions in SPSS version 11.0 (SPSS Inc., Chicago, IL), with the leave-one-out option to exclude the individual to be assigned from the calculations (i.e. cross-validation). We first performed the analysis by considering each of the seven populations as a distinct group, and then by clustering the populations according to the results of the *K*-means analysis.

To establish whether the morphometric differentiation of populations could be explained by geographic variables, we computed a multiple regression analysis on distance matrices (Legendre *et al.* 1994). We calculated Euclidean distances between the population centroids with the R Package, and we used this matrix as the dependent variable in the multiple regression computations. We also defined six independent variables. The first was the matrix of geographic distances among populations, calculated from the GPS coordinates of the sampled sites. Three binary matrices representing the Linta, Menarandra and Manambovo rivers were also created (in each case, the value 0 was given to pairs of populations located on the same side of a river and the value 1 for populations from opposite sides). In the same way, we constructed another binary matrix corresponding to the cliff of the Mahafaly plateau, which isolates southwestern coastal populations (Lavavolo, Besily and Kilibory) from other populations. Finally, we included a non-geographic independent variable in the computations to account for population abundance. This was quantified at each site on a scale from 0 to 2, and the corresponding distance matrix was added as the sixth variable. Based upon field observations, sampling effort and interviews with villagers, we gave the value 0 (few tortoises) to the populations of Bezaha-Mahafaly and Ifotaka, 1 to the population of Antsifitse, and 2 to the populations of Lavavolo, Sakotoavo, Besily and Kilibory. We computed the multiple regression model with the program Permute! (Legendre *et al.* 1994). The standardized regression coefficients (*b*), which are equal to the partial Mantel correlation coefficients, and the coefficient of

determination (R^2) of the model were tested with 1000 permutations. We employed a backward elimination procedure (p-to-remove = 0.05) to select the significant variables in the model when accounting for collinearity between independent variables.

The mean size of tortoises at harvested and unharvested sites was compared with permutational t tests to verify the trend described in O'Brien *et al.* (2003), i.e. that smaller body sizes are found in exploited populations.

4.5 Results

The size-constrained PCA revealed that most of the variation within the dataset was associated with size (74.1%), but that considerable shape variation was expressed over the other 38 principal components. The graphical representation of the variation on the first two shape components shows no clear differentiation with respect to within- and among-population distances (Figure 4.3). However, the Mantel test supports the hypothesis that within-population distances are significantly lower than among-population distances (r = 0.136, p = 0.001). When applying the K-means algorithm with two groups, the majority of the individuals from Bezaha-Mahafaly were classified in a group while the majority of the other six populations were assigned to the other group (Table 4.1). Additional information was given by the solution for K = 3, as a third group containing the majority of the individuals from Antsifitse and Ifotaka was also formed. For K = 4 or higher, no supplementary tendency could be denoted, since the additional groups only comprised a minority of individuals coming from various populations. Consequently, the most relevant clustering solution was considered to be the three-group hypothesis.

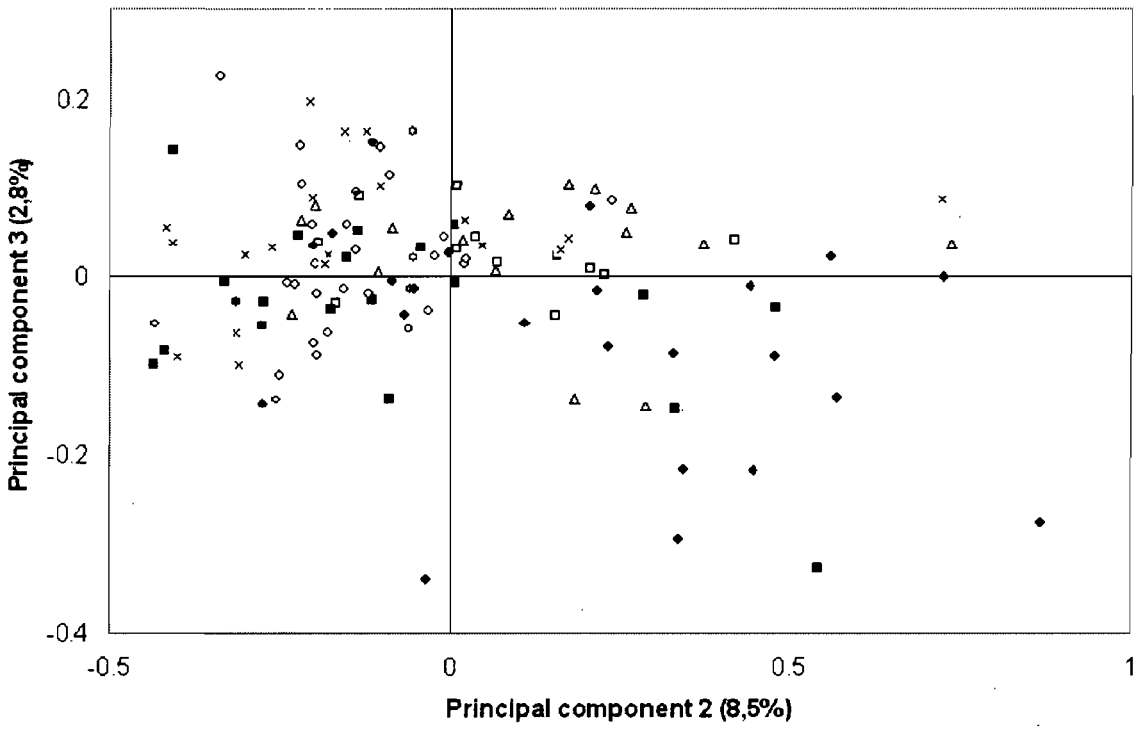

Figure 4.3 Bidimensional scatterplot defined by principal components 2 and 3 from the size-constrained PCA of shell morphometric data for *G. radiata* individuals from seven populations: Bezaha-Mahafaly (◆), Sakotoavo (■), Lavavolo (○), Besily (●), Kilibory (x), Antsifitse (Δ), Ifotaka (□). The first isometric size component accounted for 74.1% of the total variation.

Table 4.1 Classification of 121 *G. radiata* individuals from seven populations with *K-means* partitioning for $K = 2$ and $K = 3$. The values in each cell represent the number of individuals classified in a given group, along with the corresponding percentage of the population in parentheses. The best classification of populations among groups is underlined for both K values.

	$K = 2$		$K = 3$		
Population	Group 1	Group 2	Group 1	Group 2	Group 3
Bezaha-Mah.	11 (85%)	2 (15%)	8 (61%)	1 (8%)	4 (31%)
Sakotoavo	5 (25%)	15 (75%)	3 (15%)	12 (60%)	5 (25%)
Lavavolo	1 (3%)	30 (97%)	0 (0%)	24 (77%)	7 (23%)
Besily	3 (27%)	8 (73%)	2 (18%)	6 (55%)	3 (27%)
Kilibory	3 (16%)	16 (84%)	1 (5%)	14 (74%)	4 (21%)
Antsifitse	8 (50%)	8 (50%)	3 (19%)	5 (31%)	8 (50%)
Ifotaka	4 (36%)	7 (64%)	1 (9%)	3 (27%)	7 (64%)

The discriminant analysis allowed for the correct assignment of merely 50% of all individuals to their respective population, when considering the seven sites as distinct groups. However, by clustering the populations in three groups according to the solution of *K*-means partitioning (Bezaha-Mahafaly and Antsifitse/Ifotaka as two distinct groups from the other populations), the classification success rate reached 82.6% (Table 4.2). Figure 4.4 illustrates the power of the two discriminant functions to classify the tortoises among these three groups. The multiple regression model computed from distance matrices rejected all geographic variables as explanatory variables of the morphometric distances among populations. The only significant variable was the abundance of tortoises at sampling sites ($b = 0.479$, $R^2 = 0.229$, $p = 0.021$). The mean body sizes were lower at harvested sites; the average carapace length was 237.6 mm in Bezaha-Mahafaly and 268.5 mm in Antsifitse/Ifotaka, while it was 309.6 mm in Lavavolo/Sakotoavo/Besily/Kilibory (highly significant differences, $t = 3.97$, $p = 0.001$).

Table 4.2 Results of the discriminant analysis when clustering the seven sampled populations of *G. radiata* in three groups. The classification success rate (%) for each group is presented, and values on the diagonal (underlined) indicate correctly assigned individuals.

	Bezaha-Mah.	Sak,/Lav./Bes./Kil.	Ant./Ifo.	(%)
Bezaha-Mahafaly	10	2	1	76.9
Sakotoavo/Lavavolo/Besily/Kilibory	3	68	10	84.0
Antsifitse/Ifotaka	1	4	22	81.5

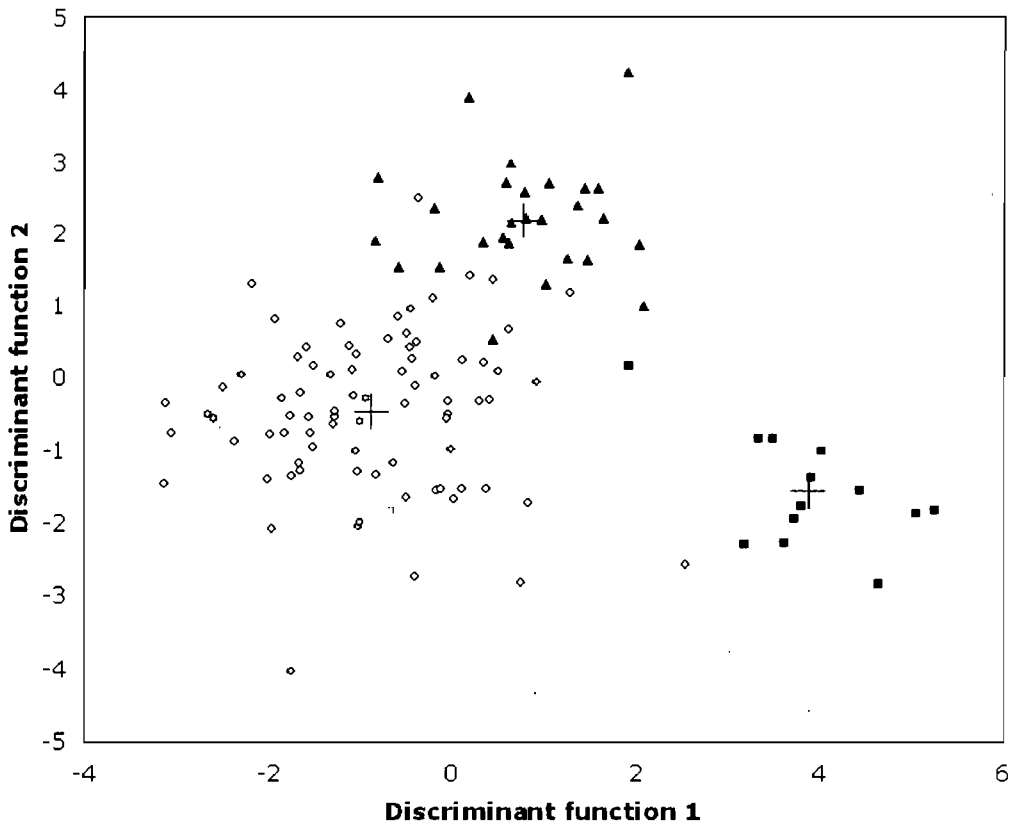

Figure 4.4 Bidimensional scatterplot defined by discriminant functions 1 and 2 to distinguish three groups of populations of *G. radiata*: Bezaha-Mahafaly (■), Lavavolo/Sakotoavo/Besily/Kilibory (○) and Antsifitse/Ifotaka (▲). Group centroids are also indicated (+).

4.6 Discussion

4.6.1 Three distinct groups

First, it is worth pointing out that the term population is used synonymously with sampling site throughout this paper, although it is not known if the sites actually represent different populations. Thus, we did not necessarily expect to find as many as seven areas to which tortoises could be assigned with satisfactory success. Indeed, the methodology employed has allowed for the identification of three distinct groups of populations that represent three geographic areas. The first of these geographic clusters corresponds to the Bezaha-Mahafaly population, located in the northernmost part of the species' range, the second group comprises the four populations from the core of the range in the southwest (Lavavolo, Sakotoavo, Besily and Kilibory), and the third one consists of the eastern populations (Antsifitse and Ifotata). The classification success rate of individuals among these three groups reached a value of 83%, whereas previous molecular work involving the use of 13 polymorphic microsatellites provided an assignment rate of 87% (Chapitre 3). In the latter study, three genetically distinct groups were also distinguished. The results obtained with the morphometric method are thus particularly impressive with respect to those achieved with molecular markers. Furthermore, the possibility that alien repatriated tortoises may have been included in the samples could increase the misassignment rate, although precautions regarding the chosen sampling sites were taken to minimize this bias. In fact, sampled populations were located far from regions where known repatriations of confiscated tortoises have occurred recently (e.g. Andohahela National Park, Leuteritz *et al.* 2005)

Unfortunately, because the sampled populations in the molecular study were not identical to those used in the present paper, it is difficult to compare the results from both studies concerning the delimitation of distinct units within the species' range. Nevertheless,

the genetic breaks represented by rivers that were revealed by the genetic analyses (Chapitre 3) are not significant in the analyses of morphometric differentiation.

4.6.2 Exploitation as an evolutionary force?

The multiple regression results are particularly noteworthy. The abundance of tortoises at sampling sites can be considered as an indicator of exploitation intensity for the local tortoise population. It has been demonstrated that tortoise abundances are much lower where the species is harvested, in comparison with regions where the taboo still protects tortoises (O'Brien 2002; Lingard *et al.* 2003; O'Brien *et al.* 2003). The sites of Bezaha-Mahafaly and Ifotaka are located near Antanosy territories, where the tortoise populations have been exploited for perhaps centuries, and the site of Antsifitse is close to an important road that facilitates the poaching and smuggling of tortoises. As explained previously, abundances at these three sites were lower compared to those obtained at the other sites. The multiple regression analysis confirmed that this factor was correlated to the morphometric differentiation of populations. It is also known that the average size of tortoises in harvested populations is significantly lower (O'Brien 2002), which was confirmed in our dataset. This may be explained by the removal of the largest individuals in the exploited populations, as observed for other exploited tortoise species (Znari *et al.* 2005). However, this explanation cannot justify the differences in shape (or scute proportions) noticed in our analyses. There have been some studies concerning the role that human exploitation may play in the rapid evolution of populations (Reznick & Ghalambor 2005, and references therein). The most striking evolutionary change in those populations is a smaller body size at maturity, with exploitation selecting against large individuals.

Although we do not know how shell proportions are related to body size in radiated tortoises, we believe that exploitation may have been the force driving morphometric differentiation. Significant phenotypic changes in populations can occur within few

generations if the mortality attributed to exploitation is high enough (Reznick & Ghalambor 2005). Archaeological evidence suggests that extant tortoise species in Madagascar have been hunted since the 8th century (Dewar 1997) and Antanosy people have occupied their present territories in southeastern Madagascar and in the Tulear region since the 9th and 15th centuries respectively (Bernard 1978; Wright & Rakotoarisoa 1997). This timeframe would allow sufficient time for our hypothesis to operate. Moreover, mature radiated tortoises do not have natural predators in Madagascar, so collecting surely represents a very high proportion of the total adult mortality in harvested populations. In any case, the trend in smaller body sizes is especially worrisome in light of recent studies confirming that larger female tortoises lay larger eggs, a correlate of hatchling size (Pedrono *et al.* 2001; Leuteritz & Ravolanaivo 2005), and that larger hatchlings have greater chances of surviving their first year (O'Brien *et al.* 2005).

4.6.3 Morphometrics as a management tool

Methods developed to correct for the effect of body size in morphometrics have been widely debated in the literature (e.g. Mosimann & James 1979; Humphries *et al.* 1981; Reist 1985; Somers 1986; Jungers *et al.* 1995). As suggested by Reist (1985), many authors have used a regression-based approach (including ANCOVA with body length as a covariate) to correct measurements for the effect of body size (e.g. Lamb & Lovich 1990; Germano 1993; van Dam & Diez 1998; Bonnet *et al.* 2001; Carretero *et al.* 2005). In the present case, preliminary analyses using residuals of size-correcting regressions yielded poorer results than those obtained with the methodology described above. The discriminatory power of these size-adjusted variables was lower, successfully classifying only between 60 to 70% of the individuals among the three groups. The idea of using PCA to discriminate size from shape was introduced by Jolicoeur & Mosimann (1960). In their classic paper, these authors suggested that the first principal component of log-transformed data corresponds to size variation, because all descriptors are positively correlated with it,

and this approach has enjoyed frequent subsequent use (e.g. Kamezaki & Matsui 1995; McLuckie *et al.* 1999). However, it has been noted that shape in addition to size variation is incorporated in the first principal component, because the loadings of each descriptor, although all positive, are not always equal (Humphries *et al.* 1981). In the present study, loading scores of the 39 variables on the first principal component varied from 0.13 (curvilinear length of carapace) to 0.25 (anal fork width), which advocated the use of the size-constrained PCA.

It is worth mentioning that a few statistical drawbacks to the method we used have been noted (Somers 1989; Sundberg 1989; Cadima & Joliffe 1996). The absence of correlation between pairs of principal components is a property of regular PCA that is lost when applying the size-constrained method. Indeed, Cadima & Joliffe (1996) have noted correlations as high as 0.81-0.92 between the isometric size component and the third principal component for some datasets. In those cases, considering this third component as shape variation only is, to say the least, hazardous. Correlation coefficients between the first isometric component and the 38 subsequent components for our *G. radiata* data did not reveal such strong correlations, with the highest being 0.51 for the 25th component. Thus, this potential drawback is not a serious problem in our case. Certain transformations could have been applied to the data had this been the case (Somers 1989; Sundberg 1989).

We have shown that the methodology outlined here allowed the distinction of three groups of populations with satisfactory classification success. Aside from the size-removal procedure, another feature of our method increased the statistical power in the analyses. At first both sexes were analyzed separately, following several studies in chelonian morphometrics (Lamb & Lovich 1990; Ernst & Laemmerzahl 2002; Carretero *et al.* 2005). Doing so, the assignment rates among the three groups varied between 65 and 75%. Small sample sizes for some populations for one of the sexes probably accounted for these poorer results. For instance, there were only three males sampled in Bezaha-Mahafaly, and five

females in Ifotaka. Hence, the computation of the discriminant functions was based on few individuals, thus explaining the greater number of assignment errors. The simple transformation we used to correct data for sexual dimorphism enabled us to achieve better results.

4.7 Conclusion

Comparing the results from several morphometric studies is not possible if the methodologies are different, as the various transformations affect the data in different ways (Reist 1985). We believe that the methodology we have described in this paper could be applied to any chelonian species. In the case of the radiated tortoise, the incorporation of additional individuals and populations might increase the classification results or help distinguish other groups. Nonetheless, using the data presented in this study, we have created an Excel spreadsheet that automatically assigns individual tortoises to one of the three putative geographic areas, given their measurements in millimeters. The procedure follows the methodology outlined above, i.e. correction for sexual dimorphism, addition of the individual to the PCA, removal of the size component, and calculation of the discriminant scores. We believe that this easy-to-use tool (available online at doi:10.106/j.biocon.2006.08.022) could be useful for the management of the radiated tortoise. Because large numbers of tortoises from the same region are usually caught simultaneously, the classification success would be even greater, since all individuals of the same seizure would be assigned to the group to which the majority of individuals would be assigned. We also hope that the development of such tools and the scientific interest devoted to *G. radiata* may encourage local authorities to increase law enforcement actions in southern Madagascar. Furthermore, although our study was not designed to thoroughly investigate the factors underlying morphological differentiation, the hypothesis we have proposed concerning the potential role of exploitation in the differentiation of populations

deserves additional examination. Further work will focus on patterns of genetic variation among exploited and unexploited populations of *G. radiata*.

4.8 Acknowledgements

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CHAPITRE 5

Genetics of a decline in a long-lived species: disentangling historical and contemporary forces explaining patterns of diversity in the critically endangered Malagasy radiated tortoise (*Astrochelys radiata*)[1]

5.1 Résumé

L'efficacité de diverses approches en génétique de la conservation est susceptible d'être influencée par les caractéristiques de l'histoire naturelle des espèces. Par exemple, la détection de goulots d'étranglement peut être plus ardue chez les espèces qui possèdent un long temps de génération. Ici, nous fournissons une analyse génétique complète de la tortue radiée (*Astrochelys radiata*) du sud de Madagascar qui inclut des populations fortement exploitées et des populations non-exploitées, en accordant une attention particulière à la détection de goulots d'étranglement. L'utilisation de différents types de marqueurs génétiques (13 microsatellites et deux fragments de l'ADNmt, cyt-*b* et d-loop) nous a permis d'établir un scénario global de l'histoire évolutive récente de cette espèce en déclin. Même si la diversité génétique était significativement supérieure pour les populations non-exploitées, d'autres résultats (détection de goulots d'étranglement, simulations) suggèrent que les activités anthropogéniques récentes ne sont probablement pas responsables de cette différence. En effet, les données des séquences de la région de contrôle de l'ADNmt révèlent que *A. radiata* a connu une importante expansion au cours du Pléistocène supérieur, et que cette expansion s'est déroulée à l'ouest du fleuve Menarandra, un cours d'eau qui constitue la plus importante barrière phylogéographique chez cette espèce (tel que

[1] Rioux Paquette, S., Mandimbihasina, A.R., Brenneman, R.A., Lapointe, F.-J.& Louis Jr., E.E. Genetics of a decline in a long-lived species: disentangling historical and contemporary forces explaining patterns of diversity in the critically endangered Malagasy radiated tortoise (*Astrochelys radiata*). Soumis, sous forme abrégée, à *Molecular Ecology*.

supporté également par les analyses des microsatellites). Nous proposons par conséquent un scénario où la diversité génétique réduite des populations situées les plus à l'est du Menarandra est attribuable à des tailles efficaces de populations historiquement plus faibles, dans un paysage naturellement fragmenté qui ne pouvait pas supporter des densités de tortues aussi élevées que du côté ouest. Nos résultats mettent aussi en évidence certains doutes à propos de la fiabilité de la méthode du *M*-ratio, et révèlent que les allèles nuls aux locus microsatellites introduisent un biais dans l'estimation des valeurs de *M* et des valeurs de différentiation génétique. Finalement, la comparaison des estimations de la taille efficace historique de *A radiata* et de son espèce sœur *A. yniphora*, la tortue terrestre la plus rare du monde, suggère que les deux espèces étaient similairement abondantes dans le passé, ce qui devrait être un sérieux avertissement pour la conservation de la tortue radiée.

5.2 Abstract

The efficiency of various approaches in conservation genetics may be affected by the life history traits of species. For instance, bottleneck detection may be hindered in species with long generation times. Here, we provide a thorough genetic analysis of the radiated tortoise (*Astrochelys radiata*) from southern Madagascar that includes heavily exploited and undisturbed populations, with special emphasis on bottleneck detection. The use of different types of markers (13 microsatellites and two mtDNA fragments, cyt-*b* and d-loop) allowed us to establish a comprehensive picture of the recent evolutive history of this declining species. Although genetic diversity was significantly greater in non-exploited populations, other results (bottleneck detection, simulations) suggest that recent anthropogenic impacts may not be responsible for this difference. Indeed, control region sequence data reveal that *A. radiata* experienced a large population expansion in the late Pleistocene and that this expansion took place west of the Menarandra River, the main phylogeographic break in the species (as revealed by microsatellite analyses). We thus hypothesize that lower diversity in eastern populations is due to historically lower

population sizes, in a naturally fragmented landscape that could not harbor tortoise densities as high as on the western side. Our results also emphasize concerns about the reliability of the *M*-ratio method, and reveal that microsatellite null alleles introduce a bias in both *M* values and population differentiation estimates. Finally, the comparison of historical effective size of *A. radiata* and of its sister species *A. yniphora*, the world's rarest tortoise, suggest that they were similarly abundant in the past, which should be a warning for radiated tortoise conservation.

5.3 Introduction

The relevance of genetics in the decline and extinction of endangered species was introduced in the literature more than 25 years ago (Frankel & Soulé 1981) and since, the field of conservation genetics has rapidly emerged as a focal discipline of conservation biology. The role of genetic data in wildlife management is diverse (reviewed in DeSalle & Amato 2004), and most principles remain valid across taxa and conservation cases. For instance, the substantial reduction of the effective size of a population will inevitably impact the level of genetic diversity it may harbor, which might in turn jeopardize its viability and long-term adaptability (Coltman *et al.* 1998; Saccheri *et al.* 1998; Reed & Frankham 2003). Detecting this phenomenon may be the first step towards applying appropriate conservation initiatives to prevent genetic variation from reaching dramatically low levels, e.g. by providing adequate habitat corridors among isolated subpopulations of a species in a fragmented landscape. Hence, the study of population bottlenecks has become an important aspect of conservation genetics through the development of detection methods (Cornuet & Luikart 1996; Luikart *et al.* 1998; Garza & Williamson 2001) and empirical evaluation in both natural (Hoelzel 1999; Spear *et al.* 2006; Busch *et al.* 2007) and simulated populations (Williamson-Natesan 2005; Leblois *et al.* 2006). Resolving population structure, defining conservation units, and identifying genotypes for translocations and captive breeding programs are among other important facets of

conservation genetics that have been investigated with the same interest (DeSalle & Amato 2004).

However, in species with peculiar life history traits, genetic approaches may fail to fulfill their role. The occurrence of recent bottlenecks, as an example, in species with particularly long generation times, may remain undetected (Kuo & Janzen 2004; Lippe *et al.* 2006). Nonetheless, these species may also be affected by the negative impacts of a demographic bottleneck, but the reduction in genetic diversity may be masked for decades (perhaps centuries) if generations also span over decades. Among vertebrates, chelonians (order Testudines) are the longest-living taxa, with giant tortoises (*Chelonoidis* and *Dipsochelys*) reported to live well over 150 years (Pritchard 1979). This extreme longevity is associated with particular demographic and reproductive strategies that include a very poor survivorship of eggs and hatchlings, a very low mortality rate among adults, and a tremendously delayed sexual maturity (Gibbs & Amato 2000). These traits make turtles and tortoises especially vulnerable to human exploitation: studies have shown that population viability is not substantially affected by the removal of eggs or hatchlings, but that an increase in adult mortality rates rapidly leads to steep declines and increased extinction risks (Congdon *et al.* 1993; Heppell 1998; but see Fordham *et al.* 2007). Noticing this incompatibility between turtle life history and human harvest has prompted Congdon *et al.* (1993) to suggest that the notion of sustainable exploitation of turtle populations may be an oxymoron. It is thus not surprising to realize that approximately half of the 320 turtle species are currently considered as threatened or vulnerable; exploitation is directly involved in the decline of 66% of all chelonian species listed on the IUCN Red List (Klemens & Thorbjarnarson 1995).

The radiated tortoise (*Astrochelys radiata*, formerly *Geochelone radiata*) is endemic to the semiarid spiny forest of southwestern Madagascar. The island of Madagascar is one of the world's highest priorities among biodiversity hotspots (Mittermeier *et al.* 2004) and

the ongoing anthropogenic environmental crisis has not spared the spiny forest. Imminent threats to this unique biome include slash-and-burn agriculture, production of charcoal and fuelwood, and livestock grazing (Seddon *et al.* 2000; Casse *et al.* 2004). Despite deforestation, however, the persistence of the radiated tortoise, or sokake in Malagasy, is mainly imperiled by human overexploitation. The patterns of tortoise harvest in southern Madagascar have been described previously (O'Brien *et al.* 2003; Lingard *et al.* 2003) and offer an ideal situation to study the genetics of a decline in a long-lived organism. In some parts of the species range, the land is inhabited by Mahafaly and Antandroy people, who respect an ancestral cultural taboo prohibiting tortoise collection or consumption (Figure 5.1). On the other hand, the remainder of the range is occupied by Vezo and Antanosy people who do not observe this belief, and tortoise meat is either considered a delicacy or constitutes a significant part of the diet in those tribes. Nowadays, the radiated tortoise is restricted to Mahafaly and Antandroy territories, as a result of an estimated 50% range reduction over the last 150 years, with an accelerated pace over the past 30 years that resulted in the local extirpation of the species in Vezo and Antanosy territories (O'Brien 2002; O'Brien *et al.* 2003). The radiated tortoise has an extremely slow growth rate (sexual maturity is reached at 16 years) and low breeding potential (mean clutch size of 2-3 eggs) (Pedrono 2008), which further increase its vulnerability to anthropogenic threats.

In this paper, a special emphasis will be directed towards the putative effects of human exploitation on the genetic signature of radiated tortoise populations: diversity indices will be compared between harvested and non-harvested populations, and two bottleneck detection methods will be employed to evaluate their ability to detect substantial effective size reductions in populations close to Antanosy territories. The main hypotheses are that exploited populations harbor reduced levels of genetic diversity, and that bottleneck detection methods are able to detect demographic bottlenecks in these populations. Additionally, if indeed differences among the two groups of populations are found, we will investigate the hypothesis that recent human exploitation is the main force explaining this

pattern. In order to do so, a thorough analysis of historical and contemporary genetic structure in *A. radiata* will be needed. Relying on a limited sampling, Rioux Paquette *et al.* 2007 (Chapitre 3) reported that the Menarandra and Manambovo rivers, which are seasonal rivers, separate conservation units of the radiated tortoise, with modest levels of differentiation at microsatellite loci. Here, we provide a more complete sampling to resolve the genetic structure of the species that also incorporates mtDNA sequence data from a subset of genotyped individuals in order to elucidate the demographic history of *A. radiata*. Finally, these sequence data will be analyzed in conjunction with sequences from its sister species, the ploughshare tortoise *Astrochelys yniphora*, another Malagasy endemic species and the world's rarest tortoise, in order to test the hypothesis that *A. radiata* has a larger ancestral effective size than *A. yniphora*.

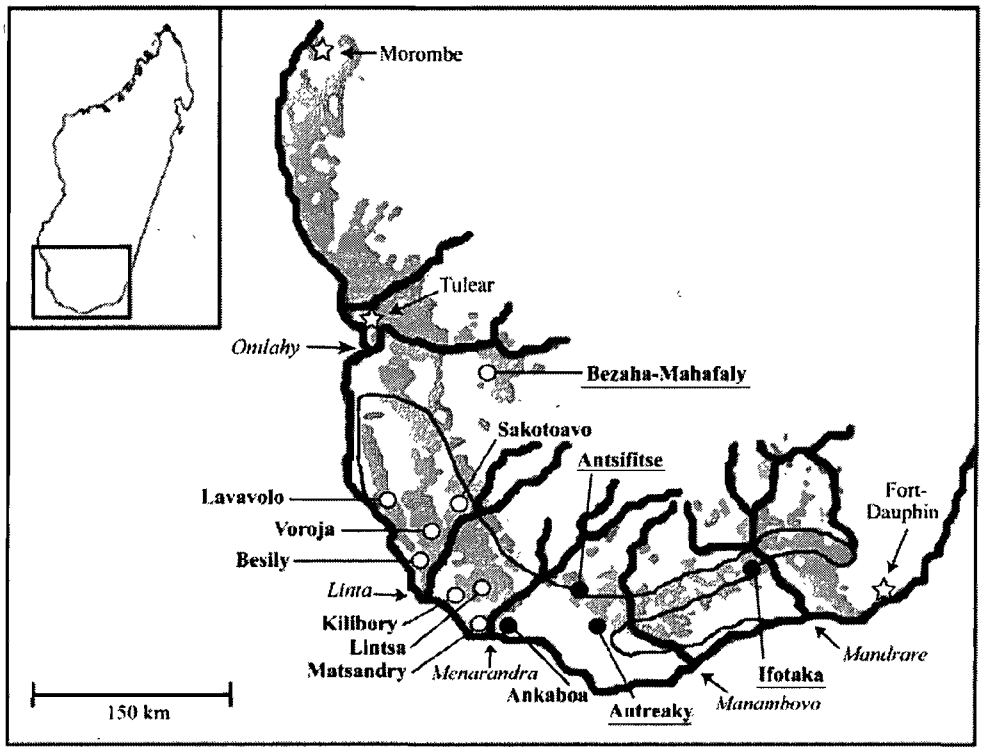

Figure 5.1 Map of southern Madagascar indicating the 12 locations where radiated tortoises were sampled in this study. Open circles correspond to populations located on the west side of the Menarandra River, whereas black circles indicate eastern populations (as in subsequent figures). Names in italic indicate rivers, star symbols represent main cities, and underlined locations indicate exploited populations. The extant range of *A. radiata* is delimited by the thin black line, while the remaining spiny forest vegetation is represented by the light grey areas (from Du Puy & Moat 1996).

5.4 Materials and methods

5.4.1 Sample collection

The sampling in this study incorporates 141 blood samples collected from wild radiated tortoises at six locations in November and December 2000 that were genotyped previously (Chapitre 3). Moreover, between February and April 2004, additional skin samples were scraped from the front limbs of 161 tortoises from eight sites (six new sites and two revisited from 2000 to complete sampling). These tissue samples were preserved in 95% ethanol until DNA could be extracted. In total, 302 individuals from 12 different sites selected across the present species range were used here (Figure 5.1; see Table 5.1 for samples sizes). Eight of these locations were considered as non-exploited based on testimonies from villagers and population structure data. These sites were all located well within Antandroy and Mahafaly territories. The remaining four locations were treated as exploited populations; they included two sites very close to Antanosy territories (Bezaha-Mahafaly and Ifotaka) and two sites adjacent to the main road facilitating tortoise poaching and smuggling in south central Madagascar (Antreaky and Antsifitse, Figure 5.1).

5.4.2 DNA extraction and molecular analyses

Total DNA extraction was performed with QIAGEN DNeasy extraction columns (QIAGEN, Valencia, CA 91355) according to the directions of the manufacturer. All samples were genotyped at 13 microsatellite loci following the procedure described elsewhere (Chapitre 2; Chapitre 3). These microsatellite markers included eight *A. radiata* specific markers (RAD14, RAD27, RAD284, RAD313, RAD573, RAD891, and RAD932; Rioux Paquette *et al.* 2005), four markers developed for its sister species *A. yniphora* (YNIP14, YNIP239, YNIP405, and YNIP604; Mandimbihasina 2004), and one Emydid marker (GMuD87; King & Julian 2004). Several samples that had been genotyped

Table 5.1 Sample sizes and summary genetic statistics of the 12 radiated tortoise (*Astrochelys radiata*) populations sampled in southern Madagascar for this study.

Pop. #	Sampling site	*n*	k_m	*Ar*	H_O	H_E	*n* mtDNA	D-loop haplotypes
1	Bezaha-Mahafaly	18	10.0	8.3	0.770	0.810	-	-
2	Sakotoavo	23	11.5	8.8	0.782	0.803	-	-
3	Lavavolo	47	15.8	9.4	0.748	0.847	5	A, J, K, L, P
4	Voroja	10	8.6	8.6	0.789	0.842	3	E, H, M
5	Besily	19	11.4	9.2	0.840	0.845	-	-
6	Kilibory	26	13.2	9.6	0.813	0.858	-	-
7	Lintsa	20	12.1	9.5	0.711	0.841	8	A, E, H, I, N, O
8	Matsandry	34	12.8	8.9	0.728	0.853	10	A, E, H, M, O, Q, R
9	Ankaboa	46	11.8	7.7	0.725	0.820	10	A, B, C, E, H, G
10	Antsifitse	22	8.7	7.0	0.751	0.781	-	-
11	Antreaky	12	6.5	6.4	0.703	0.758	7	B, C, E
12	Ifotaka	25	6.7	5.6	0.672	0.753	5	D, E, F
Total	-	302	22.2	-	0.749	0.822	48	-

n, sample size for microsatellite analyses (number of individuals); k_m, mean number of alleles per locus; *Ar*, allelic richness; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity; *n* mtDNA, sample size for mtDNA analyses; D-loop haplotypes, list of control region haplotypes uncovered in each population.

previously were reanalyzed to ensure allele size consistency, and no discrepancy was noted in comparison with the original dataset. Furthermore, sequence data from the mitochondrial control region (d-loop) and the cytochrome *b* gene (cyt-b) were obtained for a subset of genotyped individuals (48) from seven populations (Table 1). The cyt-b gene was amplified with primers *Cytb-Glu* 5'-TGACATGAAAAAYCAYCGTTG-3' (Paabo 1990) and *Cytb-H15149* 5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' (Kocher *et al.* 1989), whereas the d-loop fragment was amplified with primers *DL4* 5'-TTATTT(AG)CCACTAGCATAT-3' (Dutton *et al.* 1996) and *HDCM1* 5'-GCAAGTAAAACTACCGTATGCCAGGTTA-3' (Allard *et al.* 1994). These fragments were amplified using the same reaction mix and PCR profile as that employed for microsatellite amplification. Sequences were generated using the amplification primers in cycle sequence reactions. These were carried out in 10.5 µl volumes with 2.5 µl of sequencing buffer, 0.5 µl of the forward or reverse primer, 1.0 µl of BigDye, 6.0 µl of d_2H_2O and 0.5 µl of template DNA. Cleaned products were electrophoresed on an ABI 3100 automated sequencer (Applied Biosystems), and sequences were checked with the software 4PEAKS (http://mekentosj.com). Alignments were produced with the software CLUSTALX (Thompson *et al.* 1997) and visually verified in SE-AL (http://tree.bio.ed.ac.uk/software/seal/). Complete sequences were deposited in Genbank under accession numbers EU556344-EU556432.

5.4.3 Data analysis

5.4.3.1 Microsatellite diversity and population structure

Conventional analyses of genetic variation and differentiation were performed with the microsatellite dataset following the methodology thoroughly described in Chapitre 3. Namely, parameters of within-population diversity (observed (H_O) and expected (H_E) heterozygosity, mean number of alleles per locus k_m, allelic richness *Ar*), tests of linkage

disequilibrium, tests of deviation from Hardy-Weinberg equilibrium (HWE), verification for the presence of null alleles and estimation of their frequency, calculation of pairwise F_{ST} values, STRUCTURE (Pritchard *et al.* 2000) analyses for K = 1 to 12, and analyses of molecular variance (AMOVA; Michalakis & Excoffier 1996) were computed following the methodology described in Chapitre 3. Additionally, unbiased pairwise R_{ST} values were estimated with RSTCALC (Goodman 1997). For all tests involving multiple comparisons, significance levels were adjusted with a sequential Bonferroni procedure (Rice 1989). Relationships among populations were also represented with a neighbor-joining (NJ) tree (Saitou & Nei 1987). To do so, pairwise chord distances D_{CE} (Cavalli-Sforza & Edwards 1967) were computed in MICROSAT (Minch 1996) and the NJ tree was determined with the PHYLIP 3.5 package (Felsenstein 1993). Bootstrap values were obtained for this tree by resampling microsatellite loci 1000 times in MICROSAT.

5.4.3.2 Null alleles and population differentiation

To verify that our results about population differentiation were not biased because of the presence of null alleles in the microsatellite dataset, we computed population pairwise F_{ST} values derived from markers without null alleles and compared these to F_{ST} values obtained from markers that harbored significant null allele frequencies. Statistical assessment was performed by comparing 95% confidence intervals (CI) around each F_{ST} estimate. Bootstrap CIs were generated by resampling loci (among either markers with or without null alleles) 1000 times with the software GDA (Lewis & Zaykin 2001). Additionally, paired Student's *t*-tests were computed to compare pairwise F_{ST} values obtained with both sets of markers.

5.4.3.3 Comparing diversity among populations

Genetic diversity at the population level was compared between exploited and non-exploited populations. Differences between these two groups for H_O, H_E, Ar, and the mean

relatedness (R; Queller & Goodnight 1989) among individuals were tested with the permutation test implemented in FSTAT (Goudet 1995), and 1000 iterations were performed for each test. One-tailed tests were performed because exploited populations were expected to exhibit lower heterozygosity and allelic richness values, and higher relatedness. Tests were repeated excluding data from markers with null alleles, to ensure that results were not influenced by possibly inaccurate estimates of genetic diversity due to high frequencies of null alleles. The same precaution was also taken when performing the bottleneck detection tests described below.

5.4.3.4 Bottleneck detection and simulations

The detection of bottleneck genetic signatures in sampled populations was attempted using two methods. The first method is a test of heterozygote excess computed across all loci; the rationale of this test is that a severe decline in effective population size will lead to a faster loss of alleles (especially rare alleles) than the parallel reduction in heterozygosity (Cornuet & Luikart 1996). Thus, recently bottlenecked populations are expected to exhibit heterozygote excesses with respect to the expected heterozygosity values at equilibrium derived from the observed number of alleles. To test the hypothesis of heterozygote excess across all loci in each population, the Wilcoxon signed-rank test implemented in BOTTLENECK (Piry *et al.* 1999) was used assuming a two-phase model (TPM; Di Rienzo *et al.* 1994) of microsatellite mutation, which is a modification of the strict stepwise mutation model (SMM; Kimura & Ohta 1978) that incorporates a proportion of multi-step mutational events (p_m). In our case, tests were carried out with $p_m = 0.12$, based on the results of a review of fully resolved microsatellite mutations (Garza & Williamson 2001), but we also tried $p_m = 0.30$, which corresponds to a model that departs from SMM to a larger extent.

The second bottleneck detection approach that we used is the "M-ratio" method (Garza & Williamson 2001). The value of the parameter M for a locus is calculated as

$M = k / r$ where k is the number of alleles and r is the size range (in number of repeats) of alleles. In a declining population, M is expected to be smaller than in stable populations because k decreases faster than r due to the loss of rare alleles from intermediate size classes by genetic drift (Garza & Williamson 2001). In order to test the significance of M in a population, the observed value is compared to a theoretical distribution generated by simulating 10 000 times a population at equilibrium, based on the empirical allele frequencies of the studied population. This operation was performed with the software M_P_VAL (Garza & Williamson 2001), which provides a p value for M that corresponds to the proportion of simulated values that were inferior to the real value. Additionally, the distribution of M depends on three parameters: p_m, the average size of multi-step mutations (Δ_g), and $\theta = 4N_e\mu$, where N_e is the population effective size and μ the microsatellite mutation rate. For μ, we assumed a common figure for microsatellite loci of 5×10^{-4} mutation/generation (Weber & Wong 1993), which is also within the range of values found in another chelonian species, the green turtle *Chelonia mydas* (FitzSimmons 1998). We performed the analyses with several combinations of p_m (0.12 and 0.30 as explained previously for the heterozygote excess method), Δ_g (2.8, the average found in a literature survey, and 3.5, a more conservative value; Garza & Williamson 2001), and θ (2, 10, and 25, which correspond to N_e values of 1000, 5000, and 12 500 individuals, respectively).

Finally, we conducted bottleneck simulations in order to determine what scenarios could best explain differences in genetic diversity between exploited and non-exploited populations of *A. radiata*. Several population genetics programs exist to simulate the effects of bottlenecks (e.g. Balloux 2001; England & Osler 2001), but these assume discrete generations. However, tortoises in general are characterized by a tremendous longevity, and the overlap among generations is thus particularly great. Therefore, we employed BOTTLESIM (Kuo & Janzen 2003), a program that was especially designed to account for overlapping generations in bottleneck simulations. Simulations were carried out by simulating a population with an effective size of 5000 individuals (random mating with

equal sex ratio) that undergoes a 90% size reduction over a 225 years period. Two bottleneck schemes were analyzed: a steady decline from the first year to the last (i.e. reduction of 20 individuals every year), and an abrupt decline 30 years before the end of the simulations. Allele frequencies at the start of the simulations were the observed frequencies in undisturbed populations. Age at maturity was set to 16 years (Pedrono 2008), a longevity of 60 years was assumed (maximum longevity may be much higher, but very few individuals were estimated to be older than 40-50 years during field surveys, even in undisturbed populations), and the percentage of generation overlap was set at 95%, where 0 would correspond to discrete generations and 100 would indicate that all individuals that do not reach longevity limit are reassigned a random age value for the next year (Kuo & Janzen 2003). A total of 100 replicates were generated for each set of parameters, and simulations were repeated with a 99% size reduction instead of 90%. Results were compiled and analyzed with FSTAT in order to assess what proportion of the original genetic diversity (H_O and Ar) was retained in each bottleneck scenario.

5.4.3.5 Mitochondrial sequences

Basic measures of sequence diversity (nucleotide diversity (π), haplotype diversity, average number of differences) were obtained with the software DNASP (Rozas *et al.* 2003). To investigate the demographic history of the species, the mismatch distribution, i.e. the frequency distribution of mutational differences among pairs of sampled individuals (Rogers & Harpending 1992), was computed. It is possible to estimate the expected distribution under models of constant population size and population expansion/decline (Rogers 1995), and compare it to the empirical mismatch distribution. This operation was performed in DNASP. Another visual indication of population expansion can be noticed in haplotype networks, in which it would be visualized as a 'star-like' pattern. We constructed a network representing haplotype relationships using the statistical parsimony algorithm implemented in TCS (Clement *et al.* 2000). This method is useful to illustrate fine-scale

genetic relationships by depicting the number of mutations between haplotypes, whereas phylogenetic trees allow for a broader understanding of ancestor-descendent relationships (Posada & Crandall 2001). Furthermore, phylogenetic analyses also allow for the identification of reciprocally monophyletic lineages ('evolutionarily significant units' (ESUs) from Moritz 1994) within species. Thus, phylogenetic trees were also generated in PAUP (Swofford 2001) using maximum parsimony (MP) and neighbor-joining (NJ) algorithms. Distances were corrected according to the HKY85 model of sequence evolution for NJ analyses. Three sequences from *Astrochelys yniphora* (from Mandimbihasina 2004) were included as outgroups.

The estimation of divergences times (t) within the haplotype network was performed with the calculation of $t = \rho u$. In a network based on n sequences with a putative root and m different links from it, where l_i represents the number of mutations along the ith link, ρ corresponds to the average distance to the root, or $(n_1 l_1 + n_2 l_2 + \ldots + n_m l_m)/n$ (Saillard *et al.* 2000). To obtain the substitution rate u, we compared our d-loop data with sequences from the ploughshare tortoise *A. yniphora* (Mandimbihasina 2004), also endemic to Madagascar. We found a mean sequence divergence of 20.7% between the two species. Relying on other mtDNA genes, the divergence time between *A. radiata* and *A. yniphora* has been estimated at 10 million years (Caccone *et al.* 1999). Considering the length of the d-loop fragment sequenced here, the estimated $2.07\% \cdot \text{myr}^{-1}$ substitution rate translates into one substitution approximately every 132,000 years. The variance of t was estimated with $\sigma^2 = (n^2_1 l_1 + n^2_2 l_2 + \ldots + n^2_m l_m)/n^2$ (Saillard *et al.* 2000).

The program IM (Nielsen & Wakeley 2001) was used to further investigate the history of the radiated tortoise with respect to its sister species, *A. yniphora*. The isolation-with-migration (IM) model is useful to estimate gene flow (m), effective population sizes (θ) and time since splitting (t) between two taxa (e.g. Hey & Nielsen 2004; Won & Hey 2005). One of the fundamental assumptions of this model is that the two compared groups

are sister taxa. Although the relationship between the two Malagasy *Astrochelys* species has been questioned (Le *et al.* 2006), most molecular and morphological evidence suggests that these species are sister taxa (e.g. Caccone *et al.* 1999; Gerlach 2001; Palkovacs *et al.* 2002; Fritz & Bininda-Emonds 2007). D-loop sequences generated here for *A. radiata* were compared to 35 *A. yniphora* d-loop sequences from previous work (Mandimbihasina 2004) with IM. Preliminary Markov chain Monte Carlo with Metropolis-Hastings coupling (MC_3) simulations were conducted to determine appropriate maximum values for uniform priors. The final run consisted of 8 chains swapping 24 times every step, simulated for 3 million total iterations, discarding the first half million as burn-in. To convert estimates of θ to demographic scales ($\theta = 4N_e\mu$), a generation time of 40 years (Randriamahazo *et al.* 2007) was assumed along with the previously calculated substitution rate of 2.07×10^{-8} bp·yr^{-1}. We were essentially interested in comparing estimates of θ for the two species, since reciprocally monophyletic datasets are not informative for the estimation of t or the ancestral (before splitting) population size parameter (J. Hey, personal communication). Estimates of θ were considered as significantly different if their 90% highest posterior density (HPD) intervals did not overlap (Won & Hey 2005).

5.5 Results

5.5.1 Microsatellite diversity and population structure

Microsatellite diversity at the population level was high, both in terms of allelic richness and heterozygosity. Summary statistics (H_O, H_E, and Ar) are provided for each population in Table 5.1. Across loci, the overall number of alleles detected in this study was 289, ranging from 12 to 32 alleles per locus. Comparisons among all pairs of loci did not detect significant linkage disequilibrium, and 13 out of the 156 locus/population combinations revealed significant departures from HWE expectations. However, all HWE deviations were detected among the five loci (RAD313, RAD891, RAD932, YNIP14, and

YNIP239) that had significant frequencies of null alleles. The estimated null allele frequency for these five markers was, respectively, 0.189, 0.106, 0.074, 0.078, and 0.108. Interestingly, the mean number of (visible) alleles in loci without null alleles was 19.3 whereas it was 27.0 in loci with null alleles, and this difference was statistically significant (t-test results: $t = 2.14$, $p = 0.027$). No other genotyping artifacts (i.e. stuttering or large allele dropout) were noticed in the microsatellite dataset.

Pairwise F_{ST} and R_{ST} values are provided in Table 5.2. Results were globally consistent between the two measures, indicating differentiation between populations from both sides of the Menarandra River. Additionally, the Ifotaka population (F_{ST} and R_{ST}), and the Bezaha-Mahafaly and Sakotoavo populations (F_{ST}) were significantly differentiated from all other populations, except from the Voroja population, which had the smallest sample size. Bayesian clustering analyses in STRUCTURE clearly supported a $K = 2$ solution. This solution was obtained not only when considering the highest value of the "log probability of data" $L(K)$, but also with the more appropriate ΔK, a measure based on the second order rate of change in $L(K)$ (Evanno *et al.* 2005). These results are shown in Figure 5.2a and support the role of the Menarandra River as a barrier to gene flow. This split was also emphasized in the NJ tree depicting relationships among populations based on chord distances derived from microsatellite data (Figure 5.2b). The among-group variation was highly significant ($p < 0.001$) when computing an AMOVA with those two groups but the among-group variation represented only 4.4% of the toal variance. Even while assuming five distinct groups based on significant F_{ST} values (i.e. Bezaha-Mahafaly, Satokoato, Lavavolo/Voroja/Besily/Kilibory/Lintsa/Matandry group, Ankaboa/Antsifitse/Antreaky group, and Ifotaka), the among-group variation was merely 3.9 % of the total variance.

5.5.2 Null alleles and population differentiation

Excluding markers with prevalent null alleles did not affect results of pairwise population differentiation analyses, thus justifying their inclusion in analyses described above (i.e. significance level of F_{ST} values was not altered). Nonetheless, when comparing F_{ST} estimates obtained from the eight loci without null alleles and those obtained from the other five loci, markers with null alleles provided slightly superior estimates on average (paired t-test results: $t = 2.12$, $p = 0.038$). This bias was amplified when considering only comparisons involving significantly differentiated populations from both sides of the Menarandra River ($t = 2.79$, $p = 0.009$; Figure 5.3). However, non-overlapping 95% CIs of F_{ST} estimates computed from both subsets of markers were found in only one out of the 66 population pairwise comparisons (results not shown), and this case involved the two populations with the smallest sample sizes (Voroja and Antreaky).

5.5.3 Comparing diversity among populations

Results of permutation tests indicated significant differences in genetic diversity indices between harvested and non-exploited populations (Table 5.3). The only parameter for which the test was not significant was H_O. However, it was significant when excluding loci with null alleles (Table 5.3).

Table 5.2 Pairwise F_{ST} (above diagonal) and R_{ST} values (below diagonal) among 12 populations of radiated tortoises derived from data of 13 microsatellite loci. Statistically significant values after a sequential Bonferroni correction are shown in bold.

	Bez.-Mah.	Sakotoavo	Lavavolo	Voroja	Besily	Kilibory	Lintsa	Matsandry	Ankaboa	Antsifitse	Antreaky	Ifotaka
Bez.-Mah.	-	**0.022**	**0.027**	0.020	**0.030**	**0.026**	**0.028**	**0.038**	**0.068**	**0.106**	**0.102**	**0.108**
Sakotoavo	0.003	-	**0.012**	0.008	**0.013**	**0.014**	0.011	**0.019**	**0.058**	**0.099**	**0.097**	**0.092**
Lavavolo	0.008	0.001	-	0.007	0.006	0.005	0.006	0.009	**0.040**	**0.074**	**0.070**	**0.071**
Voroja	0.001	-0.007	-0.002	-	0.007	0.002	0.001	-0.005	**0.036**	**0.075**	**0.069**	**0.079**
Besily	-0.015	0.007	0.009	-0.004	-	0.007	0.006	0.014	**0.041**	**0.078**	**0.078**	**0.082**
Kilibory	0.014	0.020	0.010	-0.009	-0.003	-	0.001	0.005	**0.029**	**0.058**	**0.059**	**0.059**
Lintsa	0.033	0.024	0.021	-0.010	0.023	0.009	-	0.005	**0.036**	**0.064**	**0.063**	**0.060**
Matsandry	0.013	0.031	0.018	0.000	0.003	0.004	0.012	-	**0.019**	**0.050**	**0.046**	**0.052**
Ankaboa	**0.046**	**0.068**	**0.049**	0.018	**0.048**	**0.026**	**0.046**	**0.021**	-	0.008	0.011	**0.023**
Antsifitse	**0.051**	**0.112**	**0.079**	0.023	**0.046**	**0.042**	**0.066**	**0.036**	0.016	-	0.004	**0.026**
Antreaky	**0.048**	**0.083**	**0.072**	0.030	**0.056**	**0.041**	**0.064**	0.030	-0.005	0.010	-	**0.044**
Ifotaka	**0.075**	**0.093**	**0.086**	0.048	**0.080**	**0.066**	**0.073**	**0.058**	**0.061**	**0.067**	**0.054**	-

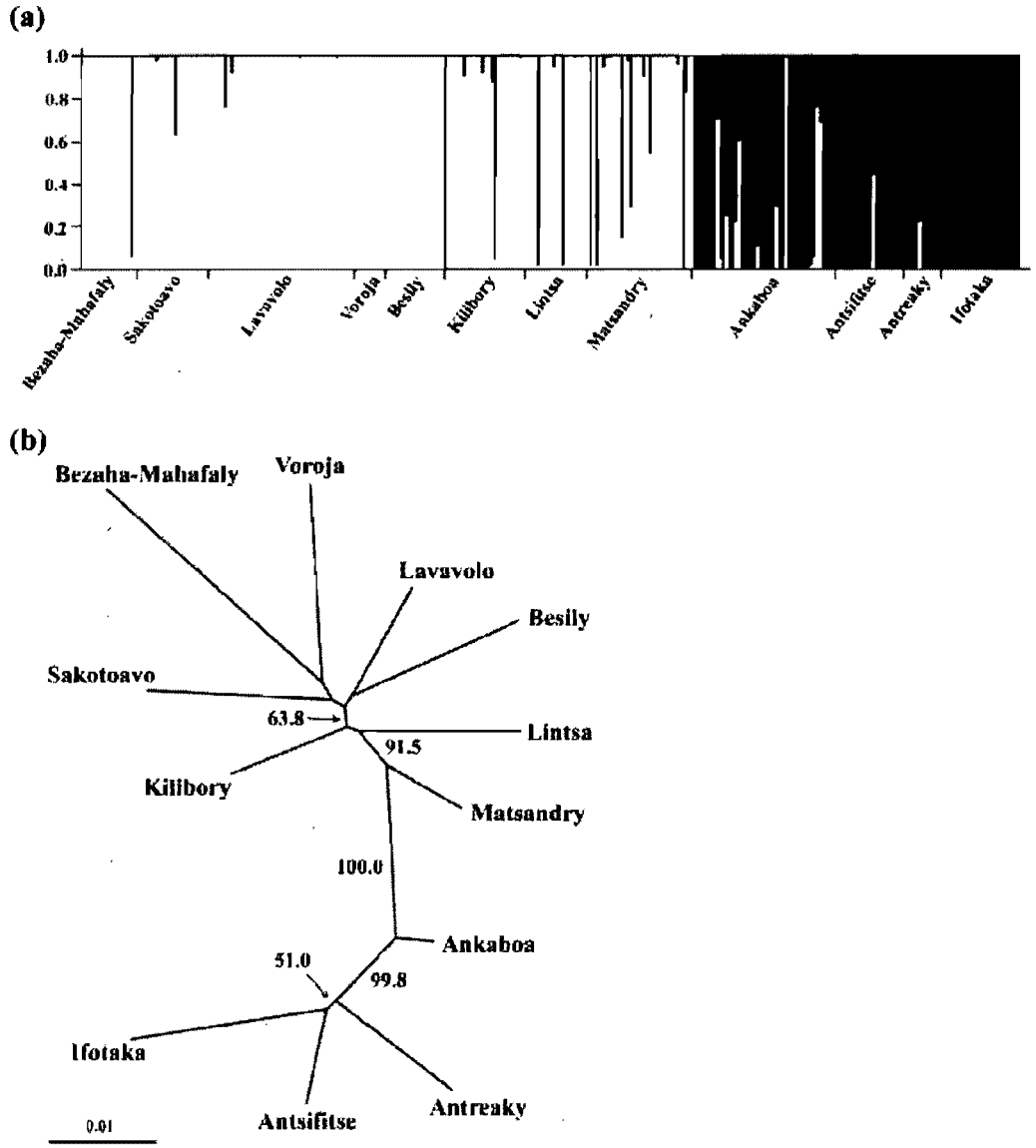

Figure 5.2 Population structure of *A. radiata* based on microsatellite data. (a) Results of the Bayesian clustering analyses indicating the repartition of all sampled individuals among two genetically distinct groups separated by the Menarandra River (West = white, East = black). (b) NJ tree derived from D_{CE} distances, illustrating the relationships among A. radiata populations. Bootstrap values > 50 are indicated along branches. The longest internal branch (with a bootstrap value of 100) corresponds to the Menarandra River.

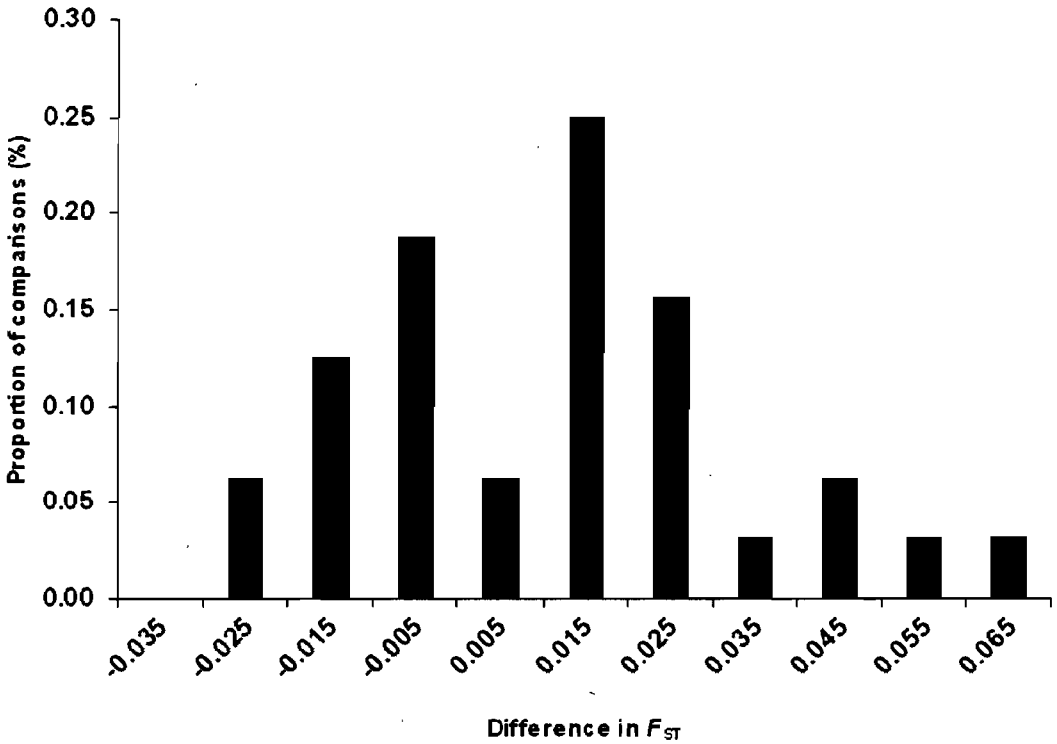

Figure 5.3 Distribution of the differences in estimated population pairwise F_{ST} values (for significantly differentiated populations of *A. radiata*) calculated from eight microsatellite markers without null alleles and five markers with null alleles. A positive difference indicates that the value derived from markers with null alleles was superior.

Table 5.3 Summary statistics of genetic diversity in exploited and non-exploited populations of radiated tortoises, with results of permutations tests assessing the statistical significance of differences.

	Non-exploited populations	Exploited populations	*p*
All loci			
Observed heterozygosity (H_O)	0.755	0.723	0.130
Expected heterozygosity (H_E)	0.839	0.777	**0.005**
Allelic richness (*Ar*)	9.035	6.824	**0.004**
Mean relatedness (*R*)	0.033	0.117	**0.016**
Only loci without null alleles			
Observed heterozygosity (H_O)	0.802	0.752	**0.021**
Expected heterozygosity (H_E)	0.822	0.771	**0.004**
Allelic richness (*Ar*)	8.628	6.742	**0.007**
Mean relatedness (*R*)	0.038	0.117	**0.013**

p, probability of test; $p < 0.05$ indicates significant difference between the two groups of populations (in bold).

5.5.4 Bottleneck detection and simulations

Analyses to detect heterozygote excesses due to population bottlenecks were not significant in any population when assuming a small proportion of multi-step mutations ($p_m = 0.12$), and only in the Ifotaka population ($p = 0.016$) when increasing p_m to 0.30. The exclusion of loci that comprised high frequencies of null alleles did not alter results at all. However, *M*-ratio values were greatly influenced by these markers; in every population, the *M* value was lower when computed across the five markers with null alleles than the value calculated with the other eight markers (Table 5.4), and this difference was highly significant (paired *t*-test results: $t = 4.49$, $p < 0.001$). Not surprisingly, every test conducted with data from all loci was significant regardless of the assumed population size. Furthermore, the same results were also obtained after the exclusion of the five questionable loci, when using $p_m = 0.12$ and $\Delta_g = 2.8$. Increasing either p_m to 0.30 or Δ_g to

3.5 had almost identical effects on results, and the outcome of the tests varied extremely depending on the chosen value for θ, ranging from being significant in all populations for θ = 2 to only one significant test (Bezaha-Mahafaly) for θ = 25 (Table 5.4). None of the tests were significant when $p_m = 0.30$ and $\Delta_g = 3.5$ were used together.

Table 5.4 Results of the *M*-ratio test to assess the occurrence of recent bottlenecks in twelve radiated tortoise populations.

Populations	*M*		*p*			
	Nulls	No nulls	θ = 2	θ = 10	θ = 25	θ = 25*
Bezaha-Mahafaly†	0.4895	0.5384	**0.0008**	**0.0051**	**0.0386**	**0.0242**
Sakotoavo	0.6011	0.6652	0.0539	0.1807	0.4811	0.3711
Lavavolo	0.6370	0.7267	0.1487	0.2135	0.3577	0.3472
Voroja	0.4735	0.4827	**0.0004**	**0.0067**	0.1282	0.0583
Besily	0.5556	0.6272	**0.0231**	0.1020	0.3679	0.2730
Kilibory	0.6334	0.7182	0.1796	0.4180	0.7251	0.6379
Lintsa	0.6177	0.6456	**0.0352**	0.1464	0.4438	0.3402
Matsandry	0.5784	0.6240	**0.0058**	**0.0177**	0.0504	0.0501
Ankaboa	0.5098	0.7053	0.0853	0.1193	0.2221	0.2300
Antsifitse†	0.5223	0.6278	**0.0186**	0.0664	0.2612	0.1994
Antreaky†	0.5307	0.5335	**0.0013**	**0.0238**	0.2132	0.1206
Ifotaka†	0.5386	0.5901	**0.0043**	**0.0140**	0.0642	**0.0487**

M, values of the mean *M*-ratio calculated from five microsatellites with high frequencies of null alleles (Nulls) and eight microsatellites without null alleles (No nulls); *p*, probability of the *M*-ratio test, using only markers without null alleles, with $p_m = 0.30$ and $\Delta_g = 2.8$, for three different values of θ, with results < 0.05 in bold.

* Results when $p_m = 0.12$ and $\Delta_g = 3.5$, shown for comparison with results obtained with the preceding set of parameters.

† These four populations are exploited populations.

The results of the bottleneck simulations are provided in Table 5.5. The extent of the loss of genetic diversity, even in worst-case simulation scenarios, was considerably lower than the observed differences in sampled populations, especially for *Ar* (Table 5.5).

Table 5.5 Proportion (%) of the original genetic diversity maintained in four different bottleneck simulation scenarios of a radiated tortoise population of 5000 individuals, along with proportion of diversity found in sampled harvested tortoise populations with respect to non-exploited populations. Statistically significant reductions ($p < 0.05$) are shown in bold.

	Constant 90% decline	Abrupt 90% decline	Constant 99% decline	Abrupt 99% decline	Real exploited populations
H_O	98.7	99.3	99.2	98.3	**93.8**
Ar	**96.7**	98.0	**96.6**	**92.9**	**78.1**

H_O, observed heterozygosity; *Ar*, allelic richness

5.5.5 Mitochondrial sequences

Amplification of the cytochrome *b* gene generated a 485 bp fragment. Variation was astonishingly low, with only two polymorphic sites detected among all individuals: seven individuals (five from Matsandry, one from Lavavolo and one from Ankaboa) had a G→A transition at position 417, and two of these also had a C→T transition at position 375. Data from cyt-*b* were not utilized in further analyses.

In contrast, the control region analyses yielded a 367 bp fragment that comprised 24 polymorphic sites, which defined 18 haplotypes in 44 individuals (four of the 48 sequenced individuals had incomplete sequences and were thus discarded). Overall, haplotype diversity in d-loop was equal to 0.896, π was 0.0085, and the average number of pairwise nucleotide differences was 3.1. The occurrence of a historical population expansion was strongly supported by the analysis of the mismatch distribution (Figure 5.4). This was also corroborated by a star pattern in the haplotype network (Figure 5.5b) that pointed towards an expansion originating from haplotype A. The phylogenetic tree (Figure 5.5a) suggested that the most basal divergence within *A. radiata* created a group comprising haplotypes {B, C, D}, which are restricted to individuals from the eastern side of the Menarandra. This split preceded the expansion that resulted in a considerable number of new haplotypes,

which are almost entirely restricted to individuals from the western side of the Menarandra River, with the exception of the {E, F,G} clade. The estimated time of the expansion is approximately 189,000 ± 21,000 years ago. Analyses in IM revealed that *A. radiata* has an impressively large historical effective size estimated at 11.9 million individuals (90% HPD: 7.1 – 17.7). Very surprisingly, this did not significantly differ from the estimate for *A. yniphora* (7.9 million individuals; 90% HPD: 4.2 – 12.4). The distribution of posterior probabilities of effective size parameters was well-defined and single-peaked, while migration parameters between the two species had very cleans peaks at $m = 0$.

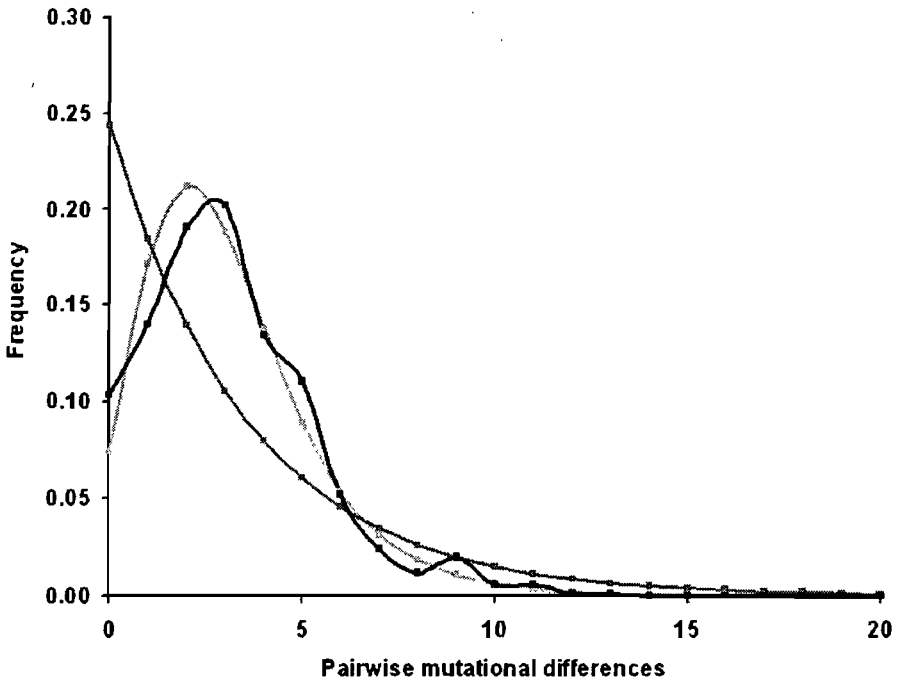

Figure 5.4 Mismatch distribution of mitochondrial control region sequences in *A. radiata*. The dark grey line corresponds to the expected distribution under constant population size; the light grey line is the expected distribution after population expansion; the black line represents the observed distribution in *A. radiata* d-loop sequences.

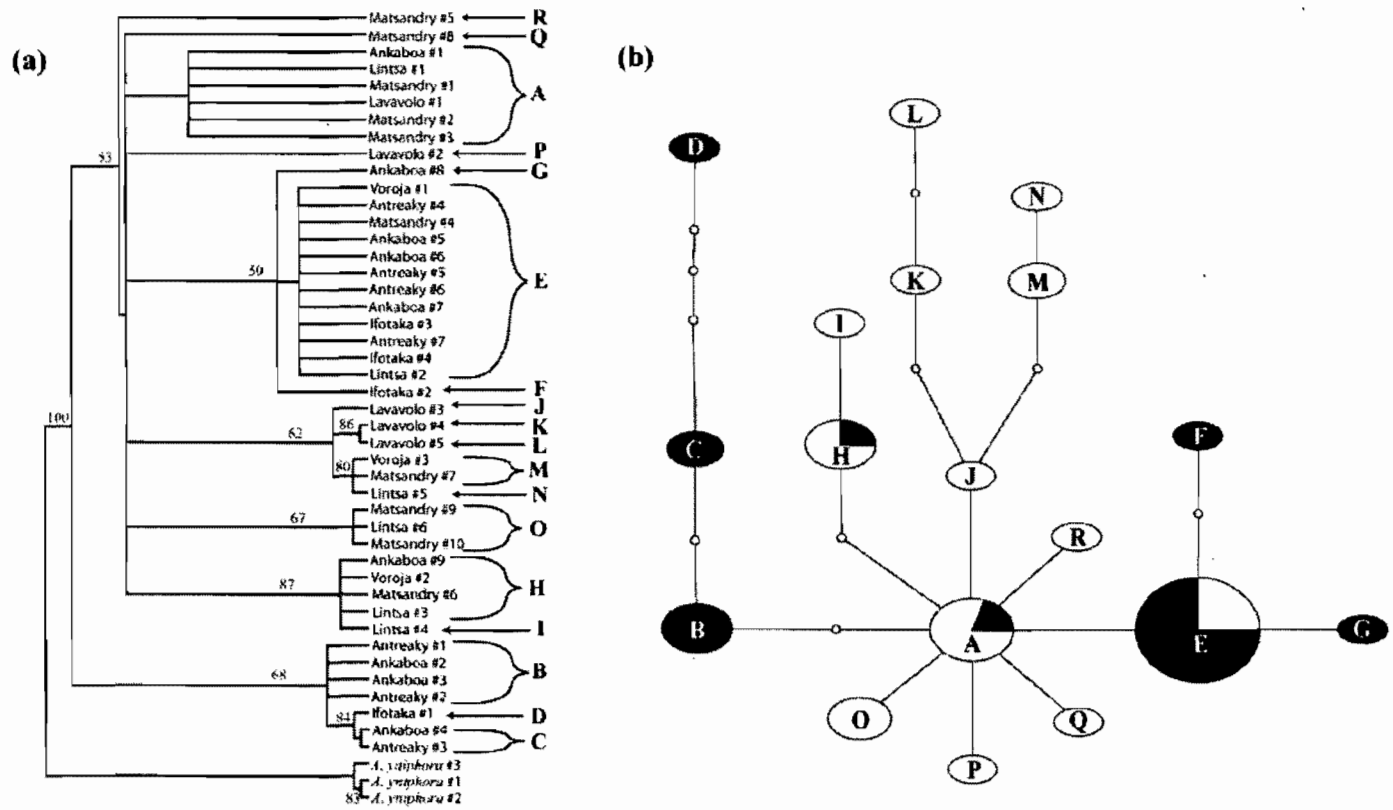

Figure 5.5 Mitochondrial control region analyses in *A. radiata*. (a) Phylogenetic tree representing ancestor-descendant relationships among *A. radiata* individuals and d-loop haplotypes (letters). Bootstrap values > 50 are provided along branches (MP tree is shown, but NJ tree had same topology). (b) Haplotype network depicting the mutational relationships among d-loop haplotypes, where each branch corresponds to a single substitution. Letters identify distinct haplotypes. The size of ovals is proportional to the frequency of haplotypes (smallest = one individual), and white and black colors indicate the proportion of individuals that were sampled on the western side of the Menarandra River (white) and eastern side (black).

5.6 Discussion

The combined use of different markers and analyses provides a highly detailed description of the patterns of genetic diversity and population structure in the radiated tortoise across southern Madagascar. Some findings are particularly relevant for understanding the historical and contemporary evolution of *A. radiata* populations, while others reveal processes of broader interest. In an effort to assimilate results into an integrative account, the following discussion will depart from the order of previous sections and focus on the global interpretation of the main findings of this study.

5.6.1 mtDNA variation and demographic history of *A. radiata*

A previous assessment that relied strictly on microsatellite data with limited sampling emphasized the predominant role of the Menarandra River as a putative barrier to gene flow (Chapitre 3). Here, our complete microsatellite dataset strongly supports this assertion (e.g. Figure 5.2). Interestingly, in the spider tortoise *Pyxis arachnoides*, a small-sized tortoise sympatric with *A. radiata* and also restricted to the spiny forest, the Menarandra River is the boundary between the species' two southernmost subspecies, *P. a. arachnoides* and *P. a. oblonga* (Pedrono 2008). In a preliminary genetic study using cyt-*b*, these two subspecific taxa were found to be reciprocally monophyletic, with three substitutions between them (Chiari *et al.* 2005). For *A. radiata*, no such signal was obtained, and cyt-*b* variation was even lower than in *Pyxis*. This is not a surprising result, considering that *Pyxis* exhibits a faster mtDNA evolutionary rate with respect to other tortoise species (Palkovacs *et al.* 2002). Additionally, our results are consistent with observations that cyt-*b* harbors excessively low levels of variation in several tortoise (Palkovacs *et al.* 2003; Gaur *et al.* 2006) and other chelonian species (e.g. Rosenbaum *et al.* 2007; Vargas-Ramirez *et al.* 2007). However, although there is a widespread notion that evolutionary rates are reduced in Testudines due to long generation times and slow

metabolism (Avise *et al.* 1992; Rand 1994), there is a growing number of counterexamples (Seddon *et al.* 1998; Weisrock & Janzen 2000) including in tortoises (Caccone *et al.* 2004). Indeed, our estimated substitution rate for the non-coding d-loop region is similar or even slightly higher than that found for the same fragment in several mammal groups (Pesole *et al.* 1999).

So how can one reconcile the extremely low variation in cyt-*b* with the impressive diversity at microsatellite loci? Our analyses of d-loop reveal that *A. radiata* most likely underwent a rapid expansion during the late Pleistocene (189,000 years ago) which could explain the low variation in the slower-evolving cyt-b gene and the high polymorphism noticed at microsatellite loci. The dry spiny forest biome is thought to be Madagascar's oldest remaining forest biome, originating while Madagascar was sitting astride 30°S during the Cretaceous through the Eocene, and when the whole island was experiencing dry condition (Wells, 2003). Since the Eocene, the spiny forest has regressed to the southwestern tip of the island as Madagascar drifted further north. However, during the Pleistocene, when glaciation cycles were prevalent on the planet, the climate in Madagascar probably changed several times, affecting all biomes of the island. In fact, glacier features have been discovered in Madagascar (Vidal Romani *et al.* 2002), and the presence of dry riverbeds and subfossil remains of giant lemurs and pygmy hippos in what is now semidesert suggest drastic climate changes in the spiny forest region (Burney 1997). Unfortunately, paleoecological data for this region reach back only five millennia (Burney *et al.* 2004). Thus, it remains impossible at this time to determine precisely what events favored the expansion of *A. radiata*, and future molecular work on other spiny forest taxa may help in formulating general hypotheses concerning the Pleistocene evolution of this unique biome. Nonetheless, patterns of genetic diversity in *A. radiata* suggest that *A. radiata* underwent a quick expansion during late Pleistocene glaciations.

5.6.2 Rivers, plateaus, and the genetic structure of the sokake

In Chapitre 3, we asserted that both the Menarandra and Manambovo rivers were genetic boundaries between *A. radiata* populations, even if these are seasonal rivers that are completely dried out several months every year. Here, strong microsatellite evidence supports this hypothesis for the Menarandra River. On the other hand, we do not find conclusive evidence in the case of the Manambovo River. Contrarily to the Menarandra River, for which sampling sites were within a few km of the shores, the Ifotaka population is located approximately 100 km from the Manambovo River, which makes the interpretation of its putative role in the genetic differentiation of that population hypothetical at best. Furthermore, in the Androy region, which spans between the Menarandra and Mandrare rivers, spiny forest vegetation is highly fragmented (Fig. 5.1; Fenn 2003*a*; Bodin *et al.* 2006), and this fragmentation may also contribute to population divergence.

In addition, our thorough sampling across the range of *A. radiata* reveals that for this species, rivers are not necessary to observe significant differentiation (Table 5.2): both the Sakotoavo and Bezaha-Mahafaly populations are divergent from coastal populations. Located approximately 50 km inlands from the southwestern coast, the escarpments of the Mahafaly Plateau (between Onilahy and Linta rivers) and of the Karimbola Plateau (between Linta and Menarandra rivers) may reduce gene flow. These are limestone plateaus that reach 250 meters above sea level; the Bezaha-Mahafaly population is on the eastern side of the Mahafaly plateau, while the Sakotoavo population is settled on top of it. Although Leuteritz *et al.* (2005) mentioned that tortoises could travel across steep cliffs and that movements were observed between plateaus and the coast, our microsatellite data suggest that these physiographic features suffice to maintain significant differentiation between coastal and inland populations. Nonetheless, it clearly appears that there are two

main populations in *A. radiata* (western and eastern with respect to the Menarandra River), while Mahafaly Plateau populations or Ifotaka represent peripheral subpopulations of these.

5.6.3 Evaluating the performance of bottleneck detection methods

We definitely know, because of the cultural taboo protecting some radiated tortoise populations (in Mahafaly and Antandroy territories), and also from conversations with villagers and data on population abundance, size structure and age structure (Chapitre 4; Chapitre 7; O'Brien 2002), that some populations have been heavily exploited while others have not been altered yet. We did not find compelling evidence of bottleneck genetic signatures in the four exploited populations (Bezaha-Mahafaly, Antreaky, Antsifitse, and Ifotaka). A heterozygote excess was only detected in the Ifotaka population. This population is located at the boundary between Antandroy and Antanosy territories, and it is one of the easternmost remnant populations of *A. radiata*. It has probably been exploited more extensively as tortoises started to disappear around Fort-Dauphin, the ancestral Antanosy territory, over the last two centuries (Chapitre 4). Other exploited populations likely were impacted more recently: there were still tortoises in the Tulear region (the other Antanosy stronghold) 30 years ago (O'Brien et *al.* 2003), so the incentive to go as far as the Bezaha-Mahafaly area to collect sokake is recent. Because the Antsifitse and Antreaky populations are located well within the taboo zone (Androy region), their exploitation is also very recent, as tortoise poachers from Fort-Dauphin were increasingly forced to venture in the Androy to find tortoises over the last few decades.

The *M*-ratio method was very sensitive to changes in model parameters, which has been noticed previously (Kuo & Janzen 2004): all populations exhibited significant values if "conservative" TPM parameters were used (i.e. small p_m and Δ_g values) or if a small θ was assumed. In contrast, completely opposite results were obtained if a more "relaxed"

TPM or higher θ values were used. Neither of these extreme solutions appears reasonable, and we had to alter parameters in order to find more realistic, although not completely concordant, results (e.g. θ = 10 in Table 5.4). Of course, finding the right settings (i.e."species-specific" parameters referred to by some authors) *a posteriori* is not acceptable, and even not possible if one does not have prior knowledge of past demographic trends, which prompts us to doubt altogether of the usefulness of this method. Similarly, in almost all studies that relied on this method, either all surveyed populations had a significant *M*-ratio or all had non-significant values (e.g. Abdelkrim *et al.*2005; Spear *et al.* 2006; Busch *et al.* 2007; examples in chelonians: Cunningham *et al.* 2002; Edwards *et al.* 2004; Hauswaldt & Glenn 2005; Pearse *et al.* 2006), even if other bottleneck tests often suggested different results. In several studies, the conclusion was that bottlenecks had occurred, and that the discrepancy between methods was because heterozygote excesses remain detectable only within 5-6 generations of the bottleneck, whereas low *M* values persist much longer (Garza & Williamson 2001). Our study demonstrates that complete disagreement may be obtained between bottleneck detection methods, depending on the chosen settings for the *M*-ratio tests, even when this explanation can de discarded (bottlenecks in *A. radiata* have certainly occurred recently).

Busch *et al.* (2007) noticed the same tendencies when analyzing pre- and post-bottleneck samples: relaxed TPM settings caused all samples to have non-significant *M*-ratios, while leaning towards a pure SMM resulted in the opposite outcome. We thus believe that the ability to correctly discriminate different demographic histories among populations of a given species is very poor for the *M*-ratio method. We have no reason to believe, however, that this is a phenomenon particularly associated with organisms with long generation times, since the same pattern was also observed in short-lived species. Further studies on microsatellite mutation processes may help in defining more adequate parameters, thus enhancing the efficiency of the method, but these vary considerably across species and markers (Ellegren 2004).

5.6.4 Is exploitation responsible for reduced diversity?

Casual comparison of genetic diversity between exploited and non-exploited populations suggests that exploitation reduced diversity in impacted populations (e.g. Table 5.3). But other analyses suggest otherwise; first, as discussed above, there was no clear evidence for the occurrence of bottlenecks in most exploited populations with the heterozygote excess method. If bottlenecks are too recent to be genetically detected, it is unlikely that they were responsible for the observed diversity differences. Furthermore, our simulations indicate that even the most pessimistic bottleneck scenarios could not account for the extent of differences in diversity between the two groups of populations (Table 5.5).

D-loop analyses may hold the answer to this intriguing problem. We have mentioned previously that *A. radiata* experienced a population expansion approximately 200,000 years ago. A more detailed analysis of the distribution of d-loop haplotypes among populations reveals that, with respect to the Menarandra River (the predominant phylogeographic barrier in *A. radiata*), the expansion most likely occurred on the western side of the river (Figure 5.5). As mentioned above, this area is mainly constituted of tertiary limestone rocks, which bear dense spiny forest vegetation all across Mahafaly territories, between the Onilahy and Menarandra rivers (Du Puy & Moat 1996). In opposition, to the east of the Menarandra, soils are mostly unconsolidated sands, which support patchy vegetation among sandy dunes (Fig. 5.1; Du Puy & Moat 1996). Although anthropogenic fragmentation has occurred there, we believe that forest cover in that region has never been as high as on the other side of the Menarandra, and that tortoise densities in the East probably could not reach the numbers from the western side even prior to human fragmentation. The strong genetic evidence supporting the expansion of *A. radiata* west of the Menarandra supports this hypothesis. Furthermore, if we compare microsatellite diversity measures between western and eastern population instead of exploited and non-exploited populations, the differences between the two groups are even larger than those

indicated in Table 5.3 (results not shown). Thus, our proposed scenario accounts for the higher diversity in western populations without resorting to recent bottlenecks. It also emphasizes that historical habitat fragmentation, rather than exploitation, may have been the main force explaining lower diversity in eastern populations, which is consistent with findings by DiBattista (2008). In a meta-analysis including data from 220 population genetics studies covering a broad range of taxa, this author concluded that habitat fragmentation consistently decreased genetic variation, whereas exploitation did not have a significant impact.

5.6.5 Microsatellite null alleles: bias in F_{ST} and *M*-ratio

Although the impact of null alleles on parameters of within-population diversity is reasonably obvious (inflation of homozygosity and inbreeding coefficients; Charkraborty *et al.* 1992; Brookfield 1996), their effect on estimates of population differentiation had remained unassessed until very recently (Selkoe & Toonen 2006). Chapuis & Estoup (2007) conducted simulation analyses and found that F_{ST} values were overestimated when populations were significantly differentiated. Our results clearly support their findings (Figure 6.3), but this bias did not interfere with the statistical conclusions of the analyses, and significant F_{ST} values remained significant even after removing questionable loci. To our knowledge, this is the first empirical support of Chapuis & Estoup's (2007) conclusions, and it would be interesting to see if the direction and extent of this bias is constant in other datasets.

Our microsatellite dataset also provided the opportunity to assess the effect of null alleles on *M*-ratio. We found clear evidence that null alleles introduced an underestimation bias in *M* (Table 5.4), which has not been reported previously. This may further affect the reliability of *M*-ratio tests. We propose a schematized explanation of this bias in Figure 5.6. This finding implies that null alleles are not restricted to visible alleles, and that they

probably correspond to several allele sizes, which had also been found in simulations (Chapuis & Estoup 2007). Additionally, we found a very strong relationship between levels of polymorphism and prevalence of null alleles: the most polymorphic loci were the ones with high frequencies of null alleles. High instability of flanking regions has been documented in the past (e.g. Grimaldi & Crouau-Roy 1997; Meglecz *et al.* 2004), but our results further indicate that there may be a direct correlation between the mutation rate of flanking regions and the mutation rate of the microsatellite repeat motif itself.

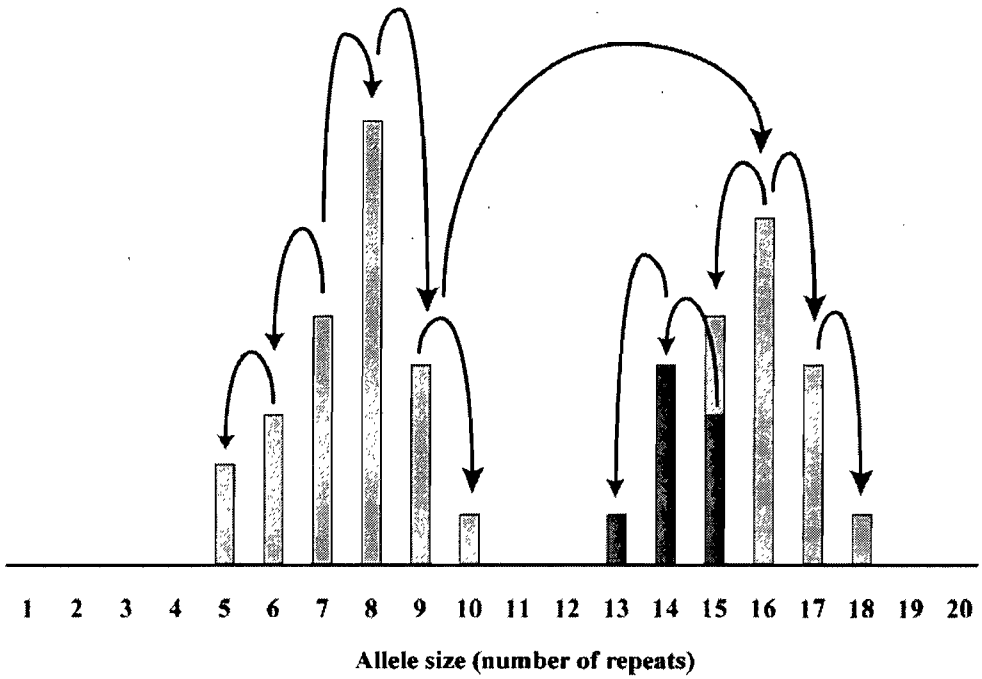

Figure 5.6 Allele distribution in a hypothetical population illustrating how microsatellite null alleles may introduce bias in *M*-ratio estimates. Arrows indicate mutations, of which most are single-step, but may also be multi-step (i.e. from allele 9 to allele 16). The size range (*r*) of alleles is 14, while the number of alleles (*k*) is 12. If no null alleles occurred, the *M*-ratio (*k*/*r*) would be 0.86. However, if we suppose that a mutation introduces null alleles (dark bar for allele 15), that this allele becomes more frequent in the population than the visible allele, and that repeat length mutations occur in this null allele rather than in the original allele 15 (leading to invisible (null) alleles at 14 and 13), than the overall number of visible alleles is reduced to 10, and the *M*-ratio is 0.71.

5.6.6 Interpreting findings in a conservation perspective

For intensively exploited species, the recognition of management units (MUs: Moritz 1994) is crucial to both conservation and exploitation efforts (Ruzzante *et al.* 2000). Because genetic differentiation is indicative of demographic isolation, distinct MUs (or "stocks" in fisheries) may have distinct population dynamics, and the failure to recognize population structure may lead to local overexploitation or the depletion of less productive populations (Ruzzante *et al.* 2000; Knutsen *et al.* 2003). Although these principles are mainly applied in marine fisheries, they are pertinent in all taxa, including, in light of our results, *A. radiata*. Our analyses not only indicate the presence of two main genetic populations of sokake, separated by the Menarandra River, but also suggest that these are characterized by different demongraphic histories. It would thus be important to formulate distinct conservation guidelines for these two different populations. Furthermore, even within these two groups, several subpopulations are characterized by different immediate threats, particularly those that are peripheral (e.g. Bezaha-Mahafaly, Ifotaka) due to their proximity to Antanosy villages and the absence of the cultural taboo in those areas, so appropriate local conservation strategies should also be established (as discussed in Chapitre 3)

Additionally, it was recommended in Chapitre 3 that conservation efforts should take into account the different MUs of the species to preserve its genetic integrity. One of the main problems facing conservation organizations in southern Madagascar is the management of the hundreds of tortoises confiscated from poachers and smugglers every year, for which the locality of origin is often unknown (Chapitre 4). When this information is available, releasing individuals in their home range is desirable and can be performed without additional expenses. However, based on results from this chapter and considering the sparse resources available for conservation in that region, we no longer believe that "preserving the genetic integrity" of the species should be a predominant issue and we feel

that allocating efforts to further genotype confiscated tortoises or tortoises that are part of captivity breeding programs would be ill-advised. First, the inter-population variation represents only a tiny portion (4%) of the overall microsatellite genetic variability of the species. Secondly, our mtDNA analyses did not find evidence of reciprocally monophyletic lineages or ESUs corresponding to geographically discrete populations. Finally, levels of genetic diversity are very high, even in exploited populations, so the need to enhance genetic variability in wild populations is not a concern. Thus, there appears to be little evidence that would justify investing more resources in genetic management issues in *A. radiata*.

It is surprising to notice that the historical effective size estimates of the radiated and ploughshare tortoises are similar, based on indices of diversity in control region sequences. *Astrochelys yniphora* is the rarest tortoise species in the world, with only a few hundred adult individuals remaining in the wild. Although archaeological evidence suggests that this species has been commercially exploited for at least 900 years (Pedrono 2008), there is a common belief that this species has somewhat always been relatively rare. Our results suggest that it may have been much more abundant historically than previously envisioned. This should serve as a warning for the radiated tortoise: not so long ago, it was considered by some specialists as the most abundant tortoise species on Earth (Tortoise & Freshwater Tortoise Specialist Group, personal communication), but it is now estimated to count between 1.6 and 4 million individuals and, assuming current rates of exploitation, all population viability analyses predict the extinction of the species in the wild within 45 years (Randriamahazo *et al.* 2007). This catastrophic decline, worsened by the breakdown of the cultural taboo in several areas, has lead to the recommendation of listing *A. radiata* as *Critically Endangered* at a recent IUCN workshop (Mittermeier *et al.* 2008), something that would have been unthinkable 30 years ago.

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CHAPITRE 6

Microsatellite analyses provide the first evidence of sex-biased dispersal in tortoises (Chelonia: Testudinidae)[1]

6.1 Résumé

La dispersion inégale des sexes est bien documentée chez les oiseaux et les mammifères, mais a rarement été étudiée chez d'autres taxons, incluant les reptiles en général, et plus particulièrement les tortues aquatiques et terrestres. Chez ces espèces, les observations de fidélité au site de ponte sont fréquentes mais peu ont démontré qu'elles étaient associées à la fidélité au site natal. Ici, nous avons testé l'hypothèse que la dispersion devrait être biaisée vers les mâles chez la tortue radiée (*Astrochelys radiata*) du sud de Madagascar, un résultat qui pointerait fortement vers la fidélité au site natal au sein de cette espèce. Grâce à un jeu de données de 13 marqueurs microsatellites, nous avons examiné la structure génétique des mâles et femelles tortues radiées de deux populations distinctes. Nos résultats démontrent clairement que les femelles tortues radiées géographiquement proches les unes des autres sont plus apparentées que les mâles. Tous les tests de Mantel réalisés entre les matrices de distances génétiques ou de coefficients de parenté et les matrices de distances géographiques ont indiqué une structure génétique significative chez les femelles et l'absence de structure chez les mâles. De plus, deux approches analytiques conçues pour évaluer les tendances générales de dispersion inégale entre les sexes ont largement appuyé l'hypothèse de dispersion biaisée vers les mâles dans les deux populations étudiées de tortues radiées. Ces excitants nouveaux résultats devraient constituer la base de futures recherches sur la fidélité au site natal chez les tortues terrestres. Ils mettent aussi en évidence l'importance d'identifier les sites de ponte pour la conservation de la tortue radiée, et devrait également être considérés dans les plans de

[1] Rioux Paquette, S., Louis Jr., E.E. & Lapointe, F.-J. Microsatellite analyses provide the first evidence of sex-biased dispersal in tortoises (Chelonia: Testudinidae). Sera soumis à *Journal of Heredity*.

rétablissement des populations réduites par l'exploitation, qui sont caractérisées par un sexe ratio fortement biaisé vers les mâles en raison de la préférence des braconniers pour les femelles.

6.2 Abstract

Sex-biased dispersal is well documented in birds and mammals, but has seldom been investigated in other taxa, including reptiles in general and, more specifically, non-marine chelonians. In these species, nest-site fidelity observations are frequent but remain to be associated with natal homing, a behavioral trait linked to male-biased dispersal. Here, we tested the hypothesis that dispersal should be male-biased in the radiated tortoise (*Astrochelys radiata*) from southern Madagascar, a result that would point towards natal homing in this species. Relying on data from 13 microsatellite markers, we investigated the genetic structure of male and female tortoises from two distinct populations. We found clear evidence that geographically close female tortoises are more related than males. All Mantel tests conducted with matrices of genetic distances or relatedness values and matrices of geographic distances indicated significant structuring in females and the absence of structure in males. Furthermore, two analytical approaches designed to assess general trends in sex-specific dispersal strongly supported male-biased dispersal in the two radiated tortoise populations. These exciting new results should form the basis of future research on natal homing in tortoises. They also highlight the importance of identifying nesting sites for conservation initiatives, and should also be considered in recovery plans of depleted radiated tortoise populations, characterized by male-biased sex ratios due to the selective harvesting of females by poachers.

6.3 Introduction

The differential propensity of males and females to disperse, or sex-biased dispersal, is a life history trait closely related to the mating system of species (Greenwood 1980). Theoretical models based on kin competition and inbreeding avoidance predict that females should be philopatric in polygynous systems (i.e. male-biased dispersal), whereas female-biased dispersal should be predominant in monogamous systems. However, the main focus of dispersal studies has traditionally been the comparison of dispersal patterns between mammals (characterized by polygynous systems) and birds (mostly monogamous), rather than comparing species with diverging mating systems (Greenwood 1980; Johnson & Gaines 1990), and there is a general lack of data in other taxa (Goudet *et al.* 2002). For instance, there are only a few documented studies of sex-biased dispersal in reptiles, including in lizards (Doughty *et al.* 1994), iguanas (Rassman *et al.* 1997), snakes (Rivera *et al.* 2006), and crocodilians (Tucker *et al.* 1998).

In chelonians, data are also sparse and come almost exclusively from sea turtles (reviewed in Bowen & Karl 2007). Marine turtles are characterized by a complex structure: females exhibit strong homing to their natal beach, but perform tremendous migrations between their feeding and nesting areas. Genetic analyses have revealed that gene flow among nesting beaches is male-mediated, and opportunities for gene flow probably occur when adult populations overlap in feeding areas and migratory corridors (Bowen & Karl 2007). This original structure emphasizes the distinction to be made between *ecological dispersal* and *genetic dispersal* as defined in Johnson & Gaines (1990): ecological dispersal is simply the movement of individuals from one place to another (including migrations), whereas genetic dispersal refers to individuals moving from their natal population to another population where they successfully breed. Population genetic approaches, such as the one employed in the present study, have become powerful tools to detect sex-biased dispersal (Goudet *et al.* 2002; Prugnolle & de Meeus 2002), but their detecting capacity is

of course restricted to genetic dispersal, since ecological dispersal does not affect the genetic structure of populations. Henceforth, here we use the term dispersal synonymously with genetic dispersal.

Natal homing, such as exhibited by sea turtles, is expected if female fitness is affected by nest-site quality and females transmit the site to their female offspring (Reinhold 1998). Species with this behavioral trait are thus expected to display male-biased dispersal. In freshwater and terrestrial chelonians, several nest-site fidelity observations have been made, but it remains unknown whether they are associated with natal homing (Freedberg *et al.* 2005). Here, we investigate sex-biased dispersal in the Malagasy radiated tortoise (*Astrochelys radiata*), an endemic species of the spiny forest of southern Madagascar, with microsatellite data. We hypothesize that dispersal in *A. radiata* is male-biased, a prediction that would be condordant with greater female philopatry and natal homing. Although many tortoise species (family Testudinidae) have been the target of population genetics studies, the topic of sex-biased dispersal has never been specifically addressed in these animals, despite the importance of this knowledge in our understanding of their evolutionary history and its ramifications for conservation. A strong signal for male-biased dispersal in the radiated tortoise would emphasize the necessity to further study natal homing and nest-site fidelity in this species.

6.4 Material and methods

6.4.1 Sampling and DNA analyses

Blood or tissue samples from 180 wild adult radiated tortoises were used in this work (87 males and 93 females). Other samples from juveniles or from individuals for which the identification of sex was uncertain were ignored. A list of all analyzed samples along with geographic coordinates is provided as supplementary material (Annexe I), and

additional details on sample collection are provided elsewhere (Chapitre 3; Chapitre 5). DNA extraction and genotyping at 13 highly polymorphic microsatellite markers were performed as described in Chapitre 5.

6.4.2 Data analyses

We analyzed isolation by distance (IBD) at the individual level for both sexes. It has been noticed previously (Chapitre 5, Fig. 5.2) that there were two genetically distinct populations in *A. radiata*, separated by the presence of a genetic barrier, the Menarandra River. We thus divided the data in two groups, called the West and East populations based on their geographic location with respect to the Menarandra River, and performed the analyses described below separately in both populations. In the Western population, there were 61 males and 58 females, while 26 males and 35 females were sampled in the Eastern population.

Mantel tests (Mantel 1967) were used to compare matrices of pairwise geographic distances (computed from geographic coordinates) and matrices of genetic distances for both sexes. Two different estimators of inter-individual genetic distances were derived from microsatellite data for these comparisons: first, Rousset's distance measure *a*; it is the equivalent of the $F_{ST}/(1-F_{ST})$ ratio but for pairs of individuals instead of pairs of populations, and it was conceived to study IBD at the individual level (Rousset 2000); and secondly, Queller & Goodnight's relatedness coefficient *R*; it is based on the proportion of identical alleles between two individuals (Queller & Goodnight 1989). Matrices of *a* and *R* values were computed with the software SPAGEDI (Hardy & Vekemans 2002). Matrices of *R* were converted to distance matrices (*D*) in order to compute Mantel tests with the operation $D = 1 - R$.

Mantel tests were performed with the VEGAN library (Dixon 2003) in R language (R Foundation for Statistical Computing, Vienna, Austria), and significance of Mantel correlation coefficients (r_m) was assessed with 10,000 permutations. Comparing sex-specific values of r_m can not be done with traditional statistical methods because of pseudoreplication issues: the number of pairwise comparisons in each matrix ($n(n-1)/2$) used to calculate r_m is higher than the actual number of observations n, which artificially inflates the number of degrees of freedom (Prugnolle & de Meeus, 2002). To circumvent this problem, two kinds of approaches developed by Knight *et al.* (1999) were employed to compare results from males and females. Both methods ensure that the number of compared values correspond to the number of observations.

In the first approach, for each individual, a regression of genetic distance a on geographic distance (d) was calculated using all pairwise comparisons that involved that individual and all others of the same sex in the population. Thus, the number of calculated regressions was 61 for males and 58 for females in the Western population, for instance. These regressions were computed with a function written in R language. The slope values (b) of these regressions were then compared between the sexes with a Mann-Whitney U-test computed in STATISTICA (Statsoft Inc.). This analysis was repeated with R. For regressions of a, high slope values indicate strong structuring with respect to geographic distance. On the other hand, for regressions of R, highly negative values indicate that related individuals are likely to be found in close proximity (Knight *et al.* 1999).

The second approach was based on the comparison of the means of ranked a values and their corresponding d values for all individuals of the same sex in each population (Knight *et al.* 1999). For each individual, a values were ranked in descending order and corresponding d values were re-ordered accordingly. Then, for each sex and population, the first data point was calculated as the mean of the highest a values of all individuals of the same sex and the mean of corresponding d values. The second point was the mean of the

second-highest *a* values and corresponding *d*, and so on. These data points were computed with a R function. The regression of mean *a* on mean *d* was calculated in each population, and slopes were compared with Student's *t*-tests for slope comparison (Zar 1996). A stronger, positive slope of *a* on *d* for females would indicate stronger genetic structuring, i.e. related females are more likely to be found in close proximity than related males, thus suggesting male-biased dispersal. These analyses were repeated with *R*, and in that case, the opposite result was expected, i.e. stronger negative slopes of mean *R* on mean *d* in females.

6.5 Results

In both populations, Mantel correlation coefficients were much higher in females than in males for either *a* or 1-*R* (Table 6.1). Furthermore, all four r_m values were highly significant in females, whereas none was significant in males (Table 6.1), suggesting greater IBD in females.

Table 6.1 Results of the Mantel correlation tests between pairwise genetic and geographic distances among individual radiated tortoises (*Astrochelys radiata*).

Population	Genetic distance	Sex	r_m	*p*
Western	*a*	Males	-0.0054	0.5349
		Females	0.2521	<0.0001
	1-*R*	Males	0.0339	0.1538
		Females	0.1291	0.0020
Eastern	*a*	Males	-0.0365	0.6446
		Females	0.2333	<0.0001
	1-*R*	Males	-0.0416	0.6634
		Females	0.1903	0.0006

r_m, Mantel correlation coefficient; *a*, Rousset's genetic distance; *R*, relatedness coefficient

The comparison of individual regressions of genetic distances on geographic distance also supported this difference between sexes. Results of the Mann-Whitney *U*-tests

are provided in Table 6.2, and we illustrate results for *a* in the Western population in Figure 6.1, where females had significantly higher values of *b*. The same tendency was obtained in the Eastern population (Table 6.2). Conversely, for *R*, *b* values were significantly more negative in females, as expected (Table 6.2).

Table 6.2 Results of the Mann-Whitney *U*-tests comparing the slopes of regressions of genetic on geographic distance for individual male and female radiated tortoises.

Population	Genetic distance	Sex	Mean rank	Sum of ranks	*U*	*z*	*p*
Western	*a*	Males	47.61	2904	1013	4.02	<0.0001
		Females	73.03	4236			
	R	Males	76.10	4642	835	-4.97	<0.0001
		Females	43.07	2498			
Eastern	*a*	Males	19.96	519	168	4.19	<0.0001
		Females	39.20	1372			
	R	Males	37.69	980	281	-2.54	0.0112
		Females	26.03	911			

In addition, the method based on the comparison of means of ranked *a* and *R* values generated very similar results. In the two populations, the regression slope of mean *a* on mean *d* was greater in females, and the regression slope of mean *R* on mean *d* were negatively greater in both cases also (Figure 6.2). Statistical tests to assess the significance of the differences in regression slopes between sexes revealed that for mean *R*, the difference was significant in both populations (Western population: $t = -2.41$, $p = 0.017$, df = 113; Eastern population: $t = -2.87$, $p = 0.006$, df = 55), whereas it was only significant in the Eastern population for *a* (Western population: $t = 1.55$, $p = 0.125$; Eastern population: $t = 2.68$, $p = 0.009$). Correlation coefficients (*r*; indicated in Figure 6.2) were significantly greater for females in all four cases. These results strongly support our hypothesis of male-biased dispersal in *A. radiata*.

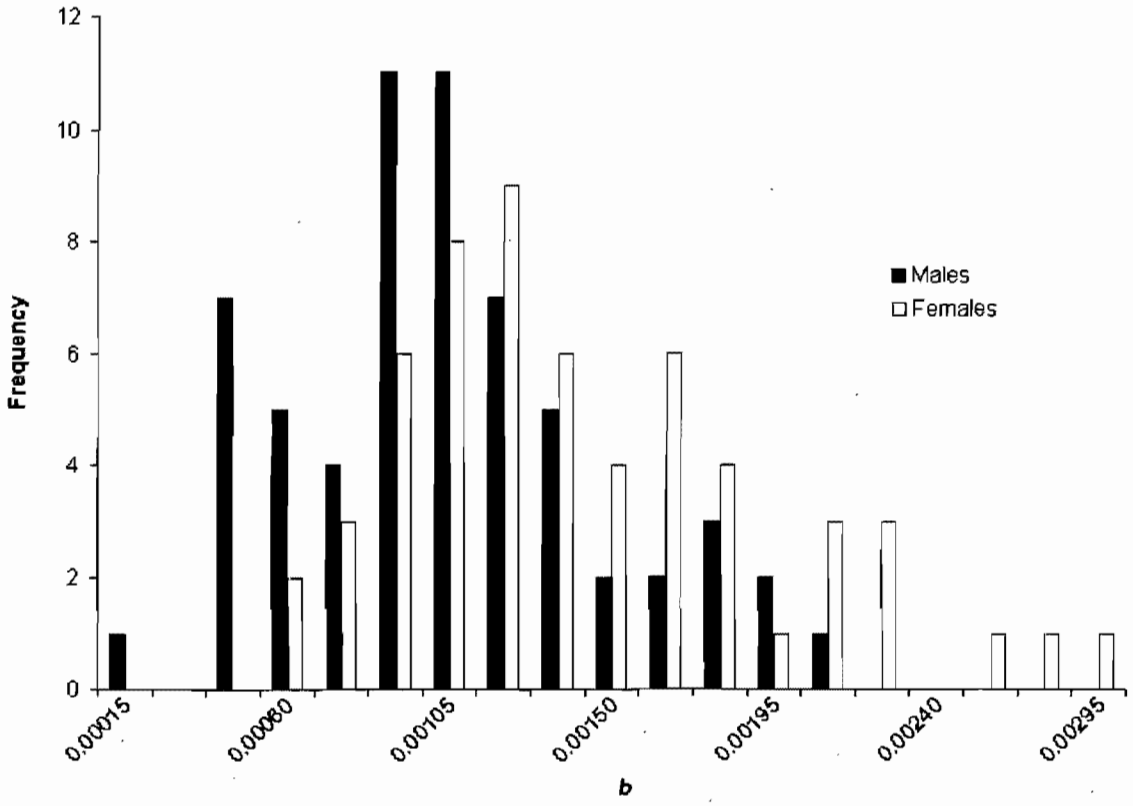

Figure 6.1 Frequency distribution of the regression slope values (b) of genetic distance a on geographic distance in male (n=61) and female (n=58) radiated tortoises in the Western population. The difference of slope values between sexes was highly significant ($p < 0.0001$ in U-test). (Summary statistics of the same analysis with R instead of a, along with results from the Eastern population, are provided in Table 6.2.)

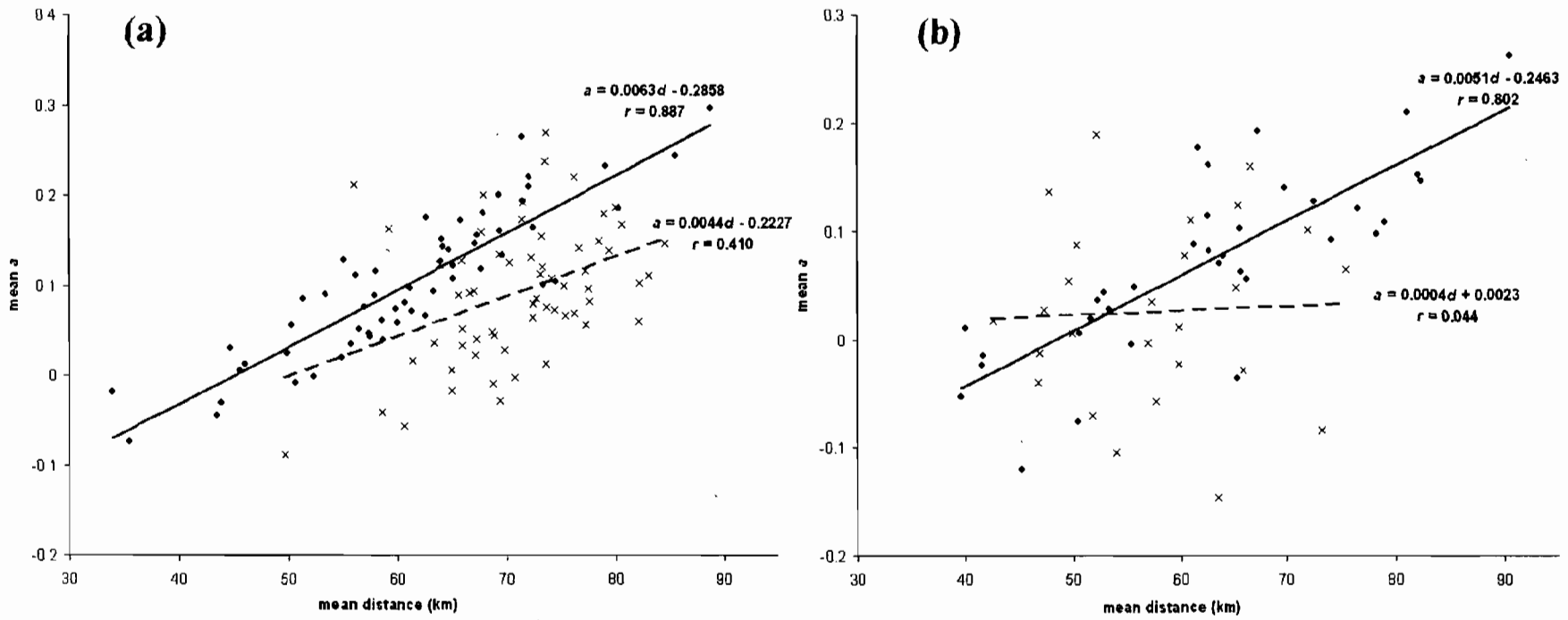

Figure 6.2 (continued on next page) Relationship between the means of ranked *a* values (a, b) or ranked *R* values (c, d) and corresponding mean geographic distances calculated separately in male and female radiated tortoises from the Western (a, c) and Eastern (b, d) populations. • symbols indicate data points from females, x symbols indicate points from males, black lines represent female regressions and dash lines represent male regressions. Regression equations and their corresponding correlation coefficients are indicated next to regression lines.

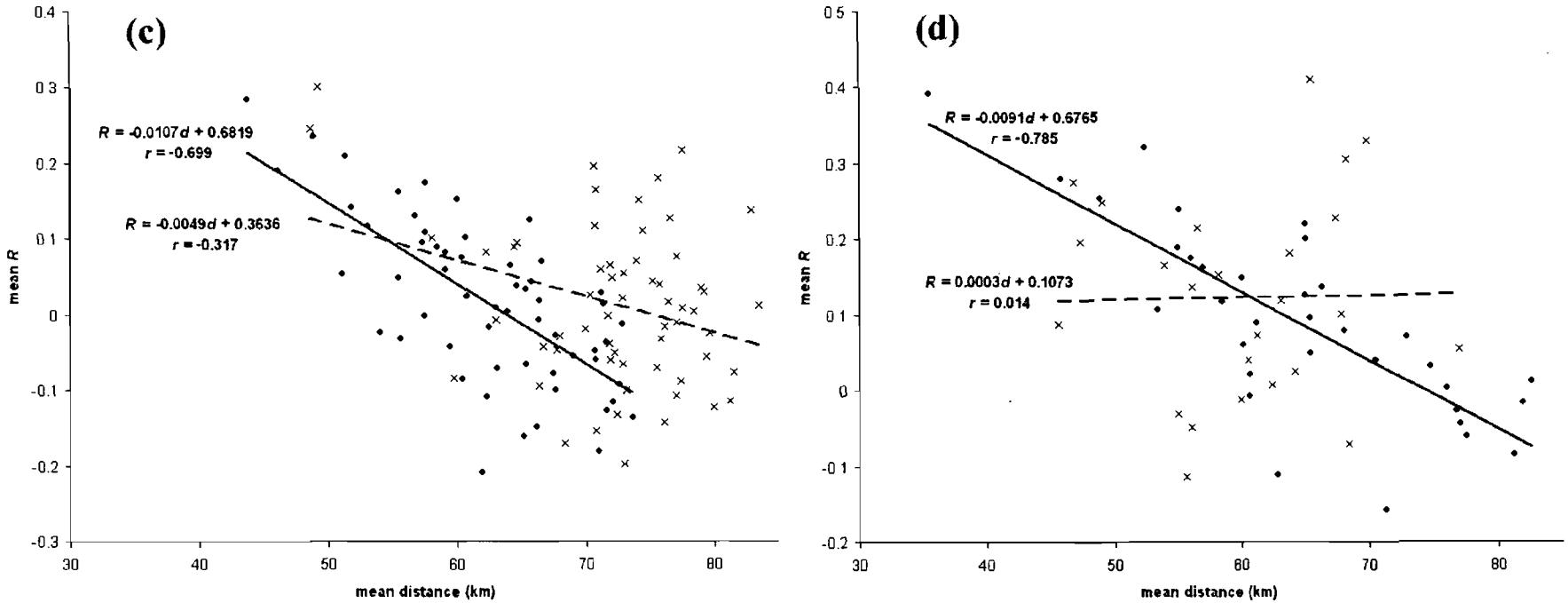

Figure 6.2 (continued from previous page).

6.6 Discussion

All results clearly support our initial hypothesis of male-biased dispersal in radiated tortoises. Mantel test results indicate that IBD at the individual level is only significant in females, and other analyses also reveal a greater tendency of females to be genetically related to other geographically close females. The fact that similar trends were consistently found with both statistical approaches in both populations with two genetic estimators appears to make a strong case for male-biased dispersal and greater female philopatry. Difficulties may arise when studying dispersal in a small subsample of a large population, especially if we consider that microsatellite data can lead to noisy relatedness estimates (e.g. Fontaine & Dodson 1999). Tests based on the comparison of accurate pairwise values (like Mantel tests) are prone to be affected by these problems (Knight *et al.* 1999). On the other hand, the two additional analytical methods employed here are robust to these issues and allow an evaluation of broad dispersal patterns based on general trends in genetic distance or relatedness values, in which significant results can simply not be found by chance (Knight *et al.* 1999).

The absence of IBD among males was unexpected considering that even within the two analysed populations, significant genetic differentiation, albeit very small, was noticed among some sampling sites (Chapitre 5). Thus, it appears that genetic structuring within these populations is mostly due to female philopatry, compensating for extensive male dispersal.

Nevertheless, female philopatry does not necessarily correlate with greater ecological dispersal by males. Although there are observations of larger home ranges and movements in males of some tortoise species (e.g. McRae *et al.* 1981; Diemer 1992; O'Connor *et al.* 1994; Eubanks *et al.* 2003; Tuberville *et al.* 2005), opposite observations have also been made (e.g. Longepierre *et al.* 2001; Lagarde *et al.* 2003), including in

radiated tortoises, in which females travel great distances outside their usual home range to lay eggs (Leuteritz 2002). Furthermore, because juveniles may be more prone to disperse from their natal area (e.g. Tucker *et al.* 1998), analyzing movement patterns in adults may not always be helpful to uncover patterns of genetic dispersal and philopatry. Thus, the role of genetic markers in dispersal studies is stressed by the discrepancy between our results and known movement patterns in adult radiated tortoises.

Tortoise species are characterized by a polygynous mating system, and theoretical models predict male-biased dispersal in such species as a mechanism to avoid either kin competition or inbreeding (e.g. Perrin & Mazalov 2000). However, these appear as unlikely explanations of male-biased dispersal in radiated tortoises. Because densities of this species in undisturbed populations are exceptionally high, with a mean value of 25 adult tortoises/ha (Pedrono 2008), local competition for resources or mates among related males is certainly minimal, and local effective sizes are so large that inbreeding is not expected to occur. Although these explanations are not mutually exclusive, natal homing coupled with nest-site fidelity appears as the main force maintaining greater female philopatry. As explained in the introduction, if female fitness is affected by the choice of nesting sites and these sites are transmited to female offspring, natal homing is expected to occur (Reinhold 1998). Olfaction appears to be involved in the homing behaviour of displaced tortoises (Chelazzi 1992), so olfactory imprinting could be the involved mechanism in natal homing and nest-site fidelity, although this has never been assessed. Further studies of natal homing in tortoises and freshwater turtles are needed, not only to clearly demonstrate the likely intrinsic relationship between male-biased dispersal and natal homing in these species, but also because they provide important insights into sex allocation theory and the evolution of sex ratio and environmental sex determination (Reinhold 1998; Freedberg *et al.* 2005).

Finally, the existence of male-biased dispersal and possible natal homing in radiated tortoises emphasizes the importance of locating nesting sites and identifying nest-site

preferences in order to implement effective conservation schemes for this species. Overexploitation has been identified as the main threat to the survival of this species (O'Brien *et al.* 2003; Randriamahazo *et al.* 2007), but habitat loss, which is currently worst in the spiny forest than elsewhere in Madagascar (Harper *et al.* 2008), may significantly impact the species if suitable nesting sites, to which females were homing, are destroyed. Furthermore, female philopatry may hinder the recovery of depleted populations, even if poaching is completely stopped. Sex ratios are highly male-biased in exploited populations as a result of poacher preference for females because they sometimes contain eggs and their meat is fattier (Pedrono *et al.* 2000). Consequently, if very few female immigrants disperse from nearby populations, the recovery of impacted populations may take longer than anticipated.

6.7 Acknowledgements

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CHAPITRE 7

Conservation genetics of the radiated tortoise population from Andohahela National Park, southeast Madagascar, with a discussion on the conservation of this declining species[1]

7.1 Résumé

La tortue radiée (*Astrocehlys radiata*), endémique à la forêt épineuse du sud de Madagascar, n'avait pratiquement jamais été étudiée dans la nature jusqu'à la fin des années 1990. Plusieurs récents projets de recherche ont contribué à évaluer l'ampleur du déclin de cette espèce, et il semble maintenant clair que *A. radiata* est sérieusement menacée d'extinction si les tendances actuelles ne sont pas inversées. Dans le cadre d'un projet de recherche portant sur la génétique et la conservation de cette espèce, nous avons étudié la diversité génétique et la différentiation de la population isolée de tortues radiées du Parc National d'Andohahela (PNA), considéré comme un des derniers refuges de l'espèce dans le sud-est, où les habitants d'origine Antanosy consomment de la viande de tortue. La diversité génétique était similaire à celle d'autres populations, mais le niveau de différentiation était le plus élevé, ce qui met en évidence l'importance de conserver cette population. Toutefois, le plus inquiétant de nos résultats est le fait que la majorité des tortues capturées étaient des mâles subadultes, ce qui laisse croire que le recrutement puisse être presque nul. Nous avons trouvé des preuves concrètes indiquant que des braconniers Antanosy prélèvent les plus grands individus de la population malgré la protection officielle dont elle devrait bénéficier en raison du statut du Parc. Nous poursuivons cet article en discutant de plusieurs aspects de la conservation de *A. radiata*, incluant le rôle des aires

[1] Rioux Paquette, S., Ferguson, H.B. & Louis Jr., E.E. Conservation genetics of the radiated tortoise population from Andohahela National Park, southeast Madagascar, with a discussion on the conservation of this declining species. Sera soumis pour publication à *Chelonian Conservation and Biology*.

protégées, l'importance de comprendre le tabou envers la tortue chez les communautés Antandroy et Mahafaly, et la pertinence du statut actuel de l'espèce sur la CITES.

7.2 Abstract

The radiated tortoise (*Astrochelys radiata*), endemic to the spiny forest of southern Madagascar, had virtually never been studied in the wild until the late nineties. Recent research projects have contributed in defining the extent of the decline of the species, and it now appears that *A. radiata* faces serious extinction risks unless current trends are halted. As part of an ongoing conservation genetics project on the species, we investigated genetic diversity and differentiation of the isolated tortoise population from Parc National d'Andohahela (PNA), considered as the last remaining sanctuary of the species in the Southeast, where Antanosy people consume tortoise meat. Genetic diversity was similar to other populations, but differentiation was the greatest, emphasizing the conservation value of this population. However, our most important finding was that the majority of captured tortoises were male subadults, indicating that recruitment may be almost non-existent. We found physical evidence that Antanosy poachers are removing the largest individuals from the population, despite the official protection associated with the status of the park. We summarily discuss several conservation issues related to *A. radiata*, including the role of protected areas in Madagascar, the importance of understanding the taboo towards tortoises in Antandroy and Mahafaly communities, and the pertinence of the current CITES listing of the species.

7.3 Introduction

Exceptional levels of endemism due to an early Jurassic separation from continental Africa (Storey *et al.* 1995), coupled with socioeconomic factors contributing to an accrued dependence on natural resources and the intensification of deforestation (Durbin *et al.*

2003), have earned Madagascar the designation of being among the world's highest priority areas for biodiversity conservation (Myers *et al.* 2000). Although the ongoing environmental crisis is widespread across the island and impacts all biomes and taxa, the focus of biologists has mainly been directed at well-known, highly diverse emblematic groups such as lemurs and chameleons. Nevertheless, the five endemic Malagasy chelonian species have also been severely impacted over the past few decades, and attention has recently been called to the bleak future of these species unless current trends are somehow halted (Randriamahazo *et al.* 2007; Mittermeier *et al.* 2008). Three of these species (ploughshare tortoise *Astrochelys yniphora*, flat-tailed tortoise *Pyxis planicauda* and Madagascar big-headed turtle *Erymnochelys madagascariensis*) are among the world's 25 most endangered chelonians (TCF 2002), whereas the other two (radiated tortoise *Astrochelys radiata* and spider tortoise *Pyxis arachnoides*) are experiencing dramatic declines (O'Brien *et al.* 2003; Randriamahazo *et al.* 2007).

The radiated tortoise, or sokake in Malagasy, is endemic to the spiny forest of southern Madagascar (Fig. 7.1). This region is inhabited by people from different Malagasy cultural lineages, and the area is shared among four main tribes: the Vezo along the western coast near Tulear, the Antanosy around Tulear and in the southeast, the Mahafaly in the southwest, and the Antandroy in the southcentral spiny forest. Antandroy and Mahafaly people respect an ancestral taboo prohibiting tortoise consumption or collection, whereas tortoise meat is part of the diet in Vezo and Antanosy communities. Not surprisingly, the radiated tortoise underwent a considerable range contraction throughout the 20th century (Juvik 1975), going locally extinct where it overlapped with Vezo and Antanosy territories. However, its most severe decline occurred over the last few decades, driven by increasing demands for tortoise meat at the national level, and rising interest at the international level for derived products (e.g. liver) and for the pet trade (e.g. Behler 2002; O'Brien *et al.* 2003). Current exploitation rates are clearly unsustainable, and all population viability analysis scenarios predict the extinction of the species in the wild within 45 years if

harvesting is not greatly reduced (Randriamahazo *et al.* 2007). Paradoxically, perhaps because of the perception of its seemingly secure status until recently, and probably also because of the rough fieldwork conditions one has to cope with in the spiny forest, very little research on the radiated tortoise in the wild had been carried out until two doctoral students undertook projects on the sokake in the late 90s (Leuteritz 2002; O'Brien 2002). Their work provided important data on the distribution, harvest, and reproductive ecology of the radiated tortoise (e.g. O'Brien *et al.* 2003; Leuteritz & Ravolanaivo 2005; Leuteritz *et al.* 2005). In 2003, we undertook a conservation genetics research project to gather molecular data on the radiated tortoise. This project has resolved patterns of genetic and morphometric variation across the current range of the species, and highlighted the role of the Menarandra River as a major barrier to gene flow, with the Manambovo River and the Mahafaly Plateau providing secondary barriers (Chapitre 3; Chapitre 4; Chapitre 5).

As part of this project, an expedition to Andohahela National Park or *Parc National d'Andohahela* (PNA; Fig. 7.1) was carried out in 2007. The PNA first came into existence as a natural reserve in 1939, and retained this status until 1997, when it became a national park managed by ANGAP, the *Association Nationale pour la Gestion des Aires Protégées* (Fenn 2003*b*). Although several Antandroy villages are located around PNA, it is located in a mostly Antanosy region. With the exception of the tortoise population located within the limits of PNA, the sokake has almost been extirpated from the region east of the Mandrare River, mainly to supply the demands for tortoise meat in Fort-Dauphin, the largest Antanosy city, and second largest market for tortoises in southern Madagascar after Tulear The main objective of this work was to collect tortoise samples in order to evaluate the genetic variation of the tortoise population within PNA, which may have been impacted by its isolation, and to determine the extent of genetic differentiation with respect to populations west of the Mandrare River (sampled in previous studies).

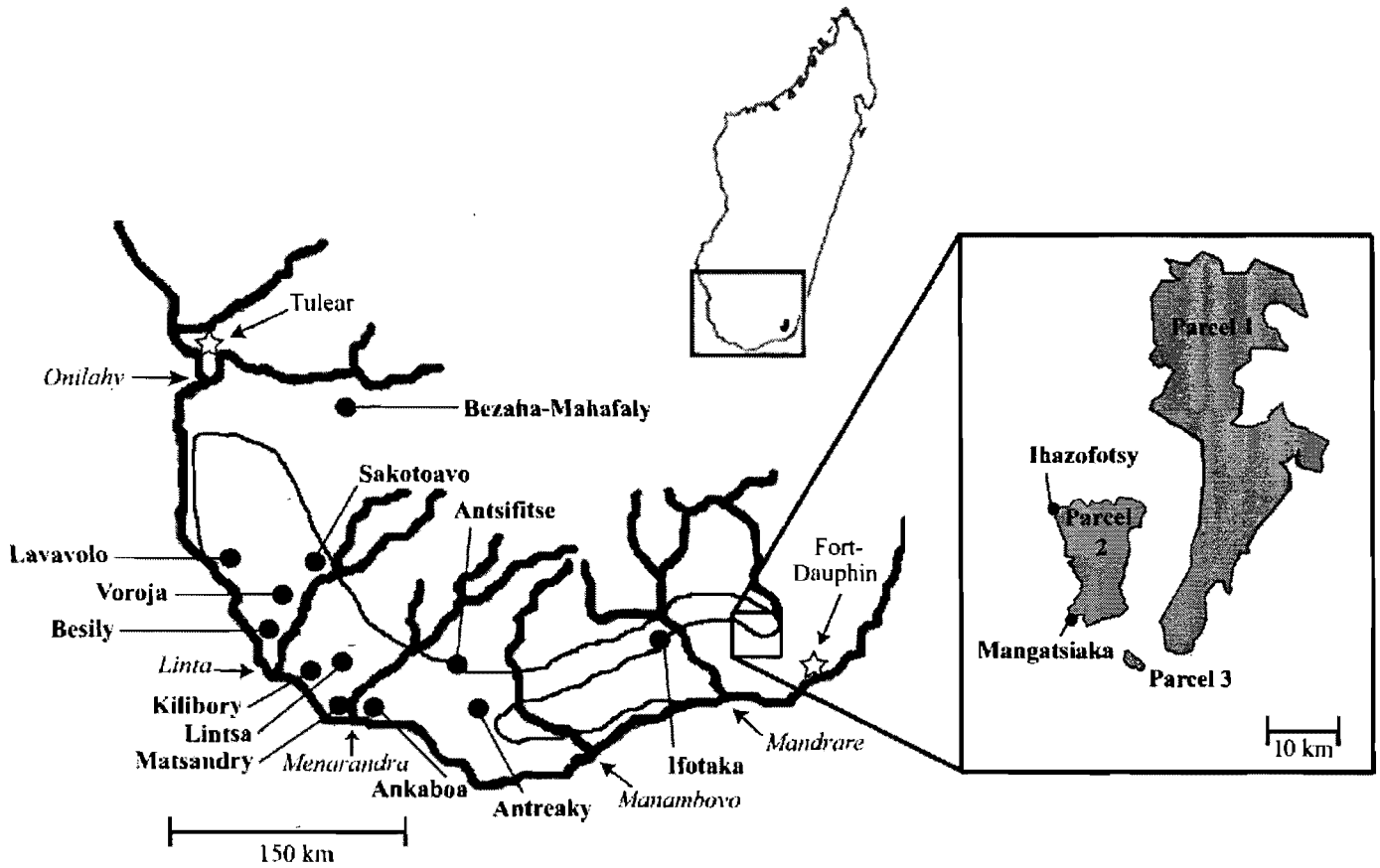

Figure 7.1 Map of southern Madagascar, with a larger view (frame) of the three parcels of Parc National d'Andohahela (PNA). The twelve localities indicated with black circles on the left are tortoise populations sampled in previous work from which genetic and demographic data were compared here with PNA. The extant range of *A. radiata* is delimited by the thin black line, and the name of major rivers is indicated in italics.

We also intended to study some reproduction parameters of the species: among others, we had planned to monitor nests throughout incubation and sample hatchlings in order to investigate multiple paternity in this species. Based on information from wildlife management staff in Fort-Dauphin, we were expecting to find a large, healthy tortoise population, protected by the status of PNA, which has strictly forbidden access and harvesting of biological resources within the park for several decades. However, upon our arrival at PNA, we rapidly realized that this objective would not be fulfilled, due to the extreme paucity of tortoises in PNA. Thus, in addition to relevant genetic results, we will seize this opportunity and include here comments on the conservation of the sokake, relying on our observations and experiences from four different field seasons, during which we have traveled throughout southern Madagascar, from Tulear to Fort-Dauphin, and interacted extensively with local communities and actors involved in biodiversity conservation. We believe this discussion may provide some insights to consider when planning conservation actions for the sokake.

7.4 Material and methods

7.4.1 Data collection

PNA is composed of three parcels that rest on both sides of the Anosyennes Mountains in extreme southeast Madagascar. This chain of mountains constitutes a boundary between eastern tropical forest and semiarid spiny forest that acts as a remarkable rain barrier separating the two biomes (Fenn 2003*b*). The largest parcel (#1; Fig. 7.1) represents eastern humid forests while the smallest (#3) is in the transitional zone between humid and arid forests. These parcels do not correspond to radiated tortoise natural habitat and accordingly, sokake does not occur in that parcel. On the other hand, parcel 2 (dry spiny forest) mostly comprises suitable habitat for the sokake, and ANGAP staff members

had confirmed before our expedition that tortoises still occurred in this parcel, with some people suggesting that they were even abundant. We carried out field work in parcel 2, searching for tortoises with a team of 5-7 people for four days in the vicinity of Mangatsiaka (Fig. 7.1) and six days in the area west of the village of Ihazofotsy, in March 2007. Searches were carried out everyday between 7:00 and 11:00 in the morning, and between 3:00 and 6:30 in the afternoon. Different portions of the park were surveyed every day. People searching for tortoises were lined up and walked in parallel, with ~20 m between each person. Habitat was thoroughly inspected, especially features where radiated tortoises usually hide (bushes, fallen trees, depressions in the ground, etc). Contrarily to line transects and distance sampling (Buckland *et al.* 1993), this method does not allow for an estimation of the overall population size, but was the only possible choice considering the scarcity of tortoises in PNA. Furthermore, it has been shown that distance sampling greatly overestimates population sizes if an insufficient number of captures are made (Freilich *et al.* 2005), which may explain surprisingly high estimates recently obtained for the flat-tailed tortoise *Pyxis planicauda* in the Menabe region of western Madagascar (Young *et al.* 2008). For every encountered radiated tortoise, sex and weight were noted, a blood sample (0.1-0.5 cc) was drawn and stored in blood storage buffer (Longmire *et al.* 1992), marginal scutes were notched with a triangular file to provide a unique visual identification code for each tortoise (Cagle 1939), and a PIT or passive integrated transponder tag (Gibbons & Andrews 2004) was injected in a hind limb (for every tortoise with a body weight > 0.5 kg).

In order to compare indices of genetic diversity and evaluate the extent of population differentiation, we used all available genetic data from other populations that have been analyzed elsewhere (Chapitre 3; Chapitre 5). Previously sampled localities are all indicated on Figure 7.1. DNA extraction, PCR amplification and microsatellite genotyping at 13 loci were performed following the methodology described in Chapitre 3 and Chapitre 5. PNA population size structure data were also compared to data from

previously sampled exploited and non-exploited tortoise populations (Chapitre 4). Results from PNA were compared with exploited and unexploited population data with χ^2 tests, and a departure from a 1:1 sex ratio was also tested with a χ^2 test.

7.4.2 Genetic analyses

Based on microsatellite allele frequencies (Chapitre 5), previous analyses have found two main populations of *A. radiata* separated by the Menarandra River. For comparison purposes, in addition to these two groups (henceforth designated as "West" for all coastal populations west of the Menarandra River and "Central" for all populations comprised between Menarandra and Manamobovo Rivers), we also compared results with other peripheral populations showing significant levels of differentiation: Bezaha-Mahafaly north of the Mahafaly Plateau, Sakotoavo on the Plateau, and Ifotaka, just west of the Mandrare River (Fig. 7.1). Pairwise F_{ST} values were computed among these five groups and the PNA population with the software Arlequin (Schneider *et al.* 2000) and significance was assessed with 1000 permutations. Furthermore, three indices of genetic diversity were computed for PNA and the other populations: heterozygosity (both observed H_O and expected H_E), allelic richness *Ar* (a measure of the number of alleles corrected for different sample sizes), and the inbreeding coefficient F_{IS}. These analyses were performed with FSTAT (Goudet 1995) and GENEPOP (Raymond & Rousset 1995), and 95% confidence intervals (CI) were calculated for each parameter.

The radiated tortoise population from PNA was initially not sampled in previous surveys because it has often been claimed that PNA serves as a release area for confiscated tortoises in the Fort-Dauphin region (e.g. Leuteritz *et al.* 2005). This would have introduced a bias in studies aimed at depicting natural patterns of genetic variation in the species (Chapitre 3). However, since the objective of the present study is to describe the variation in PNA, we were interested to see whether we could detect these released individuals in the

mutlilocus dataset. Thus, genetic assignment tests were computed with the program WHICHRUN (Banks & Eichert 2000), using the six groups of populations as putative source populations. The ratio between the most likely allocation and the second most likely solution was used to determine statistical significance of results for each individual. A likelihood ratio > 2, which corresponds to a maximum chance of error of 0.01 (Banks & Eichert 2000), was assumed to reflect statistically significant assignments, whereas individuals that had a ratio ≤ 2 could not be assigned unambiguously and were simply classified as "unassigned".

7.5 Results

Despite intensive fieldwork during 10 days, only 31 tortoises were captured in PNA. In Mangatsiaka, we were able to find seven tortoises, while 24 were found in Ihazofotsy. Based on our sample, it appears that the Andohahela population is characterized by a skewed, male-biased sex ratio: excluding unidentifiable juveniles, 17 males and 8 females were found, or a male:female ratio of 2.1:1. However, due to the small sample size, this result was only barely statistically divergent from a 1:1 ratio ($\chi^2 = 3.24$, degrees of freedom = 1, $p = 0.07$). The size or weight class distribution of sampled tortoises is skewed towards smaller tortoises (Fig. 7.2); it is statistically different from the observed distribution in non-exploited populations ($\chi^2 = 30.51$, df = 4, $p < 0.0001$), but comparable to the distribution in exploited populations ($\chi^2 = 8.38$, df = 4, $p = 0.07$).

Levels of genetic diversity in PNA were similar to other radiated tortoise populations despite the apparently small size and remoteness of the population (Table 7.1). Heterozygosity and allelic richness were even slightly higher than in the Ifotaka population, the closest population, located on the other side of the Mandrare River. As far as genetic differentiation is concerned, PNA exhibited particularly high F_{ST} values (Table 7.2). For instance, the F_{ST} between PNA and Ifotaka was higher than the F_{ST} between the West and

Central populations, which are separated by the Menarandra River, the most important genetic barrier recognized in the range of *A. radiata*. Although this difference in F_{ST} is not statistically significant as indicated by the overlap of 95% CIs (Table 7.2), results of the assignment tests also suggest a greater differentiation of PNA (Table 7.3). In fact, 29 of the 31 individuals were correctly reassigned in PNA, which represents, along with the Ifotaka population, a much higher success rate than in other populations. There was no evidence of translocated individuals in PNA in assignment test results.

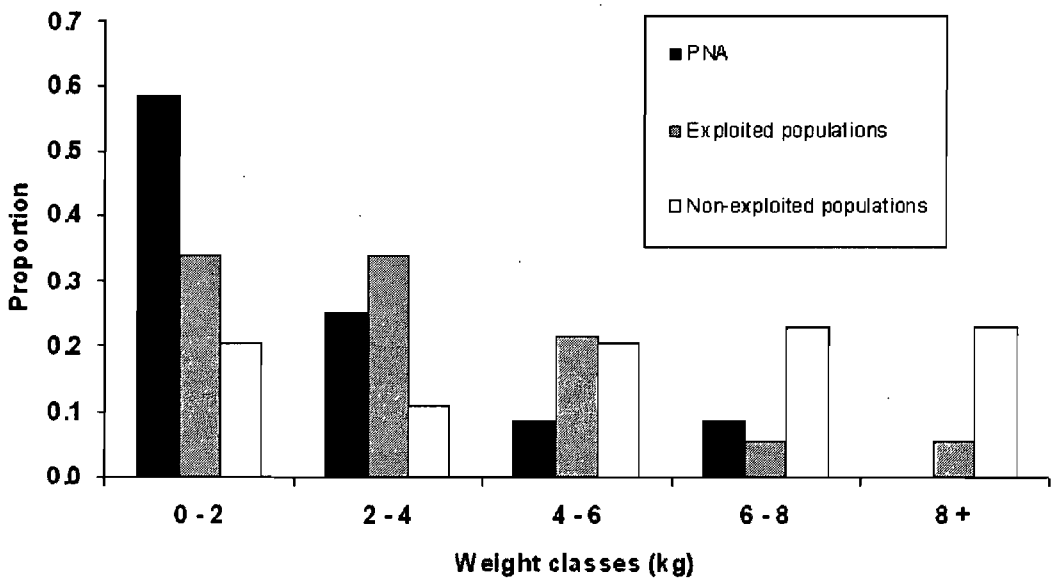

Figure 7.2 Size distribution of captured radiated tortoises (*Astrochelys radiata*) in PNA, in comparison with exploited populations and non-exploited populations sampled in previous work.

Table 7.1 Genetic diversity indices in PNA and five other populations of radiated tortoises across the range of the species.

	n	$H_O \pm 95\%$ CI	$H_E \pm 95\%$ CI	$Ar \pm 95\%$ CI	$F_{IS} \pm 95\%$ CI
Bezaha-Mahafaly	18	0.770 ± 0.112	0.804 ± 0.088	9.50 ± 1.90	0.043 ± 0.094
Sakotoavo	23	0.808 ± 0.096	0.812 ± 0.096	10.54 ± 1.90	0.004 ± 0.037
West	146	0.777 ± 0.059	0.856 ± 0.059	11.53 ± 1.84	0.092 ± 0.051
Central	80	0.747 ± 0.063	0.812 ± 0.039	8.78 ± 1.27	0.080 ± 0.076
Ifotaka	25	0.697 ± 0.067	0.757 ± 0.051	6.34 ± 0.96	0.082 ± 0.074
PNA	31	0.742 ± 0.071	0.779 ± 0.043	7.14 ± 1.04	0.046 ± 0.094

n, sample size; H_O, observed heterozygosity; H_E, expected heterozygosity; Ar, allelic richness; F_{IS}, inbreeding coefficient

Table 7.2 F_{ST} values among six genetically distinct radiated tortoise populations, including PNA. All values were statistically significant following a Bonferroni correction.

	Sakotoavo	West	Central	Ifotaka	PNA
Bezaha-Mahafaly	0.020 (0.007 ~ 0.032)	0.026 (0.015 ~ 0.033)	0.080 (0.058 ~ 0.103)	0.109 (0.081 ~ 0.132)	0.126 (0.079 ~ 0.171)
Sakotoavo		0.011 (0.004 ~ 0.017)	0.068 (0.045 ~ 0.098)	0.090 (0.065 ~ 0.115)	0.108 (0.061 ~ 0.162)
West			0.039 (0.029 ~ 0.050)	0.057 (0.049 ~ 0.072)	0.067 (0.045 ~ 0.100)
Central				0.022 (0.015 ~ 0.035)	0.060 (0.045 ~ 0.081)
Ifotaka					0.047 (0.027 ~ 0.068)

Table 7.3 Results of genetic assignment tests, indicating to which putative radiated tortoise population (columns) individuals from each original population (rows) were assigned.

	Bezaha-Mah.	Sakotoavo	West	Central	Ifotaka	PNA	Unassigned
Bezaha-Mahafaly	**12**	2	0	0	1	0	3
Sakotoavo	5	**11**	1	0	0	0	6
West	36	28	**33**	4	6	3	36
Central	0	0	1	**45**	19	1	14
Ifotaka	0	0	0	1	**24**	0	0
PNA	0	0	0	0	1	**29**	1

7.6 Discussion

7.6.1 Genetic health and distinctiveness of PNA tortoises

Contrarily to expectations, the PNA population does not exhibit particularly reduced genetic diversity, and does not appear to be threatened by inbreeding or the loss of genetic variability, for the next few generations to the least. Similarly, it has been noticed in previous work that even highly depleted populations did not display the typical genetic signature of demographic bottlenecks, which is most likely a consequence of the extremely long generation time of these organisms (Chapitre 5). This trait confers the capacity to maintain high levels of genetic diversity for longer periods after bottlenecks to chelonian populations but, on the negative side, it may mask the effects of population size reduction and henceforth cause the necessity of conservation actions to be overlooked (Kuo & Janzen 2004). Indeed, our genetic results do not suggest that the PNA population may be close to extinction.

The release in PNA of confiscated individuals from other areas of the species' range could be a source of added diversity, but assignment tests decisively rule out this

possibility, unless individuals from unsampled, remnant populations east of the Mandrare River were involved. Assignments in PNA almost reached a 100% success rate (with one unassigned individual and one individual putatively originating from the nearby Ifotaka population), so it appears that genotypes from PNA tortoises can be identified unambiguously. Genetic differentiation of the PNA population is the highest among all populations, and the F_{ST} value across the Mandrare River (between PNA and Ifotaka) is the largest reported across any river in the range of the radiated tortoise (Chapitre 5). Thus, according to criteria usually applied for conservation unit recognition (Moritz 1994; Chapitre 3), the PNA population easily qualifies as a distinct management unit. This is yet an additional argument for ANGAP wildlife managers to promote the value of the sokake population in PNA, already considered as perhaps the last remaining population in southeast Madagascar.

Considering this last statement though, on a positive note, recent radiated tortoise sightings have been confirmed from at least four different sites located in the Mandrare River Valley, west of the river, during field surveys conducted as part of an initiative to create a landscape-scale reserve in the area (Ferguson 2008). Further fieldwork will be necessary to determine whether these sightings correspond to remnant viable populations, but some of these observations have been made in newly established protected areas like the Nord-Ifotaka reserve (22,000 ha, thus only slightly smaller than parcel 2 (30,000 ha) of PNA), situated just across the river from the Ifotaka population that we have sampled in previous work (Fig. 7.1). In view of the relatively high genetic differentiation between Ifotaka and PNA, and bearing in mind that the Mandrare Valley represents one of the largest remaining areas of connected forest in southern Madagascar, that region may provide an interesting opportunity to examine the role of rivers in the establishment of genetically distinct populations of *A. radiata*.

7.6.2 Where have all the large tortoises gone?

As explained in the introduction, we were expecting to find in PNA a much more abundant tortoise population. The observed sex ratio and size structure suggest that the PNA population is in fact harvested. Numerous field studies have shown that undisturbed populations were characterized by an equal sex ratio, whereas the sex ratio in exploited population was generally male-biased (Leuteritz 2002; O'Brien 2002; Seui 2006). This is a result of the poachers' preference for females because of the texture of their meat, which contains more fat (Pedrono *et al.* 2000). Clearly, the 2:1 ratio obtained for PNA hints at exploitation. Furthermore, the vast majority of captured tortoises were relatively small, and the size structure of the population is very similar to that of exploited populations (Fig. 7.2).

After several days of fieldwork, we asked inhabitants of the Antandroy village of Ihazofotsy about the global absence of large tortoises in PNA. They decided to lead us to the answer. After a 45 minute walk in the spiny forest, we reached a small cave formed by two large boulders leaning on each other, which created a shelter underneath. Inside the cave we found piles of broken carapaces, bones and scutes, apparently from adult radiated tortoises. Hidden next to the cave, we found an enclosure made of piled up stones. Our guides explained that hunters from nearby Antanosy villages store the live tortoises they collect in this enclosure. Then, several times a year, most often in preparation for a holiday like Christmas or Easter, they gather for the killings, mostly making dried meat, which is easier to transport back to the villages and easily concealed from police checks. The guides estimated that the piles of broken shells accounted for approximately 50 large tortoises, and informed us that at least another cave like this was located within walking distance. Not surprisingly, the five largest tortoises we captured in PNA were among the seven individuals sampled in Mangatsiaka, which is considerably far from the Antanosy caves and Ihazofotsy. Interestingly, after seeing us inject PIT tags in captured tortoises, our

guides told us that if Antanosy people became aware of these tags, they may be reluctant to eat tortoises of the park in the future.

7.6.3 Protected areas and the Durban Vision

Antandroy villagers from Ihazofotsy believe that the sokake should not be hunted because of their cultural taboo. We asked them if they sometimes tried to intervene when they saw Antanosy people hunting tortoises in the park, and they answered that they did not want to cause trouble and feared being injured or even killed if they opposed poachers, who carry weapons with them. We also interviewed ANGAP staff at PNA and Fort-Dauphin. One former park ranger said that when he first started as a park employee, he denounced people he had caught illegally logging trees within the boundaries of the park. However, he quickly realized that in most cases, law enforcement officials either completely ignored this problem, or accepted bribes to call off or reduce the punishment of offenders. After a while, the same wrongdoers would come back in the park and begin logging trees again, scoffing at rangers, knowing that they could repeat the same transgressions again without serious consequences. The former ranger also confirmed what villagers had said: the violence of altercations with poachers or loggers is increasing, and park rangers do not have the necessary resources or support to assure their security if altercations occur. He concluded by saying that spending almost everyday and night in the park, away from his family, witnessing what goes on within the park for a slim salary probably was not worth it, and he said he was looking for a better job with the mining companies in Fort-Dauphin.

Recruitment in the PNA tortoise population is undoubtedly very low, since the vast majority of individuals that we captured were subadults. The number of tortoises that reach sexual maturity before being poached is certainly too low to assure the viability of the population, despite the official protection it receives. At the World Parks Congress in Durban, South Africa, in 2003, President Ravalomanana committed to tripling the

country's protected area network within five years (a commitment accordingly dubbed the "Durban Vision"). This initiative was applauded worldwide by conservationists, and the President has so far kept his promise: over a million hectares of remaining forest cover have either already been added to the network of national parks and reserves, or are in the midst of being included (Mittermeier *et al.* 2006). However, it has also raised skepticism among experts on Malagasy socio-economic, cultural and political questions. To cite Horning (2008), [the notion] *that resources are better protected when they are placed under the formal protection of the state is, at best, questionable.* The situation of the PNA tortoise population clearly shows that creating protected areas does not necessarily provide protection for species inside.

This is not an exclusive problem to PNA: for instance, during our first trip to Bezaha-Mahafaly Special Reserve in 2004 (a protected area with similar status to ANGAP parks and reserves, but managed by the School of Agronomic Science of the University of Antananarivo), we were able to locate 18 radiated tortoises, mostly subadults. This was a relatively impressive effort considering that only 16 tortoises had been marked by reserve rangers over the previous two years. Three years later, with a larger field team, we did not encounter a single tortoise after several days of intensive work. Although the human population around the reserve is mainly composed of Mahafaly and Antandroy people (Ratsirarson 2003), there are a few Antanosy settlements in the area. Reserve staff believe that the most devastating blow to the tortoise population took place prior to our first visit, between 2001 and 2003, when the reserve was temporarily closed due to a lack of funding, during which Antanosy people massively collected tortoises. Nonetheless, according to reserve employees, Mahafaly people of the region sometimes accept to sell tortoises to Antanosy even if they are taboo, and poaching has continued. Our experience shows that the tortoise population there went from uncommon to almost extinct at best within a few years. The PNA population may be awaiting the same fate in the near future.

A few years ago, there were four protected areas within the extant range of the sokake: PNA, Bezaha-Mahafaly Special Reserve, Cap Sainte-Marie Special Reserve and Tsimanampetsotsa National Park. In order to increase survival probabilities of the radiated tortoise, Nussbaum & Raxworthy (2000) recommended establishing additional natural reserves on the Mahafaly and Karimbola Plateaux, at the core of the sokake's range in the Southwest. This recommendation is in the midst of being respected through the Durban Vision, as Tsimanampetsotsa will be expended from 43,200 ha to over 200,000 ha, covering most of the Mahafaly Plateau north of the Linta River. In addition, as mentioned above, new protected areas have been recently created in the Mandrare Valley as part of a larger project that would protect, if implemented in its entirety, over 300,000 ha of connected forest in that region, and there are several other planned protected areas within the range of the sokake.

Unfortunately, this expanded network may not provide much protection for tortoises under current policies; the Durban Vision will not achieve its objective unless essential conditions for efficient biodiversity conservation within and around protected areas are met. These have been discussed elsewhere (e.g. chapter 14 in Goodman & Benstead 2003) and undeniably involve the development of sustainable resource management strategies (including sokake) in tight partnership with local communities. There are examples among the network of national parks where benefits of the protected area may exceed those that would come from unsound resource management and failing to maintain a protected core area. For instance, the famous Ranomafana National Park in tropical eastern Madagascar now generates substantial income for local residents and the park through tourism and research activities, and there is a sense of great pride towards the conservation of biodiversity in the surrounding community. The situation in Ranomafana and PNA is, of course, very different: the number of visitors in Ranomafana has reached over 10,000 annually in the late nineties (Wright & Andriamihaja 2003), whereas in PNA, less than

2000 people have visited the park in total between 1997 and 2003 (Fenn 2003*b*). And even Ranomafana has a history punctuated with frictions and conflicts (e.g. Peters 1998).

7.6.4 The absence of financial alternatives

There are virtually no economic activities in the extreme South of Madagascar, including very little tourism. Discussing this topic in details may go beyond the scope of this article, but it is crucial to recognize that economic development and conservation often go hand in hand. The view that Malagasy peasants and foragers are as intrinsic to the solution as they are to the environmental problems in Madagascar has greatly progressed in the past decade (Kaufmann 2006), and ensuring that local populations can meet their most basic needs would remove tremendous pressure from biological resources and habitats. As an example, the illegal fishing of marine turtles along the southwestern coast provides strong support for this position. In spite of official attempts to protect green turtles (*Chelonia mydas*) in that region dating back to 1923, conservation efforts have been deemed a failure (Lilette 2006). One of the main reasons for this failure is that there are no financial incentives to limit the exploitation of marine turtles, and fishermen have expressed that if there were alternatives to earn more money while protecting turtles, they would certainly do it (Lilette 2006). In Northwest Madagascar (e.g. island of Nosy Iranja), marine turtles have been integrated to ecotourism tours, and the conservation value of these animals has been improved. Unfortunately, as explained before, tourism in the South is of much smaller extent.

Among others, improving the deplorable condition of roads in southern Madagascar would certainly favor economic development and tourism in that region. But roads are a double-edged weapon with respect to sokake conservation. In addition to the direct tortoise mortality that they induce (Goodman *et al.* 1994), large numbers of tortoises are collected when first seen from vehicles on the road, as reported by Nussbaum & Raxworthy (2000),

and increased traffic would correspond to more opportunities for collecting tortoises. Furthermore, the status of remaining tortoise populations would probably be worst nowadays if a better road network had facilitated both the access to remote regions where tortoises are abundant and the transportation of great numbers of tortoises to population centers. Nonetheless, one would have to be oblivious to the needs of the people of southern Madagascar not to recognize the importance of building a decent road network in the region, and when that happens, extra measures like enhanced road checks and superior anti-corruption strategies will have to be taken to minimize ill-fated impacts on tortoises.

7.6.5 The fady

Another aspect of sokake conservation is the understanding of the taboo, or fady in Malagasy, in Mahafaly and Antandroy communities. Blaming the breakdown of the traditional taboo due to the increasing immigration of people from different ethnic groups, experts often claim that reinforcing the fady in local communities is important to promote tortoise conservation (Lingard *et al.* 2003). Traditional taboos often act as efficient *de facto* protection means for certain species or forests (Jones *et al.* 2007; Tengo *et al.* 2007), but it may be naive to believe that reinforcing them either with legal instruments or awareness campaigns would be effective. One has to consider past experiences of Antandroy and Mahafaly communities with the *outside world* and the fact that any given rural community from southern Madagascar will have experienced several, if not all, of the following: violence and pillaging from the colonial French; racist attitudes by a central authority or administration since French colonial rule; destruction of the prickly pear cactus, or raketa, by colonial administration despite it being an essential food source in times of drought, and resulting subsequent famines (Middleton 1999); theft of livestock by bandits armed by Malagasy military (Rasamoelina 2007); oppression exerted by corrupt officials at multiple levels; repeated promises by the government and NGOs concerning improvements to water supply problems or other projects that do not meet their targets; and researchers

investigating the most private and sensitive issues in their lives. Thus, external modifications or reinforcements of taboos that have usually remained untainted by the *outside world* may be seen as threats. Moreover, meddling with tradtitional institutions may cause their cultural erosion, and the self-enforcement that they once procured may simply break down (Jones *et al.* 2007).

In addition, the manifestation of the fady varies noticeably among ethnic groups and even among villages of the same group, In 2004, we spent a week in the small village of Sakotoavo, on top of the Mahafaly Plateau just north of the Linta River, where a sacred forest harbors a rather large, undisturbed sokake population. The Mahafaly King of the village granted us the permission to sample tortoises around the forest, without venturing inside. The fady, in that community, was motivated by respect for the spirits of the ancients, which are believed to dwell inside the tortoises that inhabit the sacred forest. We had to be very discreet when taking tissue samples to prevent any offense towards the villagers, who would not even accept to shake our hands knowing we had touched sokake. Through conversations with peasants, we learnt that the male nurse working in the village, a man of Antanosy descent, was secretly collecting tortoises to sell them in Ejeda, the closest town, but villagers did not interfere out of fear of losing their only medical specialist. Aside from this exception, the taboo in Sakotoavo is the strictest we have seen during our journeys in southern Madagascar. On the other hand, respect is not always at the origin of the fady, and in several Mahafaly communities, the taboo is mainly imputable to loathing rather than respect. There is a widespread legend about an ancestor who tried to boil a live tortoise in a clay kettle, causing the tortoise to kick at the kettle and shatter it (Leuteritz 2002). Because of that legend, several Mahafaly and Antandroy tribes consider that attempting to kill or eat tortoises would lead to unfortunate events. Their motivation for the taboo is thus of a very different nature than that described previously. For instance, while we were in Kilibory, on the southwestern littoral south of the Linta River, we quickly realized that Mahafaly peasants considered sokake as pests because of the damage they did

to their crops. Touching tortoises is not prohibited by the taboo there, so they did not hesitate to throw tortoises out of their fields. Accordingly, we found an unusually high incidence of shell fractures among tortoises from Kilibory (Rioux Paquette & Lapointe 2007). There is no doubt that poachers proposing to rid fields of crop-eating tortoises in Kilibory would be welcome to do so. Some people are hasty putting the blame for the dramatic decline of the sokake on Mahafaly and Antandroy people, implying that they are forgetting about their traditions. In many cases, this assessment reveals a misunderstanding of the taboo. Although several communities respect their own fady, they simultaneously allow others the right to follow their own norms.

7.6.6 CITES listing and the tortoise trade

The last point we wish to raise concerns the legislation protecting the radiated tortoise. The legitimacy of listing *A. radiata* on the Appendix I of the CITES in 1975 has often been questioned, including by Nussbaum & Raxworthy (2000) who, at the time, had no reason to believe that the species was threatened. Unfortunately, considering the evidence gathered since then, the perception on the status of the sokake has dramatically changed, and it is now firmly believed that the species could be pushed to extinction in the wild very quickly (Randriamahazo *et al.* 2007). Additionally, Nussbaum & Raxworthy (2000) specified that in order to be beneficial to the species, downgrading *A. radiata* would have had to be accompanied by careful exportation controls. The experiment with the spider tortoise *Pyxis arachnoides* shows that it probably would not have been possible: while *P. arachnoides* was listed on Appendix II between 1980 and 2004, Madagascar consistently failed to report accurate export numbers in the CITES reporting system, which may be attributed to corruption (Walker *et al.* 2004).

At first glance, the inclusion of *A. radiata* on Appendix I in 1975 would only appear sensible considering that the collection of radiated tortoises was completely prohibited in

Madagascar since 1961 through a national decree (# 60126). The ensuing turn of events emphasizes that total prohibition can only be successful when backed up by an effective law enforcement system. For the radiated tortoise, the lack of such a system has lead to the establishment of a completely parallel, illegal structure for both national and international markets. This situation has made it almost impossible to monitor what goes on. Two different studies based on interviews with poachers have estimated that approximately 50,000 radiated tortoises are taken to Tulear every year (O'Brien *et al.* 2003; Bidaud & Randria 2008). On his first day in Tulear in 2003, it took the first author of this paper (SRP) less than 30 minutes to buy a radiated tortoise. After asking a gem dealer if it was possible to find sokake in town, he was lead to a house and offered a basket full of spider tortoises and juvenile radiated tortoises. After he bought a sokake for $1, he took it back to officials of conservation agencies, and their disbelief after hearing this story hints at the general state of denial that was reigning in Tulear at that time.

Legislation should favor the sustainable use of resources and ensure they are valued at fair rates (Allen & Edwards 1995). In the case of radiated tortoises, it is safe to say that it has lead to completely opposite results. First, the discrepancy between the amount paid to collectors by intermediaries and the final price charged by exporters is staggering: it is estimated that collectors receive 0.08% of the final price of a tortoise (Bidaud & Randria 2008). By comparison, chameleon collectors received 6.5% when export controls were liberalized in the early nineties (Carpenter *et al.* 2005). Furthermore, no compensation at all is provided to Mahafaly and Antandroy communities. Finally, in Western countries, it is possible to legally acquire captive-bred radiated tortoises ($1500 for a juvenile, $4500 for an adult), but this trade does not provide any income to Madagascar (Bidaud & Randria 2008). This apparently contradicts clause 15 of the Convention on Biological Diversity, which advocates sharing profits related to the exploitation of genetic resources.

7.7 Conclusion

Successful conservation schemes for the radiated tortoise will inevitably have to incorporate ways to generate financial incentives. Captive breeding centers, managed by local communities, could be a solution. Through regulated trade, income generated this way could be shared among communities. Not surprisingly, the idea of commercial breeding centers garnered great interest among Malagasy parties at the last IUCN/TFTSG meeting in Antananarivo. However, this can only be successfully implemented in combination with genuine efforts to ensure that authorities charged with enforcement are themselves enforced. Over the years, we have been told on too many occasions of stories and anecdotes indicating that law enforcement forces are often at the forefront of the flagrant disregard for the laws concerned with the poaching and smuggling of *Astrochelys radiata*. Unless this situation can be improved, commercial farming would only legitimize the current illegal operations. In addition, measures to ensure the conservation of wild tortoise populations will need to be established in parallel. Part of the income generated through farming could serve as funding for monitoring programs. Such programs could reward communities that maintain abundant wild tortoise populations, but payment/reward systems are difficult to enforce and may be impossible to implement considering the complex property rights system of southern Madagascar. Thus, several aspects would have to be thoroughly pondered, and local communities themselves would probably be instrumental in the formulation of guidelines for projects like this. Fortunately, there may still be a few million sokake remaining in the wild (realistic estimates range between 1.6 and 4 million), so it may not be too late to launch audacious and innovative projects.

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CHAPITRE 8

Conclusion

Malgré sept chapitres et plus de 150 pages sur le sujet, il serait sans doute possible de continuer les analyses pour répondre à de nouvelles questions sur l'évolution ou l'écologie de la tortue radiée. Néanmoins, cette thèse illustre bien la variété des questions auxquelles il est possible de répondre avec des outils moléculaires, et la manière dont les résultats peuvent être utilisés pour orienter les efforts de conservation. En guise de conclusion, je présente dans ce dernier chapitre un bref retour sur les objectifs originaux en discutant brièvement des principaux résultats et de ce qui pourrait être fait dans le futur. Toutefois, au-delà de ces considérations, la conclusion primordiale à tirer de cette thèse, selon moi, est que l'importance de mes travaux ne provient pas seulement des résultats, mais également du projet lui-même. En effet, comme l'écrivaient Nussbaum & Raxworthy (2000) quelques années avant le début de mes travaux, il est incroyable, en considérant la grande popularité de la tortue radiée auprès des herpétologistes et des éleveurs, que cette espèce n'ait pratiquement jamais bénéficié de l'intérêt des scientifiques dans la nature. Mon projet de doctorat a été l'occasion de tourner l'attention de la communauté scientifique internationale vers la forêt épineuse de Madagascar et la tortue radiée. À plusieurs reprises dans cette thèse, j'ai insisté sur le fait que jusqu'à récemment, la tortue radiée était considérée si abondante que son extinction n'aurait jamais été envisageable, une vision qui s'est complètement transformée pendant mes travaux. La culmination de cette prise de conscience fut atteinte lors d'un atelier spécial de l'UICN/TFTSG tenu à Antananarivo en janvier 2008 pour réévaluer le statut des chéloniens malgaches, où il fut recommandé à l'unanimité d'élever *Astrochelys radiata* au statut CR (*critically endangered*). Puisque mes travaux ne portaient pas directement sur l'analyse du déclin de l'espèce, ce sont probablement davantage mes observations et mes expériences sur le terrain que mes résultats génétiques qui ont interpelé ceux qui ont à coeur la conservation de la sokake. La

démonstration que des recherches doctorales puissent avoir de telles conséquences, qui transcendent les implications directes des résultats attendus, est un argument de taille pour justifier le financement de projets de recherche en biologie de la conservation, un domaine où l'inaction des chercheurs au profit de la quête absolue de connaissances est trop souvent pointée du doigt (Whitten *et al.* 2001).

Les résultats aux analyses réalisées dans cette thèse n'en conservent pas moins leur importance et leur valeur pour la conservation de la tortue radiée. Évidemment, chez une espèce longévive comme *A. radiata*, certains enjeux, tels que le maintien de la variabilité génétique des populations, ne sont pas aussi préoccupants, dans l'immédiat, que chez des espèces qui ont un temps de génération beaucoup plus court. De plus, en considérant que le calcul des chances de survie de l'espèce prédise son extinction au cours des 45 prochaines années (Randriamahazo *et al.* 2007), soit une génération de tortue, il y a peu de chances que les facteurs génétiques influencent significativement le destin de cette espèce, du moins à court terme. Néanmoins, certaines informations tirées des analyses génétiques ont permis d'élucider des traits de son histoire naturelle qui auraient été difficiles, voire impossibles, à étudier autrement. Le chapitre 6 en est un bon exemple: les résultats génétiques indiquent clairement que les femelles tortues radiées sont davantage phylopatriques que les mâles, un trait possiblement associé à la fidélité au site de ponte natal. Ce résultat constitue une excellente piste de départ pour de futures recherches et représente un élément notable à considérer lors de l'élaboration de projets de conservation pour cette espèce.

Parmi les objectifs initiaux, la description de la structure génétique de la tortue radiée était un objectif clé. Grâce au développement de marqueurs microsatellites très polymorphes au chapitre 2, qui furent par la suite utilisés tout au cours de la thèse, cette description fut amorcée au chapitre 3, puis mieux définie au chapitre 5. Globalement, la tortue radiée affiche une différentiation génétique modérée, ce qui surpasse les attentes que plusieurs auraient pu avoir au début du projet, compte tenu de la très grande taille des

populations et de l'absence apparente de barrières géographiques à la dispersion des individus à travers l'aire de répartition de l'espèce (Leuteritz *et al.* 2005). Cette dernière supposition reposait sur le fait que même les plus grands fleuves du sud de Madagascar sont complètement asséchés plusieurs mois par année, laissant libre passage aux tortues. Or, pas moins de six populations génétiquement distinctes ont été décelées (incluant la population d'Andohahela au chapitre 7), chacune étant séparée des autres par l'escarpement du Plateau Mahafaly ou le lit d'un cours d'eau (Menarandra, Manambovo et Mandrare). Le fleuve Menarandra semble être l'élément physiographique prédominant dans la structure génétique de *A. radiata*, et les résultats des analyses de l'ADNmt et des microsatellites indiquent qu'il représente une importante barrière historique et contemporaine au flux génique (chapitre 5). Ce n'est sûrement pas une coïncidence que ce fleuve, chez la tortue araignée, une espèce sympatrique avec *A. radiata*, soit la frontière entre la distribution des deux sous-espèces *Pyxis arachnoides arachnoides* et *P. a. oblonga* (Pedrono 2008).

Il semble donc que les tortues radiées, qui sombrent en latence pendant la saison sèche, lorsque nourriture et eau se font rares dans la forêt épineuse, aient en réalité très peu d'opportunités de traverser le lit des fleuves. Le fleuve Linta, qui s'assèche plus longtemps que les autres, offre probablement plus d'occasions au passage des tortues radiées avant qu'elles ne deviennent inactives, ce qui expliquerait que ce fleuve ne joue aucun rôle dans la structure génétique de l'espèce. Aucune étude n'a été effectuée pour définir l'ampleur du domaine vital et des déplacements chez la tortue radiée, mais la constatation que des barrières physiques soient requises pour limiter le flux génique entre populations suggère que les individus se déplacent probablement plus que ce que l'on croyait à partir de résultats préliminaires de télémétrie (O'Brien 2002). Je crois que des études de terrain sont inévitablement requises pour mieux comprendre l'utilisation de l'habitat, le domaine vital et les déplacements des tortues radiées, des données qui semblent pourtant fondamentales à l'élaboration d'une stratégie de conservation efficace.

Par ailleurs, il est à souhaiter que les résultats obtenus pour la tortue radiée motiveront d'autres chercheurs à étudier la phylogéographie des espèces de la forêt épineuse. Le nombre d'études phylogéographiques à Madagascar a littéralement explosé au cours de la dernière décennie, révélant du même coup une biodiversité encore plus élevée qu'envisagée auparavant. C'est ainsi que, depuis 1994, le nombre d'espèces de lémuriens est passé de 32 à 68, les amphibiens de 170 à 235, et les reptiles de 290 à 370 (Mittermeier *et al.* 2006; Glaw & Vences 2007). Toutefois, presque aucune de ces nouvelles espèces ne provient du sud de l'île, et l'échantillonnage dans cette région est souvent déficient, voire complètement absent des analyses. De plus, la phylogéographie comparée d'espèces ayant des traits écologiques différents, en combinaison avec de nouvelles données paléoclimatiques pour la région, pourrait permettre, entre autres, de trouver une explication à l'importance du Menarandra dans la structure génétique des tortues terrestres.

L'analyse des effets de la fragmentation de l'habitat et de l'exploitation sur la diversité génétique de la tortue radiée figurait également parmi les objectifs de départ. Le chapitre 5 a bien illustré qu'il peut être assez difficile de clairement départager les impacts de ces deux facteurs. La variété des analyses effectuées a permis de mettre en évidence des résultats qui auraient pu être interprétés de façon erronée autrement. Ainsi, alors que les populations exploitées semblaient exhiber une diversité génétique inférieure aux autres populations, de plus amples analyses, telles que la détection et la simulation de goulots d'étranglement, suggèrent plutôt que la fragmentation historique de l'habitat, à l'est du fleuve Menarandra, est vraisemblablement à l'origine de cette différence. Tel que mentionné plus haut, et aussi démontré par des simulations, le grand temps de génération de *A. radiata* fait en sorte que la simple surexploitation relativement récente de certaines populations ne peut être considérée comme la cause de la diversité plus faible des populations exploitées. De plus, l'analyse de l'ADNmt indique que l'espèce a vécu une grande expansion démographique à l'ouest du Menarandra lors du Pléistocène, où elle a pu accumuler les mutations et maintenir des niveaux de variabilité supérieurs à ceux des

populations de l'est, où l'habitat, fragmenté dû à la nature des sols dans cette région, ne pouvait pas supporter d'aussi grandes populations. Ces résultats laissent croire que la destruction de l'habitat, qui se poursuit à un rythme inquiétant dans la forêt épineuse (Harper *et al.* 2008), pourrait avoir des effets pour le moins aussi graves que l'exploitation sur la diversité génétique de l'espèce à long terme. La surexploitation est souvent ciblée comme étant la principale menace à la survie de *A. radiata*, avec raison d'ailleurs, mais cela ne devrait pas éclipser la nécessité de développer des stratégies durables de gestion des ressources forestières et de préservation des habitats dans le sud malgache.

La désignation d'unités de conservation a fait l'objet d'une discussion assez exhaustive aux chapitres 3 et 5 (sections 3.6.3 et 5.6.6). Je ne désire pas revenir ici sur ce point, et je réitère simplement ma conviction que l'identification de telles unités pour *A. radiata* est nécessaire, pour les mêmes raisons qui justifient la discrimination de différents stocks chez une espèce exploitée dans le domaine des pêcheries. Toutefois, je souhaite apporter une perspective additionelle concernant les unités de conservation. Un des arguments souvent invoqués pour légitimer l'importance de désigner de telles unités est de s'assurer du maintien de l'ensemble de la diversité génétique (inter-populationelle) de l'espèce, afin de préserver les adaptations locales et son potentiel adaptatif. Naturellement, la prémisse sous-jacente à cet argument est que les patrons de variation génétique neutre reflètent les patrons d'adaptations locales. Or, des exemples récents s'accumulent dans la littérature pour démontrer que ce n'est pas le cas, et que les approches actuelles de délimitation des unités de conservation sont inefficaces à préserver la variation génétique réellement pertinente au maintien du potentiel adaptatif des espèces. Prenons l'étude de Hemmer-Hansen *et al.* (2007): en analysant la différentiation entre populations de flets (*Platichthys flesus*) au niveau du gène Hsc70, le gène d'une protéine responsable de la résistance aux chocs thermiques, ces auteurs ont obtenu des patrons de divergence complètement différents de ceux suggérés par des marqueurs neutres. L'accumulation d'observations similaires a mené à l'émergence d'une nouvelle discipline, la génomique

des populations, qui s'intéresse à la comparaison d'un grand nombre de régions du génome, incluant des gènes sous sélection, afin de mieux comprendre la contribution des différentes forces évolutives (dérive génétique, flux génique, sélection et mutation) à la variation génétique des espèces (Luikart *et al.* 2003). Grâce au perfectionnement continuel des équipements et des méthodes d'analyse, cette approche est promue à un brillant avenir, et s'ajoute aux arguments déjà formulés (section 5.6.6) qui m'ont poussé à écrire que pour la tortue radiée, les ressources devraient être allouées à d'autres enjeux que celui de préserver à tout prix l'intégrité de la structure génétique de l'espèce révélée par les marqueurs microsatellites.

Parmi toutes les analyses réalisées dans cette thèse, celles qui se rapprochent le plus d'une description de la variation adaptative des populations de tortues radiées sont possiblement les analyses morphométriques du chapitre 4. À l'image de la différence qu'il peut y avoir entre les patrons de divergence pour régions neutres et adaptatives du génome, il n'y a aucune corrélation entre les distances génétiques et les distances morphométriques calculées entre les individus échantillonnés au chapitre 4 (r = 0.09; p = 0.37). La morphologie des organismes est fortement influencée par la sélection naturelle, et il a été discuté de la valeur adaptative potentielle des variations de la forme de la carapace des tortues radiées à la section 4.6.2. La pression causée par l'exploitation sélective des grandes tortues a été invoquée, mais d'autres facteurs, comme des différences locales au niveau de la composition de l'habitat, pourraient aussi être responsables de ces variations. Pour élucider cette question, il faudrait être en mesure de caractériser ces variations morphologiques, ce qui est impossible avec les analyses dites traditionelles que j'ai employées. L'une des avancées majeures qui a véritablement révolutionné l'analyse morphométrique au cours des années 1990 fut la réalisation que la base de l'analyse comparative de la forme des organismes ne repose pas sur la comparaison de plusieurs "variables indépendantes" (telles que considérées dans les analyses multivariées classiques, incluant l'approche utilisée au chapitre 4), mais plutôt sur la comparaison de la forme elle-

même, représentée par plusieurs points de référence ou *landmarks* (MacLeod 2005). Cette prise de conscience a mené à l'apparition d'une nouvelle approche, l'analyse morphométrique géométrique (Rohlf 1990; Zelditch *et al.* 2004), qui permet entre autres de visualiser directement les variations de la forme, décrite par des *landmarks* en deux ou trois dimensions. Des photographies du plastron et de la carapace des tortues échantillonées au chapitre 4 ont été prises et auraient pu être numérisées pour effectuer une analyse géométrique avec des *landmarks*, mais malheureusement, les précautions nécessaires n'ont pas été respectées sur le terrain (l'angle de la caméra étant variable d'une photo à l'autre). D'ailleurs, l'analyse géométrique se prête très mal aux données récoltées sur le terrain, et les photographies sont généralement prises dans des conditions de laboratoire, compte tenu de l'équipement utilisé et des précautions à suivre. Toutefois, une méthode récente permet la reconstruction tri-dimensionelle de la forme des organismes à partir d'images digitales prises sous des conditions de terrain (Chiari *et al.* 2008), alors il devrait être possible dans le futur d'étudier en détail la variation morphologique chez la tortue radiée.

J'en arrive à la fin de ce survol des objectifs et des principaux résultats de cette thèse. Il reste sans contredit beaucoup à faire. D'une part, au niveau de la conservation de la tortue radiée, la bataille ne fait que commencer et des projets tels que celui proposé à la section 7.7 devront être mis de l'avant pour assurer la survie de *A. radiata*. D'autre part, plusieurs aspects scientifiques demeurent inexplorés. Par exemple, le rôle écologique joué par la tortue radiée au sein de la forêt épineuse n'a jamais été étudié. Lorsqu'une espèce herbivore de grande taille est très abondante sur une île, on peut s'attendre à ce qu'elle exerce un rôle clé dans l'écologie et l'évolution des plantes qu'elle consomme, comme les tortues géantes de l'atoll d'Aldabra (Eskildsen *et al.* 2004). Il ne serait donc pas surprenant que la tortue radiée soit vitale à l'équilibre écologique de la forêt épineuse, ce qui fournirait un argument supplémentaire de poids pour valoriser sa conservation.

À l'hiver 2008, un nouveau projet de recherche portant sur la population de tortues radiées du village de Lavavolo a été lancé en collaboration avec l'Université de Tuléar. Pendant deux ans, des étudiants suivront de près la population de sokake; ils analyseront les déplacements des individus, évalueront la taille de leur domaine vital, et effectueront un suivi des nids et des juvéniles, avec la participation quotidienne des villageois. Les outils moléculaires que j'ai développés seront utilisés pour répondre à de nouvelles questions concernant la taille efficace de la population et la fréquence des paternités multiples chez *A. radiata*. Cette initiative se veut en quelque sorte un projet pilote pour évaluer les impacts positifs attendus qui résulteront de cette étroite collaboration avec la communauté locale. Lorsque des questions d'ordre scientifique permettent de soutenir de tels projets, il ne fait aucun doute que la génétique est vraiment au service de la conservation.

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Annexe I

Geographic coordinates of all radiated tortoises included in analyses of Chapitre 7.

Tortoise ID	Population	Sex	Latitude (S)	Longitude (E)
201	Bezaha-Mahafaly	Male	23°39'54.8"	044°36.42.7"
202	Bezaha-Mahafaly	Male	23°39'22.4"	044°37'44.7"
203	Bezaha-Mahafaly	Male	23°39'43.9"	044°37'30.6"
204	Bezaha-Mahafaly	Male	23°39'43.9"	044°37'30.6"
206	Bezaha-Mahafaly	Male	23°39'28.7"	044°37'53.5"
207	Bezaha-Mahafaly	Male	23°39'22.4"	044°37'44.7"
208	Bezaha-Mahafaly	Male	23°39'46.3"	044°37'21.5"
209	Bezaha-Mahafaly	Female	23°39'31.8"	044°37'26.4"
210	Bezaha-Mahafaly	Female	23°39'29.2"	044°37'29.4"
211	Bezaha-Mahafaly	Male	23°39'26.1"	044°37'29.7"
212	Bezaha-Mahafaly	Female	23°39'26.1"	044°37'29.7"
213	Bezaha-Mahafaly	Male	23°39'24.6"	044°37'30.8"
214	Bezaha-Mahafaly	Female	23°39'22.4"	044°37'44.7"
215	Bezaha-Mahafaly	Female	23°39'25.0"	044°37'29.0"
218	Bezaha-Mahafaly	Male	23°39'22.4"	044°37'44.7"
301	Sakotoavo	Male	24°29'51.5"	044°27'04.1"
302	Sakotoavo	Female	24°29'51.5"	044°27'04.1"
303	Sakotoavo	Female	24°29'51.5"	044°27'04.1"
304	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
305	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
306	Sakotoavo	Male	24°30'14.4"	044°26'48.8"
307	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
308	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
309	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
310	Sakotoavo	Male	24°30'14.4"	044°26'48.8"
311	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
312	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
313	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
314	Sakotoavo	Female	24°30'14.4"	044°26'48.8"
315	Sakotoavo	Male	24°30'14.4"	044°26'48.8"
316	Sakotoavo	Male	24°30'14.4"	044°26'48.8"
318	Sakotoavo	Male	24°29'43.8"	044°27'29.4"
320	Sakotoavo	Female	24°29'43.8"	044°27'29.4"
321	Sakotoavo	Female	24°29'43.8"	044°27'29.4"
322	Sakotoavo	Female	24°29'43.8"	044°27'29.4"
323	Sakotoavo	Female	24°29'43.8"	044°27'29.4"
VORO191	Voroja	Female	24°29'22.3"	044°13'16.9"
VORO192	Voroja	Female	24°29'22.3"	044°13'16.9"
VORO193	Voroja	Male	24°29'22.3"	044°13'16.9"
VORO194	Voroja	Male	24°29'22.3"	044°13'16.9"
VORO198	Voroja	Female	24°29'22.3"	044°13'16.9"
VORO199	Voroja	Male	24°29'22.3"	044°13'16.9"

Tortoise ID	Population	Sex	Latitude (S)	Longitude (E)
601	Lavavolo	Male	24°38'15.1"	044°56'48.6"
602	Lavavolo	Male	24°38'15.1"	044°56'48.6"
603	Lavavolo	Male	24°38'15.1"	044°56'48.6"
604	Lavavolo	Male	24°38'15.1"	044°56'48.6"
605	Lavavolo	Male	24°38'15.1"	044°56'48.6"
606	Lavavolo	Female	24°38'15.1"	044°56'48.6"
607	Lavavolo	Male	24°38'15.1"	044°56'48.6"
608	Lavavolo	Male	24°38'15.1"	044°56'48.6"
609	Lavavolo	Female	24°38'15.1"	044°56'48.6"
610	Lavavolo	Male	24°38'15.1"	044°56'48.6"
611	Lavavolo	Male	24°38'15.1"	044°56'48.6"
612	Lavavolo	Male	24°38'15.1"	044°56'48.6"
613	Lavavolo	Male	24°38'15.1"	044°56'48.6"
614	Lavavolo	Female	24°38'15.1"	044°56'48.6"
615	Lavavolo	Female	24°38'15.1"	044°56'48.6"
616	Lavavolo	Male	24°38'15.1"	044°56'48.6"
617	Lavavolo	Female	24°38'15.1"	044°56'48.6"
618	Lavavolo	Male	24°38'33.4"	044°56'50.2"
619	Lavavolo	Male	24°38'33.4"	044°56'50.2"
620	Lavavolo	Female	24°38'33.4"	044°56'50.2"
621	Lavavolo	Male	24°38'33.4"	044°56'50.2"
622	Lavavolo	Male	24°38'33.4"	044°56'50.2"
623	Lavavolo	Male	24°38'33.4"	044°56'50.2"
624	Lavavolo	Male	24°38'33.4"	044°56'50.2"
625	Lavavolo	Female	24°38'33.4"	044°56'50.2"
626	Lavavolo	Female	24°38'33.4"	044°56'50.2"
627	Lavavolo	Female	24°38'33.4"	044°56'50.2"
628	Lavavolo	Male	24°38'33.4"	044°56'50.2"
629	Lavavolo	Male	24°38'33.4"	044°56'50.2"
630	Lavavolo	Male	24°38'33.4"	044°56'50.2"
631	Lavavolo	Female	24°38'33.4"	044°56'50.2"
632	Lavavolo	Male	24°38'33.4"	044°56'50.2"
633	Lavavolo	Female	24°38'33.4"	044°56'50.2"
634	Lavavolo	Female	24°38'33.4"	044°56'50.2"
501	Besily	Female	24°59'04.4"	044°06'23.9"
502	Besily	Male	24°59'04.4"	044°06'23.9"
504	Besily	Female	24°59'04.4"	044°06'23.9"
505	Besily	Male	24°59'04.4"	044°06'23.9"
506	Besily	Male	24°59'04.4"	044°06'23.9"
507	Besily	Female	24°59'04.4"	044°06'23.9"
508	Besily	Male	24°59'04.4"	044°06'23.9"
509	Besily	Female	24°59'04.4"	044°06'23.9"
510	Besily	Male	24°59'04.4"	044°06'23.9"
511	Besily	Female	24°59'04.4"	044°06'23.9"
512	Besily	Female	24°59'04.4"	044°06'23.9"
514	Besily	Female	24°59'04.4"	044°06'23.9"
402	Kilibory	Male	25°04'27.1"	044°21'12.4"

Tortoise ID	Population	Sex	Latitude (S)	Longitude (E)
403	Kilibory	Female	25°04'27.1"	044°21'12.4"
404	Kilibory	Female	25°04'27.1"	044°21'12.4"
405	Kilibory	Female	25°04'27.1"	044°21'12.4"
406	Kilibory	Male	25°04'27.1"	044°21'12.4"
407	Kilibory	Male	25°04'27.1"	044°21'12.4"
408	Kilibory	Male	25°04'27.1"	044°21'12.4"
409	Kilibory	Female	25°04'27.1"	044°21'12.4"
410	Kilibory	Female	25°04'27.1"	044°21'12.4"
411	Kilibory	Female	25°04'27.1"	044°21'12.4"
412	Kilibory	Female	25°04'27.1"	044°21'12.4"
413	Kilibory	Male	25°04'27.1"	044°21'12.4"
414	Kilibory	Male	25°04'27.1"	044°21'12.4"
415	Kilibory	Female	25°04'27.1"	044°21'12.4"
416	Kilibory	Female	25°04'27.1"	044°21'12.4"
418	Kilibory	Female	25°04'27.1"	044°21'12.4"
419	Kilibory	Male	25°04'27.1"	044°21'12.4"
420	Kilibory	Male	25°04'27.1"	044°21'12.4"
421	Kilibory	Female	25°04'27.1"	044°21'12.4"
422	Kilibory	Female	25°04'27.1"	044°21'12.4"
MAT52	Matsandry	Female	25°14'36.2"	044°28'14.3"
MAT53	Matsandry	Female	25°14'36.2"	044°28'14.3"
MAT54	Matsandry	Male	25°14'36.2"	044°28'14.3"
MAT55	Matsandry	Male	25°14'36.2"	044°28'14.3"
MAT59	Matsandry	Male	25°14'36.2"	044°28'14.3"
MAT65	Matsandry	Female	25°14'36.2"	044°28'14.3"
MAT68	Matsandry	Male	25°14'36.2"	044°28'14.3"
MAT73	Matsandry	Male	25°14'36.2"	044°28'14.3"
MAT75	Matsandry	Female	25°14'36.2"	044°28'14.3"
MAT79	Matsandry	Male	25°14'36.2"	044°28'14.3"
MAT81	Matsandry	Male	25°14'36.2"	044°28'14.3"
BOA131	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA139	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA146	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA150	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA153	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA154	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA155	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA159	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA163	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA164	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA165	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA166	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA167	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA171	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA172	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA176	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA178	Ankaboa	Male	25°09'30.0"	044°34'29.9"

Tortoise ID	Population	Sex	Latitude (S)	Longitude (E)
BOA179	Ankaboa	Female	25°09'30.0"	044°34'29.9"
BOA180	Ankaboa	Male	25°09'30.0"	044°34'29.9"
BOA183	Ankaboa	Male	25°09'30.0"	044°34'29.9"
701	Antsifitse	Female	24°46'57.8"	045°05'51.3"
702	Antsifitse	Female	24°46'57.8"	045°05'51.3"
703	Antsifitse	Female	24°46'57.8"	045°05'51.3"
704	Antsifitse	Male	24°46'57.8"	045°05'51.3"
705	Antsifitse	Male	24°46'37.2"	045°05'55.7"
706	Antsifitse	Male	24°46'47.1"	045°06'05.9"
707	Antsifitse	Male	24°46'47.1"	045°06'05.9"
708	Antsifitse	Male	24°46'47.1"	045°06'05.9"
709	Antsifitse	Female	24°46'47.1"	045°06'05.9"
710	Antsifitse	Male	24°46'47.1"	045°06'05.9"
711	Antsifitse	Female	24°47'10.5"	045°06'09.1"
712	Antsifitse	Male	24°47'10.5"	045°06'09.1"
713	Antsifitse	Female	24°47'10.5"	045°06'09.1"
714	Antsifitse	Male	24°47'10.5"	045°06'09.1"
717	Antsifitse	Female	24°47'10.5"	045°06'09.1"
718	Antsifitse	Female	24°47'10.5"	045°06'09.1"
719	Antsifitse	Female	24°47'28.7"	045°06'19.8"
720	Antsifitse	Female	24°47'28.7"	045°06'19.8"
721	Antsifitse	Female	24°47'28.7"	045°06'19.8"
722	Antsifitse	Female	24°47'28.7"	045°06'19.8"
AA90	Antreaky	Male	25°07'51.8"	045°10'47.7"
AA91	Antreaky	Female	25°07'51.8"	045°10'47.7"
AA92	Antreaky	Male	25°07'51.8"	045°10'47.7"
AA96	Antreaky	Female	25°07'51.8"	045°10'47.7"
AA97	Antreaky	Female	25°07'51.8"	045°10'47.7"
AA98	Antreaky	Female	25°07'51.8"	045°10'47.7"
AA100	Antreaky	Male	25°07'51.8"	045°10'47.7"
AA106	Antreaky	Female	25°07'51.8"	045°10'47.7"
SAT124	Ifotaka	Male	25°18'15.1"	045°55'15.1"
802	Ifotaka	Female	24°46'00.3"	045°59'09.7"
803	Ifotaka	Female	24°46'00.3"	045°59'09.7"
804	Ifotaka	Male	24°46'06.7"	045°59'09.2"
805	Ifotaka	Female	24°45'40.6"	045°58'48.2"
806	Ifotaka	Female	24°45'47.5"	045°59'18.3"
807	Ifotaka	Female	24°45'55.1"	045°59'00.0"
809	Ifotaka	Female	24°45'55.1"	045°59'00.0"
810	Ifotaka	Male	24°45'55.1"	045°59'00.0"
811	Ifotaka	Female	24°45'55.1"	045°59'00.0"
812	Ifotaka	Female	24°45'55.1"	045°59'00.0"
813	Ifotaka	Male	24°45'55.1"	045°59'00.0"
816	Ifotaka	Female	24°46'42.9"	046°00'12.2"